MONASH University

ANTIBODY-ANTIGEN INTERACTIONS IN BLOOD TYPING FOR PAPER DIAGNOSTICS AND SURFACE PLASMON RESONANCE ANALYSIS

Thesis in the fulfilment of the requirement for the degree of Doctor of Philosophy in Chemical Engineering

by

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FEBRUARY 2016

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Dedicated to my family

~ Poh Poh, Dad, Mum, & Zhen ~

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GENERAL DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Thesis including published works General Declaration

Declaration for thesis based or partially based on conjointly published or unpublished work

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 3 original papers published in peer reviewed journals and 3 unpublished publications. The core theme of the thesis is blood group typing and analysis using paper diagnostics. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Chemical Engineering, Monash University under the supervision of Prof. Gil Garnier and Prof. Marie-Isabel Aguilar.

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

In the case of 5 chapters my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
1	Paper Diagnostics in Biomedicine Antibody-Antigen Interactions for Blood Group Tying	Published Manuscript (In Progress)	Literature Review Literature Review
2	The Detection of Blood Group Phenotypes using Paper Diagnostics	Published	Key ideas, experimental works, analysis of results, writing up. (44%)
3	Quantitative Blood Group Typing using Surface Plasmon Resonance	Published	Key ideas, experimental works, analysis of results, writing up.
4	Quantitative Detection of Weak D Antigen Variants in Blood Typing using Surface Plasmon Resonance	Submitted	Key ideas, experimental works, analysis of results, writing up.
5	Duffy Blood Group (Fy^a & Fy^b) Detection Using Surface Plasmon Resonance	Manuscript (In Progress)	Key ideas, experimental works, analysis of results, writing up.

I (have)/ have not (circle that which applies) renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] **Date:** 15/02/2016

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student and co-authors' contributi[redacted]

Main Supervisor signatur [redacted] **Date:** 15/02/2016

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ACKNOWLEDGEMENTS

Four years ago, when I first began my PhD journey I had high expectations and little true understanding of where this fulfilling yet arduous road would take me. I could never have imagined the steps it would take to get here. The achievements, the failures, the triumphs and the disappointments – all contributed to the knowledge I gained during this time, not only of my research topic, but of life.

Firstly, I would like to express my sincere gratitude and thanks to my main supervisor Prof. Gil Garnier for his encouragement, constant support, guidance and patience throughout my PhD candidature. This PhD has shaped me in more ways that I could describe, and that in large part is due to Prof. Gil Garnier. Through his guidance, I have learned to think independently and critically, to push expectations with innovative ideas, and opened the door to opportunities to present my work both locally and internationally. I will be eternally grateful for his passion and motivation for research, and his unwavering faith in my abilities throughout every stage. His infectious enthusiasm for research and life allowed me to believe that I could, in fact, do what sometimes seemed impossible.

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ABSTRACT

Paper-based analytical devices (PADs) can provide a quick, inexpensive and simple detection method for medical diagnostics. Currently, applications such as blood typing require complex technical facilities and specialized staff for accurate interpretation. Rapid and reliable blood typing during transfusions and transplantations is of the utmost importance. A paper diagnostic test for rapid blood typing has been designed using haemagglutination principles. Previous studies have successfully used paper to detect major blood groups ABO and RhD; however, other blood group phenotypes, including weak reactions, remain unexplored. This research aims to better understand the antibody-antigen interactions of such phenotypes in order to develop a robust paper diagnostic which can uniformly detect all blood types.

This thesis has shown proof of concept with promising results. However, the development of a robust paper diagnostic is heavily reliant on the structure of the corresponding antibody used. Due to its five-pronged structure, immunoglobulin M (IgM), lends itself better for antigen binding than immunoglobulin G (IgG), which only has two binding sites. Many clinically significant blood group antibodies are available as IgM. Direct agglutination using paper diagnostics was achieved for all clinically significant IgM antibodies tested. However, some blood group antibodies, such as the Duffy system, are only available in an IgG structure. Therefore understanding, detecting, quantifying and optimising antibody-antigen interactions are the key to designing a functional, cheap, simple and rapid blood typing paper diagnostic.

Current available blood typing methods, such as the column agglutination test (CAT), are well established. However, methods quantifying these antibody-antigen interactions are limited. This is particularly important for identifying and characterising weak interactions between weak subgroup variants. Such weak interactions are often difficult to determine by the naked eye and can be potentially be overlooked.

Surface plasmon resonance (SPR) can quantify interactions between biomolecules. Previous studies have shown SPR detection of blood group antigens; however, these methods used solely IgM antibodies and showed poor regeneration. The platform

showed a loss of functionality after a single use due to the inability to fully desorb bound material.

In this thesis, a fully regenerable, multi-functional platform for quantitative blood group phenotyping via SPR detection was achieved for the first time by covalently immobilising an antibody, anti-human IgG, to the sensor chip surface. Anti-human IgG recognizes and binds to the Fc region of human IgG antibodies. The functionalised surface can therefore provide a platform capable of quantifying blood group interactions between RBCs and any IgG antibodies. This novel approach was validated with human-sourced whole blood samples to demonstrate an interesting alternative for quantitative blood grouping using the RhD blood group as an example. Over 100 regenerations of the SPR sensor surface was also achieved showing full reversibility of adsorption with nominal degradation. This opens a new paradigm in efficient, fast and cheap human IgG antibody quantification by SPR that can be easily up-scaled for commercialisation.

Furthermore, the detection of weak and partial D variants was achieved for the first time using this fully regenerable SPR platform, demonstrating sensitivity and quantification capabilities currently unavailable using modern blood typing techniques.

This SPR platform was also applied to a clinically significant blood group that is notoriously difficult to detect in the blood banking industry. Duffy antigens, Fy^a and Fy^b were detected using SPR, thereby validating the concept. However, full reproducibility was not feasible due to rapid dissociation of the very weak antibody-antigen complex. The instances of successful detection showed potential for this SPR platform; however, further development is required to meet blood banking criteria.

The initial development stages of this novel SPR platform provided a means to show label-free quantification of blood group antigens. Further development could allow for a fuller understanding of the different mechanisms and effects of not only the structure and density of antigens and their corresponding antibodies, but also the additives which are used to enhance blood group antibody-antigen interactions. This would be invaluable for the development of paper diagnostics for blood group detection with uniform clarity regardless of these differences.

LIST OF PUBLICATIONS

Peer-Reviewed Journal Papers

The following published and submitted papers are included in the body of this thesis as individual chapters. The sections of these published papers have been renumbered in order to generate a consistent presentation within the thesis. Papers in the published format are included as Appendix 1.

1. **Then, W.L.** and Garnier, G., Paper diagnostics in biomedicine, *Reviews in Analytical Chemistry,* 2013. **32**(4): p. 269-294
2. **Then, W.L.**, Li, M., McLiesh, H., Shen, W., Garnier, G., The detection of blood group phenotypes using paper diagnostics, *Vox Sanguinis*, 2015, **108**: 186–196.
3. **Then, W.L.**, Aguilar, M. I., and Garnier, G., Quantitative blood group typing using surface plasmon resonance, *Biosensors and Bioelectronics,* 2015, **73**: 79-84.
4. **Then, W.L.**, Aguilar, M. I., and Garnier, G., Quantitative detection of weak D antigen variants in blood group typing using surface plasmon resonance, *Submitted,* 2016

The following published papers are not included in the main body of this thesis and can be found in their published format in Appendix 2.

5. Ngo, Y.H., **Then, W.L.,** Shen, W., and Garnier, G., Gold nanoparticles papers as a SERS bio-diagnostic platform, *Journal of Colloid and Interface Science,* **409:** 59-65.
6. Garnier, G., and **Then, W.L.**, *Paper Microfluidics: Applications and Perspectives*. Advances in Pulp and Paper Research. Cambridge, 2013. **2**: p. 541-583.
7. Li, M., **Then, W.L.,** Li, L., Shen, W., Paper-based device for rapid typing of secondary human blood groups, *Analytical and Bioanalytical Chemistry*, 2014, **406**: 669–677.

Patents

1. Ngo, Y.H., Then, W.L., Li, D., Simon, G.P., and Garnier, G., “Metallic Nanoparticle Treated Cellulosic Substrate as a SERS Biodiagnostic Platform”, Australia Provisional Patent, AU2012902470, 28 June, 2012.

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
AHG	Anti-human globulin
AKD	Alkyl-ketene-dimer
ALP	Alkaline phosphatase
Anti-	Antibody/antisera
ARCBS	Australian Red Cross Blood Service
ASA	Alkeynl succinic anhydride
ASSURED	Affordable, Sensitive, Specific, User-Friendly, Rapid and Robust, Equipment-free, deliverable
AST	Aspartate aminotransferase
AuNP	Gold nanoparticles
BIA	Biospecific interaction analyses
BS	Blood spot
CAT	Column agglutination test
CD	Cross machine direction – short axis of the paper sheet
CMC	Carboxymethylcellulose
CPAM	Cationic poly(acrylamide)
DNA	Deoxyribonucleic acid
D^{w+}	Weak D antigen
E	Elution method
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enyzme-linked immunosorbent assay
EP	Elution pathway
FACS	Fluorescence-assisted cell sorting
FFMU	For Further Manufacturing Use
FLASH	Fast Lithographic Activation of Sheets
HBS-EP	HEPES buffered saline (
HBsAg	HepB surface antigens
HBV	Hepatitis B virus
hCG	Human chorionic gonadotropin
HCl	Hydrogen Chloride
HCV	Hepatitis C virus
HDFN	Haemolytic disease of the foetus and newborn

HEBES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HIV-1	Human immunodeficiency virus type 1
HPLC	High pressure liquid chromatography
HTR	Haemolytic transfusion reaction
HRP	Horseradish peroxidase
IAT	Indirect anti-globulin test
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ISBT	Internation Society of Blood Transfusion
L	Length
LISS	Low ionic strength solution
LOD	Limit of detection
MD	Machine direction – long axis of the paper sheet
MFC	Micro-fibrillated cellulose
$MgCl_2$	Magnesium chloride
NaCl	Sodium chlorine
NCC	Nano-cellulose crystals
NHS	N-hydroxysuccinimide
NISS	Normal ionic strength solution
ODR	Optical density ration
o-PADs	Origami paper-based analytical device
PAA	Polyacrylic acid
PAD	Paper-based analytical device
PAE	Polyamideamine epichlorohydride
PBS	Phosphate buffered saline
PCC	Precipitated calcium carbonate
PCR	Polymerase chain reaction
PDMS	Poly-dimethyl-siloxane
PEG	Polyethylene glycol
PEO	Polyethylene Oxide
*Pf*HRP2	*Plasmodium falciparum* histidine rich protein 2
PN	Paper network
POC	Point of care
PSA	Prostate specific antigen

RAM	Rapid antibody medium
RAMPEG	Pegylated-RAM
RBC	Red blood cell
RCA	Rolling circle amplification
RI	Refractive index
S	Sample zone
SEM	Scanning Electron Microscopy
SERS	Surface Enhanced Raman Scattering
SNP	Single nucleotide polymorphism
SPR	Surface Plasmon Resonance
TFC	Thin film chromatography
TiO2	Titanium dioxide
TLC	Thin layer chromatography
TR	Text-reporting method
UA	Uric acid
UV	Ultraviolet
WHO	World Health Organization
µPAD	Microfluidic paper-based analytical device
µPED	Microfluidic paper-based electrochemical device
1D	One dimensional
2D	Two dimensional
3D	Three dimensional
^{131}I	Radioactive isotope of iodine-131

LIST OF NOMENCLATURE

AUD	Australian dollar
C	Coulomb
c	electrolyte concentration
e	electron charge
J	Joule
K	degree Kelvin
k	Boltzmann constant (1.38 x 10^{-23} J/ K)
L	Litre
M	molar
m	metre
mg	milligram
min	minute
mL	millilitre
mm	millimetre
mol	mole
nm	nanometre
N_A	Avogadro's constant (6.02x 10^{23} 1/mol)
RU	binding response unit
s	second
t	time
v	volume
z	charge number
ϵ	permittivity of the solvent
Ƙ	integration constant from integration of the surface potential
µg	microgram
µL	microliter
˚C	degree Celsius
%	percentage

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

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Declaration for Thesis Chapter 1

Declaration by candidate

In the case of Chapter 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Initiation, key ideas, review of updated literature, development and writing up of papers,	80%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Gil Garnier	Key ideas, paper reviewing and editing	Supervisor

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature	[redacted]	**Date** 15/02/2016
Main Supervisor's Signature	[redacted]	**Date** 15/02/2016

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1.1 INTRODUCTION

Accurate, reliable and efficient detection is the first step towards treating a disease. Paper-based analytical devices (PADs) have the capability to provide rapid, cheap and simple detection methods for biological and chemical analysis. As such, the past decade has seen an enthusiastic increase in interest due to the numerous advantages of using paper-based diagnostics, especially in the field of biomedicine. Paper has many advantages which lend itself to a point-of-care (POC) platform over current conventional techniques. Although PADs hold great promise as a biodiagnostic platform, there are still many limitations preventing its commercial applicability as evidenced by the lack of marketable products after ten years of research and development. Currently, applications such as blood group typing require complex and fully equipped technical facilities, as well as qualified and trained staff for accurate interpretation.

Typing of human blood groups requires accuracy, efficiency and reliability, particularly during medical emergencies. Mismatched blood typing can lead to a haemolytic transfusion reaction (HTR), the side effects of which can range from mild to severe, and can even lead to patient death. Traditionally, an individual's blood type is determined via two procedures conducted in parallel. The first method analyses the presence or absence of antigens on the surface of their red blood cells (RBCs), while the second detects the presence of antibodies within human whole blood, more specifically the blood plasma. Blood group antibodies have two structures, Immunoglobulin M (IgM) and Immunoglobulin G (IgG). IgM is larger with a pentameric structure lending itself to faster and stronger agglutinate formation. IgG, however, is smaller and only has a monomeric structure, and therefore cannot form agglutinates when used alone. Instead an additional binding agent is required.

Utilising the principles of haemagglutination, the most prevalent modern technique for blood typing is the column agglutination test (CAT), where specific antibodies are contained within a gel or glass microbead column. Positive-antigen RBCs form haemagglutinates which are unable to move through the column during centrifugation. Conversely, negative-antigen RBCs remain unbound and can move freely, forming a pellet at the base. While fast and accurate, the CAT technique requires fully equipped

laboratories with refrigeration and centrifugation facilities which are unsuitable for employment in remote locations or in-the-field situations.

More recently, the same haemagglutination principles were implemented for rapid blood typing using a new paper diagnostic test designed at the Australian Pulp & Paper Institute (APPI). The paper platform is an extremely cheap and simple alternative which is portable and easily disposable.

Previous works have successfully used paper to determine blood typing of the well-known blood groups, ABO and RhD, though the latter is more commonly known as "+" or "-". However, there are anomalous occurrences which show weaker binding for certain blood types, such as weak AB and weak D. Furthermore, although blood group detection had been successfully demonstrated on paper for ABO and RhD blood groups, blood group typing for other RBC antigens, such as those in the Rh, Kell, Duffy, and Kidd systems, are known to exhibit weaker binding reactions during testing and have not yet been explored on paper. As of 2016, there are 36 recognised blood group systems, comprising over 300 antigens. Nine of the blood group systems are considered clinically significant, and have been previously unexplored using paper diagnostics.

The broad aim of this research is to map the full potential and limits of paper diagnostics in biomedicine, particularly in the context of extending the achievements of previous studies to include the full range of clinically significant blood groups. This is namely the detection of non-ABO and non-RhD blood group phenotypes using paper diagnostics, which all behave uniquely due factors such as antigen density, antigen structure, antibody structure, and binding affinity between antibody-antigen complexes. However, understanding of the underlying mechanisms of these differences have not been widely explored. Therefore, the overall objective of this research is to investigate the antibody-antigen interactions of such phenotypes – not only on paper but at a biomolecular level. The aims include optimising the properties of diagnostic design, testing methodology, and the best conditions for each individual antigen so all blood group types can be uniformly detected regardless of any weaker antibody-antigen interactions. This includes examining the structural properties of the paper itself, and how the directional flow of analytical reagents can affect the overall clarity and sensitivity of the paper test.

In an effort to better understand the antigen-antibody interactions for blood group typing using IgG antibodies, the focus of this thesis shifted to using a bioanalytical technique, surface plasmon resonance (SPR). SPR is a label-free diagnostic tool capable of measuring real-time interactions. These measurements could be used to design better, more functional, cheap, simple and rapid blood typing paper diagnostics. It also has the potential for sensitive and specific analysis of interactions on a biomolecular scale.

While current blood typing methods are well established, results are often subjective and heavily reliant on interpretation by trained personnel. Techniques for quantifying blood group antibody-antigen interactions are also very limited. Many modern biosensing systems rely on SPR detection to quantify biomolecular interactions. SPR is a commonly used, label-free optical bioanalytical technique which relies on a polarised laser to measure the change of adsorbed mass on the surface of a metal chip by monitoring the changes in refractive index. While SPR has been widely used for characterising antibody-antigen interactions, measuring antibody interactions with whole cells is significantly less common. As SPR analysis is usually coupled with a microfluidic system for the delivery and detection of analytes, SPR analysis of whole cells has been limited. In terms of blood analysis, this is because the RBC is of a similar length scale to the diameter of the microfluidic system in which the behaviour of RBCs has always been challenging, especially under coagulating conditions. Previous studies have utilised SPR for blood group antigen detection, however, they showed poor regeneration and an inability to desorb bound material, causing loss of functionality after a single and very expensive use. SPR sensor chips consist of a metal surface (eg. gold or silver) which can be functionalised to detect specific analytes and facilitates SPR analysis through reflection of the polarised laser to a detector. However, SPR sensor chips are also notoriously expensive (costing around AUD200 in 2016) and have a short life span. Also, these previous studies only showed binding using IgM antibodies; IgG antibodies have never been explored. Furthermore, studies of whole cells using SPR have been limited due to: (a) the microfluidic channels used in conjunction with SPR, and (b) the small detection field of the polarised laser (~300nm). RBCs, however, while still comparatively large, have the main functional purpose of distributing oxygen

throughout the entire body, travelling from the heart via the large blood vessels of the circulatory system to the outer most extremities. In order to facilitate this function, RBCs are able to change conformation to move through even the smallest micro-capillaries. This attribute can theoretically allow for RBC movement through the microfluidic channels often coupled with SPR detection.

When compared, paper diagnostics and SPR analysis each have its own advantages and disadvantages. While paper is cheaper, easy-to-use and portable it lacks a sufficient system for quantifying binding interactions observed between the antibody-RBC complexes. SPR analysis, on the other hand, is a very sensitive, real-time and label-free bioanalytical technique capable of quantifying the detection and kinetic interactions of biomolecules. Despite potential for multiplex testing, it requires expensive laboratory equipment and may be constrained by the limits of the size and viscosity of human RBCs.

The objectives of this chapter is two tiered. First, a brief overview of the thesis structure is provided (section 1.2). Next, a review summary (section 1.3) highlights the gaps in knowledge (section 1.4) for the development of a fully comprehensive paper diagnostic for blood group phenotyping, and the uses of SPR in blood analysis. Following (section 1.5), is an outline of the overall and specific aims of this doctoral research for the understanding of blood group antibody-antigen interactions in both paper diagnostics and SPR analysis. The second objective is an in depth literature review of the structure and applications of paper diagnostics in biomedicine (section 1.6), as well as the current standing of transfusion medicine (section 1.7), particularly methods for the detection and analysis of blood group antibody-antigen interactions, including surface plasmon resonance analysis.

1.2 THESIS OUTLINE

This thesis is presented in the format of "Thesis with Publications" based on the Monash University guidelines for Doctoral and MPhil Degrees 2016 and the Thesis with Publications guidelines. It consists of three publication chapters (all published), and two traditional thesis chapters, which are also manuscripts-in-preparation. All published papers are reformatted for a consistent presentation whilst the content remains unchanged. The original publications are provided in Appendix I.

A chapter-by-chapter outline based on the research aims, the conducted research studies and the successful outcomes is presented.

- **Chapter 1 – Introduction & Literature review**

  (Then, W.L. and Garnier, G., Paper diagnostics in biomedicine, Reviews in Analytical Chemistry, 2013. 32(4): p. 269-294)

  The purpose of this chapter is to review the literature of both paper diagnostics and surface plasmon resonance (SPR), and their applications in blood group phenotyping and blood antibody-antigen interaction analysis. Two reviews are contained within. The first review is an in-depth analysis of the structure, properties and applications of paper diagnostics in biomedicine. The second review is a study on the current methods for detection and analysis of blood group antibody-antigen interactions in transfusion medicine. Critical gaps in knowledge are discussed, followed by the overall and specific objectives on this thesis.

- **Chapter 2 – The detection of blood group phenotypes using paper diagnostics**

  (Published: Then, W.L., Li, M., McLiesh, H., Shen, W., Garnier, G., The detection of blood group phenotypes using paper diagnostics, Vox Sanguinis, 2015, 108: 186–196.)

  As all blood group phenotypes are unique, some show different intensities of agglutination on paper due to weaker antibody-antigen interactions. This second chapter focuses on the detection of clinically significant non-ABO and non-RhD blood group phenotypes using paper diagnostics and the performance there-in,

comparing two different techniques using the mechanisms from within the paper structure, and evaluating the effect of different testing variables on the robustness and clarity of the results. Using an elution method, which operates much like chromatography, and a direct flow-through method, which uses a filtration-like mechanism, several clinically significant blood group antigens were tested for positive and negative detection. In addition, the effects of reaction time, reagent concentration, and antibody type/structure were compared.

- **Chapter 3 – Quantitative blood group typing using surface plasmon resonance**

(Published: Then, W.L., Aguilar, M. I., and Garnier, G., Quantitative blood group typing using surface plasmon resonance, Biosensors and Bioelectronics, 2015, 73: 79-84.

Techniques for analysing blood group antibody-antigen interactions, particularly binding quantification and kinetic analysis, is limited. This third chapter commences the development of a surface plasmon resonance (SPR) platform for the detection and quantification of such interactions. SPR is an analytical tool commonly used to measure and observe binding kinetics of biomolecules. Binding responses are also concentration-dependent, capable of quantitative analysis. This concept explores the functionalisation of an SPR sensor surface with anti-human IgG. Anti-human IgG has the specific capability of binding to the Fc region of human IgG antibodies. Positive pre-sensitized red blood cells (RBCs) with IgG antibodies are detected and reported as binding responses to quantify the binding strength of the antibody-antigen interactions. Using blood group RhD as an example, this approach was validated using both positive-RhD and negative-RhD human-sourced whole blood samples and achieved complete regeneration of the anti-human IgG surface. This preliminary study demonstrates an interesting alternative for quantitative blood group typing.

- **Chapter 4 – Quantitative detection of weak D antigen variants in blood group typing using surface plasmon resonance**

(Submitted: Then, W.L., Aguilar, M. I., and Garnier, G., Quantitative detection of weak D antigen variants in blood group typing using surface plasmon resonance, 2016)

This fourth chapter further explored the use of surface plasmon resonance (SPR) for the quantitative detection of blood group antigens, focusing on weak and partially expressed RhD variants. There are many blood group variations between different individuals, not only phenotype and antigen density, but also antigen structure. Individuals that express weak D variants have red blood cells (RBCs) with a much lower D antigen density, however, the antigen expressed is that of a normal D structure. Conversely, partially expressed D variants may have an antigen density within a normal range, but the structure of the antigen itself is incomplete. There are nine classes of partial D antigens. While most a relatively rare within the Melbourne population, its detection is no less important. As a sensitivity study, this research demonstrates the ability to detect both weak and partial D antigens using an SPR sensor platform functionalised with anti-human IgG.

Chapter 5 – Duffy Blood Group (Fy^a & Fy^b) Detection using Surface Plasmon Resonance

This fifth chapter extends the surface plasmon resonance (SPR) platform analysis to blood groups other than RhD, namely Duffy blood groups, Fy^a and Fy^b. While RhD antibodies are available in both IgM and IgG structures, and are known as potent and strong antibody-antigen interactions, Fy^a and Fy^b are only available as IgG antibodies and show weaker binding affinities. Furthermore, Duffy antigens are expressed at a much lower density with a unique antigen structure which is very different to that of RhD. The ability to detect Fy^a and Fy^b antigens would signify the true capabilities of this SPR platform for multiplex and quantitative blood group antigen detection.

- **Chapter 6 – Perspectives and Conclusions**

This final chapter summarizes the major contributions of this thesis and provides perspectives where this research sits in both the fields of paper diagnostics for blood group typing and the use of surface plasmon resonance in blood group typing applications.

1.3 REVIEW SUMMARY

It is clear that there is great potential for paper diagnostics as a cheap and robust alternative to current laboratory-intensive techniques. In particular, the field of blood group typing is vast, especially with 36 blood group systems and over 300 blood group antigens, most of which have been untested using paper diagnostics thus far. As the antibody-antigen interactions of each individual blood group, particularly of those from the 9 systems classified 'clinically significant', are unique due to antigen density, antigen structure, antibody structure, and bioaffinity between these biomolecules, their performance on paper is currently undefined.

At present, the progress of developing a rapid paper diagnostic for blood typing is stalled by a limited understanding of why phenotypes such as weak AB, weak D and partial antigens do not show the same reactivity when tested using the paper diagnostics. Furthermore, while the ABO and RhD blood groups have IgM antibodies available for testing, this is not the case for all blood groups, particularly for the Duffy system which can only be tested using IgG antibodies.

Blood group typing using paper was previously reported using two different washing methods, an elution method and a flow-through method. Both use different mechanisms within the paper for blood group antigen detection. The elution method operates much like chromatography, using the microfluidic capillary structure of the paper during testing. On the other hand, the flow-through method operates much like filtration. While the understanding of ABO and RhD blood group detection by haemagglutination is progressing, that of the other clinically significant blood groups remains unexplored.

Furthermore, there is also a poor understanding of the effects caused by variations in antibody formulation and antigen type, especially for weakly or partially expressed antigens. Though much rarer among the population, accurate detection is imperative for successful blood transfusions.

There is also a lack of biomolecular analysis for blood group typing. Each phenotype behaves uniquely, with the differences ranging from small to vast. There is currently

limited quantification and kinetic analysis techniques that are robust and capable of real-time and label-free analysis. This has led to: (a) poor quantification of antibody-antigen interactions, especially in the context of haemagglutination; (b) poor kinetic understanding of haemagglutination; and (c) poor quantification of the effect of stoichiometry ratio between the RBC antigen and antibodies, temperature, pH, ionic strength and time.

Of particular interest is surface plasmon resonance (SPR) analysis which is one of the most commonly used analytical techniques for biomolecules. The antibody-antigen interactions are examples of such biomolecules. However, analysis involving whole cells is much rarer. While there have been some previous studies for blood group detection using SPR, its uses in blood analysis has been largely unexplored. As SPR has not been widely investigated with whole cells, critical questions arise such as the practicality of testing with RBCs, the potential for the detection and quantification of antigens upon the RBC surface, the sensitivity and specificity of detecting RBC antigens, and finally, cost effective regeneration capabilities.

1.4 GAPS IN KNOWLEDGE

The following literature review has highlighted a series of significant gaps in scientific knowledge that have hindered the efficient development of a paper diagnostic for use in the blood group phenotyping industry.

Of particular interest and concern are:

1. The unexplored avenue of typing clinically significant non-ABO and non-RhD blood groups using paper diagnostics.
2. The factors affecting blood group typing using paper diagnostics, including: antigen type, antigen density, antibody structure, antibody serum formulation, binding affinity between antibody-antigen complexes, and properties of the paper and test reagents.
3. Poor quantification of antibody-antigen interactions, particularly for weakly or partially expressed antigens
4. The limited understanding of the potential of surface plasmon resonance (SPR) for blood analysis, including: the practicality of use with red blood cells, antigen detection and quantification, sensitivity and specificity, and regeneration capabilities,
5. A lack of robust quantification technique sensitive enough for weak and partial antigen detection

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1.5 RESEARCH OBJECTIVES

The broad aim of this research is to map the full potential and limits of paper diagnostics in biomedicine, focusing in particular on quantifying and better understanding the antibody-antigen interactions in blood group typing for improved design and application. As SPR is a long-standing and sensitive analytical tool for biointeractions, with capabilities of real-time and label-free detection, it has the potential to be applied to antibody-antigen interactions involved in blood group typing. However, SPR has not previously been widely applied to whole cell interactions. In particular, use of SPR for blood analysis of RBCs has been grossly under explored, with poor regeneration capabilities. Through SPR analysis, the aims to observe, quantify, and analyse the binding events between blood group antibodies and antigens could be achieved. Thus allowing for the improvement of paper diagnostics for the previously unexplored non-ABO and non-RhD blood groups, including weak and partial antigen phenotypes.

In particular, the specific objectives of this research are:

1. To examine and quantify the typing of clinically significant non-ABO and non-RhD blood groups using paper diagnostics.

2. To define and optimize the factors affecting blood group typing using paper diagnostics, including: antigen type, antigen density, antibody structure, antibody serum formulation, binding affinity between antibody-antigen complexes, and properties of the paper and test reagents.

3. To compare the testing methodologies of paper diagnostics for blood group typing – i.e. the elution method vs. the flow-through method

4. To develop and explore the limits of a new testing platform utilising the bioanalytical tool, surface plasmon resonance, for blood typing applications including quantitative detection and improved sensitivity.

5. To quantify antibody-antigen interactions, particularly for weakly or partially expressed antigens.

6. To investigate and better understand the antibody-antigen interactions involving IgG antibodies, particularly for the Duffy blood group system, for detection and application with paper diagnostics.

7. To compare the use of paper diagnostics for blood group typing with the quantitative and sensitive bioanalytical technique, surface plasmon resonance.

1.6 REVIEW: PAPER DIAGNOSTICS IN BIOMEDICINE

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1.6.1 Abstract

Paper diagnostics are devices made of paper and cellulosic materials to recognize and quantify biomolecules and chemical agents affecting health. There has been a rapid increase in interest for paper-based diagnostic methods in recent years. This is because paper is cheap, widely available, easily engineered, biodegradable, combustible, biocompatible, sterilisable, hydrophilic, and easy to functionalise and process into diagnostic devices. Paper is a very attractive substrate to develop as a low-cost diagnostic platform. Paper can serve four functions in a diagnostic: (1) transport and measurement of sample and analytes, (2) reaction support, (3) separation of reactants from products, and (4) communication of results. When an aqueous analyte, such as blood, saliva, urine or faeces is tested using a paper diagnostic analytical devices (PAD), the fluid is driven by capillary flow induced by the porous and hydrophilic structure of the cellulose fibres, causing wicking through the paper inter-fibre space. Should hydrophobic barriers be formed onto or within paper, microfluidic systems can be created allowing for the passive transport of the analyte.

This article has four sections. The first presents paper as a composite and highlights its properties and attributes with respect to modern paper diagnostics needs. Functional printing is presented as a technology to manufacture paper diagnostics. The second section analyses paper diagnostic design. PADs based on 1 dimension (1D), 2D and 3D flow, the methods of reporting and the principles of detection are reviewed. The third section investigates applications in health and medicine for paper diagnostics in terms of clinical diagnostics, physiological disorders and pathogenic diseases. The last section presents an analytical perspective of some of the critical issues. It is the objective of this

article to analyse paper as a viable technology for producing low cost medical analysis and to delimit the range of applications and potential best suited to paper diagnostics.

1.6.2 Keywords

Paper diagnostics, bioactive paper, analytical, paper structure, sensitivity, biomedicine

1.6.3 Introduction

The first step towards treating a disease is accurate and efficient detection. Paper-based analytical devices (PADs) can provide a quick, inexpensive and simple detection method for biological and chemical analysis, which can easily be employed in medical diagnostics. Currently, applications such as blood typing and pathogenic detection require a technical infrastructure operated by qualified staff for accurate interpretation. Paper diagnostics could offer a cheap, simple and convenient method for the user to directly test and interpret results such as the pregnancy test and electronic blood glucose tests. The past five years has seen the rise of a strong interest in paper-based diagnostic methods. Although PADs hold great promise as a biodiagnostic platform, there are still many limitations preventing its commercial applicability as evidenced by the lack of new products on the market after five years of intense research and development.

Paper diagnostics are devices made of paper, cellulosic films, cardboard and fabrics to recognize and quantify biomolecules and chemical agents affecting health. While paper-based analysis has been readily used in biomedicine since the 19th century, its potential in modern medicine has yet to be fully realized [1-3]. The past five years has seen a rapid increase in interest due to the compelling advantages of using paper-based diagnostic methods. A key advantage is its ubiquitous nature. Paper is a staple in everyday life all over the globe, which is manufactured on a large-scale into a plethora of different structures and properties. Paper is cheap, easily engineered, biodegradable, combustible, biocompatible, sterilisable, hydrophilic, and easy to functionalise and

process into diagnostic devices. Paper is a very attractive substrate to develop as a low-cost diagnostic platform (Table I).

Table I: The desirable ASSURED properties of paper as a medical diagnostic.

Requirement	**Paper is:**	
Affordable	Cheap & Cost Effective	Inexpensive manufacturing process Minimal use of reagents and analytes
Sensitive	White-background	A good contrast medium for colorimetric tests
Specific	Dried Reagents	Reagents can be dried and stored within paper fibres
User-friendly	Single Use Autonomous Minimal step-processes	 Does not require external power sources or equipment To eliminate as many unnecessary steps (e.g. washing) as possible
Rapid and Robust	Fast acting	Generally faster analysis compared to conventional testing methods
Deliverable	Portable	Easy to manufacture, stack, store and transport
Other	Disposable Particulate filter	To protect end-users from exposure to biohazardous wastes fibres can trap unwanted particulates in loading zone, preventing movement to detection zone

With these attributes, paper can become an ideal platform for point-of-care (POC) diagnostic devices and eliminate or restrict the need for external equipment and specialized technical personnel; this would be ideal in developing countries, and military, emergency and humanitarian field operations [1]. As defined by the World Health Organization (WHO), diagnostics in developing countries should be ASSURED: affordable, sensitive, specific, user-friendly, rapid and robust, equipment free and deliverable (Table I) [4]. Additionally, POC diagnostics need to be disposable, portable, sterilized and rugged [4]. These requirements help to overcome the unpredictability exhibited during in-the-field testing caused by the surrounding environment [4]. This includes limited clean water, unreliable electricity, high temperatures and humidity [4]. The versatility of paper and its many advantages make it more desirable as a POC platform than others technologies such as thread and magnetic levitation [5]. Another requirement of modern paper diagnostics is ease of use and the direct testing and interpretation by users.

Paper can serve four functions in diagnostics: (1) transport and measurement of sample and analytes, (2) reaction support, (3) separation of reactants from products and (4)

communication of results. When an aqueous analyte, such as blood, saliva, urine or faeces is tested using a PAD, the fluid is driven by capillary flow induced by the porous and hydrophilic structure of the cellulose fibres, wicking through the paper inter-fibre space. Should hydrophobic barriers be formed onto or within paper, microfluidic PADs can be created (μPADs) [6], allowing for the passive transport of the analyte without any further equipment required [7]. This represents a major advantage over traditional microfluidic materials, such as glass, silicone, poly-dimethyl-siloxane (PDMS) and other polymers, which require external pumping [2, 7]. Paper microfluidics are achieved by patterning hydrophilic channels that are defined by hydrophobic barriers; the micro-channels allow the testing of small samples with minimal reagents, thus reducing cost [2, 7].

Even though paper has many of the desirable attributes of a diagnostic platform, the current commercial PADs, such as immuno-chromatographic strips, have limited quantitative capabilities [7]. This has generated interest into coupling paper devices with electrical reporting methods, from basic telemedicine [8] to electrochemical sensing platforms [9]. Another disadvantage is the simplistic nature of the paper device which has restricted multiple-sample, multiple-analysis or multi-step assays [7].

Despite its recent inception in 2007, the growing interest in paper-based biodiagnostics has already resulted in several review papers, each detailing the desired properties, patterning methods and potential applications in health, food and the environment [1, 2, 6, 10, 11], as well as reviews in microfluidics, in which paper is included [4, 7, 12]. However, few have critically analysed the potential and limitations of paper as an engineered material to manufacture low-cost biomedical diagnostics.

While paper-based medical diagnostics have a single beneficiary, they are used in widely different contexts of applications, each with their specific requirements. Three broad categories can be mapped. The first are generic high throughput diagnostics for routine tests. Cholesterol analysis, basic ABO blood typing, and blood sugar measurement are examples. These generic tests are performed by specialized scientists operating well-automated analytical instruments in dedicated laboratories. Speed of analysis, high throughput and low cost are the main drivers. The second category of

diagnostics regroups the series of patient specific analyses required for a particular diagnostic. Examples are specific antibody identification, complete blood phenotyping for a transfusion or elemental blood analysis. The main requirements are flexibility and speed. These tests require laboratories with efficient management, a wide analytical expertise and instrumentation, exclusive to modern medical laboratories. Sample preparation and chemical/biochemical reactions are often required. The last category consists of diagnostics for stand-alone remote testing. These tests are often performed either in the privacy of home or in difficult conditions either by the patient himself or by untrained personnel in absence of technical or information support. A single specific test is usually performed. Robustness, ease of use, direct quantification/interpretation of results and speed are four important requirements.

Paper diagnostics cannot pretend to address all medical analytical needs. With the explosion in research development and the strong media interest surrounding paper tests, the medical world and community at large are often left in confusion as to the state of development and potential paper diagnostics offered for medical applications. This article attempts to address these issues and provide an analytic roadmap; it is critical to distinguish between elegant prototypes and research prowess from promising technologies able to impact the medical community. An engineering approach is adopted here as it best segregates science from development and clearly highlights the strengths and limitations of paper technology in medical diagnostics.

This manuscript consists of four sections. The first presents paper as an engineered composite and highlights its properties and attributes with respect to paper diagnostics needs. Functional printing is introduced as a complementary technology for paper in the manufacturing of paper diagnostics. The second section analyses paper diagnostic design. PADs based on 1 dimensional (1D), 2D and 3D flow are analysed, followed by a review of the methods of reporting and the principles of detection. The third section reviews high impact applications in health and medicine for paper diagnostics. Applications are studied in terms of clinical diagnostics, physiological disorders and pathogenic diseases. The last section presents a critical perspective of the current developments, the missing links and the potential of paper diagnostics. It is the objective of this article to analyse paper as a viable technology for producing low cost

medical analysis and to delimit the range of applications and potential best suited to paper diagnostics.

1.6.4 Paper as Diagnostic Substrates

Paper is a porous and flexible composite made from cellulosic fibres and functional colloids that can be tailor-made for a plethora of applications (Figure 1). While the near totality of paper diagnostics has been made using filter paper, this substrate represents a negligible fraction of the paper production, and of the range of structures and properties achievable. Furthermore, filter paper is among the most expensive grade of paper. The choice of filter paper has been dictated by the quest for a standard to avoid unknown variables from cellulosic materials engineering. Most filter papers are made of a single type of fibre, mostly cotton, uniformly distributed in all directions. However, bioactive paper engineering relies not only on the fibrous structure, but also on the structure of the void fraction; fibres control the reactants and biomolecules distribution while the void fraction dictates liquid transport.

Paper is made by uniformly distributing and draining a suspension of cellulosic fibres onto a moving wire passing over a series of drainage elements, and then into a series of three presses, and finally through a long dryer. Paper is a specific type of non-woven material, manufactured by a wet laid process, which involves four steps: (1) fibre preparation, (2) forming, (3) bonding, and (4) surface treatment. *Fiber preparation* consists of selecting and preparing cellulosic fibres to optimize the properties of the paper: the selection determining whether recycled or virgin fibres are used. Recycled fibres are avoided for bioactive paper and paper diagnostics due to the high risk of contaminants associated (dirt, oils, polymers, ink and organic residue, microbial growth). For wood based paper, the fibres can be long softwood or short hardwood fibres. They can also be made by a chemical or by a mechanical pulping process. Chemical processes dissolve most of the extractives (fatty and resin acids, tannin, lignin and hemicellulose), basically producing almost pure cellulose fibres. Mechanical pulping processes rely on a combination of shear, heat and plasticisation by water to

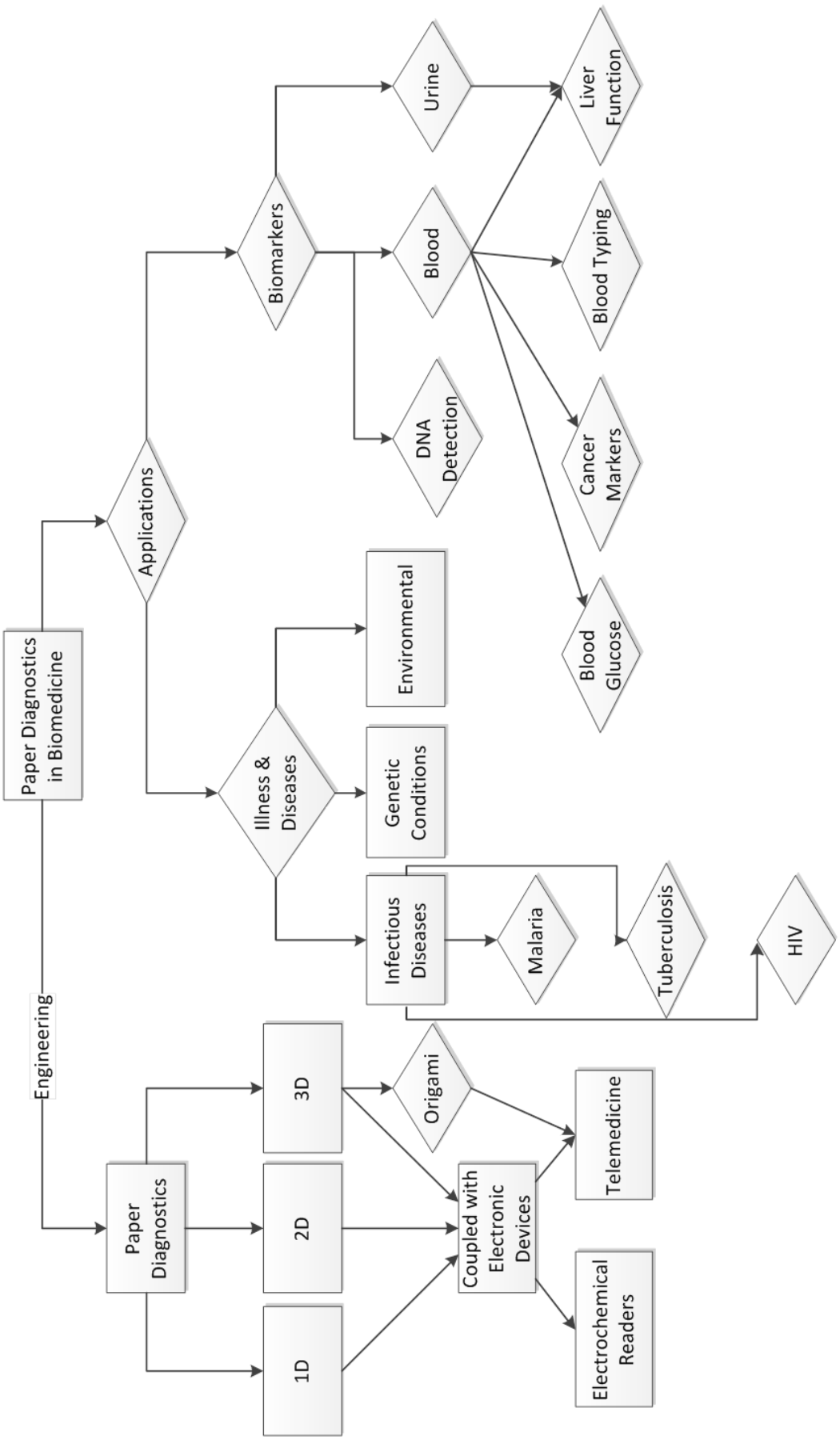

Figure 1: Schematic roadmap of Paper & Cellulosic Diagnostics in Biomedicine.

separate fibres from wood by fracturing the middle lamellae; the resulting mechanical fibres are made of cellulose, hemicellulose and lignin. The fibres are then lightly refined to provide the optimal surface area and bonding ability. Plant fibres from cotton, linen, flax and hemp are sometimes used for specialty papers. Additives, such as polymers, micro-particles (calcium carbonate, clay, kaolin, titanium dioxide) and dyes, are often added to the fibrous suspension to control the strength and the optical properties of paper. A cationic hydrophobic colloid, referred to as internal size, is often adsorbed onto pulp fibres prior to papermaking to control paper wettability and ensure sharp printability. The typical internal sizes are: alkyl ketene dimers (AKD), alkenyl succinic anhydride (ASA) and rosin. Sizing agents are mostly used in office paper and packaging paper. *Forming* transforms the pulp suspension into paper, a uniform and continuous network of cellulosic fibres and functional colloids. The fibre suspension is impinged onto a moving wire where it is drained and pressed to give fibre orientation, thickness and the basic structure of paper. Fibre orientation in paper is affected by the difference of velocity between the moving forming wire and the impinging jet of pulp fibres. *Bonding* links the discontinuous fibres into a continuous non-woven paper network, which is achieved by pressing and drying. The 'Hydrogen Bond' between hydroxyl groups forms a network between the cellulosic surfaces and provides the bonding between fibres. Polymeric strength agents can increase the paper's wet and dry strength. *Surface treatments* allow paper specific properties. This is achieved by calendering, surface sizing, coating, spraying or other processes. Calendering passes the paper through a series of roll nips of increasing pressure to decrease paper porosity, and increase thickness uniformity and surface smoothness. Surface sizing applies a polymer solution, usually starch with a surface active polymer or latex, onto both surfaces of paper, which creates an interphase when the fluid polymer solution penetrates a few μm within the surfaces of paper. Coating applies a viscous solution of inorganic (filler) and organic (latex) colloids, which smooth the surfaces of paper and provide a surface of controlled micro-porosity, surface energy and wettability [13].

The paper properties are in large part defined by the choice of fibers and the papermaking process. Fiber dimension and chemistry depend on the type of lignocellulosic material pulped and the pulping process. The fiber orientation in the X-Y

plane and in the paper thickness (Z) are controlled by process. Process and fiber selection affect the porosity (1/paper density), and size and orientation of pores in paper. This means that the fiber orientation in the machine direction (MD- long axis of the paper sheet) differs from those of the cross machine direction (CD- short dimension of the paper sheet). Basic properties for important grades of paper are presented in Table II.

Many different structures and interphase morphologies of paper can be achieved using conventional paper technology (Figure 2). Refining and blending fibres, such as nano-cellulose crystals (NCC) or micro-fibrillated cellulose (MFC) fibres, can increase the internal surface area, bonding and density, while reducing internal pore sizes of paper (Figure 2a). Functional colloids, such as gold nanoparticles (AuNP), can be adsorbed either as individual particles (Figure 2a) or as aggregates of controlled size (Figure 2b) [14]. By controlling the conditions of the process at the size press and the properties of the solution, polymer layers of different thickness and concentration gradients can be deposited and pressed into both surfaces of the paper (Figure 2c) [15-17]. The colour contrast by SEM (Figure 2c) was achieved using a marker (e.g. potassium iodide, KI) [17]. The polymer sizing solution can contain a hydrophobic surface active polymer such as styrene maleic anhydride polymer derivatives or a latex that can migrate to the surface of paper to minimize differences in surface energies. The polymer can assemble into polymer domains, as for styrene-maleic anhydride copolymers (Figure 2d) [18, 19]. Therefore, controlled paper structures having a complementarity of length scales in the nm, the μm and the mm ranges can be engineered with the current paper technology.

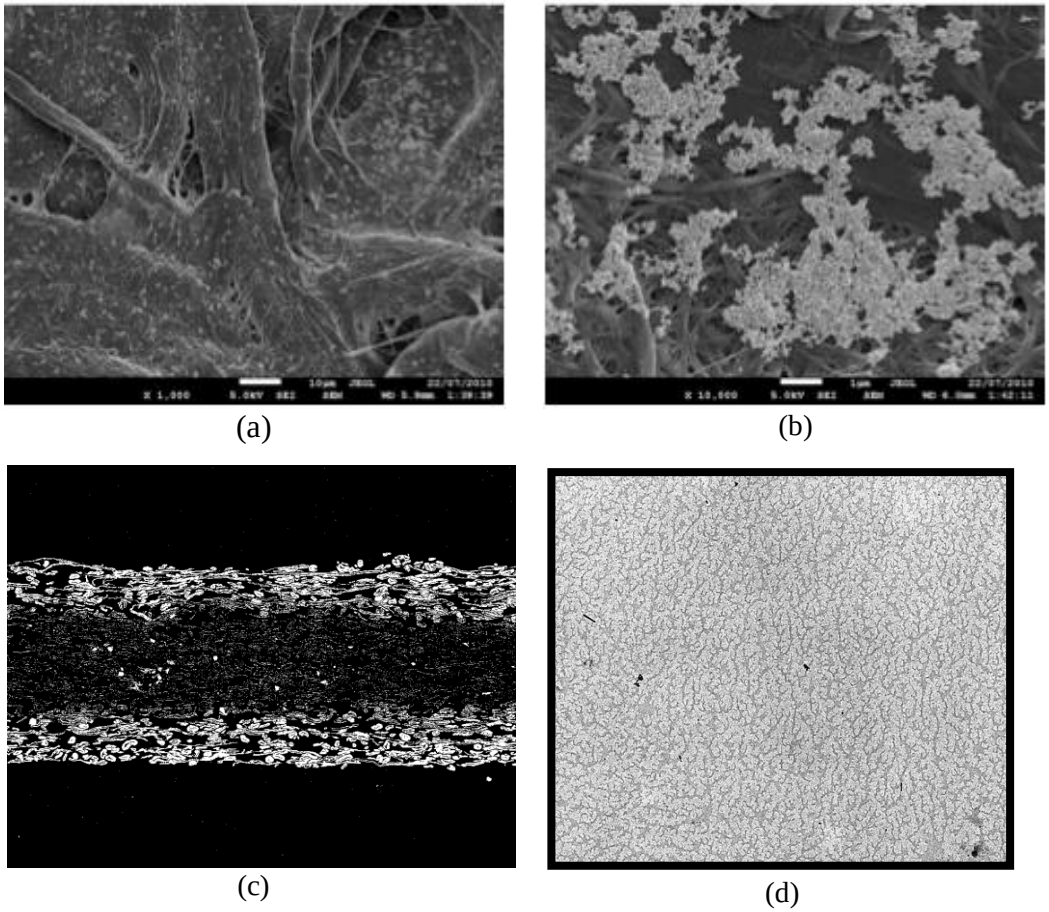

Figure 2: **Paper as an advanced material: (a) cellulosic fibre bonding and micro-fibrillation, (b) retention of aggregated gold nanoparticles on paper, (c) creation of an inter-region on paper: surface sizing a starch-polymer solution, (d) creation of surface active assembled alternating copolymers.**

Table II: Type and structures of paper.

Paper Type	Basis weight	Structure	fibres	Additives
Filter paper	90-200 g/m2	Random fibre orientation, variable porosity and controlled pore size (1-20 µm)	Mostly cotton; sometimes bleach kraft softwood.	Wet strength agent (such as polyamideamine epichlorohydride[PAE]).
Tea bags	15-30 g/m2	High porosity, low density, high strength	bleach kraft softwood	High concentration of wet strength agent
Uncoated fine paper	80-120 g/m2	Dense, low porosity High MD fibre orientation,	Blend bleach chemical softwood (spruce, pine, fir) hardwood fibres (eucalyptus, maple, aspen) and sometime recycled fibres	Filler (10-20%- precipitated calcium carbonate [PCC], clay, or TiO2). Retention aid, internal size, surface size starch and latex or polymer
Coated fine paper	80-120 g/m2	Dense, low porosity High MD fibre orientation,	Blend of mechanical, chemical and recycled fibres. Mostly long softwood fibres	Same as above, plus latex and PCC for coating.
Newsprint	42-48 g/m2	Dense, low porosity High MD fibre orientation,	Blend mechanical softwood and recycled fibres. Resin and fatty acid impurities from wood	Filler from recycled paper, UV brighteners, yellowing inhibitors, retention aids.
Facial tissue	15-20 g/m2 per ply, 2 to 3 ply per tissue	Low density/high porosity, excellent formation, moulded or creped structure. 1, 2 or 3 layers per ply, 1 ,2 or 3 plies	Layered eucalyptus/ softwood/ fibres structured	Wet (PAE) strength agents, softener. Sometimes silicone (PDMS) applied as surface treatment.
Bath tissue	15-32 g/m2 per ply 30-40 single ply	Low density/high porosity, excellent formation, moulded or creped structure.	Layered eucalyptus/ softwood/ fibres structured. Recycled fibres sometimes used	Temporary wet strength agents, (glyoxylated polyacrylamide) softener.
Towels	30-50 g/m2	Moulded or creped low density and absorbent structure	Blended or Layered eucalyptus/ softwood / fibres structured. Recycled fibres sometimes used	Wet (PAE) and dry carboxymethylcellulose (CMC) strength agents, softener. Sometimes PDMS applied as surface treatment.
Packaging	80-200 g/m2	Dense, fibres aligned	Recycled and high yield chemical softwood fibres (high lignin content)	Wet and dry strength agents, sizing agents
Liner board	200-600 g/m2	Dense, fibre aligned. 2 or 3 layers can be wet pressed into a single layer	Recycled and high yield chemical softwood fibres (high lignin content)	Wet and dry strength agents, sizing agents

1.6.4.1 Functional Printing

Printing is a process which transfers controlled patterns of liquids or dye onto a surface: traditionally this has been ink on paper. Ink is made of dyes and particles dispersed in water or an organic solvent, containing additives to control viscosity and surface tension, to give required optical properties. Contact printing, such as flexography, lithography and rotogravure, and non-contact printing like ink jet or laser jet, allow a printing resolution better than 20 μm. If dye/nanoparticles are replaced by a hydrophobic wax, such as AKD, ASA or wax, hydrophobic barriers can be printed to produce microfluidic systems on paper [20]. Biomolecules such as enzyme, antibody molecules or cells in an aqueous media form a bio-ink which can be inkjeted in the microfluidic system previously printed on paper. Paper diagnostic prototypes have been entirely manufactured by printing [21].

1.6.5 Paper Diagnostics Design

Optimisation of the structures and fabrication methods for microfluidic paper devices has focused on a perceived limitation of the current μPADs: performing multiplex assays. While a simple, single step assay is invaluable in many circumstances, in some instances, multi-test ability is required. Research interest has shifted to the actual design structure of the devices, expanding from single sheets of paper to 2D networks and 3D designs, for creating diagnostics able to test multiple analytes or samples simultaneously. While more complex designs might add functionality where single-step procedures are inadequate, a balance with simplicity is required in PAD design. This section analyses PAD design.

A simple differentiation between device designs is the directional flow of fluid. For instance, 1D indicates the flow of liquid in a single direction, while 2D describes lateral flow in multiple directions on the horizontal plane (e.g. the spread of liquid from a single corner to multiple detection zones), and 3D designs add a vertical component (Figure 3).

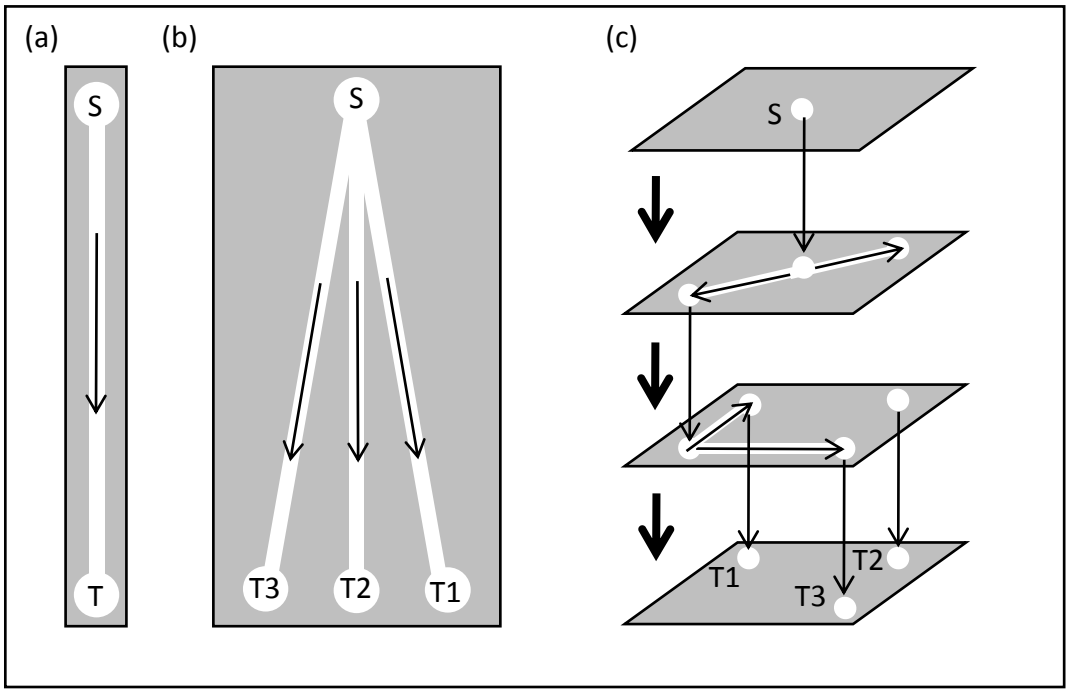

Figure 3: **Schematic representation of the different types of paper diagnostics in (a) 1D (b) 2D and (c) 3D design.**

1.6.5.1 1D Paper Diagnostics

1D lateral flow paper diagnostics, commonly known as dipstick tests, have been used for decades. These simple dipstick tests were first used to detect urinary glucose levels [22, 23]. Dipsticks are made of stiff paper and used by dipping one end of the strip into the sample, allowing fluids to be transported passively through the cellulose fibres towards the reagent zone [12]. In the early 1960's, testing evolved to a triple analyte test, adding the ability to detect protein albumin and pH levels; it has since expanded to a 10-type multi-analyte test which can assay for additional biomarkers such as leukocytes, nitrite, ketones, bilirubin and urobilirubin. Some assays can also measure and report the sample specific gravity [24].

In the 1980's, the applicability of dipsticks was increased to include immunorecognition. The spotting and immobilisation of antibodies on nitrocellulose lead to the development of a wider range of PADs which are now found on the market. Urine analysis tests include the take-home pregnancy tests, testing for human chorionic

gonadotropin (hCG), and in drug testing, such as the 9-tetrahydrocannobinol agent to detect marijuana users. Immune-based PADs can also test blood analytes for cholesterol levels, diabetes and pathological diseases such as hepatitis C and human immunodeficiency virus type 1 (HIV-1), as well as autoimmune screening [25-27]. In 1989, the need for incubation and wash steps was eliminated with the integration of capillary-driven lateral fluid transport with the dipstick technology. This amplified the total number of captured and detected bioanalytes, thus improving the lower limits of detection (LOD) [28, 29].

Typically, the reagents are dried and stored in the fleece sections of the assay during fabrication. The fleece sections allowed for different detection zones to be present on a single stick. The fluid solution dissolves the dried reagents, allowing a reaction to occur. Figure 4 illustrates a typical dipstick assay. The one dimensional flow of liquid through the stick from one end to the other was integrated into a 1D paper diagnostic. While useful, 1D paper diagnostics are limited to single step tests and lack the ability to perform multiple-step assays, often required for techniques such as based enzyme-linked immunosorbent assay (ELISA). However, these tests are cheap, reliable and easy to use.

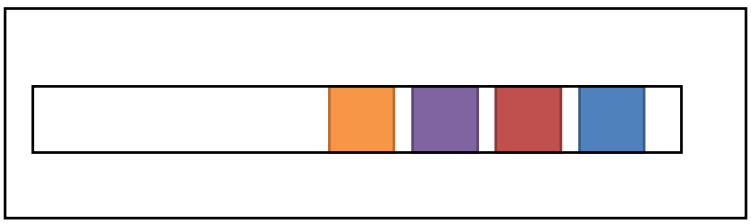

Figure 4: Example of a multiple-analyte 1D paper diagnostic on a dipstick.

1.6.5.2 2D Paper Diagnostics

1.6.5.2.1 Simple 2D PADs

The advent of patterned paper to create micro-channel designs has enabled multidirectional flow paper diagnostics. Martinez et al. (2007) [6] patterned a simple device with 3 detection zones comprising indicators for glucose and protein, as well as a control zone (Figure 5). The 'walls', emplaced by hydrophobic boundaries patterned using photolithography, allowed fluid to be directed into the 3 different zones without cross-contamination, thus adding a spatial advantage against the conventional 1D-µPAD, dipsticks.

Previous reviews have summarized patterning methods for diagnostic design. Such methods include: photolithography, plotting, inkjet or plasma etching, cutting and wax printing [2]. A summary of the patterning techniques analysis is presented in Table III. The concept is to create hydrophobic barriers onto or within the paper structure and to rely on paper capillarity for liquid flow. While the original techniques created rigid and brittle barriers, technology has nicely progressed, allowing channels as narrow as 250µm width to be created [20]. Common 2D-PAD test designs still lack the ability to perform multi-step assays for more complex applications and remain prone to contamination and fluid evaporation. However, 2D-PADs are very cheap (especially when manufactured by printing), easy to use, versatile and robust.

Table III: Analysis of µPAD fabrication by functional printing.

Paper Patterning Techniques	
Photolithography	**Description:** Patterned using chromatography paper soaked in SU-8 photo resist polymer solution before being selectively exposed to ultraviolet (UV) radiation using a patterned mask to shield desired pathways. Shielded regions remain hydrophilic and the unreacted SU-8 is washed away. Unshielded regions become hydrophobic after undergoing polymerization. **Advantages:** Convenient, useful; **Disadvantages:** Expensive chemicals and equipment, multiple steps, time consuming, reduced paper flexibility; **Examples:** Martinez et al. (2007) [6], (2008) [30]
"FLASH" printing	**Description:** Fast Lithographic Activation of Sheets (FLASH); Much like photolithography, except the paper is laminated between a transparent film and a black paper sheet. A standard ink-jet printer is then used to print a black ink mask onto the film. After polymerization the black paper and film is removed. **Advantages:** Faster, customized masks; **Disadvantages:** Expensive, multiple steps, reduced paper flexibility; **Examples:** Martinez et al. (2008) [30]
Etch Printing	**Description:** Completely hydrophobised paper, using a polystyrene toluene solution, is "etched" using a toluene solvent printed on the surface which dissolves the solution to allow for the hydrophilic channels to form. **Advantages:** Custom designs, faster; **Disadvantages:** Corrosive/flammable, chemicals; **Examples:** Abe et al. (2008) [31], (2010) [32]
PDMS Printing	**Description:** PDMS is dissolved in hexane and printed onto filter paper using a modified x-y plotter. To form the hydrophobic barriers, the PDMS solution penetrates through the paper thickness. **Advantages:** Enhanced flexibility; **Disadvantages:** Reduced channel resolution due to "creeping" solution; **Examples:** Bruzewicz et al (2008) [33]
Plasma Printing	**Description:** Paper previously hydrophobised using the cellulose reactive compound, alkyl-ketene-dimer (AKD) is patterned using metal masks that are clamped to the paper before being placed in a plasma asher. The AKD hydrocarbon chains are then oxidised by the plasma, leaving the hydrophilic channels. **Advantages:** Flexible; **Disadvantages:** Expensive, slow manufacturing rate; **Examples:** Li et al. (2008) [34], (2010) [35], (2010) [21],
Wax Printing	**Description:** Multiple techniques. The simplest involves pattering both sides of filter paper with a wax crayon before heating it. He was then melts into the substrate to form hydrophobic barriers. Also can be extended to inkjet printing for more complicated designs with higher resolution, but at an increased cost. **Advantages:** Good in resource limited settings; **Disadvantages:** Low resolution; **Examples:** Lu et al (2009) [36], Carrilho et al. (2009) [37]
Laser Cutting	**Description:** Uses a computer-controlled x-y knife plotter to cut the paper into the desired design with very high detail. Does not utilise imbibing techniques. **Advantages:** Cheaper fabrication costs, detailed design, clear labelling, can be fully or partially enclosed; **Disadvantages:** experimental technique, heating; **Examples:** Fenton et al. (2008) [24]
Inkjet	**Description:** Office or specialized inkjet printer receive hydrophobic and bioactive ink cartridges. Resolution of 20 µm or better determined by the diameter of the ink droplet. **Advantages**: low cost and flexibility of digital printing, established technology. **Disadvantages**: interaction ink-paper critical, plugging of nozzles. **Examples:** Khan et al. (2010) [20].
Lithography, Flexography, Silk Screening	**Description:** contact printing techniques. Advantages: fast, cheap, established technology. **Disadvantages**: require a mould, blanket or negative

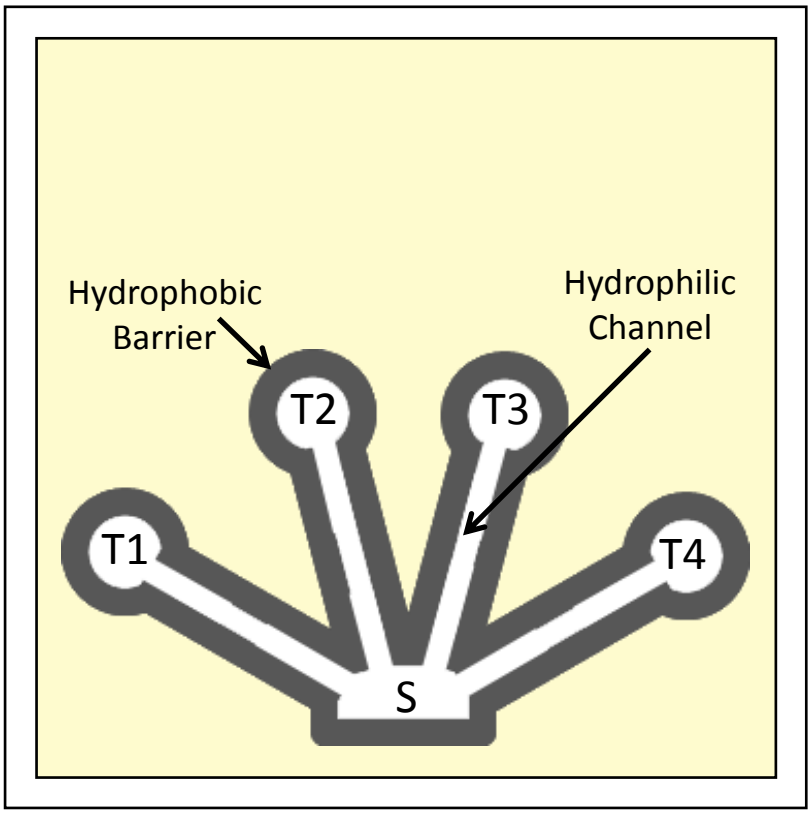

Figure 5: **Prototype design of a 2D paper-based microfluidic device that tests multiple analytes simultaneously; showing separate testing zones (T) connected to a single sample zone (S). (Redrawn from Martinez et al. (2007) [6]).**

1.6.5.2.2 Partially and Fully Enclosed PADs

A major disadvantage of the basic 2D-µPAD design is its exposed nature. Both sides of the paper devices are uncovered, resulting in the evaporation of reagents and sample, and risking contamination from the support beneath. Contact with the support can also result in loss of reagent and sample fluids. Early attempts to avoid such loss and contamination investigated adding samples and solutions onto the device held in mid-air- which is simply impractical [38].

Fenton et al. (2008) [24] proposed a method to avoid imbibing paper with hydrophobic/hydrophilic patterns. Instead the paper was shaped into one of three desired two-dimensional designs using a computer-controlled x-y knife plotter. Type 1 consisted of a single sheet of paper cut into the desired shape; for type 2, paper was

mated with one layer of polyester cover tape before being cut; and type 3, pre-cut paper was sandwiched between two layers of cover tape. Type 3 was the first example of a fully enclosed µPAD. Partially or fully enclosed PADs are reported to decrease the rates of operator error; however, this may simply be that paper cut into clearly labelled sections leave no room for error interpretation [24]. The design is cheaper to fabricate and impervious to external contaminants.

Olkkonen et al. (2010) [39] presented a partially enclosed device which flexographically printed a polystyrene xylene/toluene ink onto the back of the device, while the front was printed with the microfluidic channel design. The hydrophobic back layer provided a protective layer that prevented fluid from escaping through the back via contact with the underlying support and protected against contamination from the support. This method allows direct roll-to-roll production well suited for high throughput manufacture. The enclosed backing contributed to full penetration of the hydrophobic barrier while protecting from contaminants and loss of fluids.

More recently, Schilling et al. (2012) [38] investigated printing toner to produce fully enclosed µPADs. A thermally bonded thin plastic protective layer was printed onto paper. Similar laser printers and toner are commonly purchased for offices, and have been used extensively in the fabrication of µPADs. It is a cheap and convenient method of manufacturing µPADs. The toner had no effect on the microfluidic channels, since it does not diffuse into paper, nor does it come off when wetted. Printing four layers of toner could enclose the µPADs and resulted in faster wicking rates.

However, the heat required (180°C) during the laser printing method can affect the biological reagents that are affixed prior to printing and enclosed beneath the toner layer (Figure 6). At extreme heats, proteins undergo denaturation, lose their ability to bind to other molecules and become inactive. Schilling et al. addressed this problem and reported a 90% decrease in enzymatic function using glucose oxidase for testing glucose concentration [38]. The decreased function was reported not to affect test sensitivity and to allow detection at concentrations as low as 1mM. However, this raises serious concerns on optimisation and economics, and questions as to why a 90% decrease in enzyme activity did not affect sensitivity. An alternative was explored where

a 1mm diameter hole was designed in the surface to act as a reagent addition port, therefore allowing the addition of reagents after enclosing the µPAD, allowing wicking to the reagent-storage zones for testing (Figure 6). This resulted in no loss of reagent or enzyme function, and with the exception of the area of the port, the reagents were protected from the surrounding environment.

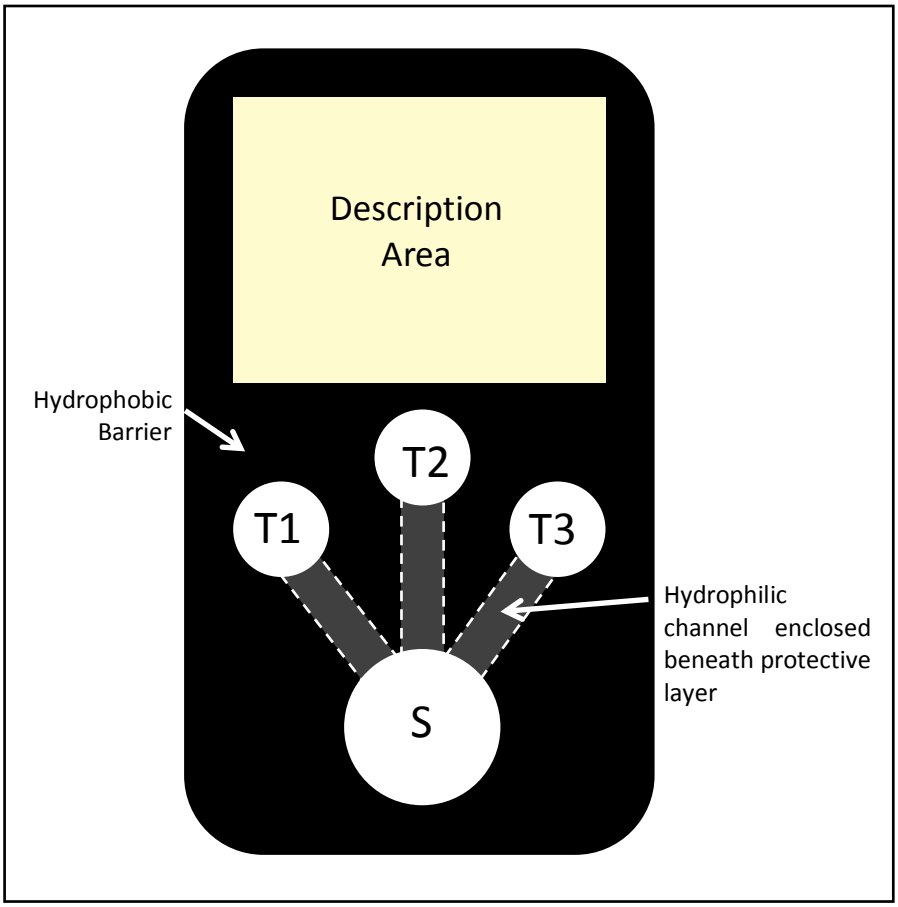

Figure 6: **Example of a fully enclosed 2D paper diagnostic; showing separate testing zones (T) connected to a sample zone (S). (Redrawn from Schilling et al. (2012) [38]).**

1.6.5.2.3 PAD Networks

A disadvantage of the 'traditional' single sheet µPADs is their limitation to single-step processes. A single-step process is ideal for the user but restricts its applicability since most laboratory-based diagnostic assays involve multi-step processes. This is the case for multi-step assays, such as the ELISA, which improve sensitivity and specificity with signal amplification and washing steps. Two-dimensional paper networks (2D-PNs) attempt to emulate multi-step assays while using a single activation step, thus retaining the simplicity and affordability of the single-step µPADs.

Fu et al. (2010) developed a 2D-PN that retains the autonomous nature of µPADs while allowing the complexity of multiple reagents to be delivered sequentially to a detection zone (Figure 7) [40]. The first reported 2D-PN used three methods that allowed multiple inlets to simultaneously converge toward a single point [40]. All methods focused on varying the delivery time of the fluid by either: (1) varying the length of paper, (2) its width or (3) creating a dissolvable barrier (trehalose) to slow the liquid in its tracks. Factors such as paper composition, pore size and surface chemistry can also affect the fluid flow rate, but were not explored. Paper was patterned using a laser cutter to fabricate the device, much like Fenton et al. (2008), and was supported by double-sided tape on a glass substrate [24, 40]. Absorbent pads were used at the inlets for reagent application, allowing the solutions to wick to the detection zone. Using dye and pH as examples, the 2D-PN design staggered the delivery of each component to a common site. Later studies used the same design for chemical signal amplification [40, 41]. The transport mechanisms of fluid through the paper networks was also explored [42]. However, the effects of paper composition, pore size and surface chemistry remained unaddressed. The added complexity of the 2D-PN design also increases the possibility of errors.

A method using a single fluid source, rather than an individual source for each of the reagents, was explored [43]. It involved 'programming' the device to disconnect each reagent in a particular order. The design used a single source well and shaped the 2D-PN by varying the length of each reagent segment. Isolated strips of different lengths were used to show the overall method. The paper strips were housed in a poly (methyl

methacrylate) (PMMA) plastic casing to reduce evoparation and provide a support from which to mount the device into the source well. Plastic cartrige and well were designed to receive a paper cartridge /strip inserted by the user. The paper strips are immersed at different depths into the fluid. The well depletes as fluid wicks paper, thereby disconnecting strips from the source well. This was controlled by: (1) the fluid depletion rate, (2) the immersion depths and (3) the cross-sectional area of the fluid source. The design also relies on a large and thick paper strip used as a regulator; without it, the lengths of paper varied only slightly. The test is very sensitive to the cross-sectional area of paper. Thin paper strips of small cross-sections are often used, which increases variability due to the high heteogeneity of paper structure at different cross sections. Using these basic principles of managing fluid flow, the 2D-PNs can be programmed to deliver fluids to a common point and disconnect from the souce well in a timed, sequential manner. This series of experiments used colored dyes, previously dried onto paper.

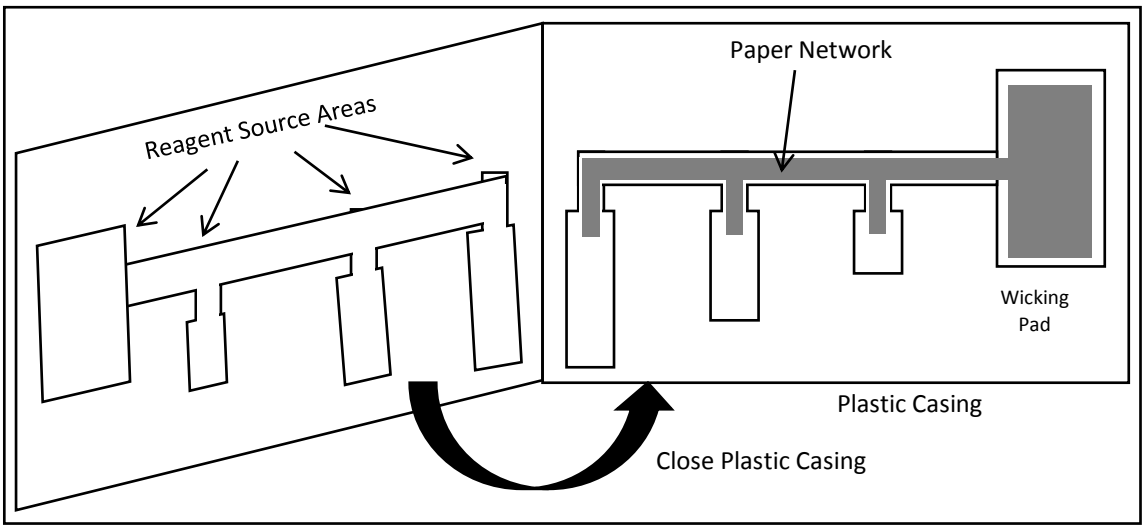

Figure 7: **Example of a 2D paper network used for the sequential testing of multi-step analysis with a single activation point. (Redrawn from Fu et al. (2012)[44]).**

1.6.5.3 3D Paper Diagnostics

The desire or need to increase the density of circuitry and testing has led to the development of 3D paper devices with often complex fluidic systems. Improving the multi-analyte capabilities of µPADs has been a major focus of development. It has culminated with the advance of multiplex 3D-µPADs prototypes, often consisting of multiple 2D layers. 3D paper diagnostics certainly offer advantages; however, it sacrifices simplicity, cost and practicality for increased functionality. Other technologies such as threads [45, 46] and traditional micro-fluidics [12] devices provide competitive alternatives at this high end market.

The design alternates layers of patterned paper and perforated double-sided tape stacked upon each other (Figure 8). This allows not only lateral flow, but also vertical flow. The perforated holes were filled with cellulose powder to connect each layer of paper, creating vertical micro-channels through which the analyte solution can wick. This design can create interweaving pathways through the device and allow the analyte to reach multiple detection zones without cross contamination. Martinez et al. (2008) [47] designed a 4 channel device, where each channel underwent 8 connections as fluid travelled through the layers at the top to the detection zones at the bottom. The purpose of each layer could vary throughout the device, including fluid distribution, filtration or combination with other reagents [47].

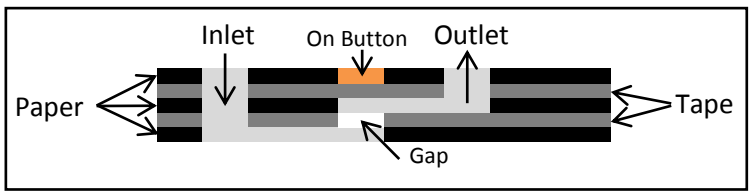

Figure 8: **Side-view representation of an example 3D paper diagnostic (Redrawn from Martinez et al. (2010) [47]).**

Martinez et al. (2010) [48] later modified the 3D-µPAD design to allow choice of specific applications. Usually the function of each test is predetermined during fabrication. However, designing a single platform to test multiple analytes enables a certain level of specificity for each patient. The testing function can be configured at the site, allowing the user to program the device by choosing only the channels needed. This might be useful when a limited sample quantity is available and only specific analytes are desired. Using a single platform allows for mass production of a "universal device" to test a wide range of samples, and eliminates the need to design each test separately. The gaps that were once filled with cellulose powder are left empty, and are instead labelled as buttons. When pushed, the "on" buttons form a bridge in the required channels through which the fluid can travel. When the button is left un-pushed, the bridge is not connected and liquid will only flow to the desired tests. Once a button has been turned "on" it cannot be turned "off" afterwards as paper deforms inelastically and remains compressed. However, ease of use and reliability tend to decrease exponentially with test complexity. While an elegant study, it is questionable whether the user might be able to identify the needs and modify the test in a time of stress or illness.

1.6.5.3.1 Origami

3D-µPADs can be constructed using origami, the art of paper folding. Liu and Crooks (2011) [49] report a method using a single sheet of folded paper to design a 3D-µPAD (Figure 9). A piece of chromatography paper is patterned using a single photolithographic step. The design consists of micro-channels, reservoirs and a frame. The frame provides the template for the paper folding and ensures correct alignment. When folded in a particular sequence, the micro-channels are matched to allow flow in both the lateral and vertical directions. The four corners are trimmed and fit in an aluminium clamp. Samples and solutions are injected through one of the four holes drilled into the clamp's top plate. An advantage origami-PADs (*o*-PADs) have over the previous designs is the elimination of double-sided tape, which can diffuse into the paper over long periods of time and decrease the hydrophilicity of the micro-channels, along with assembly tools such as the laser cutter. However, despite these advantages,

origami PADs require an aluminium clamp to ensure alignment, which increases cost of the device and decreases disposability.

Govindarajan et al. (2012) described a type of 3D-µPAD which utilises the origami principles (Figure 9) [50, 51]. However, it is used functionally rather than to simplify fabrication. The origami serves to sequence the complex processing steps by folding the layers in succession to prepare collected DNA samples for molecular diagnosis.

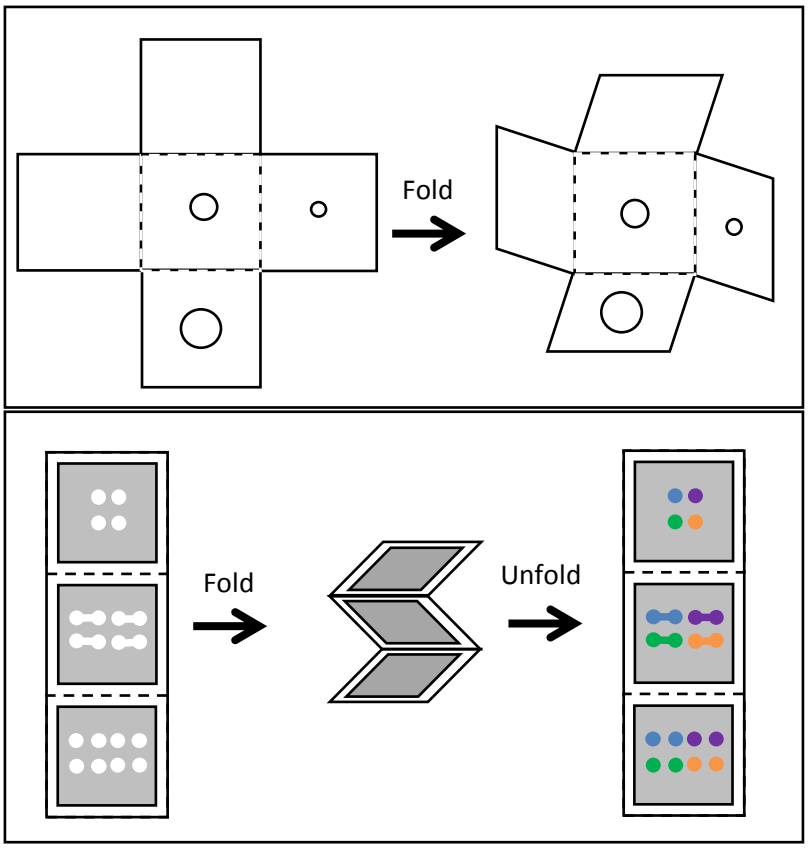

Figure 9: **Redrawn examples of origami microfluidic paper diagnostic for multi-step analysis designed by (a) Govindarajan et al. (2012) [50, 51]. (b) Liu and Crooks (2011) [49].**

The design uses stacked layers of cellulose paper and double-sided tape for assembly, but also uses single-sided tape, a repositionable adhesive layer and card paper to allow for bidirectional folding. Another difference is the way in which the device is constructed. Rather than shaping each layer individually before stacking, a laser cutter is used after stacking instead. The cutting step during fabrication serves as method for

patterning the fluid channels. This is achieved by a combination of full-pass and partial cuts in the device, before peeling away the adhesive layer, leaving fluid channels on a non-wicking substrate.

1.6.5.4 Detection Principles in Paper Diagnostics

Using PADs for the detection of a disease is a two-fold process that relies on the ability to report a specific recognition event. Both the reporting and recognition methods differ for various applications, and are discussed below.

1.6.5.4.1 Methods of Reporting

Detecting the presence of an analyte is half the challenge. Accurate and successful diagnosis also requires a reliable and simple method of relaying the result to the user. Currently, most PADs report results visually; some have attempted to incorporate electronics into the reporting process. This section provides an overview of the development and issues of the main technologies.

1.6.5.4.1.1 Colorimetry and Visual Signals

Most PAD analyses rely on a visual change within the device detection zone to communicate results. Colorimetric analysis is by far the most common reporting technique described in PAD literature. It has also been a common approach in many laboratory-based testing procedures for decades. Colorimetric reporting consists of a visual colour change that occurs after the addition of the sample, usually in the presence of the desired target. For example, the ELISA uses an immobilised biosensor on the substrate surface to capture a desired analyte, which induces a colour change after binding with a second reporting enzyme, thus showing a positive result.

The appeal for colorimetric analysis on PADs is simplicity and applicability to POC situations. Other visual reporting methods such as florescence or absorbance require analytical instrumentation for accurate interpretation. Colorimetric assays in its

simplest form can provide a qualitative binary result: whether or not a colour change is observed. Paper is an ideal substrate for colorimetric analysis due to the excellent contrast provided by the white background. Conversely, this white background and the UV brighteners present in paper (lignin residue and dyes) can interfere with fluorescence and absorbance reading methods, further supporting the use of colorimetric reporting. Smart phones with applications can also process data directly.

While colorimetric reporting is reliable and easy to implement on PADs, there are, however, a few drawbacks. Accurate interpretation, especially for quantitative analysis may require trained personnel or analytical instruments. An alternative is telemedicine – using a communication device to interpret or transmit results to a specialized centre for analysis before reporting the result back [2, 8].

The concept of printing symbols or text has been demonstrated for qualitative results to contour user-barriers. The concept behind the "writing" technique is a refinement and simplification of the previous method by Bodenhamer [52] which described a displacement assay for transparent packaging. A printed pattern of antigens is immobilised on the substrate and saturated by the corresponding antibody with a dye attached. The dye is then released from the surface when interacting with the antigenic target. The resultant disappearance of the printed pattern indicates the presence of the target, in this case a pathogenic antigen, which results from an undesired exposure event.

1.6.5.4.1.2 Reporting with Electronics

While stand-alone paper diagnostics are ideal, many reporting methods rely heavily on colorimetric results. This requires interpretation by trained medical personnel. It has been suggested that µPADs be combined with mobile phones with camera capabilities for rapid and accurate interpretation. This is a field which has been dubbed telemedicine [8]. It involves the image capture of the PAD, either by a scanner or camera, and then sending it via satellite for expert analysis. The expert would not be required on-site for interpretation, and the delay between when the test was taken and the time

it takes for the results to reach the intended party would be minimalized. An alternative is to rely on downloadable applications for direct analysis by intelligent phones.

While colorimetric reporting is the most prevalent method for PAD analysis, electrochemical reporting method have been explored [53]. Colorimetric detection is adequate but to discern an accurate result often requires trained personnel for interpretation. The use of telemedicine improves the situation; however it still relies on a person specifically qualified to correctly identify and diagnose the results. Using a metal/air battery powered biosensing platform could be promising to eliminate the need for a trained professional. The microfluidic paper electrochemical devices (μPEDs) method could provide an easily readable result for non-trained users by adding a user-friendly reporting method to paper devices.

1.6.5.4.2 Biorecognition in Paper Diagnostics

Accurate diagnosis of a disease is not solely reliant on the reporting method, but also the ability to selectively detect the presence of specific biomolecules, also known as biorecognition. The human body has an abundance of distinct analytes which can be used for the diagnosis of not only pathogenic diseases, but also physiological disorders. It all hinges on finding the correct biomolecular target and developing methodologies to detect its presence. Biomolecules, such as nucleic acids, enzyme proteins and antibodies can be immobilised onto paper, and can reportedly be dried without denaturation for this purpose. This process allows for storage and use in remote areas without access to laboratory facilities while retaining the thermal stability that is often lost with analyte solutions not properly stored at low temperature. This ability to immobilise bimolecular targets upon paper thus provides the other crucial half of the detection process. There are four main methods of biomolecule immobilisation on paper: (1) physical, (2) chemical, (3) biochemical couple and (4) bioactive pigments; Pelton provided a detailed description of these techniques [1]. A summary is provided in Table IV.

Table IV: Methods of analyte immobilisation on a paper substrate [1].

Technique	Description
Physical Immobilisation	Relies on van der Waals and electrostatic forces. Polymers can be used for bridging.
Chemical Immobilisation	Relies on covalent bonding
Biochemical Coupling	Relies on cellulose binding modules or bother biochemical binding agents
Bioactive Pigments	Coats colloidal particles that and then printed or coated onto paper

Perhaps the greatest challenge of achieving biorecognition is choosing the correct target that will detect a specific marker in the body. The job of this marker is to unequivocally detect the presence of a disease to avoid misdiagnosis of a patient. Whilst the principles of detection have been modelled after current diagnostic methods, the behaviour of biomolecules on paper is not always congruent with the behaviour exhibited in current laboratory methods.

There is no distinct advantage of a specific biomolecule over another, but is rather a function of the current methods of detection available. For example, utilising the specificity of antibody-antigen interactions is desired for applications such as blood typing, while recognition of a specific protein is required when diagnosing malaria. The type of biomolecular target will therefore differ from application to application.

Because of the wide array of type and properties of analytical targets, each must be examined separately to develop a robust detection method for the purpose of each device. The source of the analyte is an important factor in the diagnostic design. Samples can be primarily taken from urine, blood and saliva for PADs as they are already present in liquid form, but faecal matter is also a potential analytical source.

1.6.6 Applications in Health & Medicine

While some µPADs have been designed for a specific purpose, other 2D and 3D platforms have focused on performing parallel or multiplex analysis for general bioassay applications. 3D systems have been explored for paper-based ELISA tests. Both 2D and 3D array formats have been investigated, including an emphasis on converting well

established techniques from plastic to paper. This section reviews and analyses the important paper test designs and their specific medical applications.

1.6.6.1 Clinical Diagnostics

Although conventional 1D dipstick assays have been used for decades and represent a well-established example of a multiplex assay on paper (Section 3.1), the potential applications of PADs in biomedicine has grown rapidly in recent years. The following section outlines its general applications, as well as some specific examples for physiological and pathological disorders.

1.6.6.1.1 Paper Micro-zone Plates

Many medical applications rely on paper micro-zone plates [54]. Conventional micro-zone plates are usually made from plastic and consist of 96- or 384- wells in which analytes can be deposited. The result is then determined quantitatively via absorbance or fluorescence measurements using a micro-plate reader. Paper micro-zone plates can be functionally similar to its plastic predecessor, but with all the familiar benefits of using a paper substrate: cheap, easy storage and disposal, small volume requirements, etc. Like plastic plates, the paper version can be designed with 96 or 384 detection zones using photolithography. Many paper types were found suitable for the micro-zone plate format [54]. The main limiting factor was paper thickness. If the thickness was too great, the entire depth of the paper could not be properly hydrophobised. Paper thickness defines the required volume of samples and the capacity for the hydrophilic zones. It can be improved through plasma oxidation [54]. In comparison to the plastic micro-zone plates, paper showed a 40 fold increased sensitivity when read using fluorescence. However, under absorbance mode, detection was limited by light scattering caused by paper. The addition of mineral oil or similar liquids matching the refraction index of cellulose allows accurate measurements by absorbance [54]. The 2D format of the paper micro-zone plates was ideal for telemedicine reporting methods and can perform serial dilutions – a benefit unachievable with the 1D-µPAD design. A

unique advantage paper has over plastic is the ability to perform serial concentrations. A sample can be added, then a known volume of the solution can be evaporated, thus removing the excess liquid and increasing the sample concentration.

1.6.6.1.1.1 ELISA

The paper micro-zone plate format was incorporated into a 2D paper-based enzyme-linked immunosorbent assay (ELISA) design [55]. ELISA tests typically use microtiter plates or small vials; it is one of the most commonly used assays for disease marker analysis. This paper-based design uses a 96-microzone paper plate printed with a 12x8 grid of circular detection zones [55]. This allows for multiple P(aper)-ELISAs to be run in parallel. Like the micro-zone plates, the P-ELISAs are compatible with existing equipment, such as 8- or 12- channel pipettes and plate readers. The design allows a washing step by leaving the top and bottom faces open to the atmosphere. The buffer is added to the top of paper and blotted against the bottom. The perpendicular fluid flow removes any unbound reagents through the paper. However, the open bottom surface is impractical, requiring the paper be suspended in mid-air while the reagent solutions are initially added; this prevents the solutions from wicking through the test zones. A colorimetric reporting system is used, enabling the P-ELISA to be coupled with telemedicine if used in remote areas. However, the ability to perform a P-ELISA outside the laboratory environment is dubious.

There are 3 different methods for ELISAs: (1) indirect, (2) direct and (3) sandwich ELISA. An indirect P-ELISA was demonstrated to detect rabbit IgG. Indirect P-ELISAs involve five steps [55]: (1) *immobilisation* of antigens, (2) *blocking* of non-specific protein adsorption, (3) *labelling* the immobilised antigen with enzyme conjugated antibodies, (4) *washing* of unbound antibodies, and (5) the *addition of a substrate solution* for the enzyme. The results were proportional to the concentration of the rabbit IgG dilutions; however, the sensitivity decreased by 10-fold compared to traditional ELISAs. This could be due to shorter incubation periods for the antibody-antigen interactions, or non-specific interactions between antibodies and the cellulose fibres. Despite this disadvantage, the P-ELISA still has benefits; it requires a shorter completion time, less analytical reagents and can be quantified using a desktop scanner, thus retaining the

appeal of a paper-based device. The test zone concentrations increase as the solutions dries, enhancing the binding kinetics involved, however, the rate of evaporation is dependent on the surrounding environment, particularly relative humidity and temperature, and thus could affect the results [55].

The P-ELISA can be integrated with printed electrodes to improve the limits of detection (LOD) to similar sensitivity observed with the colorimetric assays [56]. Printed electrodes from graphite ink were used for an indirect P-ELISA and the voltage was measured periodically. Using this electrochemical method of detection produced a LOD similar to the conventional ELISA. However, the need for additional equipment and fabrication steps could negate the benefits of a more sensitive LOD.

Liu et al. (2011) also demonstrated an ELISA test using the 3D-µPAD design [57]. The 3D design can test multiple analytes in parallel, which is applicable to ELISA analyses. However, the 3D-µPAD adds another level of complexity to the design. The vertical component allows for the fluid transport within the device to be controlled to fit multi-step assay requirements. The difficulty with duplicating conventional ELISAs on paper is the need for multiple distinct working steps. µPADs are mostly designed for a single-step process. The 3D design adds a movable delivery strip to allow multiple reagent delivery and washing steps without cross-contamination. The strip can be manually operated. Reagents are dried and stored on the paper substrate, removing the need for pipetting reagents and buffers. This eliminates the need for operator training. The test takes around 45 minutes to complete [57]. The performance of the 3D-ELISA device was demonstrated using the indirect testing of rabbit IgG. The intensity of the colorimetric signals was proportional to the concentration of rabbit IgG, and had a 5-fold decreased sensitivity compared to conventional testing, which is an improvement over the P-ELISA. Some clinical uses could accept the reduced sensitivity of paper ELISA; however, whether the sensitivity improvement over the colorimetric techniques justifies the increased cost and complexity is not clear.

1.6.6.1.2 Paper for Sample Preparation and Storage

Rather than testing for a particular disease, paper has also been explored as a platform for preparing samples at POC. While paper for sample preparation has been mostly investigated for urine samples, it is applicable to any biofluid [58]. Parker & Cubitt (1999) developed a dried blood spot (DBS) device where blood samples could be taken, stored and transported on paper [58]. The DBS cards is an older concept and can collect blood samples using finger or heel pricks, and then be reconstituted in the laboratory for examination. This is of interest for epidemiological studies in remote regions. Even when kept in conditions varying from cold storage to humid and tropical conditions, the samples were found to remain stable after many years on paper. Additional benefits are the significantly reduced infection risk, ease of use with minimal training, the elimination of needles and syringes, and storage at ambient temperature, removing refrigerated storage and transport requirements. This method was investigated for the surveillance and detection method of HIV [58, 59]. The interaction between paper and blood is poorly understood but is critical for improving blood sample aging and preservation.

1.6.6.1.3 Sample Separation

Not all µPADs report using colorimetry, one of the simplest methods. An alternative strategy is to separate analytes for detection [60]. Carvahal et al (2010) reported an electrochemically-coupled device that separates the analytes, uric acid (UA) and ascorbic acid (AA), and amperometrically detects their presence [60, 61]. Much like a chromatography column, eluent wicks paper by capillary action to dissolve the sample. The solubility is dependent on ionic strength. At an optimal pH lying between the pKa of AA and UA, the AA becomes ionized and more soluble. The type, thickness and length of the paper control separation efficiency. Electrodes (gold) can be printed on paper for detection. The paper-based device compared to typical high pressure liquid column (HPLC) instrumentation but has a slightly longer run time, 16 minutes compared to 5 minutes; however, the benefits of paper could outweigh the extended test time. This

method could be developed not only for clinical diagnostics, but also for forensics, agriculture and environment applications.

A potential µPAD application is the separation of red blood cells (RBC) from untreated whole blood, and integration with a colorimetric diagnostic test [62]. Yang et al. (2012) described a method utilising RBC agglutination principles using antibody-A,B to form aggregates too large to flow through the porous structure of the substrate (e.g. chromatography paper). However, the plasma constituents can still flow through the pores to adjacent testing terminals functionalised with reagents. Colorimetric detection for various compositional properties of the plasma can be achieved. This principle was demonstrated for measuring glucose concentration in blood. However, this test did not account for the blood group O population.

1.6.6.1.4 Blood Group Typing

The accurate, rapid and reliable blood typing of human blood, particularly during medical emergencies, is important [63-66]. Mismatched blood typing can lead to a haemolytic transfusion reaction which can be fatal. Traditionally, an individual's blood type is determined by detecting the presence or absence of antigens on the surface of their red blood cells (RBCs), as well as the antibodies present within the blood serum, more specifically the blood plasma. The most commonly used methods for blood typing include the slide test, tube test, micro-plate method, gel column agglutination systems, thin-layer chromatography (TLC)-immunostaining, fibre optic-microfluidic device, and spin tube method, etc. [67]. The gel column test is the most prevalent in industry, however it requires centrifugation, and each of these methods requires professionally trained personnel to be reliably analysed. Developing a low-cost alternative for remote regions or home care which is user-friendly, portable and equipment free could provide a great alternative to the blood typing options currently available [68].

In recent years, several studies have explored the possibility of using paper as a blood typing diagnostic tool [68-70]. Much like the traditional blood typing tests, the paper diagnostic also relies on the principles of haemagglutination.

When an aqueous analyte, such as blood, is tested using paper the fluid is driven by capillary actions created by the structure of the cellulose fibres, wicking through the inter-fibre space of the paper [68]. This is known as capillarity microfluidics and allows for the passive transport of the analyte without any further equipment [7]. However, when blood agglutinates due to a specific antibody/antigen interaction, the resultant RBC complexes become too large to adequately transport through the paper structure and retain on paper; in absence of agglutination, RBCs are easily removed by elution. This contrast in RBC retention or elution allows for the differentiation between when agglutination does occur (specific) and when it does not (non-specific) to directly report typing [68]. This has been the primary basis for the investigation of a paper-based diagnostic tool for blood typing.

Khan et al. (2010) developed a method soaking paper strips in antibody solutions of either A, B or D antibodies [68]. Droplets of blood were then added to the centre of the paper strip and the resultant wicking behaviour of the blood was observed. A distinct difference was discernible between agglutinated and non-agglutinated blood, the former presenting a chromatographic separation of the RBCs and plasma (Figure 10(a)). When RBCs agglutinate with the corresponding antibody, very little wicking is observed, while the plasma does wick along the paper strip. In contrast, non-agglutinated blood forms no separation along the paper strips, but travels uniformly, thus creating a distinction between a positive and negative result.

Al-Tamimi et al. (2011) next developed another paper-based diagnostic for blood typing using the same principles but presenting two different testing methodologies [69]. The first described an elution-based method using the chromatographic behaviour of blood when added to paper (Figure 10(b)). This was achieved by spotting blood on the surface of the paper where known antibodies had previously been added. A TLC tank was used to elute the blood spot in saline buffer. The capillary action of the paper allowed for the buffer to travel through the paper structure, thus creating a chromatography-like test. Much like Khan et al. (2010), the fixed agglutinated blood spots showed a positive result, while non-agglutinated elution paths of unbound red blood cells were negative. Visually, there was a distinct difference between the two results, using both the density of the blood spot and the presence or absence of an elution path to make an informed analysis.

A second method, known as the spot test (Figure 10(c)), also involved spotting the blood to stock antibodies upon the paper substrate; however they were not eluted in the TLC tank. Instead, saline solution was used to wash the spot directly by pipetting. Separation occurred by filtration through the paper thickness; RBC aggregated by matching antibodies remain on the top of paper, forming an intense red dot, while non-agglutinated cells (non-specific antibody) simply wash through paper and disappear. The results of both tests accurately detected the ABO and RhD blood groups of 100 samples, including 4 weak AB and 4 weak RhD samples, thus supporting the use of paper as a robust biodiagnostic for blood typing. The effect of paper structure on blood typing performance was also investigated in a different study [71].

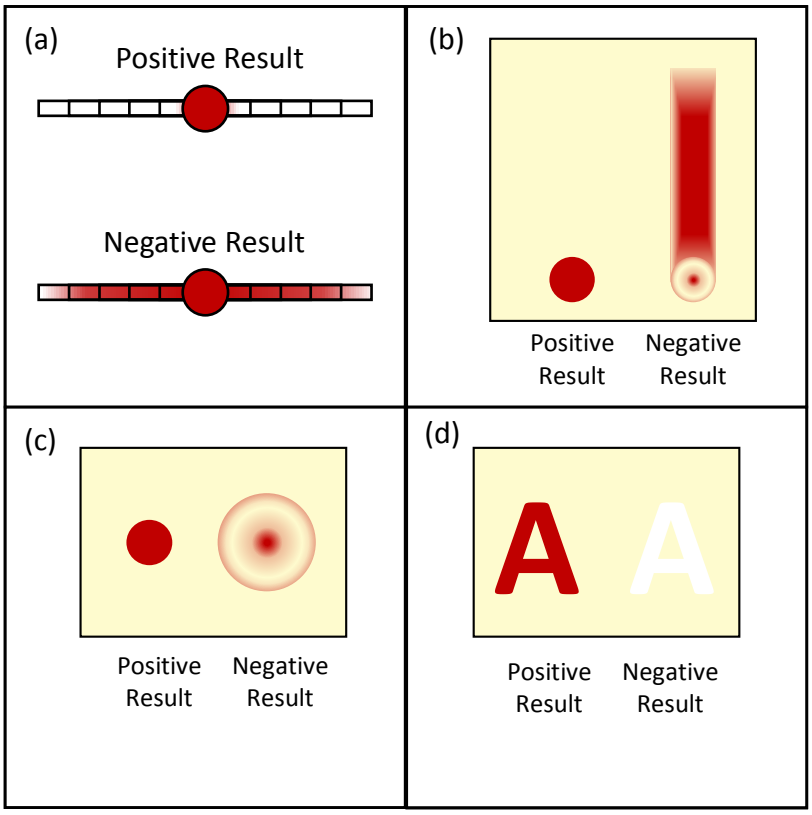

Figure 10: **Use of paper for determining blood groups relying on antibody-antigen interactions to show agglutinated and non-agglutinated RBCs: (a) via wicking (Khan et al. (2010) [68]), (b) chromatographically (Al-Tamimi et al (2011) [69]) (c) using a blood spot test (Al-Tamimi et al (2011) [69]), and (d) using the text-reporting paper substrate method (Li et al. (2012) [70]).**

Li et al. (2012) recently developed a text-reporting method [70]. The detection principle is an adaptation of Al-Tamimi's method [69] by relying on filtration through the paper thickness to retain RBC aggregated coagulated by specific antibody interaction. By patterning the paper substrate with hydrophobic boundaries to surround a hydrophilic channel in the shape of text or symbols, 'invisible writing' is present on the paper and dotted with antibodies. The addition of antibodies remains invisible to the naked eye until blood is added, which form the shape of the channel, for example an A or a B, and once washed with a saline solution – if a haemagglutination reaction has occurred – the resultant blood type will be directly printed on paper and can be "read" straight from the device (Figure 10(d)). This test successfully reported the ABO and RhD blood types of 99 samples including weak groups.

1.6.6.1.5 DNA Extraction and Detection

The genetic material of all living organisms is unique. There are well-established DNA detection techniques used for medical diagnosis of pathological diseases, such as the polymerase chain reaction (PCR) and rolling circle amplification (RCA) [72]. While sensitive, these techniques require complex methodology and elaborate equipment infrastructure. Ali et al (2009) investigated paper to fabricate a PAD capable of RCA, a technique used for DNA amplification and detection [72]. Despite the well-established PCR technique for DNA amplification, RCA is more appealing as a PAD. It can be carried out under isothermal conditions, at room temperature or 30°C, using a simple biochemical procedure involving Phi29 DNA polymerase. The Phi29 DNA polymerase enzyme can displace short DNA strands to amplify a DNA primer into long single-stranded DNA products. This increases the detection capabilities. Immobilisation of a DNA oligonucleotide on strips of paper containing poly(*N*-isopropylacrylamide) microgels creates a platform for which target DNA could ligate and undergo RCA-mediated amplification, enhancing DNA detection. The advantage of identifying a specific DNA sequence rather than other biomarkers is the specificity of the DNA itself. It provides an ultra-sensitive diagnosis tool for disease. If achievable, it can be applied to any specific pathogen, including bacteria. However, preliminary results have

identified a decreased sensitivity compared with other strategies; methods for improving sensitivity are being explored.

Govindarajan et al. (2011) used 2D-*o*PADs to demonstrate POC cell lysis and bacterial DNA extraction from raw viscous samples of *E.coli* spiked pig mucin [51]. The sequential folding of 2D surfaces created temporary circuits which dictated capillary flow without external pumping or power. The ability to detect DNA using 2D-*o*PADs can be used for diagnosis of tuberculosis. However, lysis of the *M. tuberculosis* bacteria is difficult and requires further investigation.

1.6.6.2 Physiological Disorders and Analytes

The ability to detect a wide variety of physiological biomarkers can be used to develop low-cost biodiagnostic platforms. Most initial PAD designs used glucose, pH and protein detection to demonstrate the design's validity for biomedical testing. However, more recently, specific applications of detecting other biomarkers for disorders such as cancer and liver function have also been used to show the functionalisation of PADs. Whilst the current research has only attempted these specific examples, the use of PADs is not limited to the physiological disorders mentioned below.

1.6.6.2.1 Diabetes

One of the most common validation tests used for paper diagnostics is the detection of glucose in urine [6, 24, 31]. In fact, dipstick diagnostics to detect glucose were the first functionalisation application [22]. While diagnosis and detection of diabetes in patients is well established, paper diagnostics can provide an alternative testing method. The dipstick assay relies on enzymatic reactions of glucose with glucose oxidase to produce gluconic acid and hydrogen peroxide. The hydrogen peroxide would in turn react with orthotolidine which results in a colorimetric change to a deep blue colour [24]. Later tests involved the addition of red dye and resulted in shades of purple at different intensities to estimate concentration.

1.6.6.2.2 Cancer markers

Recently, cancer biomarkers have gained interest as a potential analyte for paper diagnostics. An example is a platform for miRNA detection which does not require the use of equipment for lung cancer diagnosis [73]. miRNA regulates the mRNA function in gene transcription. The miRNA assay detects the formation of duplex and triplex species which show unique and varying optical signals to the naked eye. mir21 is a miRNA sequence specifically associated with lung cancer. Analyses showed a linear correlation between concentrations 10nM to 10mM. It is selective, displaying a purple colour for the duplex and orange colour for the triplex. While a good indicative process for lung cancer treatment, further testing is required prior to clinical testing, including an investigation into the sample pre-treatment and a sensitivity analysis compared to current standard tests [73].

1.6.6.2.3 Liver Function

Paper diagnostics have been considered to monitor liver function [5]. The overmedication of patients can induce liver toxicity. Overmedication can also lead to drug resistance, rendering the treatment ineffective. This commonly occurs in developing countries since tests for monitoring liver function are expensive, and require trained equipment and personnel. Vella et al. (2012) developed a finger-prick paper diagnostic to measure the levels of two enzymatic markers which are indicative of liver function: alkaline phosphatase (ALP) and aspartate aminotransferase (AST). The total serum protein can also be measured. The design comprises a patterned paper-chip, a filter and self-adhesive laminating sheets. It accomplishes four primary functions: (1) separation of red blood cells from plasma, (2) distributing the plasma into three regions within the paper, (3) conducting three simultaneous colorimetric assays, and (4) displaying the results for quantitative analysis using telemedicine. The results reported were consistent with the colours seen in tests for all three analytes in artificial blood; however a sensitivity analysis was not provided. Calibration curves were measured, but failed to take into account the background interference from using whole blood.

Additionally, further investigation regarding stability in warmer climates and selectivity from other potential biomarkers are required.

1.6.6.3 Pathogenic Diseases

Paper diagnostics can immobilise analytes which selectively bind to biomarkers for the detection of pathogenic diseases. Such diseases include: malaria, HIV-1, and hepatitis B and C (HBV and HCV respectively). ELISA paper diagnostics, in particular, have great potential applications. The ability to use paper diagnostics for pathogenic detection is ideally suited for diagnosis in developing countries where equipment, resources and trained personnel are scarce.

Dipstick assays can detect several pathogenic diseases, and these analyses can also be applied to other paper diagnostic designs [27]. Dineva et al. (2005) describe a dipstick test combined with multiplex reverse transcription polymerase chain reaction (RT-PCR) for the detection of HIV-1, HBV and HBC [27]. No 2D or 3D paper diagnostic prototypes for pathogenic detection has yet been commercialized; however, their applicability in the field has been demonstrated.

1.6.6.3.1 Malaria

Malaria is caused by the *Plasmodium* parasite, which is transmitted by infectious mosquito bites, displaying symptoms of fever, vomiting, and/or headaches [74]. It is endemic in 104 countries, most of which are remote with humid weather conditions.

Successful identification of the disease can be achieved by detection of the *Plasmodium falciparum* histidine rich protein 2 (*Pf*HRP2) found in malaria [27, 44]. The 2D paper network described by Fu et al (2012) demonstrated a fully automated system for detection using a sandwich format immunoassay [44]. The 2DPN diagnostic had two additional processing steps for rinsing and signal amplification using a gold enhancement reagent. The limit of detection for the amplified assay was similar to that

reported for an ELISA: 2.9±1.9 ng/mL and 4.0 ng/mL respectively. This demonstrates the applicability of the automated 2DPNs in a POC setting for malaria.

1.6.6.3.2 Human Immunodeficiency Virus Type 1 (HIV-1)

The retrovirus, human immunodeficiency virus (HIV), infects cells within the immune system by destroying or impairing their function [74]. The immune system is progressively weakened over time until the patient becomes easily susceptible to infections. While there are antiretroviral drugs which slow down the disease, there is currently no cure for HIV-1. Efficient and accurate detection may not stop the illness, but early detection can help slow progression and patient deterioration, while preventing the spread of the disease.

The dipstick assay uses reverse transcription polymerase chain reaction (RT-PCR) which test for specific nucleic acid sequences. For HIV-1, this means detection and identification of the HIV-1 genome [27]. However, the HIV-1 ribonucleic acid (RNA) must be prepared before testing. While the instrumentation is relatively inexpensive, it still limits application in remote areas and developing countries where equipment-free diagnostics are preferred.

While the dipstick device relied on signal amplification of nucleic acid hybridisation, detection of HIV-1 provides an example for the applicability of the P-ELISA [55]. The HIV-1 envelope antigen gp41 successfully detected specific antibodies in human serum by using indirect P-ELISA methodology. Serum samples from HIV-1 positive patients were tested at different concentrations against control samples of human serum without anti-gp41. The colorimetric results indicated decreased intensity signals with serum dilution, but could still distinguish a positive result, thereby showing that a complex mixture such as human serum could be analysed. Additionally, Ayele et al (2006) described a method to collect and store samples for HIV-1 testing in field conditions [59].

1.6.6.3.3 Hepatitis B & C

Both hepatitis B and C (HBV and HCV respectively) are viral infections transmitted through contact with blood or other bodily fluids, infecting the liver and causing hepatic diseases. Both vary in severity; however, HCV is known to potentially lead to liver cirrhosis or cancer. A vaccine for HBV is currently available but not for HCV [74].

Dineva et al (2005) described a dipstick method to detect HBV DNA and HCV RNA [27]. However, much like with HIV-1, the procedures and equipment used for preparation are not ideal for POC situations.

Successful HBV analysis showed the applicability of the 3D-ELISA paper device to detect HepB surface antigens (HBsAg) in rabbit serum. However, the protocol varied from the indirect ELISA methodology [57]. A primary antibody was used combined with an ALP-conjugated secondary antibody to label the HBsAg. The additional steps were achievable thanks to the µPAD's ease of fabrication and flexibility. Serum samples of HBsAg positive hosts were compared to serum controls without HBsAg, and showed that a 10-fold dilution was still discernible, thus supporting its potential for detecting HepB and other infectious diseases.

1.6.7 Perspectives

There are a few critical issues to address relating to the development of paper diagnostics for biomedical applications. These include: (1) test sensitivity, (2) test robustness, (3) single versus multiple testing, (4) validity/reproducibility and (5) simplicity of use.

1.6.7.1 Test sensitivity

Paper colorimetric tests can directly detect and visually report an analyte down to concentrations of 10^{-6} M [35]. The detection limit is influenced by the wavelength (colour) and intensity of the specific dye chemistry, as well as by the lighting conditions,

type of paper and moisture level/relative humidity. However, a concentration of 10^{-6} M might be insufficient for certain applications such as early cancer detection which might be required to detect analytes at a much lower range, possibly concentrations of 10^{-9} to 10^{-12} M. For such applications, an amplification mechanism is required. Two amplification mechanisms have been tested for paper diagnostics. These include: (1) Amplification by an enzyme producing a colour product upon detection, such as ELISA, and (2) Solid-light interaction principles. Mechanisms based on fluorescent dye are possible but necessitate special instrumentation; quenching and interaction with paper can bring complications. ELISA paper test, while possible and tested, requires multiple adsorption/washing steps that are cumbersome, time consuming and delicate operations for standalone paper tests. ELISA for paper test would only be realistic when a paper insert/test is used in conjunction with a robotized analytical plate reader apparatus.

Optical detection mechanism offers an alternative with techniques such as Surface Plasmon Resonance (SPR), Surface Enhanced Raman Scattering (SERS) and similar techniques based on solid-light principles. These techniques usually rely on some light-surface interaction. SPR is widely used in biosensors to quantify the adsorption of molecules at the solid-liquid interface. Surface Plasmon Resonance is the collective oscillation of valence electrons that occurs when light irradiates a surface, typically a metal such as gold or silver. SPR is very sensitive to the refractive index at the metal interphase (zone extending around 50 nm from the surface), thus providing a mechanism to measure adsorption of the small molecules widely used in lab-on chip sensors. SPR of metal nanoparticles, especially gold (AuNP), yields visible colour change upon molecule adsorption. Small and well-dispersed AuNPs (diameter of 10-50nm) show an intense red colour with extinction coefficients which are much higher than those of common dyes, due to their localized SPR [75]. The surface of AuNPs can be tailored by ligand-functionalisation to selectively bind biomarkers. The most general approaches are chemical functionalisation and thiol-linking of DNA of AuNPs to detect specific binding of proteins or antibodies. Upon the addition of analyte, functionalised and well-dispersed AuNPs are induced into aggregates which show a significant colour shift from red to blue (Figure 11). This is due to interparticle plasmon coupling which

occurs as the surface plasmon of the individual AuNPs combine when their interparticle distance is smaller than their diameter. To achieve an efficient SPR performance on paper, a higher coverage of AgNPs is needed on the paper surface to enhance sensitivity of the nanoparticles-functionalised paper. The paper structure must maintain the adsorption state of nanoparticles (e.g. dispersed or aggregated) upon drying. To carry out ideal colorimetric detection, the activity of nanoparticles must be preserved during storage and remain functional upon rehydration for use. Furthermore, careful control of nanoparticles' size and porosity of paper is required, so that the nanoparticles will not be entrapped inside the pores within the paper structure, which would restrict the aggregation or dispersion of the nanoparticles and accessibility of target analyte.

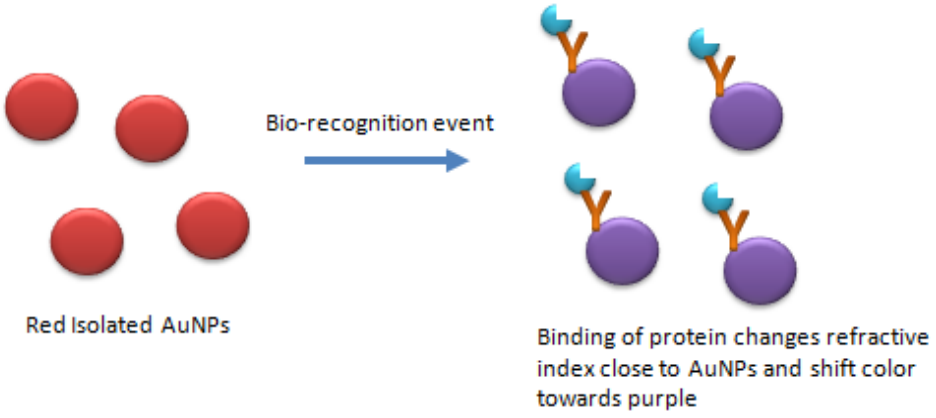

Figure 11: Colorimetric detection of biological targets by AuNPs. (redrawn from [76]).

Raman scattering is a light scattering technique in which Raman photons are scattered by interaction with vibrational and rotational transition in a molecule. However, its sensitivity is limited because the Raman signal is very weak. The development of Surface Enhanced Raman Scattering (SERS) has increased dramatically since Fleischmann et al. [77] published their discovery on enhanced Raman signals from pyridine on a rough silver electrode. This discovery of SERS has transformed Raman spectroscopy from a structural analytical tool to a sensitive single-molecule detection and nanoscale probe [78]. Intense SERS enhancement is often present at the point of contact between two or more metal nanoparticles [79]. As the metal nanoparticles are contacted to form aggregates, their transition dipoles couple to each other and the enhanced fields of

each nanoparticle coherently interfere at their contact point. When molecules are adsorbed in this contact point – often called hot spot- their Raman signals can be significantly enhanced (10^{14}–10^{15}) [80]. Aggregates of nanoparticles have more efficient SERS properties than individual nanoparticles because larger enhancements can be achieved at particle junctions of the aggregates.

Since the aggregation of nanoparticles plays an important role in enhancing the SERS signal, their adsorption and aggregation state within the paper structure is critical. Stability of nanoparticles on paper substrates must be preserved so that they can be stored for long periods between measurements. Reproducibility of their aggregation state is another important factor to achieve accurate SERS results. Since the spot size of Raman's laser beam is approximately 1µm, an ideal SERS active substrate needs to have uniform distribution of nanoparticles on a sub-micrometer scale in order to achieve high degree of reproducibility. This is a critical issue to address when paper is used as a SERS substrate, since it is a challenge to achieve uniform distribution of nanoparticles on paper due to its high roughness and porosity. In addition, because of the high sensitivity of SERS, the paper substrates must be free of additives or fillers to avoid any background or fluorescence interference during the analysis. Figure 12 illustrates the different length scales involved in SERS analysis with AuNP treated paper.

SERS using AuNPs is an exceptional technique for quantifying adsorption of target molecules on substrates, and allowing different orientations and interactions of the molecules with the substrates to be determined [81]. Single molecule detection is achievable via SERS and this concept has been used to design protein and nucleic acid biosensors [82]. A highly SERS-active substrate is the most important factor in producing efficient SERS applications. Previously, aqueous metal colloids were employed in most SERS techniques, but this limits the application of SERS since specimen to be analysed must be water-soluble. Filter paper coated with AuNPs offers a much simpler method and the compounds examined do not have to be water-soluble. Ngo et al. investigated the effect of AuNP treated paper as generic platform for SERS detection and quantification of analytes [75, 83, 84]. The effect of AuNP surface coverage, aggregation size and distribution on paper on the efficiency of SERS were quantified [75, 84]. Methods of producing AuNP paper for SERS application were optimised. Not all analytes

are SERS sensitive; a better understanding of the SERS efficiency as a function the analyte molecular structure is needed.

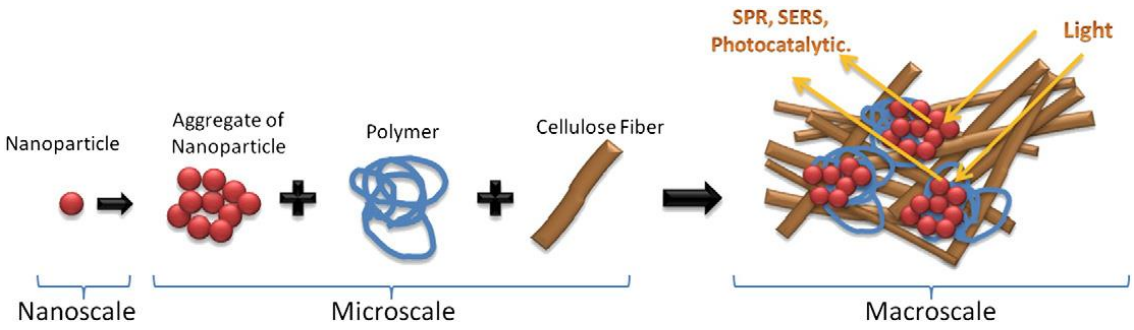

Figure 12: **Different length scaled of the consitutents of AuNP treated paper for SERS application[14].**

1.6.7.2 Test robustness

Proper life time of paper diagnostic is a major issue. This requires both the microfluidics and the bioanalytical detection system to be stable for at least 6 months, or better 1 year or more, to ensure proper time for commercialisation and distribution. A critical requirement is for the adsorbed biomolecules to be stable and to remain bioactive over paper. Enzyme, antibody and antigen are of special interest for biorecognition in diagnostics. To be practical, paper diagnostics must be stored dry until use.

Khan et al. measured the stability and the kinetics of enzyme immobilised on paper with and without polymers used as retention aids [85]. The stability of ALP and horseradish peroxidase (HRP) adsorbed on paper was found to be 2 to 3 orders of magnitude higher than in solution, but also 2 to 3 orders of magnitude slower [86]. Khan et al. [86, 87] also modelled the thermal deactivation of ALP on paper to predict enzyme activity. Heat deactivates and denatures enzymes by modifying their conformation due to increased thermal movement and decreased solvent stabilisation [86, 87]. The idea that immobilisation of an enzyme on paper prevents aggregation and retards the conformation disorder by stabilising the secondary and tertiary structures of the enzyme was studied. The effect of aging time and temperature on the relative activity

of ALP enzymatic papers, with and without polymer treatment, is illustrated in Figure 13. The enzymatic activity quickly dropped within the initial hours of thermal treatment and then gradually decreased at a slower rate. The enzyme deactivation was faster at higher temperatures. The deactivation rates of ALP enzymatic papers, treated with polymers were much faster. Rochefort et al. investigated enzyme microencapsulation to protect biomolecules [88-90]. Enzymes microencapsulated in cross-linked PEI and coated on paper using standard technology displayed a marked improvement in stability without losing selectivity [88-90]. A better understanding on the aging of biomolecules such as antibodies and antigen adsorbed or immobilised on paper is required.

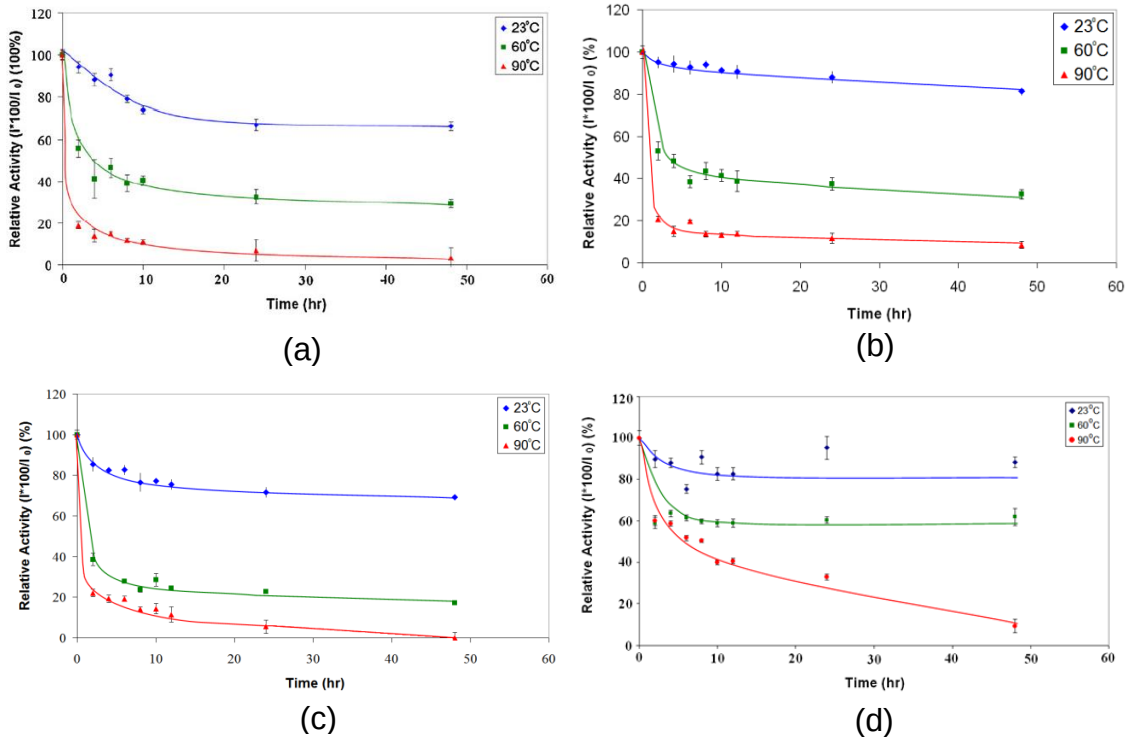

Figure 13: **Effect of time and temperature on the relative activity of enzymatic papers. ALP stabilized on paper using: (a) ALP paper without polymer, (b) – (d) ALP CPAM, PAA and PEO papers [86, 87].**

1.6.7.3 Test Design and application

An important issue is the validity of the test and understanding how design affects detection threshold and its effect on diagnostic and treatment. A consequence of increasing sensitivity can be the increase in the occurrence of false positive tests. This can be achieved by optimising the antibody conditions to react with an antigen. A lack of selectivity can also create false positives tests. The opposite is a false negative test, which can result either from a too low sensitivity i.e. level of detection, or from the lack of recognition of the analytical biomolecule. The lack of recognition can be due to poor binding specificity, but also from weak or partial antibody/antigen. Examples are the weak D and partial D antigen on the red blood cells recognition in blood typing. For blood typing test, false positive and false negative results can lead to a fatal haemolytic transfusion reaction. A false negative can results in the transfusion of a donor with blood A, B or D to a recipient of blood O-; conversely, a false positive can cause a O- recipient to be incorrectly identified as A, B or D, resulting in the same crisis upon improper transfusion. For certain applications, such as blood typing of primary and secondary groups, total accuracy and the absence of any false positive/negative results are imperative. For other applications, in which paper test is used as first detection line, reproducibility/sensitivity can be traded for simplicity and low cost allowing wider and more frequent public use. Unfortunately, very few studies have analysed the issue of false positive/negative results in diagnostics and their potential effects.

Simple detection on/off binary tests identify the presence or absence of a critical analyte. The main advantage of these tests is their simplicity and ease of use and interpretation. The best example is the pregnancy test; the patient is either pregnant or not. Of interest is the ability to control the critical analyte concentration at which a “positive” is communicated. For example, cholesterol and prostate specific antigen (PSA) are becoming health issues only over a certain concentration. A binary test only reporting concentration above these critical thresholds would be very useful for home testing.

Significant research effort has been dedicated for multiple paper test use as it had been identified as a major weakness. But is that so? The higher the test complexity, the higher the price and the lower the robustness, ease of use and interpretation. At the limit, these tests become non-competitive relative to the traditional tests and methods they were designed to replace in the first instance. Research progress has demonstrated that paper tests can be designed to provide alternatives to most diagnostics needed. However, paper diagnostic will make sense and be successful only should they provide a significantly cost saving and simplicity over their targeted replacement. Research prowess and high level publication is a facet of paper diagnostics; public acceptance and commercialisation is another. These should not be confused.

1.6.8 Conclusion

The development of paper diagnostics analytical devices (PADs) for medical applications has exploded in recent years. This is because paper is cheap, widely available, easily engineered, disposable, sterilisable, hydrophilic and easy to functionalise and process into diagnostic devices. Paper is a very attractive substrate to develop a generic low-cost diagnostic platform. Paper can serve four functions in a diagnostic: (1) transport and measurement of sample and analytes, (2) reaction support, (3) separation of reactants from products and (4) communication of results. A plethora of manuscripts present new concepts of rapid/instantaneous medical analytical tests and analysing the performance of PADs. Many ingenious and sometimes complex 1, 2 and even 3D flow prototype diagnostics have been developed. Printing has emerged as the manufacturing technique of choice for PADs, first by printing microfluidic systems on paper and then the reagents and biomolecules. From literature, paper diagnostics have become ubiquitous for medical applications; but surprisingly, very few commercial applications exist.

This article has attempted to provide a roadmap by reviewing the different types of paper tests, their principles, and medical applications and has analysed their performances and limitations. Paper tests for medical applications must meet two requirements: very low cost and ease of use. The challenge is to ensure the selectivity

and sensitivity of the diagnostics, while maintaining the simplicity and affordability that paper diagnostics attracts. The need to create more complex PADs to suit multi-step processes has often hindered the ability for many designs to be applicable in the field and commercialized. Additionally, each biomedical application differs in its own right, making it impracticable to design a single PAD to suit multiple diseases and disorders. Development is needed to reconcile the potential of very low cost, meaningful and robust paper tests for specific medical analyses with increased sensitivity, flexibility and reliability of the concept.

1.6.9 Acknowledgements

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1.7 REVIEW: ANTIBODY-ANTIGEN INTERACTIONS FOR BLOOD GROUP TYPING

1.7.1 Introduction

The role of antibody-antigen interactions in transfusion medicine has always been important. It is the accurate, reliable and efficient detection of these interactions that form the basis of modern blood banking techniques, which are critical for the successful typing of human blood, particularly during medical emergencies [63-66, 91].

The most prevalent forms of blood group typing are based on the principles of haemagglutination, a by-product of the human immune system. The capabilities of the human immune system are tremendous. From the common cold to infectious diseases, one of its roles is essential for fighting off foreign matter, often achieved by antibodies acting as a natural defence mechanism for the human body. Antibodies develop after exposure to specific foreign molecules that are not recognised by the body. This creates a complication during blood transfusions. Since the human body was not 'designed' for the transfer of donor blood, the development of antibodies after blood transfusion is seemingly inevitable [64]. Therefore, the accuracy and reliability of blood typing techniques are critical for patient safety. Furthermore, in emergent situations such as surgical or in-the-field trauma, efficiency and safety are imperative to a successful transfusion.

Red blood cells (RBCs) contain antigens on or within their cell membrane structure. Introduction of foreign RBCs into the blood stream during transfusion can result in either the interaction of antibodies already present in a patient, or trigger the formation of antibodies as a reaction to any 'unfamiliar' antigen. Incorrect blood typing and the subsequent mismatching of transfused blood could result in a haemolytic transfusion reaction (HTR), whereby the patient's body signals lysis – or cell death – of the RBCs. The severity of HTRs can range from mild to severe, and can lead to patient death.

Accurate and reliable blood typing is also critical for pregnant mothers at risk of haemolytic disease of the foetus and newborn (HDFN) [92]. HDFN is a disorder whereby maternal antibodies cross the placenta and interact with foetal antigens, destroying the red cells. As a result, the foetus can suffer from HDFN, and much like HTR, symptoms vary from mild anaemia to death in utero. Correct blood typing serves a critical role in the prediction, diagnosis, treatment and prevention of HDFN.

Since the discovery of the ABO blood groups in 1901, the traditional method for determining an individual's blood type has involved the detection of both the specific blood group antigens on the surface of their RBCs, and the antibodies present within the blood plasma [93]. DNA and molecular analysis is also capable of determining blood group types; however, it remains a very expensive and complex substitute [66]. Emerging technologies using paper diagnostics have recently been reported as an alternative for cheap, fast and easy-to-use testing, capable of implementation in hospital, laboratory and in-field situations.

A basic understanding of blood group typing is that antibodies interact with antigens on the surface of RBCs to cause haemagglutination – the formation of RBC aggregates. While much is known about the types of antigens and antibodies, and subsequently types of blood groups that are found among humans, few studies have attempted to understand the interactions on a biomolecular basis. Many factors affect the binding strength and affinity observed in such tests. Some are molecular, such as antibody and antigen structure, as well as antigen density and antibody concentration. Others are external factors during testing such as the antibody-antigen ratio, temperature, ionic strength, reaction time and pH.

This review explores the current knowledge of blood group typing; it aims at better understanding the binding behaviour and capabilities of these antibody-antigen interactions, with a particular focus on the important clinically significant blood groups. While most studies have focused on either the antigen or antibody structure and expression, little investigation has approached the nature of their interactions such as binding affinity and kinetics.

1.7.2 The Basics of Blood Group Typing

Transfusion medicine involves the successful identification of antigens and antibodies found in a patient or donor's blood [93]. The underlying principles for understanding these interactions allows for accurate interpretation when typing an individual's blood groups.

1.7.2.1 Antigens

The presence of foreign antigens in the body are detected by the human immune system. An antigen triggers the production of a specific antibody, and by doing so, initiates an immune response to eliminate the foreign matter. In the case of mismatched blood transfusion or HDFN, this could cause haemolysis – red cell death. Antigens on the surface of the donor red blood cells can propagate the immune production of a specific and corresponding antibody, or bind to an already existing antibody within the blood.

Currently, there are 36 recognised blood group systems, comprising over 300 antigens [94]. The presence or absence of blood group antigens is determined through inheritance. Certain groups, such as the ABO and RhD systems are considered universal amount all races and populations. In fact, only nine of the blood group systems are considered clinically significant. Table I lists the most clinically significant blood group systems and antigens.

1.7.2.1.1 Structure

Blood group antigens are part of larger integral proteins that are embedded within the RBC membrane. These proteins span the membrane bilayer in single or multi-pass domains, and it is on the extracellular constituents protruding from the RBC surface where blood group antigens are located. While blood group antigens are key to blood banking practices, they are also functional depending on their composition and structure, which varies. Some antigens are non-glycosylated proteins, while other are

carbohydrates which form glycoproteins. Table I lists the associated membrane structures and function of the main antigens within the nine clinically significant blood group systems. Blood group antigens are comprised of a 'parent' structure and an 'antigen' structure. Genes dictate the variability of the antigen structure, usually by the presence or absence of a particular amino acid residue. The parent structure, however, remains generally unchanged for a particular blood group within a majority of the population. It is the parent structure that holds the functional component of the antigen and is not affected by the differing amino acid residues.

Table V: List of the most clinically significant blood groups.

Blood Group System	Antigen Structure	Function	Antigens	Estimated Density of Antigens per RBC
ABO	Carbohydrates	Possibly structural/ protective	A B	800,000 – 1,200,000 [95] 600,000 – 850,000 [95]
Rhesus	Non-glycosylated Proteins	Structural/possibly transport	D C E c e	10,000 – 66,000 [96] 2,000 – 27,000 [97] 600 – 27,500 [97] 1,000 – 31,500 [97] 1,600 – 20,100 [97]
Kell	Glycoprotein	Enzyme	K k	2,000 – 6,000 [95] 2,000 – 5,000 [95]
Kidd	Glycoprotein	Urea Transport	Jk^a Jk^b	~14,000 [95] ~14,000 [95]
Duffy	Glycoprotein	Chemokine receptor	Fy^a Fy^b	~15,000 [96] ~7,5000[96]
MNS	Sialoglycoprotein	Structural	M N S s	~1,000,000 [95] ~1,000,000 [95] 170,000 – 250,000 [95] 170,000 – 250,000 [95]
P	Glycolipid	Unclear	P1	N/A
Lewis	Carbohydrate	Unclear	Le^a Le^b	N/A N/A
Lutheran	Glycoprotein	Cell adhesion	Lu^a Lu^b	N/A 850 – 4,000 [95]

The antigen structure plays an important role in the strength of the binding reaction observed. Some antigens, such as those for blood groups A and B, are believed to protrude much farther from the RBC surface in comparison to others, such as the Rh antigens [98]. This could actually result in the corresponding antibody to require less distance to span the gap between a neighbouring RBC cells, thereby allowing for easier binding.

While each different type of antigen has a unique structure and composition, some antigens are believed to be transcribed in close proximity. Models have been proposed where the antigens form a junctional complex within the membrane, which can sometimes affect the binding of other blood group antibodies (Figure 14). This is particularly true for the Rh antigen complex.

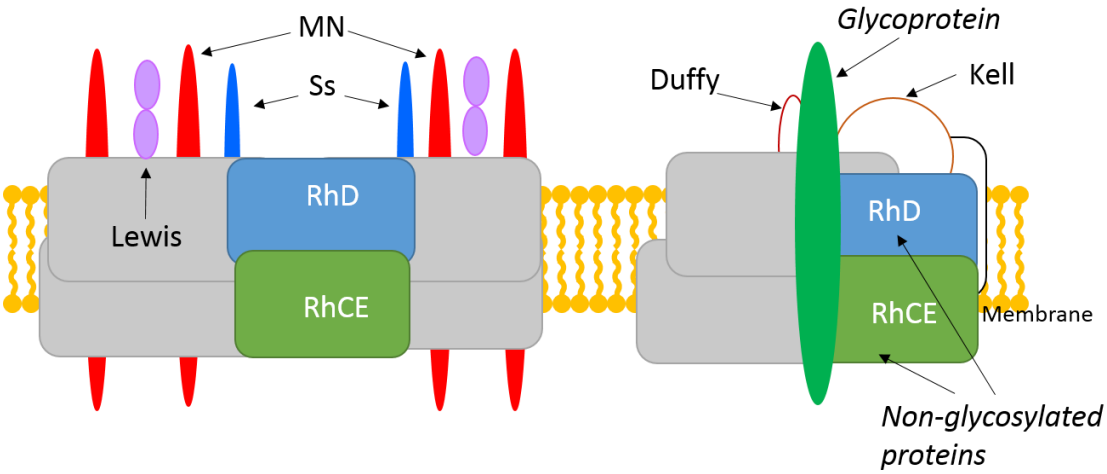

Figure 14: **Two proposed models of a junctional protein complex encompassing Rh, Kell, Duffy, Lewis and MNS blood group antigens. (Redrawn from Daniels & Bromilow, (2007) [66])**

1.7.2.1.2 Density

Blood group antigens are expressed at different densities upon the RBC surface, which affects the observed binding strength during blood banking practice [98]. The antigen expression is highly variable among the population. Table I lists the average estimated number of antigen sites per red cell for each blood group. Sparsely located antigen sites are much harder to react than antigens that are more densely populated. Furthermore, the genetics of homozygous and heterozygous alleles for a particular antigen may affect the number of sites expressed, the former resulting in more antigen sites than the latter [98]. This is known as the dosage effect.

1.7.2.2 Antibodies

Antibodies are produced as an immune response to foreign antigens in the body [99]. They consist of immunoglobulin (Ig) – biomolecules composed of 'heavy' and 'light' protein chains which are bound together by non-covalent and disulphide bonds (Figure 15a). The Ig structure is crucial for haemagglutination, both in the formation of RBC aggregates and the recognition of individual blood group antigens.

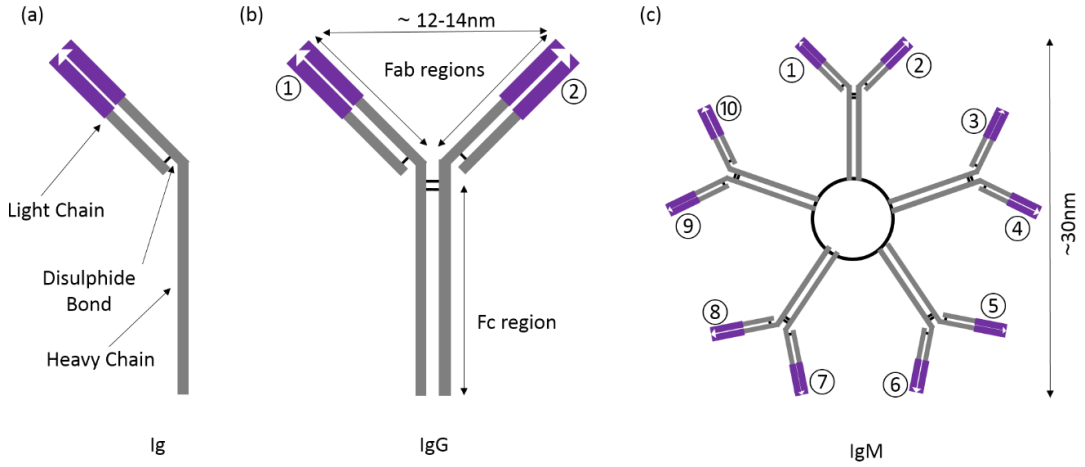

Figure 15: **(a) Basic structural component of an Ig molecule; (b) Structure of monomeric IgG molecule with 2 binding sites; (c) Structure of pentameric IgM with 10 binding sites.**

The 'heavy' protein chain largely determines the type of Ig, of which there are five classes: (1) IgM, (2) IgG, (3) IgA, (4) IgD, and (5) IgE. The different lengths of amino acids, as well as carbohydrate content, within each of the five classes provides distinct characteristics and biological activity.

The 'light' protein chain contains an antigen binding site. Its composition is highly variable, allowing for unique recognition to countless antigens that can be considered 'foreign'. In terms of blood typing, this provides the necessary specificity to identify over 300 different blood group antigens recognised by the International Society of Blood Transfusion (ISBT), which have been categorised into 36 blood group systems.

Transfusion medicine primarily involves only two classes of Ig: IgG and IgM. IgG antibodies have a monomeric structure resulting in only two binding sites, separated by a distance of approximately 12-14nm between them (Figure 15b). IgM antibodies have a pentameric structure with 10 binding sites, spanning approximately 30nm in diameter (Figure 15c). In comparison, RBCs are approximately 8-10µm in diameter (Figure 16). Due to the larger structure of IgM antibodies, serological tests are able to show direct agglutination. However, IgG antibodies are limited by their size and decreased number of binding sites, making it difficult to span the distance of the negative surface charge surrounding RBCs. This negative surface charge ordinarily prevents aggregation, and therefore, an additional binding agent is required when testing with IgG antibodies. This is achieved through the indirect anti-globulin test (IAT), which uses a reagent known has anti-human globulin (AHG). The AHG has anti-human IgG as a main component, which binds to human IgG antibodies to affect agglutination indirectly.

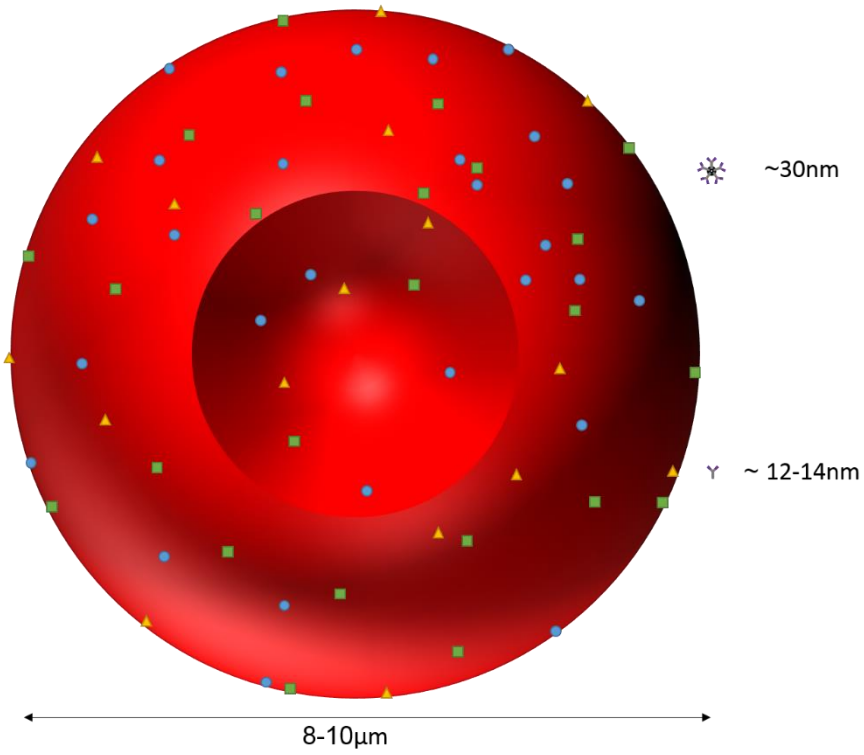

Figure 16: **Scaled comparison of a red blood cell (8-10µm) with different antigen binding sites to an IgG antibody (~12-14nm) and IgM antibody (~30nm). Note: antigen binding sites are not to scale.**

1.7.2.2.1 Polyclonal and Monoclonal Antibodies

Particular clones of antibody sera may behave differently during clinical testing. There are two types of commercially available antibodies: (1) polyclonal, and (2) monoclonal. Polyclonal antisera contain antibodies produced from multiple cell clones. While each antibody clone for a particular blood group antigen is derived from a different source, each clone has the same specificity to that antigen [98]. Polyclonal antibodies are usually of human origin and will bind to the same antigen. This is unlike monoclonal antibodies where each individual molecule is identical. Also, monoclonal antibodies are produced *in vitro* from a single immune cell line through hybridoma technology. Monoclonal antibodies may be of human or mouse origin [98].

1.7.2.3 Antibody-Antigen Interactions

Currently, the most prevalent blood typing methods are based on the principles of haemagglutination, where the antibodies bind to RBC antigens to form a three-dimensional lattice structure [95]. This antibody-antigen cross-linking results in cell aggregation and can be visualised as a clump of RBCs. The cross-linking is a reversible chemical reaction [100].

$$\text{Antigen} + \text{Antibody} \leftrightarrow \text{Antibody-Antigen complex}$$

Haemagglutination can be separated into two distinct, but interrelated stages: (1) the primary stage, and (2) the secondary stage. The primary stage involves the weak attraction forces between RBC antigen and the corresponding antibody, while the secondary stage deals with the formation of the 3D lattice structure through cross-linking of RBCs from these antibody-antigen interactions. Interactions between the antigen site upon the RBC and the specific antibody involves minute portions of the biomolecules [101]. As these binding sites each consist of just a few amino acids within a surface area between 0.4 to 8nm^2, the interactions must overcome the overall electrostatic repulsion forces between each molecule [100]. This involves the initial attraction due to long range forces, such as ionic and hydrophobic bonds, overcoming hydration energies so that van der Waals forces prevail, until the resultant binding can

be achieved solely from the suitable contact of the antibody to the antigen structure [100].

The actual bond strength between the antibody and antigen is known as the antibody affinity [98]. The affinity relates to the 'fit' of the antigen with its corresponding antibody, and different clones of antibodies may display different binding affinities when compared. Thus far, there have been no studies quantifying the interaction strength of the antibody affinity. In contrast, the ability and strength to bind to multiple antigens sites simultaneously is known as antibody avidity. The antibody avidity relates directly to its structure [98]. Although both IgM and IgG antibodies have more than a single binding site, the pentameric structure of IgM provides ten potential binding sites, whereas IgG only provides two. Therefore, IgM antibodies have a higher avidity than IgG antibodies.

1.7.2.3.1 The Primary Stage of Haemagglutination

The primary stage is also known as RBC sensitization. It involves the relatively weak attraction forces between RBC antigens and their corresponding antibodies when the two units come into contact, connecting to form an antibody-antigen complex. As this interaction is reversible, the rate of reaction is directly proportional to both the antibody and antigen concentrations before reaching equilibrium.

There are five main factors which effect the primary stage of agglutination: (1) antibody to antigen ratio, (2) temperature, (3) ionic strength, (4) pH, and (5) time.

1.7.2.3.2 Effects of antibody-antigen ratio

When blood group typing using haemagglutination, a balance between the antibody-antigen ratio is required for optimal efficiency. As a general rule, the higher the antibody concentration, the greater number of antigen sites will be bound. However, too much or too little antibodies will affect the secondary stage of the reaction. Therefore, there are three distinct phases of antibody-ratio which determine the success of agglutination:

(1) The prozone – an excess of antibody, (2) postzone – an excess of antigen, or (3) zone of equivalence – a balance of antibodies and antigens.

When there is an excess of antibodies such that all the RBC antigen sites are each bound by an individual antibody molecule, the necessary cross-linking for haemagglutination is obstructed. This is known as prozone and results in false-negative readings; this is the equivalent of steric stabilisation for polyelectrolytes on colloids. This is particularly important when IgM antibodies are used for direct agglutination. Prozone does not affect testing when IgG antibodies are used as IgG are usually incapable of direct agglutination. Instead, detection involving IgG antibodies is dependent on indirect agglutination. In laboratory practice, this is achieved using the indirect anti-globulin test (IAT). During the IAT, it is the aim of the primary stage to coat the RBCs with as many antibodies as possible, implying that the prozone is preferable. A binding reagent, anti-human globulin (AHG), is needed to affect cross-linking, therefore prozone provides more binding opportunities for the binding reagent. Conversely, postzone is when not enough antibodies are present to affect agglutination, therefore also appearing as false-negative.

The ideal situation for direct agglutination when using IgM antibodies is for a zone of equivalence where the balance between antibody and antigen concentration promote the most efficient haemagglutination. This ratio varies between antibody clones, blood groups, and even between individual patient samples. However, the traditional method of measuring the ideal ratio is by conducting a simple dilution test where the optimal titre concentration of the antisera is observed by a trained technician. This test is also subjective to the perspective and technique of the technician.

1.7.2.3.2.1 Effects of temperature

The ideal testing temperature will increase antibody binding to RBC antigens. Blood grouping antibodies can be ‘cold-reacting’ or ‘warm-reacting’. The nature of a particular antibody-antigen bond determines if the reaction is ‘cold’ or ‘warm’. Table II lists the properties of each.

Most clinically significant IgG antibodies are typically warm reacting, requiring incubation at 37°C to facilitate antibody-antigen binding [92]. This is due to the physiological nature of IgG antibodies which are often polyclonal and derived from human sources. The 37°C temperature mimics that of the human body. Conversely, IgM antibodies are generally cold reacting, displaying greater binding at low to ambient temperatures (4 – 20°C).

Table II: The effects of temperature on RBC agglutination [95]

Reaction Type	Cold-Reacting	Warm-Reacting
Optimal Temperature	4 – 20°C	37°C
Type of Bonds	Hydrogen	Non-hydrogen (e.g. hydrophobic)
Antibody Type	IgM	IgG
Effects	- Exothermic reactions - Faster reactions at lower temperatures - Warm temperatures can cause dissociation - Normally associated with carbohydrate antigens	- React stronger at warmer temperatures - Stable on cooling - Normally associated with protein antigens

1.7.2.3.2.2 Effects of Ionic Strength

A majority of substances in contact with a polar (e.g. aqueous) medium, such as water, acquire an electrostatic charge at its surface [102]. Generally, most surfaces in water are anionic as cations are more hydrated and soluble. Oppositely charged ions are attracted towards each other, while similarly charged ions are repelled. Therefore, in order to reduce the contact area with water, the less hydrated surface anions attract cations. This forms an electrostatic double layer and provides a neutralising diffusion effect of the suspended substances in the water [102].

The same is true for RBCs and antibodies in testing solutions (Figure 17) [95]. As both the RBC and antibody carry electrostatic charges, the ionic strength of the testing medium effects the rate and efficiency of binding. RBCs carry a negative charge. This negative charge attracts cations, therefore forming an electrostatic barrier. This is also known as the electrical double layer, typically quantified in terms of zeta potential [102]. On the other hand, antibodies carry a net positive charge, attracting anions to form its

electrostatic barrier [95]. The electrostatic barriers depend on the suspension medium of the test.

The strength of the diffusing ionic charge reduces exponentially from the anionic surface. This diffusing layer has a thickness defined as the distance over which 63% of the charge is decreased. This is known as the Debye length, and is defined as:

$\frac{1}{\kappa}$ where κ represents an integration constant from integration of the surface potential (Poisson equation).

$K = [2\, e^2\, N_A\, c\, z^2]^{1/2} / \epsilon kT$ where

- z is the charge number
- e is the electron charge
- N_A is Avogadro's constant (6.02×10^{23} 1/mol)
- c is the electrolyte concentration
- ε is the permittivity (c^2/Jm) of the solvent
- k is the Boltzmann constant (1.38×10^{-23} J/ K)
- T is the temperature (K).

As a higher salt concentration compresses the Debye length, a lower ionic strength solution decreases the electrostatic barrier density. This allows for easier access of the antibody to the RBC antigen, and increases the rate of binding and efficiency. Low-ionic strength saline (LISS) is often used to reduce reaction time due to the increased binding rate. However, a lower ionic strength also increases binding of non-specific and cold-reacting antibodies, leading to false-positive readings. From clinical practice, the ideal ionic strength must be above 0.03 M [95]. Normal ionic strength saline (NISS) is 0.15M [95]. Also reaction times must not exceed 20 mins and antibody reagents must be room temperature before use [95].

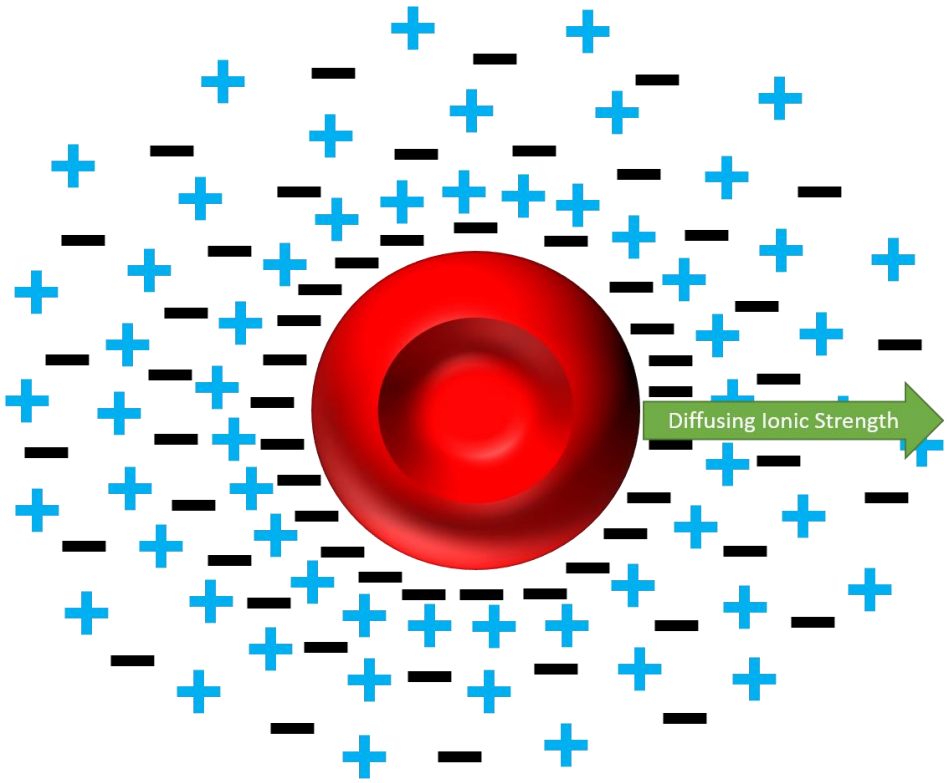

Figure 17: **A negatively charged red blood cell suspended in an aqueous medium attracting positively charged cations. The diffusing anionic charge decreases exponentially from the surface of the RBC. The diffusing charges create a neutralising effect, forming a surrounding electrostatic double layer.**

1.7.2.3.2.3 Effects of pH

The testing pH effects the efficiency of the blood typing antibodies. While the pH of *in vivo* human blood is 7.4, a slightly acidic pH is preferable for *in vitro* testing as haemagglutination occurs at an optimal pH between 6.5-7 [92, 95]. However, testing can be carried out within a range of 6 – 8 [95].

1.7.2.3.2.4 Effects of Time

The reaction or incubation period should be allowed sufficient time for enough antibody-antigen complexes to form [98]. Through industry practice, it has been determined that a short incubation period will not allow sufficient time for binding to occur between the antibody and the antigen site upon the RBC surface. This is particularly true for IgG antibodies which usually require an average incubation time of 20-30 mins, without additives. Some additives, referred to as antibody potentiators, are

reported to half that time when incubated with the antibody-RBC reactants [92]. The potentiators, such as polyethylene glycol (PEG), are known to enhance the reactivity of the IgG antibody by promoting antibody uptake. Conversely, a prolonged incubation period may result in the dissociation of the antibody-antigen complex [98].

1.7.2.3.3 The Secondary Stage of Haemagglutination

During the secondary stage of haemagglutination, the antibodies cross-link to RBCs to form aggregates. This stage is dependent on the collision rate of RBCs, which in turn is dependent on shear, RBC concentration, and to a lesser extent, the balance of attracting and repelling forces between the RBCs. Due to the surrounding electrostatic charges, there is a net repulsive force preventing contact and maintaining a minimum distance of 14nm between cells [95]. Therefore, the blood grouping antibody must at least be able to span the 14nm to cross-link with neighbouring RBCs [95].

Since IgM antibodies have a diameter of approximately 30nm between opposite binding sites, its large structure is capable of bridging the gap without requiring any additional binding agents (Figure 18a); hence allowing direct agglutination. Moreover, the IgM antibodies have 10 binding sites, affording more opportunities for RBC binding upon collision. Direct contact between cells sensitized with IgM antibodies are further aided by gravitational forces during incubation [95]. Due to sedimentation, RBCs settle at the bottom of test tubes or columns over time, allowing for more collisions and resulting in agglutination. An additional centrifugation step can significantly hasten the cross-linking process, which is the most common method of enhancing these antibody-antigen interactions.

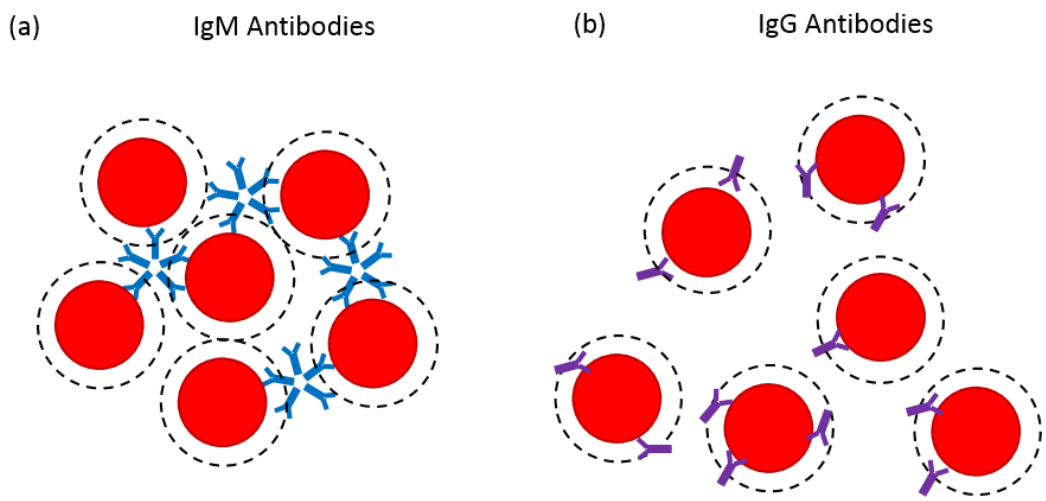

Figure 18: **Schematic depiction of: (a) IgM antibodies consisting of a larger pentameric structure with 10 binding sites, allowing for bridging across the electric double layer to enable direct agglutination; (b) IgG antibodies consisting of a monomeric structure with only 2 binding sites which are too short to bridge the distance and therefore requires an additional binding agent, such as anti-human globulin, to trigger agglutination.**

However, in most cases, as the distance between binding sites on IgG antibodies only span 12-14nm, the structure is too short to overcome the Debye length for direct agglutination (Figure 18b). Instead, the IgG antibodies merely sensitize the RBCs. Neither sedimentation nor centrifugation are useful in this situation as gravitational forces do not affect the charge stabilising the RBCs.

The majority of blood groups tested using IgG antibodies require an additional binding agent, such as bridging antibodies like those of AHG, to affect agglutination (Figure 19). The anti-human IgG component of AHG recognises and binds to the Fc regions of any human IgG antibody, overcoming the repulsion distance to form agglutinates. The addition of AHG to sensitized RBCs therefore allows cross-linking, promoting haemagglutination.

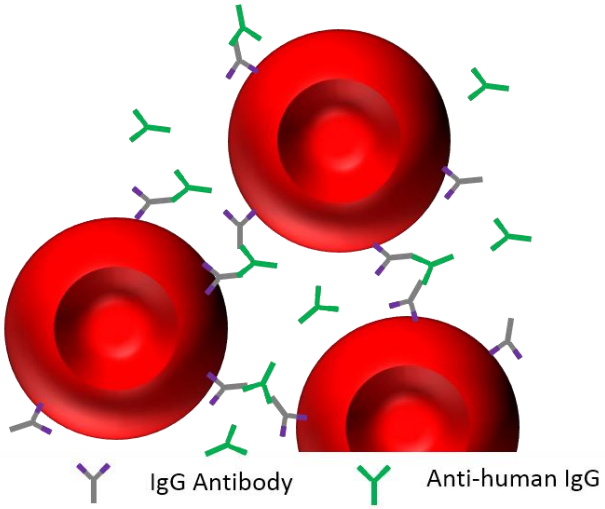

Figure 19: **As IgG antibodies are too small to bridge between red blood cells due to the surrounding electrostatic double layer, an additional binding agent, such as anti-human globulin, is required to trigger agglutination. (Redrawn from Daniels *et al.* (2007)[66])**

There are also alternative binding agents to AHG which reduce the zeta potential, such as potentiating agents or proteolytic enzymes. Polyethylene glycol (PEG), bovine serum albumin (BSA) and polybrene are examples of potentiating agents. However, the mechanisms of such additives are not fully understood, and therefore have limited practicality as the sensitivity and effectiveness varies. PEG acts to concentrate the antibodies within the testing media, and was reported to increase the sensitivity of IAT by removing water molecules [92]. This promotes the probability of RBC-antibody collisions. However, centrifugation negates the effects of PEG, implying washing is not required prior to analysing the results. BSA is reported to influence the second stage of haemagglutination by allowing sensitized RBCs to come into closer proximity of each other [92]. Proteolytic enzymes, on the other hand, cleave glycoproteins from the RBC membrane. The negative charge of RBCs is carried on glycophorin, a part of the glycoproteins, so its removal decreases the overall negative charge. This, however, is ineffective for blood group antigens with a glycoprotein structure. Both potentiating agents and proteolytic enzymes decrease the minimum distance between RBCs, resulting in agglutination.

There have been reports of direct agglutination with IgG antibodies when targets antigens on the outermost regions of the RBC surface are involved, allowing for the distance between cells to be bridged. Such cases involve IgG antibodies for anti-A, anti-B and anti-M. However, they are the rare exception [95].

1.7.3 Current Commercial Methods for Blood Typing

The most common methods for blood typing include the slide test, tube test, microplate method, and the gel column agglutination systems [67]. These techniques are largely known as serological tests based on haemagglutination. Also, with the ability to perform gene sequencing, DNA and molecular analysis has also become a viable option for blood group typing. The gel column test is the most prevalent in industry; however, it requires centrifugation and each of these methods requires professionally trained personnel for analysis. Therefore, these methods are unsuitable for non-laboratory conditions. This has prompted the development of a low-cost alternative for remote regions, home care and emergent in-field situations which is user-friendly, portable and equipment free to provide an alternative to the current blood typing options available [68].

1.7.3.1 Haemagglutination methods

The basic mechanism of blood group typing is direct agglutination. RBCs are reacted with either plasma or blood group typing antisera, and then checked for the formation of agglutinates. Direct agglutination is achieved when an antibody large enough to bridge the gap between cells collides with and binds to its corresponding blood group antigen upon the RBC surface, i.e. IgM antibodies. Due to its smaller size, IgG typically is not large enough to bridge the gap except for rare circumstances.

1.7.3.1.1 Direct Agglutination

The history of blood group typing is based on haemagglutination principles applied to different methodologies. Beginning with the simple slide or tube test, and progressing to the modern column agglutination test (CAT) and microplate systems.

For the simple test tube technique, a sample of RBCs are added to an antisera reagent, and allowed to react before centrifugation. The centrifugation promotes contact and forms a pellet of cells at the base. A positive-antigen result is observed as an agglutinate which is not easily dislodged, while a negative-antigen reaction shows an easy dissociation of cells, and appears 'cloudy'. The glass slide test is similar, except there is no centrifugation involved and instead a reaction period is observed.

Currently, the CAT is the most prevalently used method in the blood banking industry (Figure 20). The CAT was designed as a ready-made test system for typing multiple blood groups at once. Each CAT is presented as a card consisting of multiple microtubes, each preloaded with a matrix of specific antibodies and gel or glass microbeads. RBCs are added to the column and allowed to react before centrifugation. The ready-made CAT card can also be used for automated blood group typing [92]. Positive-antigen results form an aggregate at the top of the column and are unable to migrate through the microbead structure. On the other hand, negative-antigen cells are unbound and freely able to move through the structure, forming a pellet at the base of the column. The CAT can also test plasma for the presence of antibodies within the blood; this is used for reverse ABO blood typing or antibody screening.

The microplate technique is similar in that multiple groups can be tested simultaneously, where the wells are coated individually with blood grouping antibodies prior to the addition of RBCs [103]. This is known as 'solid-phase' technology as the antibodies are transfixed upon a solid medium. The cells are then allowed time to react and settle upon the well surface. From above, positive-antigen results will appear as a fully covered well after reacting with the antibodies, while negative reactions will congregate within the well trough, appearing as a dotted clump of cells.

Figure 20: **Gel column agglutination test depicting the blood type B-negative. Positive-antigen RBCs form agglutinates at the top of the gel column which are unable to diffuse through the gel after centrifugation. Negative-antigen RBCs are unbound and move freely through the gel, forming a pellet at the base. (With permission from Daniels *et al.*[104] Copyright (2015) John Wiley & Sons)**

1.7.3.1.2 Indirect Agglutination

Using IgG antibodies for blood group typing is more complex, requiring the indirect anti-globulin test (IAT). Since the IgG antibodies are too small to span the electrostatic barrier between RBCs to affect agglutination, an additional binding agent is required after sensitization. Basically, during testing an incubation step is followed by an agglutination step by adding anti-human globulin (AHG) which binds to the Fc region of IgG antibodies, which allows for adjacent cells to cross-link and form aggregates.

The IAT can be performed using both the test tube (Figure 21) and CAT methods (Figure 22); however, like with direct agglutination, the CAT is more prevalent in modern blood banking. Since any unbound IgG antibodies are also able to react to the AHG if not separated from the sensitized RBCs, analysis using the test tube method necessitates for any unbound IgG antibodies be removed prior to the addition of AHG. If not removed, the clarity of results is affected and may result in weaker binding. Previously, when using the test tube method, after RBCs were incubated with antisera/plasma at 37°C, the sensitized RBCs were then washed to remove any free antibodies before the addition of AHG.

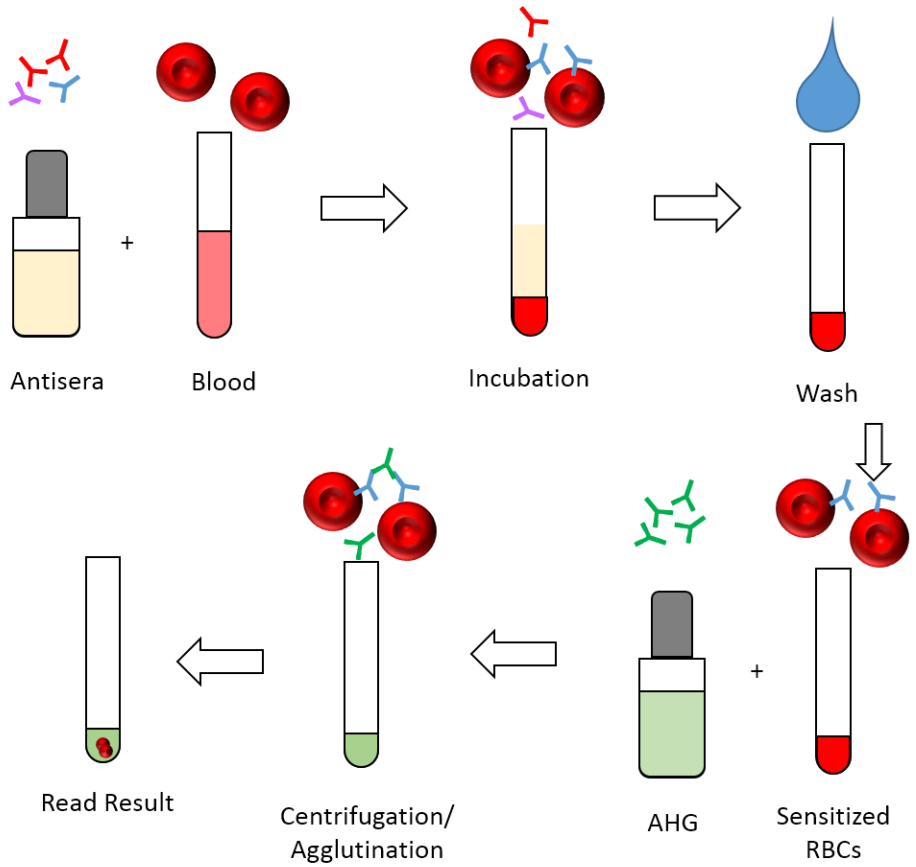

Figure 21: **Illustration of the traditional test tube method for the indirect anti-globulin test (IAT). Antisera or blood plasma is incubated with red blood cells (RBCs). After incubation, the cells are thoroughly washed to remove any unbound antibodies, so that only sensitized RBCs remain. Next, the binding agent, anti-human globulin (AHG) is added to facilitate agglutination. Finally, the RBCs are resuspended and analysed for a result. (Redrawn from Daniels *et al.* (2007)[66])**

Conversely, the CAT method eliminates the washing step completely. During IAT with CAT, the gel or glass microbead matrix is impregnated with AHG (Figure 22). Above the matrix is a reaction chamber containing the IgG antisera or plasma for testing. RBCs are added to the reaction chamber and then allowed to incubate. Instead of washing away any unbound IgG antibodies after incubation, however, the microtube is centrifuged. Due to the comparatively low molecular weight of the IgG antibodies, the centrifugation step separates the sensitized RBCs from the unbound IgG antibodies, and enters the AHG-impregnated microbead matrix for agglutination. Again, positive-antigen reactions form aggregates at the top of the microbead matrix, while negative-antigen reactions form a pellet at the base.

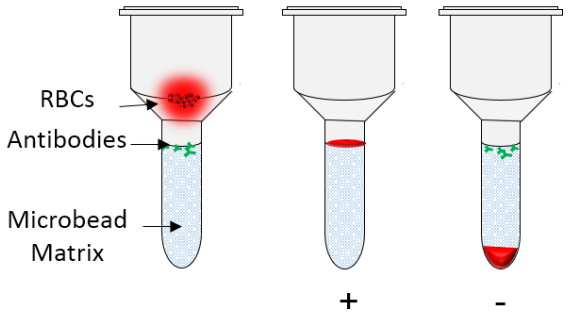

Figure 22: **The modern column agglutination test (CAT) allows for a 'no wash' system, where the red blood cells (RBCs) are added to upper chamber of the microtube which contains blood group antibodies. Here, the RBCs and antibodies are incubated. After incubation, the microtubes are centrifuged. Anti-human globulin (AHG) is contained within the matrix of microbeads, therefore allowing positively sensitized RBCs to react. Positive-RBCs form a layer of agglutinates at the top of the CAT, whilst negative-RBCs are unbound and free to move through the column to form a pellet at the base. (Redrawn from Daniels *et al.* (2007)[66])**

1.7.3.2 DNA & Genetic Analysis

As the genes of most clinically significant human blood groups have been cloned, with all molecular polymorphisms determined, it also possible to predict the phenotype of an individual's blood groups through deoxyribonucleic acid (DNA) analysis [66, 105]. The commercially available technology capable of detecting single nucleotide polymorphisms (SNPs) at a higher throughput has grown rapidly. Examples of such technology include microarrays and coloured microbeads. This makes DNA blood typing possible as a majority of blood group polymorphisms result from unique and specific SNPs. The only exceptions are the ABO and Rh blood groups systems. These SNPs can be isolated for detection, and therefore can report an individual's phenotype.

Molecular analysis can be particularly advantageous in patients who have received or require multiple blood transfusions (e.g. sickle cell disease or other haemoglobinopathies), who may potentially suffer from HDFN during pregnancy, or have autoimmune haemolytic anaemia where clinically significant alloantibodies may be masked by the presence of an autoantibody [66]. Furthermore, gene testing does not require increasingly rare serologic reagents [105]. Conversely, genetic testing can

be over analytic in some cases. A prime example of such is null phenotypes where no antigen is expressed despite the apparent SNPs encoded within the DNA, which may cause complications to arise post-transfusion. The null phenotype is caused by a mutation which renders the gene inactive; however, cases are very rare.

There is a research trend toward DNA technologies replacing haemagglutination methods. However, DNA technology is unlikely that to replace serological testing in clinical blood typing in the foreseeable future, especially where the ABO and RhD blood groups are concerned. This is because DNA testing does not guarantee compatibility while serology does; one mistake can be fatal. Comparatively, DNA analysis is slower, expensive, and more complex as ABO molecular genetics have numerous variants. Current serological techniques for ABO blood group typing are quick, simple, relatively cheap, and highly accurate.

1.7.3.3 Emerging Technology – Paper Diagnostics

An exciting, emerging technology in recent years, has been the use of paper as a blood typing diagnostic tool [68-70]. Similar to the most prevalent blood typing techniques, the paper diagnostic also relies on haemagglutination. The cellulose fibre structure of paper allows for the capillary driven action of fluids – called wicking – through the inter-fibre space of the paper [68]. This action is due to microfluidics, which allows liquid analytes, such as blood, to travel by capillary forces without any further equipment [7]. So far, focus has be set upon using whole blood, unlike in current laboratory techniques where RBCs need to first be separated from plasma before dilution to 3-5% concentration (v/v). This is particularly useful in emergent or remotely located diagnostic situations where access to equipment and refrigeration is limited. Paper diagnostics are also useful in the laboratory as testing can be much faster and more convenient.

For blood group typing, positive-antigen RBCs react with antibodies to form agglutinates, which are too large and unable to travel through the fine porosity of the paper structure. When compared to negative-antigen RBCs, the cells are unbound and

easily washed away. This forms a clear distinction between agglutination and non-agglutination, i.e. a positive and negative result, respectively [68].

The first method was developed by soaking paper strips in antibody solutions. This technique was demonstrated for the ABO and RhD blood groups using either anti-A, anti-B or anti-D [68]. Testing involved releasing a drop of whole blood (RBCs and plasma) at the paper strip centre and allowing the microfluidic wicking to transport agglutinated and non-agglutinated blood differently; a phenomenon used to distinguish between a positive and negative result. Agglutinated blood presented a chromatographic-like separation of the RBCs and plasma (Figure 23), while non-agglutinated blood travelled uniformly along the paper strips and displayed no separation. Positive-antigen RBCs interacted with a corresponding antibody and little to no wicking of cells was observed, but the plasma travelled a considerable distance along the paper strip. Negative-antigen RBCs were observed as a solid stripe of whole blood. This allowed for distinction between positive and negative results as illustrated in Figure 23.

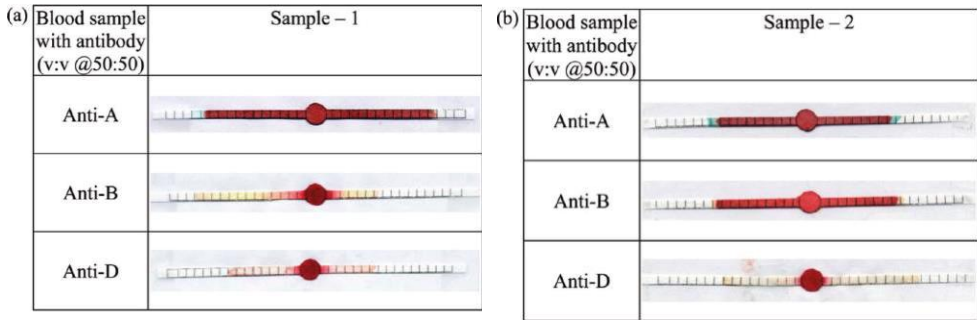

Figure 23: **Use of paper for determining blood groups via wicking of agglutinated and non-agglutinated RBCs, relying on antibody-antigen interactions. Depicted blood types are (a) B+ and (b) O+. (With permission from Khan *et al*.[68] Copyright (2010) Analytical Chemistry)**

Two alternate paper-based methodologies for blood typing were also developed using the same principles involving different approaches to utilising the paper microfluidic structure for analysis [69]. The first method was based on elution principles, displaying chromatographic-like behaviour when adding blood to antibody-treated paper (Figure 24). Droplets of whole blood were spotted onto the paper surface which had been previously treated with a series of known antibodies. Next, the blood spot was eluted

in 0.9% NaCl (saline) buffer for 10 mins by vertically hanging the paper in a thin film chromatograph (TFC) tank. The microfluidic capillary action of the paper drew the buffer up through the paper structure, acting much like a chromatography test and allowing the separation of bound positive-antigen RBCs and plasma to occur. Much like the previous studies, fixed agglutinated blood spots were unable to elute, forming a red dot at the base and leaving the paper strip white as a positive result. Paths of non-agglutinated RBCs could clearly been seen along the paper, indicating a negative result. The second method involved a spot test, where blood was spotted onto an antibody-treated paper substrate. However, instead of a TFC tank, the saline solution was washed directly onto the spot via pipette. Both methods accurately detected the ABO and RhD blood groups for 100 samples, even including 4 weak AB and 4 weak RhD samples, demonstrating the capabilities of paper-based testing for blood group typing.

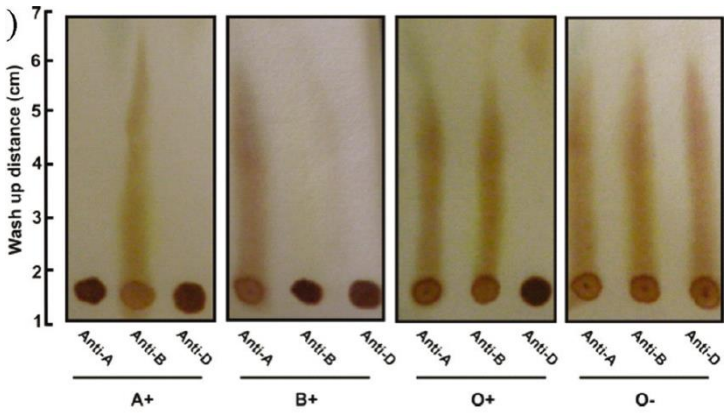

Figure 24: **Agglutinated and non-agglutinated blood tested chromatographically using paper. Tests were eluted with 0.9% NaCl (saline) buffer for 10 mins. (With permission from Al-Tamini *et al*.[69] Copyright (2011) Analytical Chemistry)**

More recently, Li et al. (2012) developed a text-reporting method. Drawing inspiration from the popular novel by J.K. Rowling, 'Harry Potter and the Chamber of Secrets' [70], the concept showed that paper diagnostics could provide an explicit result in writing. This allows any user to correctly analyse the results simply by reading the corresponding blood group letters. The paper substrate was patterned with hydrophobic boundaries surrounding a hydrophilic channel which could be shaped into text or symbols. This

'writing' appears invisible upon the paper. When paper is infused with antibodies, the addition of positive-antigen blood forms the shape of the channel, for example an A or B, and thus the blood type is legible on paper (Figure 25). If negative, once washed, the blood is easily removed, with the shapes remaining 'invisible'. This test successfully reported the ABO and RhD blood types of 99 samples, including at least one of each group (Figure 26). Among these samples, there was one weak AB and one weak D.

While promising, the dependence on patterning hydrophobic channels is problematic. In the case of alkyl ketone dimers (AKD), the channels only last for a couple of days before the AKD continues to seep through the paper thus rendering the hydrophilic channel ineffective.

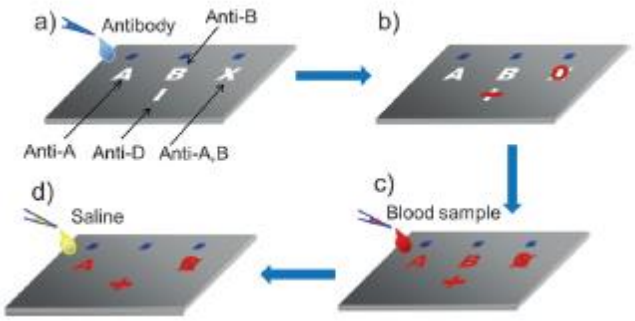

Figure 25: **Methodology of blood group typing using the text-reporting patterned paper substrate. (a) Antibody solutions are added and allowed to dry, (b) "O" and "-" symbol are printed on test over negatively pattered symbols of "x" and "|", respectively, (c) blood sample added and allowed to react, and d) washing step with saline solution. (With permission from Li *et al*.[70] Copyright (2012) John Wiley & Sons)**

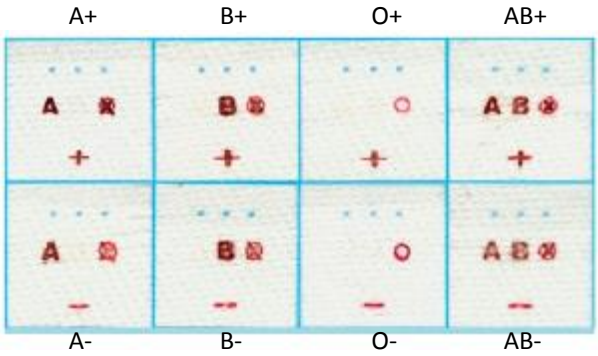

Figure 26: **An array of the ABO and RhD blood group types as tested using the text-reporting paper substrate method. (With permission from Li *et al*.[70] Copyright (2012) John Wiley & Sons)**

1.7.4 Analytical Techniques

There is much general knowledge regarding the potency and effectiveness of antibody-antigen interactions within the blood banking industry. This also includes knowledge of the rare exceptions to that general knowledge which has been gathered through many decades of experience and practice. However, it appears very little has been reported regarding the scientific explanations behind this knowledge. This is, in part, due to several reasons: (1) a lack of analytical techniques; (2) varying expression of antigens among the population; (3) varying affinity of antibodies to these antigens among the population; and (4) varying affinity of different antibody clones to the blood group antigens.

1.7.4.1 Fluorescence-Assisted Cell Sorting & Flow Cytometry

The best documented variable is the estimated antigen expressions among different populations, achieved using fluorescence-assisted cell sorting (FACS) and flow cytometry (Figure 27); which are currently the most prevalent techniques for blood group analysis.

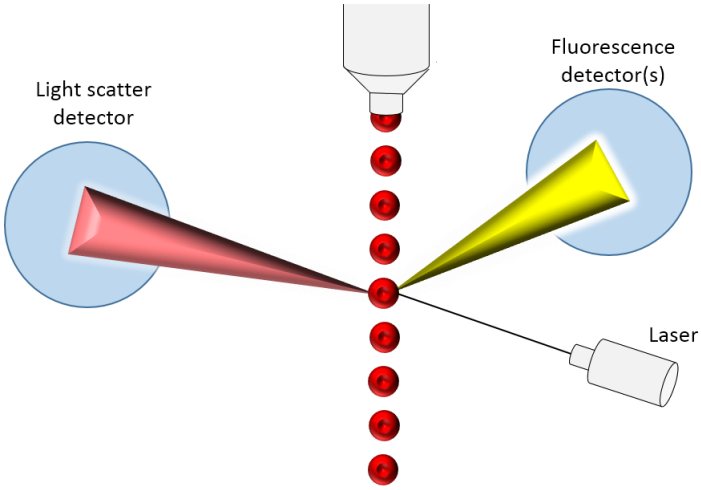

Figure 27: **A simplified representation of flow cytometry where fluorescence-conjugated antibodies are bound to red blood cells flowing in single file. A laser light is shone upon the RBCs. Positive cells cause the light to scatter and to fluoresce. Multiple fluorescence detectors can be used in conjunction to visualise differently timed 'events'. (Redrawn from Daniels *et al.* (2007)[66])**

Both FACS and flow cytometry are capable of quantitatively measuring the expression of RBC antigens. This is achieved using fluorescence-conjugation for both methods, which can affect binding. FACS analysis relies on tagged antibodies that emit a brightly fluorescent colour visualised by proper light detection microscopy. Similarly, flow cytometry also uses fluorescent-labelled antibodies grafted to blood cells, and measures the light emitted as the cells pass through a laser in single file [67, 106].

Through FACS and flow cytometry, the antigen density and some equilibrium constants of individual samples of RBC antibodies were measured [100, 107-109]. A study from 1962 demonstrated the reaction kinetics of anti-c with a single sample of blood using FACS with ^{131}I-labelled human-sourced anti-c [110]. While the rate of dissociation was measured through time delayed measurements, non-specific uptake of ^{131}I to upon the RBCs was also reported. Similar studies on samples of human-sourced anti-D, anti-K, anti-C, anti-E and anti-E were also carried out using the same methodology [111, 112]. As the human-sourced antibodies are polyclonal – and not monoclonal – binding kinetics vary widely [110]. Monoclonal antibodies only became available in 1975, when the production of monoclonal antibodies was pioneered; most surprisingly, further studies on interaction kinetics have not been pursued since [113]. This lack of

monoclonal antibodies is likely the factor for the halt in reaction kinetic studies, as individual clones have differing antibody affinities. Other factors such as a lack of techniques for real-time and label-free analysis have also hampered kinetic studies.

1.7.4.2 Surface Plasmon Resonance

While FACS and flow cytometry can successfully measure the number of blood group antigens present on the RBC surface, the kinetic interactions of the antibody-antigen complexes observed are time-delayed; these techniques are unable to conduct real-time analysis at the biomolecular level.

Surface Plasmon Resonance (SPR) is now a well-established method for biospecific interaction analyses (BIA) [114-118]. This technique has been widely utilised to: (1) detect and analyse interactions between biomolecules [119-121], (2) characterize antibody-antigen interactions, and (3) as a biosensor. SPR is one of the most common optical biosensors available [91, 122]. It is a label-free optical technique which relies on a polarised laser to measure the change of adsorbed mass on a metal chip surface by monitoring the change in refractive index (RI) (Figure 28) [123]. The laser light is directed through a medium of high refractive index, usually a prism, while a low refractive index medium is bordered by a thin layer of metal, in most cases gold. At the gold layer surface, surface plasmons are generated at a critical angle of incident light dependent on the refractive index within a few hundred nanometres from the surface. These surface plasmons absorb light, and change the concentration of reflected light. When molecules bind, the critical angle changes and so does the amount of reflected light. This decrease in intensity is measured by a sensor and reported as a binding response unit (RU), equal to a critical angle shift of 10^{-4} degrees.

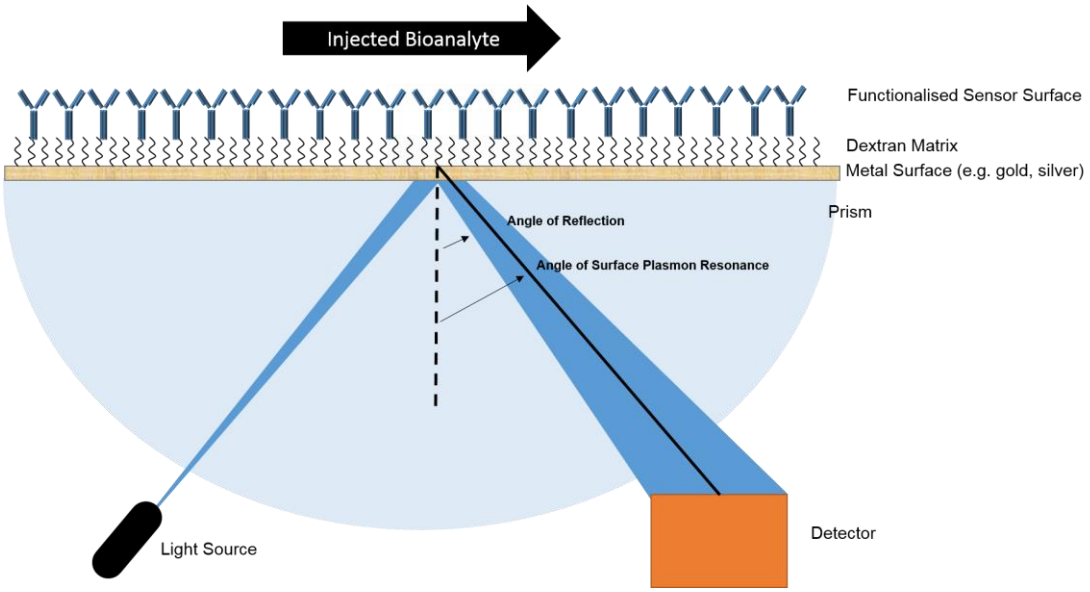

Figure 28: **Schematic diagram of a surface plasmon resonance (SPR) instrument, where a polarised laser is shone upon a noble metal surface. The angle of reflection changes in correspondence to the change of adsorbed mass upon the functionalised sensor surface. Changes in angle of reflection are the result of changes in refractive index which are detected and computed into a binding response (RU).**

Typically, the SPR can monitor intermolecular binding events in real time, which enables analysis of the interaction kinetics between biomolecules [119, 124-127], including protein-membrane analysis [122]. However, SPR investigations monitoring whole cell interactions are very rare; this is due to the average cell sizes being orders of magnitude larger (8-15µm) than the evanescent field depth (~300nm) [120, 128]. SPR detectors can be coupled with a microfluidic system which allows for real-time quantitative analysis using microliters of analytes at a pico mole sensitivity. Therefore, large cells can appear unsuitable when tested with microfluidic systems as cells can settle or congregate upon the sensor surface [129]. However, unlike most cells, RBCs are highly deformable. The primary function of RBCs is to deliver oxygen throughout the human body. Therefore, the RBC membrane structure must move through the smallest micro-capillaries with ease. This presents RBCs with a unique advantage over most other cells. Another concern is the viscosity of suspended RBCs. High viscosity samples must be avoided to minimize potential clogging in the microfluidic channels and to maximize sensitivity.

However, SPR can provide a good platform to detect and quantify antibody-antigen interactions for blood grouping. SPR was used for qualitative antigen detection for blood typing and observing antibody-carbohydrate interactions [91, 130]. Previously, SPR was shown as a tool for rapid quantification of IgG antibodies specific for blood group antigens A and B [131, 132]. Also, by immobilising IgM antibodies to a BIAcore sensor chip surface, Quinn et al. (1997) were able to capture free RBCs via antigen binding, and analyse the subsequent change in mass for detection [91]. Quinn et al. (1997) demonstrated the detection of the A and B antigens upon RBC surfaces using SPR. While successful, regeneration of the expensive sensor chip surface was poor despite trying multiple regeneration buffers at different pH. The necessary harsh regeneration conditions caused a loss of antibody/biosensor functionality after a single use. This was due to the inability to fully desorb bound material. In addition, each chip was limited to a single blood group.

Of particular interest are weak, partial, and varying phenotype antigens, as well as anti-IgG interactions, which have thus far, been unexplored. If successfully detected, SPR could be used to model the effects of kinetics, stoichiometry and antibody structure [110]. Commercial SPR systems, such as the BIAcore, can control variables to achieve this, including temperature, injection rate and reaction time. Other variables such as concentration can be controlled manually. However, little is known on the effect of RBC concentration, shape, repulsion and attraction forces, cell receptor density, and ligand distance and affinity on SPR performance.

1.7.5 Perspectives & Conclusions

There has been a vast interest in transfusion medicine ever since its discovery, where the well-established haemagglutination methods for serological testing have already been in use for several decades. Other methods, both simpler and more complex, have emerged with different advantages. However, in spite of the high medical use and clinical interest, the antibody-antigen interactions involved with blood group typing have remained largely unexplored. This includes the basic kinetics dictating how these blood group interactions reach and maintain an equilibrium, if at all. While the factors

which effect the rate of agglutination are known, their mechanisms are not always understood and have never been quantified. This would be particularly useful to improve weak interactions that are difficult to detect using current serological methods. The lack of study is, in part, due to an inability to accurately monitor the blood group antibody-antigen interactions in real-time and label-free. The best previously used analytical methods, fluorescence-assisted cell sorting and flow cytometry, offer neither real-time nor label-free analysis. Surface plasmon resonance analysis appears as the most promising method to quantify the elusive antibody-antigen interactions in blood typing. Indeed, there has been a few previous investigations which show feasibility. However, the field remains largely unexplored and most of the critical issues have remained unaddressed.

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Chapter 2

THE DETECTION OF BLOOD GROUP PHENOTYPES USING PAPER DIAGNOSTICS

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PREFACE

Paper biodiagnostics are a cheap, rapid and easy-to-use alternative to many current laboratory techniques. The application of paper as a blood group typing diagnostic tool has been readily achieved for the detection of ABO and RhD blood groups. However, there are 36 different blood group systems consisting of over 300 antigens, with nine systems classified as clinically significant. Blood group antigens other than the ABO and RhD phenotypes have yet to be explored using paper diagnostics. This chapter explores the use of paper diagnostics for the detection of clinically significant non-ABO and non-RhD blood group phenotypes. Two different techniques are used, each with contrasting and unique microfluidic mechanisms for blood group detection. The first, is an elution method based on a chromatography-like mechanism, where washing is driven by the capillary forces of the microfluidic structure. The second, is a flow-through method which operates much like filtration during washing, and reports the result in text. In addition, other factors such as reaction time, reagent concentration and antibody type and structure are compared.

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Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Initiation, key ideas, experimental works, analysis of results, writing up	44%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Mioasi Li	Experimental works, analysis of results	44%
Wei Shen	Key ideas, paper reviewing and editing	Supervisor
Gil Garnier	Key ideas, paper reviewing and editing	Supervisor

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature	[redacted]	**Date** 15/02/2016
Main Supervisor's Signature		**Date** 15/02/2016

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THE DETECTION OF BLOOD GROUP PHENOTYPES USING PAPER DIAGNOSTICS

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2.1 ABSTRACT

2.1.1 Background and Objectives:

Paper biodiagnostics for blood typing are novel, cheap, fast and easy to use. Agglutinated red blood cells cannot travel through the porous structure of paper, indicating a positive antibody-antigen interaction has occurred. Conversely, non-agglutinated blood can disperse and wick through the paper structure with ease to indicate a negative result. This principle has been demonstrated to detect blood group phenotypes: ABO and RhD. However, typing for red blood cell antigens such as Rh, Kell, Duffy, and Kidd, have not yet been explored on paper.

2.1.2 Materials and Methods:

Two paper testing methods – an elution and a direct flow-through method – were investigated to detect red blood cell antigens excluding the ABO system and RhD. Antigens explored include: C, c, E, e, K, k, Fy^a, Fy^b, Jk^a, Jk^b, M, N,S and s, P1, Le^a and Le^b. The variables tested include: reaction time, reagent concentration. The importance of antibody type/structure for successful agglutination on paper was confirmed.

2.1.3 Results:

Some blood group phenotypes showed less agglutination due to weaker antibody-antigen interactions. Most blood groups with antibodies available as IgM, such as C, c, E, e, K and k, and Jk^a and Jk^b, and P1, were successful using both methods. However, other blood groups, especially those with antibodies only available as polyclonal antibodies were unsuccessful and require further scrutiny.

2.1.4 Conclusion:

Paper can be used as an alternative blood grouping diagnostic tool for selected blood group phenotypes.

2.1.5 Key words:

Paper diagnostics, blood typing, haemagglutination, blood groups, antibodies, IgM, IgG, Rh, Kell, Kidd, Duffy,

2.2 INTRODUCTION

Although ABO and RhD are the most important blood group systems in transfusion medicine, many other blood group antibodies can cause serious or fatal haemolytic transfusion reactions (HTRs) or haemolytic disease of the foetus and newborn (HDFN). Current blood typing techniques rely on antibody-antigen interactions to cause red blood cell agglutination for positive identification. Techniques, such as gel columns, are well established. Common methods for phenotyping blood groups are the tube test and column agglutination test (CAT), which require centrifugation only available under laboratory conditions, unsuitable for in-field and remote testing [1]. Currently CATs are more widely used, relying on gels or glass beads to visualize agglutination. However, the process requires trained personnel, is more expensive, lengthy and time consuming to operate. Low-cost and equipment-free methods, such as the glass slide test, can be insensitive and are unsuitable for antigen identification, particularly for neonatal samples [2].This is especially true when testing for the wide array of clinically significant blood group phenotypes.

For many years, blood groups were the best genetic markers, and have played a key role in the mapping of the human genome [3]. Blood group antigens are found on the surface of red blood cells (RBCs). These dictate an individual's blood type. Blood group antibodies are present in the blood plasma and generally result from an immune response triggered by prior exposure, such as previous transfusion or during pregnancy, to the corresponding antigen not located on that individual's RBC surface (alloantibody). There are now 35 blood group systems recognised, with over 300 blood group antigens, many of which are clinically significant, requiring detection and the identification of antibodies to be performed prior to blood transfusions [3, 4]. The discovery of the ABO blood groups made blood transfusions safe; the disclosure of the RhD antigens led to the understanding and prevention of HDFN [2, 5, 6]. Each of the blood group phenotype systems contain antigens which are unique and have a different frequency depending on the patient's ethnicity [7]. The blood groups and their corresponding alloantibody examined in this study are summarized in Table I. Aside from these blood groups, some alloantibodies, such as Anti-M, N, P1 and Lewis antibodies, are seldom active at body

temperature (37 °C) and are generally not considered clinically significant [2]. The mismatching of a blood group antigen in the presence of a clinically significant antibody could cause immediate, delayed or severe HTRs or HDFN, necessitating tests to identify or confirm the presence of these antigens. Prior to blood transfusion, the accurate and fast identification of the blood group phenotypes is essential for patients who possess an alloantibody [8].

Table I **Blood groups and the corresponding antibodies used for blood group phenotyping on paper. The antibody structure and clone is also included.**

Blood System	Blood Type	Reagent Type	Clone
Rh	C	IgM	P3X25513G8
	E	IgM	DEM1
	c	IgM	H48
	e	IgM	MS-36 and P3FD512
Kell	K	Polyclonal	Polyclonal (Human Source)
	K	IgM	MS-56
	k	IgM	Lk1
Kidd	Jk^a	IgM	P3HT7
	Jk^b	IgM	P3.143
Duffy	Fy^a	Polyclonal	Polyclonal (Human Source)
	Fy^b	Polyclonal	Polyclonal (Human Source)
MNS	M	IgM	LM1
	N	IgM	LN3
	S	IgM	P3S13JS123
	s	Polyclonal	Polyclonal (Human Source)
P	P1	IgM	650
Lewis	Le^a	IgM	LEA2
	Le^b	IgM	LEB2

Further development in low-cost minor blood typing methods is necessary, especially for testing in developing countries and the battlefield. Novel paper diagnostic for human blood typing through the haemagglutination of the RBCs was developed [9-17]. Paper can be pre-treated with a blood typing antibody reagent. When a blood sample is subsequently introduced onto the antibody pre-treated paper, if positive, a haemagglutination reaction will occur between the RBCs and corresponding antibody. These blood agglutinates are formed within the porous structure of paper and cannot be removed when washed with solutions such as phosphate buffered saline (PBS) or 0.9% NaCl saline. Inversely, during negative reactions, the RBCs do not agglutinate and the cells are easily washed away. This phenomenon is the foundation of paper

substrates for blood typing which was used to design diagnostics for ABO and RhD blood testing [12].

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In this study, paper diagnostics were engineered to identify blood groups using haemagglutination, focusing on common clinically significant blood group phenotypes. The method for phenotyping these blood groups proved not to be as simplistic as the ABO and RhD groups; time-dependent reactions became evident [10, 17]. The influences of antibody types, reaction time, washing conditions, and antisera concentration on the identification of blood group phenotype were investigated.

Two separate testing methods were developed using paper, each using different principles regarding the interactions of the analytes, samples and washing method. One method follows the principles of chromatography, allowing the washing solution to elute unbound blood cells laterally along the paper structure (wicking). This method was used for ABO and RhD groups [11]. The second uses the flow-through method [12, 18, 19], which washes through the paper, rather than along, utilising a filtration mechanism. While each method has been successful when testing ABO and RhD blood types, this is the first report testing paper diagnostics for the detection of blood group phenotypes other than ABO and RhD using both methods.

2.3 MATERIALS AND METHODS

2.3.1 Materials and Equipment

Professional Kleenex paper towel from Kimberly-Clark, Australia, was employed as paper. Antisera blood typing antibodies were purchased from ALBA bioscience, Edinburgh, United Kingdom and used as received (Table I). Washing solutions 0.9% (w/v) NaCl saline and phosphate-buffered saline (PBS) were prepared using MilliQ water, analytical grade NaCl (Univar) and PBS tablets (Sigma-Aldrich), respectively. Alkyl ketene dimer (AKD) from BASF was used for paper hydrophobilization. Analytical grade n-heptane from Sigma-Aldrich was used to formulate ink-jet solution to print text on paper. EDTA blood samples were sourced from Australian Red Cross Blood Service (ARCBS); stored at 4°C and used within 7 days of collection.

Micropipettes (Eppendorf research®, 2.5–50 µL) were used to introduce the antibodies, blood samples, and saline solutions on paper. Millipore centrifugation tubes (50kD cutoff) from Merck, Australia were used to remove supernatant and increase concentration of the antibodies by filtration.

2.3.2 Method

Two methods were tested. The first was the elution methodology for high-throughput diagnostics in laboratory settings [11]. The second flow-through method used the direct reporting method suited for remote and emergent situations [12]. These methods differ by the direction of liquid transport within paper during the washing step. The elution method relies on the paper structure to wick saline solution laterally and separate by chromatography along paper, while direct-reporting washes via a flow-through filtration-like method.

2.3.2.1 Elution

A known antibody reagent is dotted along the bottom of paper (10µL) before the addition of mixed EDTA blood sample (3µL) [11]. After a reaction period, ranging from 30 to 120 seconds, the substrate was hung in a vertical-standing elution tank containing 0.9% NaCl saline solution at a depth of 1cm. Following an elution period of 5 min., the substrate was removed and dried in the laboratory (T = 22 ± 1˚C; relative humidity = 30-50%) (Figure 1). Qualitative evaluation of results was conducted visually. Agglutination showed a distinct bloodspot, while non-agglutinated RBCs were defined by the absence of a blood spot and by a clear elution path on paper. Quantitative analysis was achieved using ImageJ software to measure colour densities of the blood spot (BS) and elution pathway (EP).

Kleenex paper towel was selected for performance, attainability and cost. Compared with other paper, such as Whatman filter, the results achieved were clearer and more reliable [11, 16].

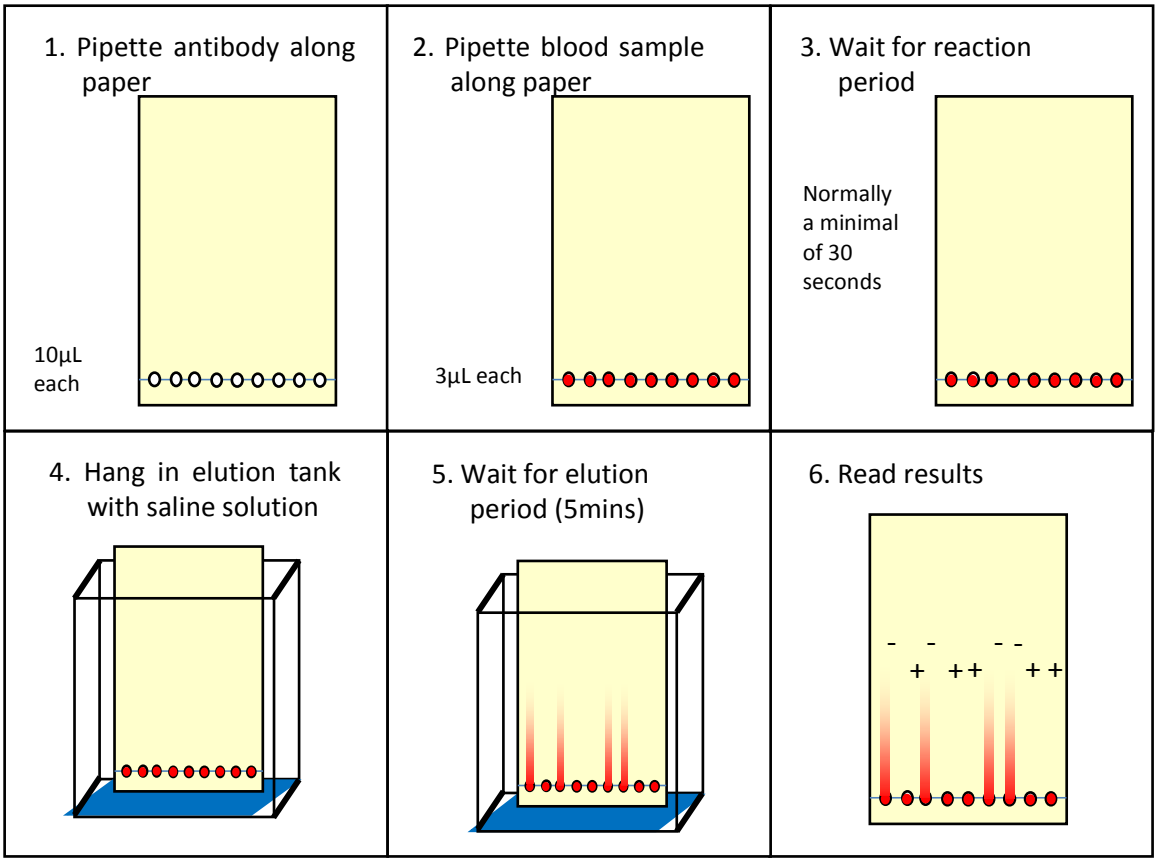


Figure 1 **Methodology for blood group phenotyping using paper via elution.**

2.3.2.2 Flow-Through Direct reporting

Agglutinated cells can form patterns to resemble text or signs. This can be achieved by hydrophobic barriers printed onto the paper prior to the addition of cells. The hydrophobic barrier guides where antisera will be absorbed into the paper. This strong hydrophobic-hydrophilic contrast can be used to border the RBC-antibody interactions on paper, forming shapes or 'text' that can be easily read when fabricating a user friendly blood group device [15]. A reconstructed Canon ink jet printer (Pixma iP3600) was used to print channels onto paper with an AKD-heptane solution (Figure 2).

Aliquots of 2.5µL of antibody solutions were introduced into the corresponding patterns. After the antibody solution was dried, 2.5µL of blood sample was introduced into the vertical channel. A reaction period was allowed for the antibodies to interact with the RBCs inside the channels. Washing was performed with two 30 µL aliquots of saline solution introduced into the patterns to wash out non-agglutinated RBCs. The blood types were then read directly. Quantitative analysis used ImageJ to measure channel density.

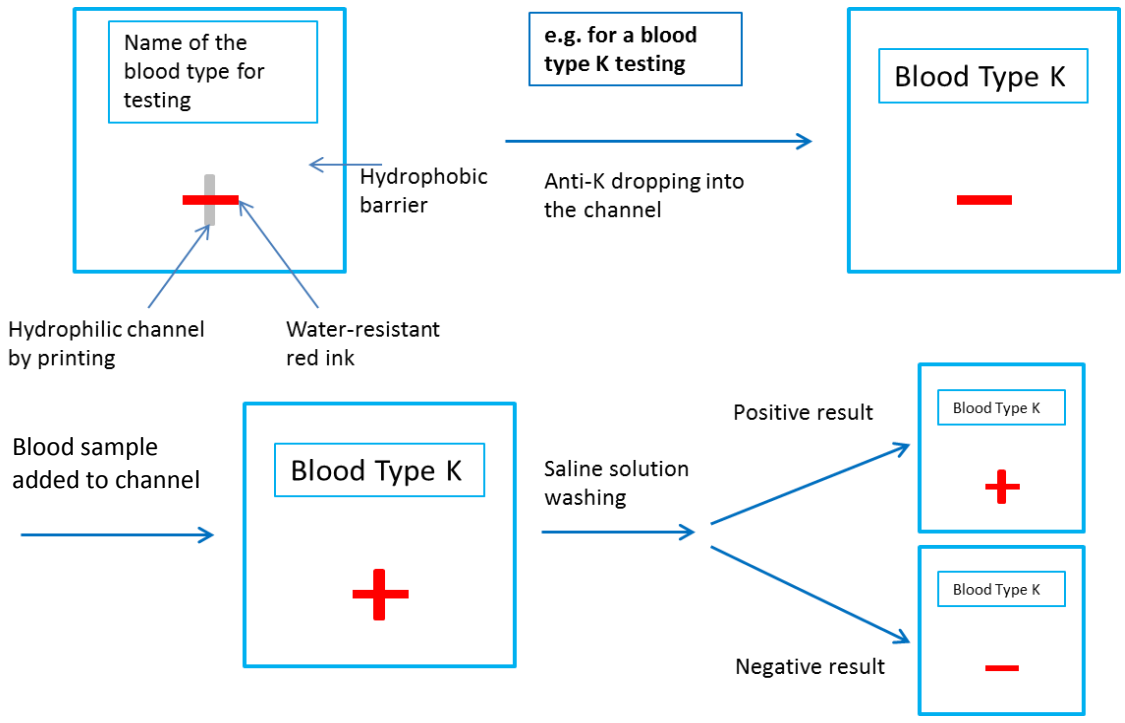


Figure 2 **Fabrication, testing procedures and result reporting of paper diagnostics for antigen detection using example: blood group K.**

2.3.2.3 Analysis Criteria

Successful testing should easily distinguish positive from negative, while preventing false positive and false negative results. For the elution method, a positive result should report a well-defined blood spot (BS) with no RBCs in the elution pathway (EP); conversely, a negative result would have no BS, but show distinct wicking in the EP. The direct reporting method prints a positive "+" sign, while a negative in absence of RBCs, leaves a negative "-" sign. 236 different patient samples were tested. However, not all samples were fully phenotyped. The number of samples for each antigen tested was restrained by blood availability, outlined in Table II.

To compare the strength of each blood group's antibody/antigen reactions and the testing conditions, the colour densities of the resulting BS were measured using ImageJ. Distinct patterns emerged when comparing positive and negative results. Higher density values indicated a positive result (usually >100), while lower values indicated negative samples (<40). Densities between this range (40-100) could be classified as weaker reactions. This distinction was used for the text-reporting method.

However, to analyse the elution method results, the BS alone was insufficient to indicate the interactions of certain blood groups. In such instances, the BS optical densities bordered the weak reaction threshold. In some cases, the BS for negative and positive looked too similar for accurate determination by the naked eye. It was the presence/absence of an EP that confirmed the result. Therefore, in addition to BS density, an area of the EP 2cm above the BS was measured. Positive results contained little or no RBCs in EP with low density values. Conversely, negative results showed high densities representing unbound RBCs travelling through paper after washing. This difference was translated into a fraction, the optical density ratio (ODR), comparing density of BS to EP. A higher ODR value (>6.0) indicated positive, while low values (< 3.0) indicated negative, with weak reactions between indicated in between (3.0-6.0).

$$ODR = \frac{BS}{EP}$$

2.4 RESULTS

A series of antibody/antigen reaction tests were performed using both procedures to detect blood group phenotypes (Figures 1 and 2). As each antibody/antigen pair behaves differently when tested, each needed to be analysed individually. This differs from the major blood groups (A, B, O and RhD), which react strongly and clearly for most patients; exceptions include the weak subgroup variants [10, 11, 16]. It was found that several factors impacted the interaction strengths observed for each antigen tested. These factors are: (1) reagent type, (2) reaction time, (3) antibody concentration, and (4) washing conditions. The washing conditions were found to affect the results of the text-reporting method, but not the elution method. This is due to the effects of directional flow during washing. Table II and Figure 3 depict the results achieved for both the elution and text-reporting method for the blood groups investigated (Table I). However, testing for Duffy groups, Fy^a and Fy^b, MNS groups, and Lewis groups, Le^a and Le^b, was unsuccessful.

2.4.1 Testing conditions

The main variables investigated for optimisation include: (1) reagent type, (2) reaction time, and (3) antibody concentration, and (4) washing conditions.

Reagent type – The effect of reagent type was investigated. Certain antibodies are only commercially available as polyclonal (human source), not immunoglobulin M (IgM). IgM antibody reagents are generally monoclonal, containing a pentameric structure, with ten binding sites. IgG antibodies are monomers, containing only two-binding sites. Whilst IgM antibodies show strong agglutination, IgG antibodies generally only sensitize cells without agglutination. This is due to electrostatic repulsion between RBCs. IgM antibodies provide over twice the bridging distance, overcoming the electrostatic double layer surrounding RBCs. IgM antibodies are expected to exhibit better binding capabilities compared to IgG antibodies, allowing direct agglutination rather than only sensitization to occur. The identification of certain blood groups by direct agglutination using paper is therefore constrained by the commercial availability of IgM antisera.

Reaction time – The time allowed for the antibody sera and RBCs to react. Haemagglutination between ABO and RhD antigens and antibodies can be achieved within 30s. However, some minor blood group antigens take longer than 30s to bind with their antibodies to show strong haemagglutination for clear identification. Thus, the time the RBC antigens had to bind was extended and compared. Reaction times 30, 60, 120 and 180s were tested, with optimum conditions reported in Table II. Reaction times beyond 180s were not explored as longer reaction times could increase the potential of false positives.

Antibody concentration – The effect of antibody concentration was explored. Antibody concentration was increased by filtering the reagent using Millipore centrifugation tubes. These tubes retain biomolecules 50kDa or larger, and remove supernatant. Increased concentration allows faster collision rate and resulted in stronger agglutination for positive tests. However, this raises the possibility of provoking the prozone effect where agglutination does not occur due to an excess of antibody or antigen.

Washing conditions – Varying the washing buffer was investigated to determine the effect of pH, ionic strength and specificity has on the elution of RBCs and clarity of results. Using the text reporting method, washing using PBS yielded clearer results, while the effects were nominal for the elution method.

Table II. **Blood group phenotyping using the Elution (E) and Text reporting (TR) methods on paper. Blood spot (BS) and elution pathway (EP) are represented as density. Optical density ratio (ODR) compares density EP:BS. Positive is denoted by high density and ODR, negative has lower densities and ODR.**

ANTIGEN		C		E		c		e		K		K		k	
Antibody Type		IgM		IgM		IgM		IgM		IgM		Polyclonal		IgM	
No. of Samples		76		67		54		8		11		53		25	
Reaction Time	(s)	30		30		E:120, TR:180		E:60, TR:180		E: 60, TR:120		120		120	
Buffer		NaCl		NaCl		PBS		PBS		NaCl		NaCl		NaCl	
		+	-	+	-	+	-	+	-	+	-	+	-	+	-
No. of Samples		31	45	17	50	40	14	41	8	2	9	12	41	23	2
Elution Method	BS	123 ±9.1	51 ±9.4	118 ±5.1	42 ±7.9	105 ±4.3	44.0 ±2.4	107 ±4.63	38 ±4.1	82 ±3.6	41 ±7.7	97.2 ±23.7	57.9 ±6.90	97 ±6.0	64 ±1.7
	EP	8.2 ±3.1	32 ±6.8	8.5 ±3.0	49 ±11	5.8 ±1.0	32 ±6.5	6.1 ±0.48	54 ±6.5	13 ±8.8	27 ±4.3	15.8 ±9.43	33.5 ±7.11	16 ±3.1	44±6.0
	ODR	17 ±2.7	2.9 ±0.55	16 ±3.6	1.0 ±1.4	12 ±3.7	1.8 ±0.39	18 ±1.5	0.72 ±0.12	8.4 ±5.5	2.0 ±0.46	0.183 ±0. 179	0.585 ±0.126	6.4 ±1.5	1.5 ±0.25
Text Method		152 ±7.4	28 ±3.8	145 ±7.1	30 ±4.9	154 ±5.7	30 ±3.7	135 ±9.2	30 ±4.2	106 ±5.0	34 ±4.0	46 ±4.1	35 ±7.1	131 ±8.3	31 ±5.0
ANTIGEN		Fy^a		Fy^b		Jk^a		Jk^b		P1		Le^a		Le^b	
Antibody Type		Polyclonal		Polyclonal		IgM		IgM		IgM		IgM		IgM	
No. of Samples		12		8		30		25		40		46		23	
Reaction Time	(s)	120		30		120		120		30		180		180	
Buffer		NaCl		NaCl		NaCl		NaCl		NaCl		PBS		PBS	
		+	-	+	-	+	-	+	-	+	-	+	-	+	-
No. of Samples		9	3	7	1	22	8	15	10	26	14	13	33	11	12
Elution Method	BS	60 ±13	60 ±23	37 ±5.6	40	106 ±8.7	48 ±4.0	100 ±7.5	67 ±8.3	128 ±16	69 ±10	N/A	N/A	N/A	N/A
	EP	39 ±15	38 ±13	33 ±5.8	35	14 ±3.6	40 ±4.2	15 ±3.6	34 ±9.1	14 ±8.8	35 ±15	N/A	N/A	N/A	N/A
	ODR	1.6 ±0.17	2.0 ±0.028	1.1 ±0.19	1.1	8.3 ±2.7	1.2 ±0.13	6.8 ±1.8	2.9 ±1.2	12 ±1.9	1.7 ±0.63	N/A	N/A	N/A	N/A
Text Method		36 ±11	27 ±4.0	N/A	N/A	95 ±6.2	37 ±4.8	122 ±9.8	34 ±6.2	140 ±5.7	31 ±5.5	68 ±3.1	35 ±6.2	116 ±5.9	37 ±6.2

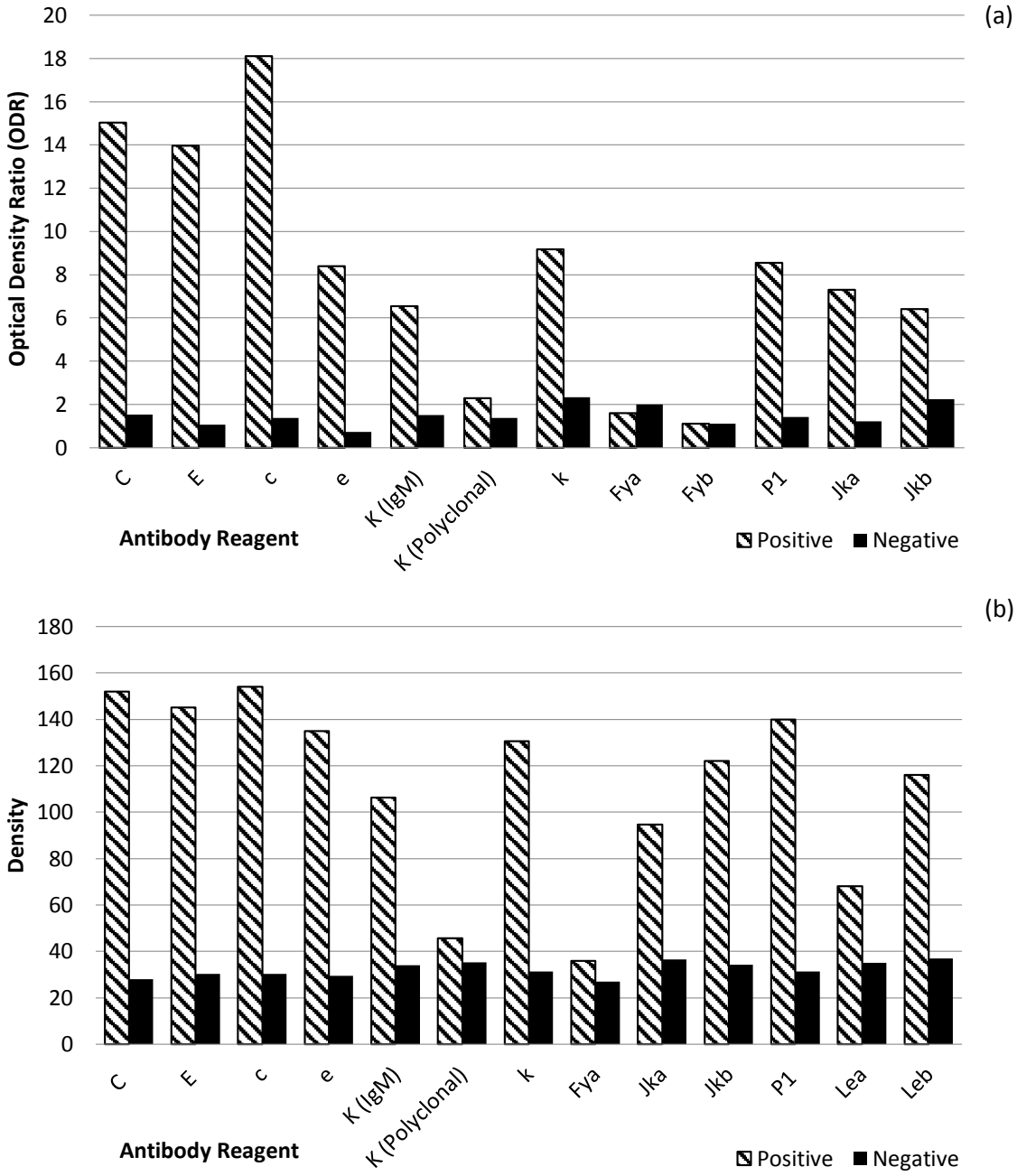


Figure 3 **Blood group phenotyping using (a) elution, and (b) text reporting methods on paper. Blood spot (BS) and elution pathway (EP) are represented as density. Extent of coagulation is represented as optical density ratio (ODR) comparing density EP:BS. Positive is denoted by high density and ODR, negative has lower densities and ODR. (Fy^b was not tested using the text reporting method.)**

2.5 DISCUSSION

Each blood group typing reaction behaved differently when tested, and therefore each required individual analysis.

2.5.1 Rh Blood Group System

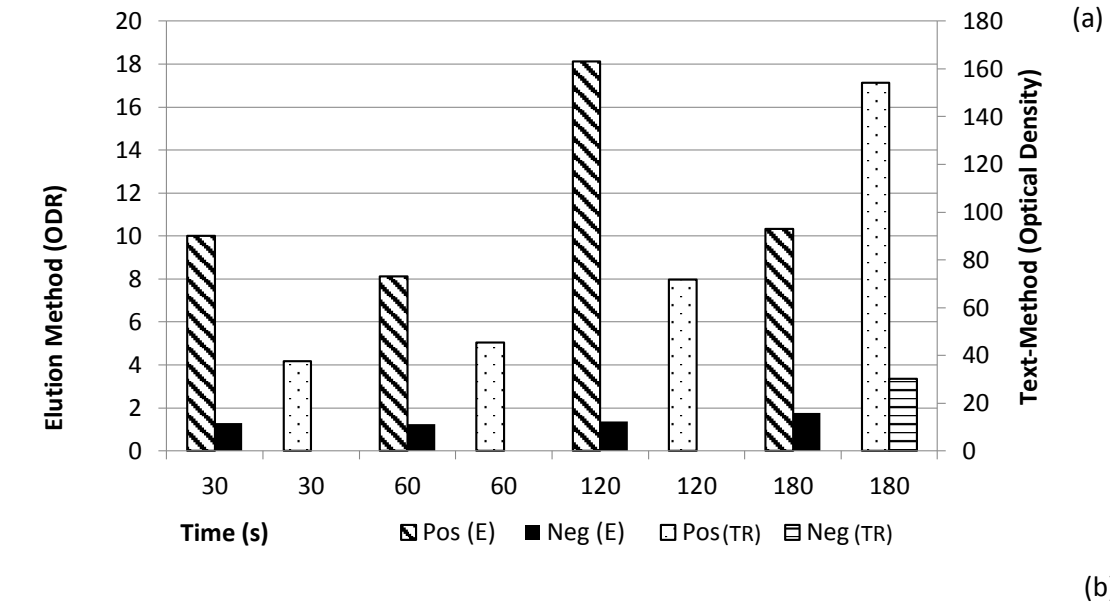
Apart from the ABO blood groups, the antibodies against the Rh blood group system are the most common clinically significant antibodies. Of these, D is the most immunogenic antigen. The additional antigens investigation within the Rh system which are important when screening patient or donor blood are C, c, E, and e. Testing using paper for these four antigens was successful using the commercial antibody reagent at a standard reaction time of 30 seconds. The Rh antisera used were all monoclonal IgM (Table I).

Using the elution method, the C and E antigens were strongly detected (ODR pos: 17±2.7, neg: 2.9 ±0.55; pos: 16±3.6, neg: 1.0±1.4, respectively) (Figure 3(a)), only needing 30s reaction time. However, c and e antigens showed a slightly less clear BS with higher density in the EP. The clarity of tests for c and e improved with longer reaction times of 120s (ODR pos: 12±3.7, neg: 1.8±0.39; pos: 18±1.5, neg: 0.72 ±0.12, respectively). Extending the RBC-antibody contact time to two minutes or more showed better agglutination with less unbound RBCs able to migrate through the paper structure.

A maximum reaction time of 180s was selected as longer reaction times could increase false positives. For the elution method for c and e, a 180s reaction time provided less distinct results with negative ODRs of 1.8 ±0.39 and 3.0, respectively. While above the threshold, a lower ODR for negative results provides a clearer result (Figure 4).

The text-reporting method was similar. C and E had clear and distinct densities (pos: 152±7.4, neg: 28±3.8; pos: 145±7.1, neg: 30±4.9 respectively). When compared to the BS data of the elution method, the text-reporting method showed a clearer distinction between positive and negative. The c and e tests similarly improved with a longer

reaction time of 180s (pos: 154±5.8, neg: 30±3.7; pos: 135±9.2, neg: 29±4.2 respectively) (Figure 4).



(b)

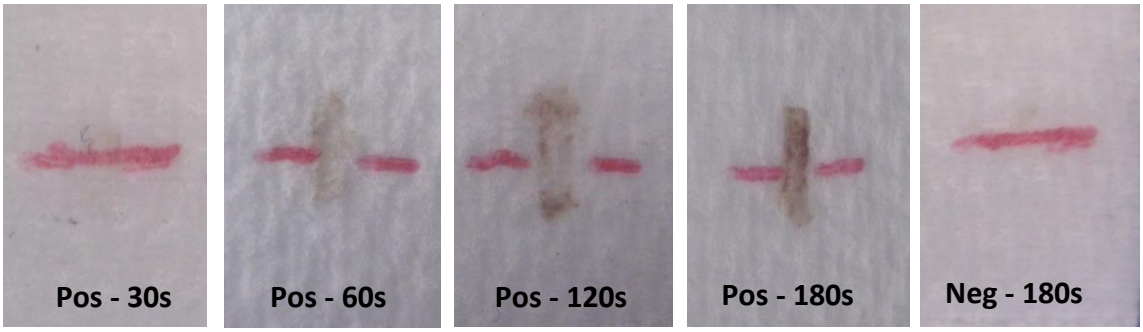


Figure 4 **(a) Effect of time on the reaction period tested with c antigen, comparing elution method (E) and text-reporting method (TR). ODR of E is compared to density of TR. (b) Effect of time on the reaction period tested with c antigen using text-reporting.**

2.5.2 Kell Blood Group System

Two clinically significant blood group antigens from the Kell system were explored: K and k. Two types of K reagents commercially available were tested: monoclonal IgM and polyclonal (Table I). The anti-K polyclonal reagents manufactured from human plasma is expected to be composed of IgG antibodies since the immune response

against the Kell system produce predominantly IgG antibodies [2]. The efficiency of both reagents was tested with both paper testing methods.

The elution method reported ODR pos: 8.4±5.5, neg: 2.0±0.46; pos: 2.3±0.11, neg: 1.4±0.39, for IgM and polyclonal respectively. The text-reporting method had densities of pos: 106±4.9, neg: 34±4.0; pos: 46±4.1, neg: 35±7.1, for IgM and polyclonal respectively. Interactions between IgM antibodies and the RBCs are strong and there is no overlap between a positive and a negative test. While both paper methods were accurate with the anti-K IgM antibody, they both failed with polyclonal anti-K (Figure 5). A clear distinction between positive and negative is fundamental for blood group testing, and only IgM antibodies have systematically achieved such distinction.

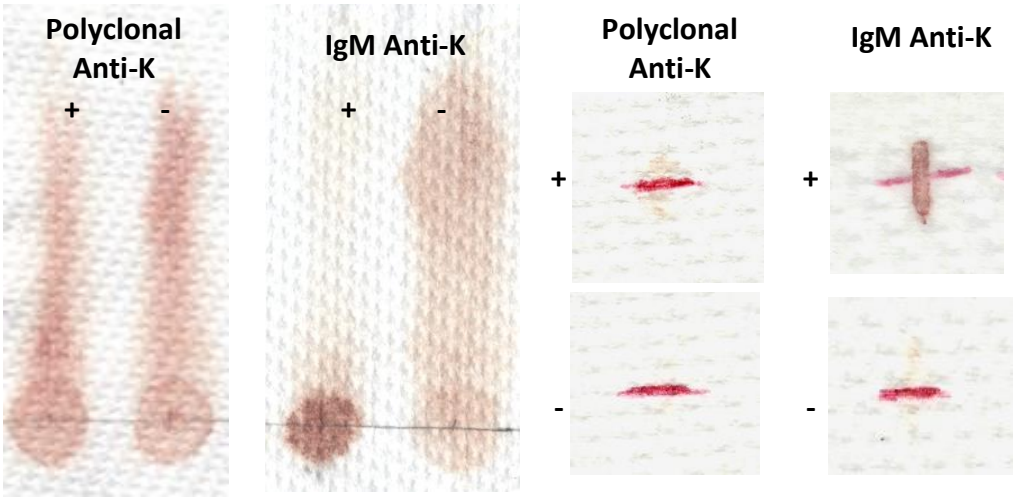


Figure 5 **Comparison of antisera (polyclonal or IgM) effecting identification of testing results for K antigen.**

In the saline tube test, IgM antibodies can directly agglutinate antigen-positive red cells, whereas IgG antibodies require anti-human globulin (AHG) to effect agglutination. For both paper testing methods, agglutination between IgG antibodies and RBC antigens without AHG led to false negative results. As in saline tube testing, this is explained by the greater size of the IgM pentamer able to bridge red cells for electrostatic stabilisation. To achieve bridging, the critical length of the antibody must be greater than the electrical layer surrounding the RBC. While IgM antibodies can bridge red cells,

where IgG monomers cannot. This allows IgM antibodies to agglutinate red cells in saline solution.

Buffer washing solution affected the clarity of results for the text-reporting method. Using Phosphate buffered saline (PBS) improved the density compared to 0.9% NaCl saline. This is likely due to the directional flow when washing. Only nominal effects were observed for the elution method.

Testing with polyclonal anti-K antisera was used to determine the effect of increasing antibody concentration. Surprisingly, increasing the concentration of the commercial polyclonal antibodies by removing excess serum enhanced reaction clarity. Antibody concentration was increased by filtering the serum to retain biomolecules 50kDa or larger.

Increasing antibody concentration improved the stoichiometric ratio between antibody and antigen (or RBCs). A higher stoichiometry ratio allows for more colloidal interaction; however, it is costly. This provides a counterpoint to reaction kinetics, as a longer reaction time decreases rapidity and could increase potential false positives.

This worked unexpectedly well, enhancing the clarity for polyclonal anti-K when concentration was doubled (pos: 6.8, neg: 1.4±0.46 with a reaction time of 60s), with optimum at 120s (pos: 7.8, neg: 0.61±0.18) (Figure 6). However, the same method applied to other polyclonal reagents was unsuccessful. Polyclonal anti-K antisera, while predominantly IgG antibodies, could contain residual IgM from the original source. Removing the excess supernatant probably increased the IgM concentration in addition to the IgG, thus promoting agglutination. To test this hypothesis, a simple immediate spin tube test and an Indirect Anti-globulin Test (IAT) were performed with the original polyclonal reagent, against K positive cells. Surprisingly, cell agglutination was observed with both tests, albeit significantly weaker in the immediate spin test. This result indicates that the polyclonal reagent used was predominantly IgG antibodies with some residual IgM antibodies present.

Drawbacks of increasing concentration are: antibody sera are expensive, and not all polyclonal reagents have an IgM component.

Testing for blood group k typing using a monoclonal IgM antibody was straightforward. Much like the Rh groups, results for both the elution and text reporting methods were clear. For the elution method, positive and negative ODR was 6.4±1.5 and 1.5±0.25, respectively. Density results for text-reporting method were 131±8.3 and 31±5.0 for positive and negative respectively (Table II and Figure 3).

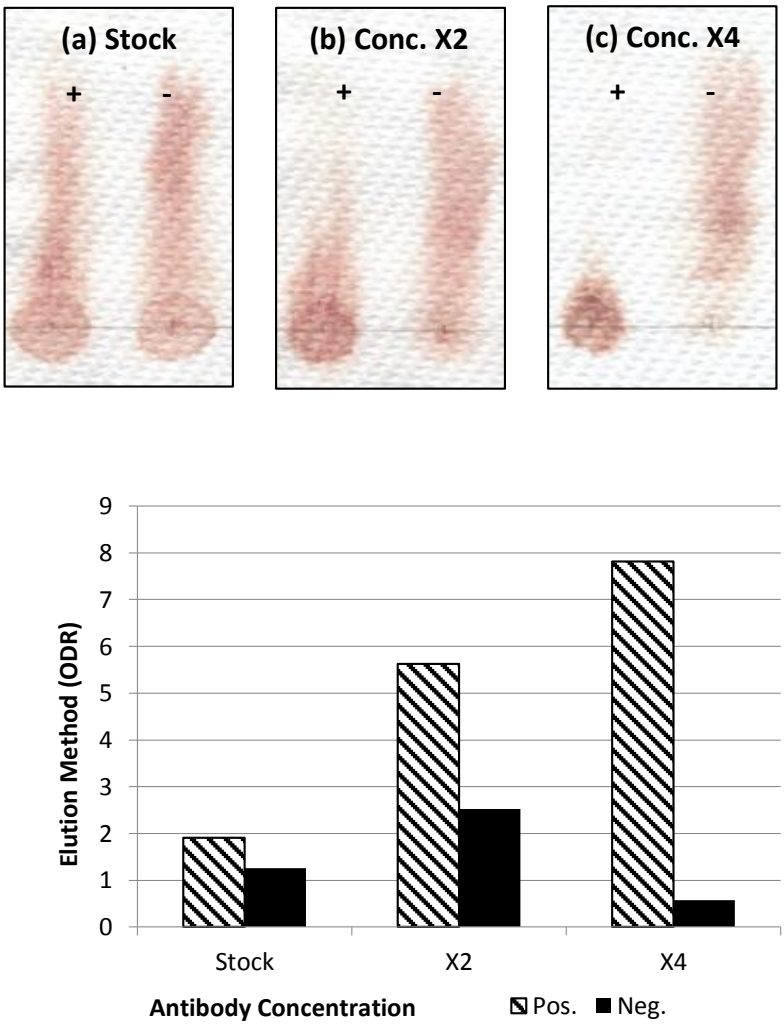


Figure 6 **Effect of anti-K polyclonal concentration on efficacy; (a) at stock solution, (b) double stock concentration by volume, and (c) quadruple stock concentration by volume.**

2.5.3 Duffy Blood Group System

Unlike the anti-K antibody reagent, Duffy antibodies, anti-Fy^a and Fy^b, are not available as IgM. Polyclonal Duffy antibody reagents are predominantly IgG [1]. No optimisation techniques could achieve clear results as positives reported as (false) negatives. When reaction time was varied, despite an increased density for positive results, tests for negatives showed an equal increase in density. Attempts to duplicate the improved results seen when the concentrating the polyclonal anti-K were unsuccessful for both anti- Fy^a and Fy^b. All results were negative, showing the inability of IgG antibodies to form RBC agglutinates retainable within paper. The antibody structure (IgM versus IgG) is crucial for successful identification.

2.5.4 Kidd Blood Group System

The Kidd blood groups studied are Jk^a and Jk^b. The corresponding typing reagents are available as monoclonal IgM antisera, and generally showed a clear distinction between positive and negative results. However, the BS achieved was not as defined as desired, requiring optimisation. Extending reaction time improved results. Reaction time of 120s was determined for group Jk^a (ODR pos: 8.3±2.7, neg: 1.2±0.13; pos: 95±6.2, neg: 37±4.8; elution and text-reporting methods, respectively) and Jk^b (ODR pos: 6.8±1.8, neg: 2.9±1.2; pos: 122±10, neg: 34±6.2; elution and text-reporting methods, respectively). Both elution and text-reporting results were accurate.

2.5.5 MNS Blood Group System

Paper testing for the M, N, S and s antigens was unsuccessful for both methods. The M and N blood groups often reported falsely as ‘weak’ positives. Numerical data are available as Supplementary Material.

2.5.6 P Blood Group System

P1 antigen was tested using a monoclonal IgM antibody. Like the C and E antigens, no special optimisation was required. The results were clear and easy to distinguish between positive and negative results for both elution and text-reporting (pos: 12±1.9, neg: 1.7±0.63 ODR; pos: 140±5.7, neg: 31±5.5 S respectively) (Figure 3a and b).

2.5.7 Lewis Blood Group System

Testing the Lewis blood system revealed the importance of the antibody solution composition. Unlike other blood groups, testing with anti-Le^a and anti-Le^b, showed anomalous patterns using the elution method. Rather than displaying a clear BS or EP, the blood dispersed irregularly with important tailing (Figure 7). This is likely due to potentiators, such as dextran, often added to formulation. Potentiators, consisting of polymers, are often used to enhance the extent of RBC agglutination for low potency antibodies. Potentiators can become problematic for paper tests should they non-specifically retain individual RBC on paper. Instead of absorbing into paper, the antibody solution created a film above the surface, affecting elution and causing irregular dispersion. There was no distinction between positive and negative. The irregular dispersion pattern in paper caused by potentiators was confirmed using "For Further Manufacturing Use" (FFMU) anti-Le^a, the raw material without potentiators. However, the FFMU anti-Le^a was still unsuccessful for distinguishing positive or negative, due to the lack of potentiators to affect agglutination.

Unlike the elution method, the flow through method distinguished both positive and negative samples. This is due to directionality and the strength of washing, unaffected by potentiators. The perpendicular washing allowed the saline solution to flow through paper and filtrate, rather than eluting RBCs. Instead, unbound cells could flow through paper. Colour density for Le^a and Le^b using the text-reporting method were pos: 68±3.1, neg: 35±6.2; and pos: 116±5.9, neg: 37±6.2, respectively.

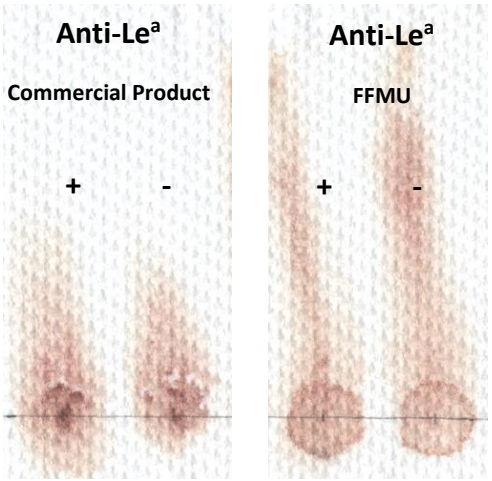


Figure 7 **Dispersion difference between the commercial product and FFMU antibody Le[a].**

2.6 PERSPECTIVE

More than half of the clinically significant blood group phenotypes explored within this study were successfully optimised; the exceptions being Fy^a, Fy^b, S and s. This demonstrates the potential for paper biodiagnostics as a viable alternative for blood group phenotyping. Both testing methods are unique and designed for varying purposes. Successful results for the elution method were more definitive than the flow-through method, better designed for high throughput testing. Meanwhile, the flow-through method is ideal for point-of-care testing, especially in remote areas or developing countries. Although in-field testing is unlikely to achieve standard laboratory conditions for temperature and humidity, paper blood typing diagnostics would still provide a cheap, fast and easy to use alternative to current conventional blood grouping methods.

2.7 CONCLUSION

Two paper methods - an elution and a flow through direct-reporting method – were investigated to determine the blood group phenotype of red blood cells. Clinically significant antigens tested include C, c, E, e, K, k, Fy^a, Fy^b, Jk^a, Jk^b, S and s antigens. The M, N, P1, Le^a and Le^b antigens were also tested. As each group behaved differently, optimisation for each antigen was required. This optimisation was achieved by controlling the main variables: antibody-antigen reaction time, antibody concentration and changing the washing buffer solution. Antibody class is of the utmost importance on paper. Most antigens with antibodies available as IgM monoclonal antisera were successful with both paper methods (Rh, K (IgM), k, Kidd, P1), though to varying degrees of clarity. Testing with polyclonal antisera was unsuccessful (K and Duffy). Unexpectedly, increasing the antibody concentration of polyclonal anti-K showed a discernible difference between positive and negative results; this was due to increasing the concentration of IgM component. Results were not replicated using polyclonal Duffy antibodies (IgG). Increasing reaction time between antibody and antigens (RBC) showed increased clarity with both methods, while changing washing solution improved testing using the flow-through method. The formulation of the antibody solution is also an important variable for paper testing; any additive able to non-specifically bind RBC to paper affects test sensitivity; this was observed with the Lewis antibodies.

While successful detection was achieved with most IgM antibodies, except Lewis, M, N and S, using both paper assays, polyclonal antibodies, consisting predominantly of IgG, resulted in inconsistent results.

2.8 ACKNOWLEDGEMENTS

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Chapter 3

QUANTITATIVE BLOOD GROUP TYPING USING SURFACE PLASMON RESONANCE

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PREFACE

In the previous chapter, it was clear that the underlying mechanisms of the antibody-antigen interactions effected the success and clarity of testing each individual blood group on paper, especially that of antibody structure. Blood group testing using IgG antibodies were completely unsuccessful. Therefore, to better understand the binding and affinity of these interactions, a more bioanalytical approach is required.

This chapter explores the use of surface plasmon resonance (SPR) – a bioanalytical tool commonly used to study the binding kinetics of biomolecular interactions – for the detection of blood group antigens using RhD as an example. As SPR is also a concentration-dependent diagnostic, it is also capable of quantitative analysis reported as a binding response. In this study, the SPR sensor surface is functionalised using anti-human IgG which is capable of binding to any human IgG antibody. Through incubation at 37°C, red blood cells (RBCs) are pre-sensitized with an IgG antibody, in this case anti-D IgG, before detection. D antigen is used as example due to the strong binding affinity with its IgG antibody counterpart, which will be useful during the development of this novel blood group detection platform. Human-sourced whole blood samples were tested for validation, showing a strong correlation between the binding response observed and the reported estimated antigen of RhD. Furthermore, the anti-human IgG surface was capable of regeneration with negligible degradation.

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Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Initiation, key ideas, experimental works, analysis of results, writing up	90%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Marie-Isabel Aguilar	Key ideas, paper reviewing and editing	Supervisor
Gil Garnier	Key ideas, paper reviewing and editing	Supervisor

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature	[redacted]	**Date** 15/02/2016
Main Supervisor's Signature		**Date** 15/02/2016

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QUANTITATIVE BLOOD GROUP TYPING USING SURFACE PLASMON RESONANCE

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3.1 ABSTRACT

The accurate and reliable typing of blood groups is essential prior to blood transfusion. While current blood typing methods are well established, results are subjective and heavily reliant on analysis by trained personnel. Techniques for quantifying blood group antibody-antigen interactions are also very limited. Many biosensing systems rely on surface plasmon resonance (SPR) detection to quantify biomolecular interactions. While SPR has been widely used for characterising antibody-antigen interactions, measuring antibody interactions with whole cells is significantly less common. Previous studies utilised SPR for blood group antigen detection, however, showed poor regeneration causing loss of functionality after a single use. In this study, a fully regenerable, multi-functional platform for quantitative blood group typing via SPR detection is achieved by immobilising anti-human IgG antibody to the sensor surface, which binds to the Fc region of human IgG antibodies. The surface becomes an interchangeable platform capable of quantifying the blood group interactions between red blood cells (RBCs) and IgG antibodies. As with Indirect Anti-globulin Tests (IAT), which use IgG antibodies for detection, IgG antibodies are initially incubated with RBCs. This facilitates binding to the immobilised monolayer and allows for quantitative blood group detection. Using the D-antigen as an example, a clear distinction between positive (>500RU) and negative (<100RU) RBCs is achieved using anti-D IgG. Complete regeneration of the anti-human IgG surface is also successful, showing negligible degradation of the surface after more than 100 regenerations. This novel approach is

validated with human-sourced whole blood samples to demonstrate an interesting alternative for quantitative blood grouping using SPR analysis.

3.2 KEYWORDS:

surface plasmon resonance, blood group typing, red blood cells (RBCs), Rh phenotype, IgG antibody, diagnostic

3.3 INTRODUCTION

Accurate and reliable typing of blood groups is of the utmost importance prior to blood transfusion. The mismatching of incompatible blood types could lead to a potentially fatal haemolytic transfusion reaction [1]. The most widely known blood groups are ABO and RhD, however there are actually 35 blood group systems with over 300 identified antigens, many of which are clinically significant. During blood typing, two types of antibodies are used: (1) pentameric IgM, and (2) monomeric IgG. Based on the haemagglutination principle, antigens upon the surface of a red blood cell (RBC) will bind in the presence of corresponding antibodies. IgM antibodies bind to multiple RBCs, facilitating agglutination and positive identification. However, not all blood group antibodies are available with an IgM structure, necessitating the use of IgG antibodies instead. RBCs binding with IgG antibodies do not agglutinate and require the use of an additional agglutination reagent, anti-human IgG, to indicate positive antigen identification. This is known as the indirect anti-globulin test (IAT)[2].

While there are many well established blood typing methods available, such as the column agglutination test (CAT), current methods for quantifying blood group antibody-antigen interactions are very limited. This is particularly important when identifying weak interactions between weak subgroup variants, which are often difficult to determine via traditional testing by simple visual analysis, and can easily be overlooked or misinterpreted [3]. There are currently only two methods available for quantitative analysis: flow cytometry and fluorescence microscopy. Flow cytometry uses time delayed measurements, while the conjugation required for fluorescence microscopy

may influence activity. This is where the advantages of surface plasmon resonance (SPR) lie.

SPR is a widely used technique for the detection and analysis of interactions between biomolecules, including antibodies and antigens. It is a label-free optical technique that can monitor intermolecular binding events in real time. SPR relies on a polarised laser to measure the change of adsorbed mass on a metal chip surface by monitoring the change in refractive index (RI), and reporting the change as a binding response unit (RU) [4]. However, SPR investigations of whole cell interactions are significantly less common [5]. This is because detectors are often coupled with a microfluidic system with a diameter similar to the average cell size which ranges from 10 to15µm. Therefore, most large cells are unsuited for use with microfluidic systems as cells are more likely to settle or congregate [6]. However, unlike most cells, RBCs are highly deformable in nature to allow for easy vascular transport, which bares similarities to microfluidics.

A few previous studies have investigated SPR for blood group antigen detection [7] and antibody detection [8, 9]. Using RBCs, Quinn et al. (1997) demonstrated the detection of A and B antigens with SPR by immobilising the corresponding blood group antibodies (IgM). RBCs were then passed over the surface with positive cells captured for detection. However, while successful, this method showed poor regeneration despite trying multiple regeneration buffers at different pH. Furthermore, the harsh regeneration conditions resulted in a loss of antibody/biosensor functionality after a single use due to the inability to fully desorb bound material.

In this study, a novel concept is explored. In contrast to the use of specific IgM antibodies, by immobilising an alternative antibody, anti-human IgG, the chip surface becomes an interchangeable platform capable of quantifying the blood group typing interactions between RBCs and antibodies (IgG). Anti-human IgG is able to recognize and bind to the Fc region of human IgG antibodies for detection. Much like the IAT, blood group IgG antibodies incubated with RBCs. The cells become sensitized with IgG and are able to bind to the immobilised anti-human IgG. This test has the potential to quantitatively detect any blood group with a corresponding IgG antibody. The D antigen, more commonly recognised as the '+' of group bloods, was detected as example using

anti-D IgG. It is the objective of this study to develop this novel concept of functionalising an SPR sensor chip with anti-human IgG to detect RBCs which have been pre-sensitized with anti-D IgG. In the first part, the method is optimized for sensitivity and selectivity. The second shows validation using human-sourced blood samples.

3.4 MATERIALS AND METHODS

3.4.1 Chemicals and Equipment

All chemicals and sensor chips were purchased from VWR International (Brisbane, Australia) unless otherwise stated. Anti-D IgG For Further Manufacturing Use (FFMU) antibodies were supplied by Quotient EU (Edinburgh, United Kingdom). Abtectcell red blood cells (RBCs), Celpresol and Celpresol Low-Ionic Strength Solution (LISS) were supplied by CSL Limited (Melbourne, Australia). Anti-human IgG Fc (Clone CBL101) was purchased from Merck Australia (Melbourne, Australia). Analytical grade phosphate buffered saline (PBS) was purchased from Sigma-Aldrich (Sydney, Australia). EDTA blood samples were sourced from the Australian Red Cross Blood Service (ARCBS) (Melbourne, Australia), stored at 4˚C and used within 7 days of collection. The BIAcore X system (GE Healthcare, Uppsala, Sweden) was used for all analyses.

3.4.2 Methods

Anti-human IgG Fc was immobilised upon a CM5 SPR chip surface using amine coupling. The CM5 chip consists of a gold surface grafted with a medium molecular weight carboxy methyl dextran layer. Firstly, the surface was activated by mixing and injecting equal volumes of 100mM N-hydroxysuccinimide (NHS) and 400mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) at 5µL/min for 7 min. Both were made with distilled water before use, aliquoted and stored at -15˚C until required. Then 5µL of anti-human IgG Fc (0.5µg/mL) ligand was dissolved in 10mM sodium acetate buffer before injection over the activated surface for 6 min at 5µL/min. Unreacted sites were then blocked by injecting 1M ethanolamine-HCl at 5µL/min for 7 min.

Reagent red cells (Abtectcells III 3%) and human-sourced red cells were used during these experiments. Reagent red cells are washed human red cells that are kept in a preservation solution at a constant concentration for standardisation. Human-sourced red cells are collected as whole blood from donors, including plasma, and stabilized with Ethylenediaminetetraacetic acid (EDTA); these cells have not been standardized.

Prior to injection, RBCs were incubated with excess anti-D IgG FFMU for 30 min at 37˚C (1:1 by volume), then washed 4 times with Celpresol LISS and centrifugation before dilution to a known concentration in Celpresol LISS for injection into the BIAcore X system. Magnesium chloride was used to regenerate the chip surface with 1 min pulses at 1µL/min.

Three variables were explored for optimisation including: experimental buffer, washing, and RBC concentration. Temperature was kept constant at 37 ˚C to maximize binding of IgG antibodies which are optimal at said temperature. A single flow cell detection mode, flow cell 2, was used for binding analysis. Sensograms were analysed with standard BIAcore X control software. The different buffers investigated include: PBS, Celpresol, and Celpresol LISS.

3.5 RESULTS & DISCUSSION

Using anti-D IgG FFMU antibodies and reagent red cells as a model, a regenerable platform for blood group detection was explored and optimized using SPR. The concept consists of producing a monolayer of anti-human IgG capable of interacting with any IgG sensitized RBC through anti-IgG/anti-human IgG interaction. If positive, the IgG antibody will specifically bind onto the RBC antigen site. These IgG covered RBCs can then be retained with the generic anti-human IgG grafted on the SPR chip through binding of the Fc region of the IgG antibody structure; negative unbound RBCs will be directly eluted (Figure 1). Several questions must be addressed to validate this concept. Firstly, what is the sensitivity and reproducibility of the technique; secondly, how well does the SPR surface regenerate; thirdly, how quantitative can the technique be; fourthly, how dependent on surface blocking/interaction is the technique; and finally, whether or not the SPR can be used to quantify the antibody-antigen interaction

binding and kinetics. Furthermore, the validity of using SPR for detection was also tested using clinically derived EDTA blood samples. Throughout this study, each sample was prepared twice, tested three times, and upon two different sensor chips to show reproducibility and reliability.

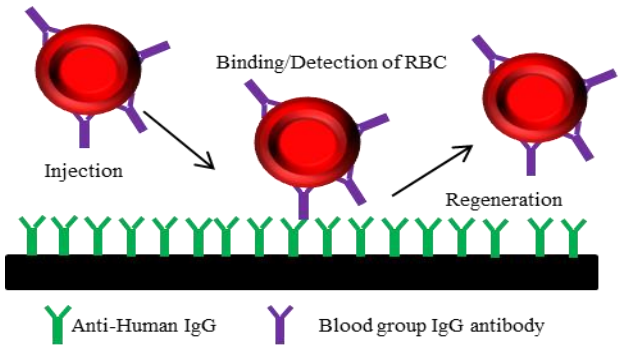

Figure 1 **Diagrammatic representation of immobilising anti-human IgG upon a chip surface for the quantitative blood group typing using SPR.**

3.5.1 Functionalisation

Anti-human IgG Fc (Clone: CBL101) antibodies were immobilised onto the CM5 sensor chip surface using EDC/NHS amine coupling. The maximum detection limit is defined by the amount of anti-human IgG immobilised as evidenced by the differing binding responses observed between sensor chip preparations (Supplementary Table S1). The achieved binding response was approximately 10,500 relative units (RU). In general, 100 RU corresponds to an adsorption of 100pg/mm^2 [10]. RU is measured from a change in refractive index which corresponds to a variation in mass adsorbed onto the chip surface. It does not determine specificity of blood group type but rather detects the presence of RBC antigen-antibody interaction. The CBL101 clone showed strong binding with fully regeneration using single 1 min pulses of 3M $MgCl_2$. Little to no degradation of the anti-human IgG monolayer was observed between each sample run, however, a prolonged use of the same surface showed a slight but steady decrease in the baseline, usually occurring after more than 100 regenerations.

A series of controls were tested to ensure that no non-specific binding was occurring (Supplementary Table S2). These controls included the injection of: (1) sensitized RBCs

and (2) anti-D IgG FFMU over a non-functionalised sensor chip (≤20 RU), indicating the necessity of the functionalisation process as neither RBCs nor anti-D IgG was detected without it; (3) non-sensitized RBCs over a functionalised sensor chip prepared in the abtectcell stabilisation buffer (≤30 RU) and (4) prepared in Celpresol LISS (<0 RU), demonstrating the importance of the sensitization process, and that no non-specific binding has been promoted in either the abtectcell stabilisation buffer, in which the reagent RBCs are packaged, and Celpresol LISS by the sensitization process. Surface blocking to prevent non-specific interactions is critical to ensure sensitivity and selectivity, thus avoiding false positive or negative readings.

3.5.2 RhD Blood Group Detection

Reagent red cells (Abtectcells) are commercial red blood cells which have been standardised for the detection of blood groups, including the D antigen, as well as a wide range of other clinically significant blood groups. Each comes with two positive D antigen samples and one negative sample. These cells were first used as a model to develop the methodology and evaluate the selectivity and sensitivity for using SPR for RBC antigen detection. The use of reagent RBCs prevents the tremendous variability expected from human-sourced EDTA blood samples. Three types of reagent RBCs were sequentially injected over the antibody-treated SPR chip and the response signal (RU) was measured as a function of time, forming a sensogram of the RBC injection (Figure 2). Reagent RBCs were used to show the successful and selective detection of the D antigen (Figure 2). The sensograms display the distinct binding differences between positive and negative RBCs against anti-D IgG FFMU. However, due to the large size of RBCs, significant dispersion was observed, as evidenced by the irregular curves (Figure 2), making it unsuitable for conventional kinetic analysis. Efforts to minimize dispersion were ineffective.

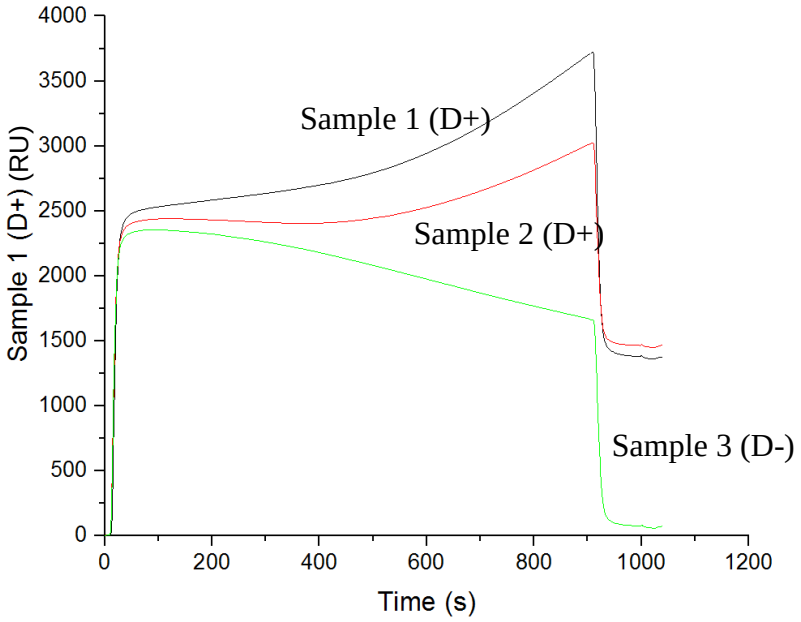

Figure 2. **SPR Sensogram: Injection of RBC at 10% concentration for 15min at 1µL/min. Cells were pre-sensitized and washed 4 times in Celpresol LISS prior to injection.**

Blood group typing using IgG antibodies requires incubation of RBCs with the desired antibody at 37˚C for 30mins. This 'sensitization' procedure is used for blood grouping due to the electrostatic repulsion between RBCs, allowing the small-sized IgG specific antibodies to bind to the RBC antigens by increasing kinetic collision and allowing ample time for binding to occur; no binding occurs with antibody non-specific to the RBC tested. Therefore, incubation of cells with the desired IgG antibody is required prior to injection. Attempts to bind an anti-D IgG to the surface anti-human IgG, and subsequently injecting RBCs over the surface was unsuccessful (Supplementary Figure S1). Sensograms showed very little binding, indicating a false negative. Most likely, the large size of the RBCs combined with their momentum as they flow over the sensor surface hindered binding of the antibody to the RBC antigen. The antibody-antigen interactions are either too weak, or the distance of bioaffinity is too short.

Prior sensitization of the RBCs with the anti-D IgG allowed for binding to the immobilised anti-human IgG, and showed distinct differences between positive (~1500 RU) and negative blood samples (~50 RU) (Figure 2). This is due to the binding of anti-D IgG to the D antigen, making the site more readily available for detection by the anti-human IgG. It is believed that the D antigen, in particular, is integrated within the RBC

membrane, making access to its binding site even more difficult without pre-sensitization [11].

Full regeneration of the sensor surface was achieved using 3M $MgCl_2$, allowing for any bound material to be completely removed with little to no degradation of the sensor surface (Supplementary Figure S1). This is due to the very high ionic strength of the regeneration solution, screening all electrostatic interactions and leaving only van der Waals attraction forces. This observation suggests that electrostatic interactions play a dominant role in the antibody-antigen interactions of RBCs. After over 100 regenerations, the sensor still accurately detected positive and negative blood samples with consistent binding strength. The ability to fully regenerate the sensor surface presents a potential multi-antibody detection technique, particularly with newer models of SPR biosensors, which can be automated and multiple samples analysed simultaneously.

3.5.3 Optimisation

The initial detection study showed the potential of the SPR technique for blood typing, especially using IgG antibodies. However, non-standardised blood samples are expected to be weaker. To ensure the best possible binding strength is observed, the effect of critical variables were tested and optimized. Reagent red cells were also used during the optimisation process, where the experimental buffer, washing, and RBC concentration were varied to determine the best conditions to define standard testing (Figure 3). Optimized injection time and flowrate were chosen as 15min and 1µL/min, respectively. Effects of injection time and flowrate are discussed in the supplementary material section. Using the D antigen as an example, the targeted response for positive detection was greater than 1000 RU, while no more than 100 RU for negative samples was observed (Figure 2). This difference of 900 RU between positive and negative samples ensures good sensitivity and clear identification. One limitation to achieving a standard response for positive and negative antigen interactions is that the binding response between blood samples differs. Differences could even be seen between the two reagent cells which are standardised for detection. Therefore, the key objective

was to achieve distinct responses between positive and negative samples. The binding response of positive samples could vary quite vastly, while negative responses should be maintained below 100 RU.

Binding efficiency during SPR analysis is dependent on factors such as experimental buffer, washing, and RBC concentration. Due to the higher activity rate of IgG antibodies, a temperature of 37°C was used at each stage. The greater mass of the anti-human IgG ligand to the sensor surface therefore increased adsorption of the sensitized RBCs when injected.

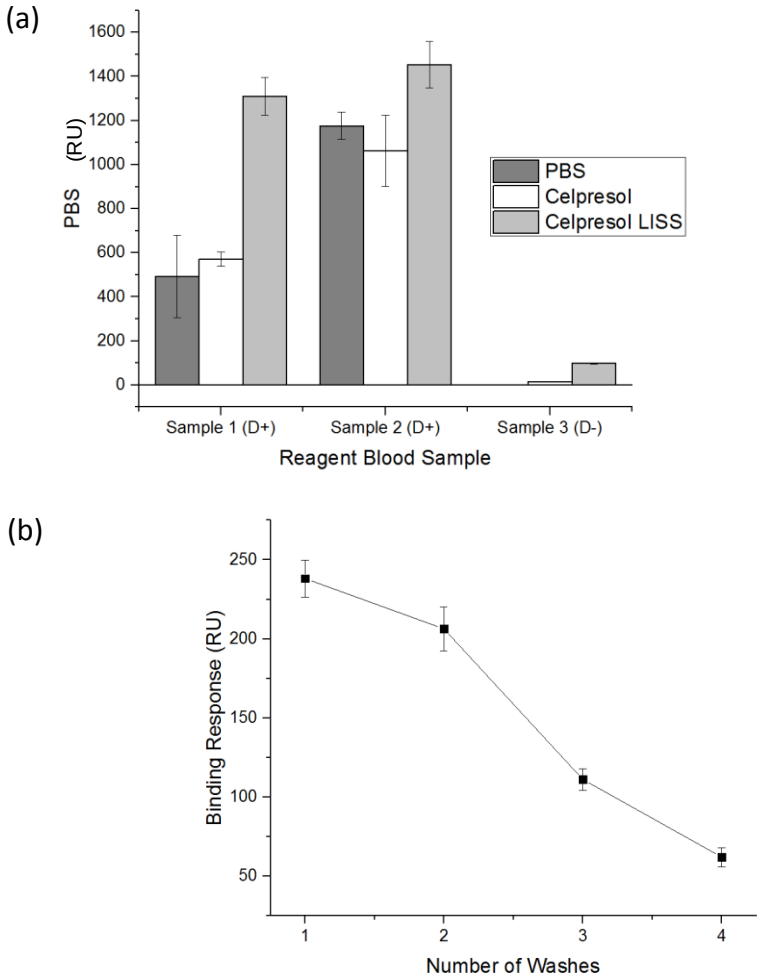

Figure 3. **Detection of the D antigen using different (a) washing buffers, (b) varying degrees of washing for sample 3 (D-). With the exception of the variable tested, each set of experiments were: washed 4 times in Celpresol LISS, and injected for 15 mins at 1µL/min.**

3.5.3.1 Effect of Washing Buffer and Washes

The effect of the washing buffer was shown to be one of the most important factors to effect binding during optimisation (Figure 3a) due to buffer pH and ionic strength. Three buffers were tested and compared: PBS, Celpresol (pH 7.05-7.25), and Celpresol Low Ionic Strength Solution (LISS) (pH 6.7-6.9). When tested using PBS, the binding response for positive D samples was weak, particularly using the Sample 1 D+ RBC. Two other experimental buffers were explored, both specifically designed for testing with RBCs; Celpresol and Celpresol LISS. Celpresol is a cell preservation solution used for long term storage of RBCs. It contains nutrients required to keep cells stable. Celpresol LISS is a similar solution; however, it has a low ionic strength to enhance antibody interactions. D antigen detection using Celpresol had similar results to PBS. Conversely, Celpresol LISS showed markedly higher improvements in binding response for positive D RBCs. While the negative D samples showed a slightly increased RU compared to PBS and Celpresol, the difference compared to the positive D samples was significant enough for blood group determination. This is likely due to the Celpresol LISS changing the isoelectric point of the RBCs and allowing less repulsion between cells and the dextran matrix onto the sensor surface. Celpresol LISS performed significantly better as the washing buffer compared to PBS and Celpresol. This is especially true for Sample 1, where the positive D antigen detection binding response was much greater than double the latter two solutions.

The contrasting binding strength observed between both positive D antigen RBCs is likely due to donor variability. However, further testing showed that the Rh phenotype of a RBC could play a role in the binding efficiency of the D antigen (section 3.4).

The number of times each sample was washed prior to dilution to the desired concentration played a key role in accuracy when comparing negative samples. A reduced number of washes resulted in higher, non-specific binding responses for the negative sample 3. This is likely due to free, unbound antibodies within the supernatant binding to the functionalised sensor surface. Anti-human IgG binds non-selectively to human IgG antibodies; therefore rigorous washing is necessary to ensure all unbound antibody molecules are removed. Four washing steps were subsequently used to

minimize binding of any free unbound IgG antibodies in the supernatant. This is standard practice for current methods using IgG antibodies, including IAT. While still distinctly lower than the binding responses seen for positive samples, more washes ensured less binding of free unbound Anti-D IgG, and higher sensitivity (Figure 3b).

3.5.3.2 Effect of RBC Concentration

Similar to previous studies, testing using varying RBC concentration showed a linear correlation, indicating its potential as a quantitative study for blood group detection (Figure 4). An optimum RBC concentration of 10% v/v was chosen. Lower concentrations resulted in lower binding, while higher concentrations push the limitations of the SPR instrument's microfluidic structure. Whole blood contains approximately 40% RBCs and is quite viscous; high viscosity increases the likelihood of false positives and must be avoided to minimize potential clogging within the microfluidic channels, therefore a lower concentration range was tested.

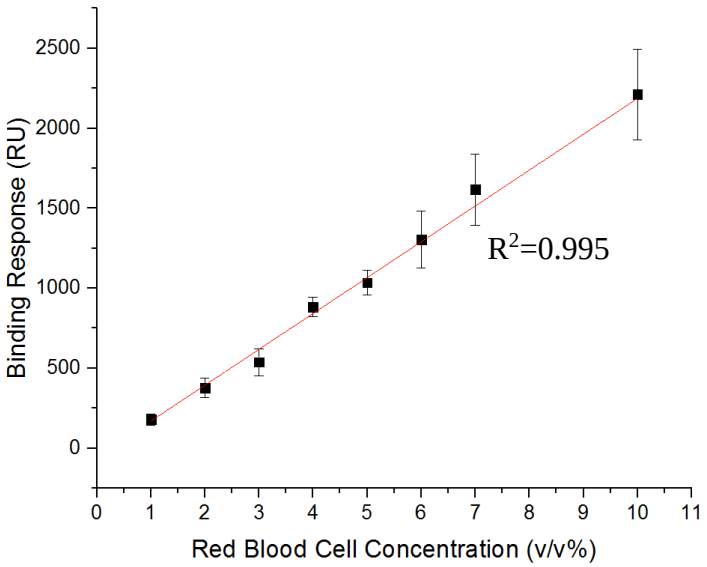

Figure 4. **Detection of the D antigen using different RBC concentrations of reagent sample 1 (D+), washed 4 times in Celpresol LISS. Injected at 1µL/min for 15 mins.**

3.5.4 Validation

Finally, 50 human-sourced EDTA samples were tested to verify the efficiency of SPR for blood group detection (Table 1 and Figure 5). In addition to not being optimized for blood group testing, unlike the reagent red cells, there are other factors affecting the binding efficacy, which show a strong correlation with a patient or donor's genetics. There was a wide spectrum of positive D antigen detection. Binding responses ranged from as low as 530 RU to as high as 1200 RU. This variance in binding strength is a recognizable trend in D antigen detection practice. Interestingly, the binding strength observed for each positive D antigen correlated with the Rh phenotype of that sample. Each Rh phenotype is a category defined by the presence or absence of antigens from the Rh blood group family. These antigens include: D, C, E, c, e [12]. Table 1 describes each Rh phenotype and the average binding response observed. Testing was limited by the constraints of the blood samples available at the time of testing, as such collation of particular phenotypes was more difficult due to scarcity, i.e. the R2R2 phenotype.

Table I. **Effect of Rh Phenotype on binding response when testing for the D antigen using anti-D IgG FFMU. Average binding responses observed for each Rh phenotype shows strong correlation to the number of estimated D binding sites. *Except for the R2R2 phenotype, all testing was completed using human-sourced EDTA samples. Data from R2R2 phenotype testing using reagent RBCs was included only as a reference to its stronger binding response.**

Rh Phenotype	Rh Antigens	No. of Samples	Ave. Binding Response (RU)	Std. Dev.	Estimated D sites per cell [12]
R1R1	DCEce	7	1202	209	14,500 – 22,800
R1R2	DCe	4	1071	248	23,000 – 31,000
R2R2*	DEc	6	1388	98	15,800 – 33,300
R1r	DCce	16	760	187	9,900 – 14,600
R2r	DEce	5	1045	296	12,000 – 19,700
rr	ce	17	78	15	N/A
r'r	Cce	1	53	N/A	N/A

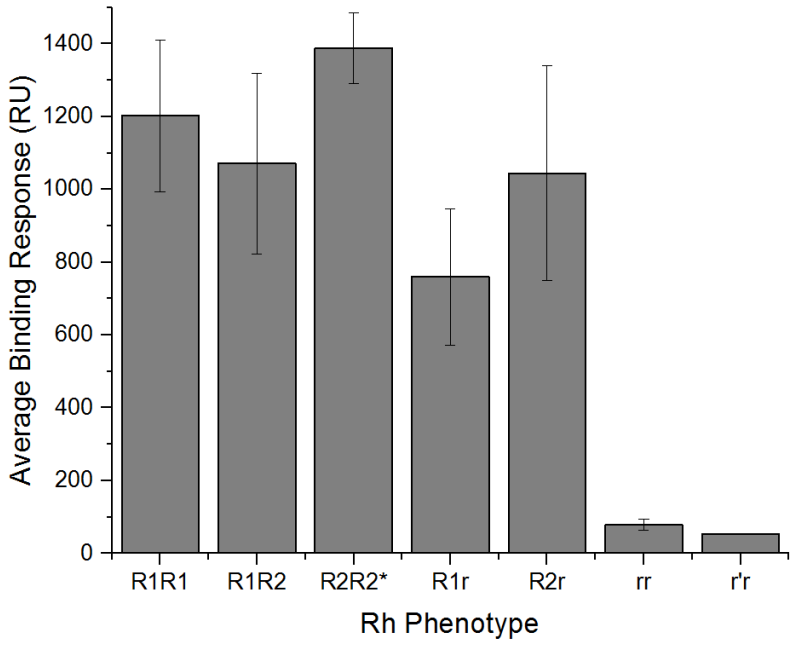

Figure 5. **Effect of Rh Phenotype on binding response when testing for the D antigen using anti-D IgG FFMU. *Except for the R2R2 phenotype, all testing was completed using human-sourced EDTA samples. Data from R2R2 phenotype testing using reagent RBCs was included only as a reference to its stronger binding response.**

Among the three reagent red cells, the Rh phenotype of each sample is R1R1 (1), R2R2 (2) and rr (3). During conventional blood typing, the R2R2 phenotype binds the strongest. This was supported during optimisation where sample 2 always showed a higher binding response. The order of binding strength during testing from strongest to weakest is as follows: R1R1, R1R2, R2r, and lastly R1r. R1r is the most commonly observed Rh phenotype in the general Melbourne population but has the weakest binding capacity. This is why a large number of the human-sourced samples tested appeared not to meet the initial 'standard' criteria for this study. R2R2 phenotypes only comprise approximately 2% of the population, and are therefore rare during random sampling. Data from the reagent red cells was included as reference to show the strong binding capabilities of the R2R2 phenotype (Table 1 and Figure 5). However, due to the standardized nature of reagent cells, it is unsuitable for accurate comparison with the results of the human-sourced EDTA samples tested.

Patient variability offers three possible explanations for the differences observed: (1) number of D antigen sites on the cell surface (2) Rh antigen conformation upon the cell surface, or (3) interference from other biological material, for example, within the plasma.

The most likely explanation for the differing binding strength between Rh phenotypes is the number of D antigen binding sites (Table 1). There is a strong correlation between average binding responses observed to the number of estimated D binding sites. Additionally, the large standard deviation observed for each Rh phenotype could be explained by the large range, and often overlapping, estimation of D antigen binding sites.

Another possible explanation is the conformation of Rh antigens within the RBC membrane. The structure of each antigen is predicted to be closely related, hence affecting the binding ability of anti-D to the D antigen [12].

Since reagent RBC samples are standardised, there is a consistency observed between different batches of reagent RBCs. However, human-sourced RBCs are not standardized, therefore, it is possible that the variation measured could be caused by interference from components in the plasma, cell concentration, condition of cells, etc.

Despite the large variability, binding responses for both D negative antigen Rh phenotypes, rr and r'r, consistently remained below 100 RU. Since the lowest observed binding response for a D positive human-sourced sample was 530RU, there is a distinct difference between positive and negative D antigen detection, indicating the potential to use SPR as a diagnostic tool for quantitative blood group typing.

3.6 LIMITATIONS & PERSPECTIVES

Despite the potential of SPR analysis as a quantitative blood typing technique, current blood typing diagnostics are both simpler and faster, which is more desirable during emergent situations. SPR biodiagnostics are also reliant on expensive equipment and consumables, such as the sensor chip, and are confined to a laboratory environment. Emerging technologies, such as the paper diagnostics for blood typing, are less reliant on equipment and trained personnel [13-15]. The one key advantage of SPR analysis is the ability to quantitatively detect blood groups, which could be especially useful when detecting weaker variants.

RBC detection using SPR is dependent on many factors. However, one of the main concerns is the large size of the RBCs compared to the sensor surface. Large responses that were expected due to the RBCs' large size were not observed. While the RBCs are deformable in nature, the evanescent field of detection using SPR only extends to a depth of 0.3µm from the sensor surface. The size of RBCs far extends this limit, therefore only partially cover the sensor surface. While detection is possible within the field, the partial coverage resulted in decreased sensitivity. RBC size also hinders the ability to monitor binding kinetics, which is of particular interest since there are no studies that have been able to accurately quantify the kinetic mechanisms of blood group detection. However, as prior incubation is necessary for detection, kinetic analyses would be between the anti-human IgG Fc and anti-D IgG FFMU, not the RBC.

3.7 CONCLUSION

This study presents a new methodology for quantifying blood typing interactions using surface plasmon resonance (SPR). An anti-human IgG antibody capable of interacting with any IgG sensitized RBC through anti-IgG/anti-human IgG binding is covalently immobilised to the sensor surface. If positive, the IgG antibody will specifically bind to the RBC antigen sites, which are then retained by the generic anti-human IgG monolayer through binding of the Fc region of the IgG antibody. Negative unbound RBCs are directly eluted. The SPR chip surface can potentially be used as an interchangeable platform to quantify the interactions between RBCs and any IgG antibody.

Using the RhD blood group as an example, a clear distinction between positive (>500RU) and negative (<100RU) D antigen RBCs is achieved with anti-D IgG. Complete regeneration of the anti-human IgG surface is also successful, showing little to no degradation of the immobilised surface after more than 100 regenerations. The technique is also validated using whole human blood samples. An interesting correlation between the binding response and Rh phenotype is reported showing the sensitivity of the technique; it is likely due to the different concentration of D antigen binding sites on the RBC surface.

This novel approach to blood typing using SPR analysis presents an interesting alternative for quantitative blood grouping. This platform has potential as a multi-functional, fully regenerable method for quantifying antibody-antigen interactions for blood group typing using SPR.

3.8 ACKNOWLEDGEMENTS

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Chapter 4

QUANTITATIVE DETECTION OF WEAK D ANTIGEN VARIANTS IN BLOOD TYPING USING SPR

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PREFACE

Following the previous chapter, the surface plasmon resonance (SPR) platform for blood group typing was further developed in this fourth chapter by focusing on weak and partial expressions of the RhD antigen. Compared to normally expressed D antigens, weak D variants have a much lower antigen density, however the antigen structure expressed is the same as of a normal D. Conversely, partial D variants can be expressed within the normal range of antigen density, but the antigen structure is incomplete. While rarer among the population, weak and partial D antigens are still very clinically significant and can elicit haemolytic transfusion reactions if wrongly typed since both weak and partial D variants can appear as false negative reactions. For the first time, this study demonstrates the sensitivity of the SPR platform through positive detection of weak and partial D antigens using human-sourced whole blood samples.

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Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Initiation, key ideas, experimental works, analysis of results, writing up	90%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Marie-Isabel Aguilar	Key ideas, paper reviewing and editing	Supervisor
Gil Garnier	Key ideas, paper reviewing and editing	Supervisor

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature	[redacted]	**Date** 15/02/2016
Main Supervisor's Signature		**Date** 15/02/2016

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4.1 ABSTRACT

Anti-human IgG antibodies were immobilised on a gold sensor surface to detect weak blood group antibody-antigen interactions using surface plasmon resonance (SPR). This sensitivity study used SPR to detect two types of weaker D variants; (1) weak D, and (2) partial D. Positive pre-sensitized cells bind to the anti-human IgG functionalised surface, and the response unit (RU) is reported (>100RU). Unbound negative cells are directly eluted (<100RU). Weak D classed cells were detected between a range of 180-580RU, due to a lower expression of antigens. Cells with partial D antigens, category D VI, were also positively identified (352-1147RU), showing expression similar to that of normal D antigens. The detection of two classes of weaker D variants was achieved using this fully regenerable SPR platform, opening up a new avenue to replace the current subjective and arbitrary methods for quantifying blood group antibody-antigen interactions.

4.2 KEYWORDS

surface plasmon resonance, blood group typing, antibody-antigen interactions, weak variants, IgG antibody

4.3 INTRODUCTION

Mismatching incompatible blood types can lead to a haemolytic transfusions reaction (HTR), the severity of which can range from mild to fatal [1]. Therefore, accurate and reliable blood typing is essential prior to any blood transfusion. Current available blood typing methods are well established. The column agglutination test (CAT), is the most common qualitative technique for blood group antigen identification. However, methods for quantifying blood group antibody-antigen interactions are currently very limited. Quantification is often subjective, relying on the perspective of trained technicians for identification (Figure 1). This can be particularly important when characterising weaker blood group interactions, such as the weak subgroup variants of the D antigen. Weaker agglutination of RBCs are visually categorised from 4+ to 1+, while negative RBCs are categorised as 0. Figure 1 represents how each category is assigned; however, analysis is rather arbitrary and completely subjective.

The RhD blood group is the most clinically significant blood group after the ABO blood system, more commonly denoted as '+' [1]. While the D antigen usually shows strong haemagglutination in the presence of the corresponding D antibody, there are weaker subgroup variants that do not react as strongly or as readily. These interactions can be difficult to identify using traditional testing since blood group typing is dependent on a simple visual analysis, and can be easily overlooked or misinterpreted [2]. While much more difficult to identify because of their partial antigen or minute interactions, these weaker groups are nonetheless as clinically significant, and can stimulate the formation of antibodies in the recipient and can still result in HTR in subsequent transfusions. This represents a major and unresolved concern in transfusion.

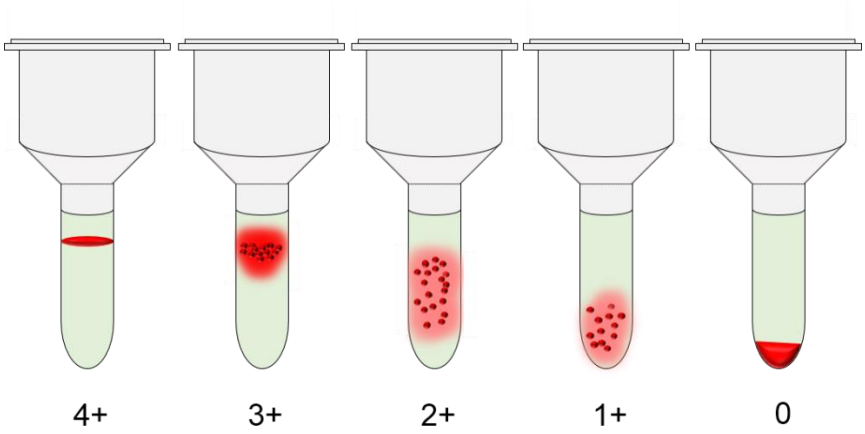

Figure 1. **Illustration of current technique used for rating blood group antibody-antigen binding strength during a column agglutination test (CAT). Strong clear positive samples are visually categorised as 4+, while weaker positive variants can be categorised between 3+ to 1+, with the latter being the weakest; Negative reactions are denoted as 0.**

Currently, there are two methods available for quantitative analysis of RBC-IgG antibody interactions: (1) flow cytometry, and (2) fluorescence microscopy. Both methods require fluorescence which may affect binding. Flow cytometry measures fluorescent-labelled antibodies attached to blood cells in suspension as the cells pass through a laser in single file [2, 3]. Surface plasmon resonance (SPR) holds advantages over these methods as it measures real-time interactions and is label-free; it can also be very sensitive.

SPR has been widely utilised for the detection and analysis of interactions between biomolecules [4-17]. SPR can monitor intermolecular binding events in real time, allowing analysis of the interaction kinetics between biomolecules. Whole cell investigations using SPR are significantly less common [12, 18]. This is because average cell sizes are orders of magnitude larger (8-15µm) than the evanescent field depth (~300nm). Large cells are also unsuitable with the microfluidic system of most commercial SPR instruments as cells can settle or congregate [19]. However, unlike most cells, RBCs are highly deformable in nature to allow for easy vascular transport, which makes its use with microfluidics suitable.

SPR for blood group antigen detection [20] and antibody detection [21-23] has been reported. Quinn et al. (1997) demonstrated the detection of A and B antigens on whole RBCs using SPR by functionalising the sensor surface using the corresponding blood

group IgM antibody. While this method successfully demonstrated selectivity for both A and B antigens, surface regeneration was poor. Harsh regeneration conditions resulted in a loss of antibody/biosensor functionality after a single use. This was due to either the inability to fully desorb bound material or partial removal of the functionalised surface.

In our previous study, the concept of using a generic anti-human IgG antibody to detect antigen positive RBCs was explored using the D antigen as an example [24]. The concept draws from the Indirect Anti-globulin Test (IAT) used to detect blood groups using IgG antibodies with the CAT [3]. As anti-human IgG is able to recognize and bind to the Fc region of human IgG antibodies for detection, functionalising the sensor surface with such an antibody allows for the detection of any human IgG antibody. Therefore, when a positive-RBC is sensitized using an IgG antibody via incubation and injected over the sensor surface, the anti-human IgG are able to detect the sensitized RBCs, and thereby detect the presence of an antigen. Furthermore, the surface was regenerable, showing little to no degradation of the functionalised surface for more than 100 regenerations.

Expression of the D antigen varies greatly from person-to-person, and as SPR is a quantitative technique, a strong correlation with the estimated amount of D antigens expressed and binding response was found. However, sensitivity of this new method for weak and partial antigen detection was not explored. Here, we optimize and validate this new technique using human-sourced blood samples to detect the weakest expressions of the D antigen [24].

There are two main classes of D variants: (1) weak D, and (2) partial D (Figure 2) [1]. Weak D variants still express the full D antigen structure, but at estimated low quantities (Table I). Partial D variants, as the name suggests, only express partially complete D antigens. As the D antigen is comprised of a mosaic of different epitopes, the absence of one or more of these epitopes often results in weak detection using commercial anti-D IgM [25].

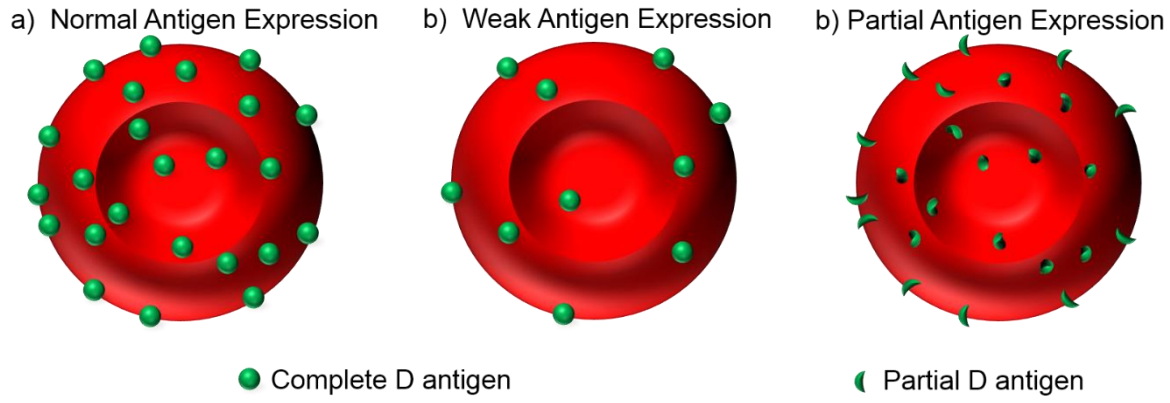

Figure 2. **Schematic representations of the different types of antigen expression on the surface of red blood cells; (a) normal, (b) weak, and (c) partial expression.**

Table I. **Number of estimated D antigen binding sites per red blood cell [26].**

D Antigen	Estimated D sites per cell [26]
Normal D	9,900 – 33,300
Weak D	66 – 5,200
Partial D VI	300 – 14,502

Most partial D antigens are divided into nine categories; however, up to 30 different partial D antigen profiles have been characterised, but are rarely seen [25]. Category DVI is one of the more prevalent partial D antigens. Both weak D and partially D variants are usually detected using the indirect anti-globulin test (IAT), which is a non-quantitative technique[25].

Here, we investigate the potential and sensitivity of SPR to detect and quantify weak and partial antigens for blood typing. The first objective of this study is to explore the concept of functionalising an SPR sensor chip with anti-human IgG to detect RBCs with weak or partial D antigen variants which have been pre-sensitized with anti-D IgG. The second is to determine and optimize the sensitivity of this technique to hopefully provide an attractive alternative that can also quantify the level of interaction. The technique is validated directly with human-sourced blood samples – not reagent blood samples. We aim at developing a reliable, sensitive and quantitative analytical method that can be automated for robust blood typing.

4.4 MATERIALS AND METHODS

4.4.1 Chemicals and Equipment

All chemicals and sensor chips were purchased from VWR International (Brisbane, Australia) unless otherwise stated. Anti-D IgG For Further Manufacturing Use (FFMU) antibodies and polyspecific anti-human globulin (AHG) antibodies were supplied by Quotient EU (Edinburgh, United Kingdom). Abtectcell red blood cells (RBCs), Celpresol and Celpresol Low-Ionic Strength Solution (LISS) were supplied by CSL Limited (Melbourne, Australia). Anti-human IgG Fc (Clone CBL101) was purchased from Merck Australia (Melbourne, Australia). EDTA blood samples were sourced from the Australian Red Cross Blood Service (ARCBS) (Melbourne, Australia), stored at 4˚C; samples were analysed within 7 days of collection. The BIAcore X system (GE Healthcare, Uppsala, Sweden) was used for all analyses.

4.4.2 Methods

Amine coupling was used to immobilise anti-human IgG Fc upon a CM5 SPR chip surface. Each CM5 chip contains a medium molecular weight carboxy methyl dextran layer grafted onto a gold surface. The surface was first activated by injecting an equal volumetric mixture of 100mM N-hydroxysuccinimide (NHS) and 400mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) at 5 µL min^{-1} for 7 min. Both NHS and EDC solutions were made with distilled water before use, aliquoted and stored at -15˚C until required. Anti-human IgG Fc (0.5 µg mL^{-1}) ligand was dissolved in 10mM sodium acetate buffer and then injected over the functionalised surface for 6 min at 5 µL min^{-1}. Unreacted sites were blocked by 1M ethanolamine-HCl, injecting at 5 µL min^{-1} for 7 min. The achieved binding response of anti-human IgG Fc was approximately 9,700 relative units (RU).

During these experiments, reagent red cells (Abtectcells III 3%) and human-sourced red cells were used. Reagent red cells are standardised human red cells which have been

washed and kept in a cell stabilisation solution with a constant concentration. Human whole blood was collected from donors by the ARCB, and stabilized with Ethylenediaminetetraacetic acid (EDTA); these human-sourced cells were washed using a cell preservation solution, Celpresol, upon receipt to for stabilisation and to remove plasma, and diluted to 3% solution using Celpresol. These cells have not been standardized.

RBCs were incubated with excess anti-D IgG FFMU for 30 min at 37˚C (1:1 by volume) to sensitize the cells. After sensitization, the cells were washed 4 times using Celpresol LISS and centrifugation before dilution to 10% concentration in Celpresol LISS for injection into the BIAcore X system.

Temperature was kept constant at 37˚C to maximize binding of IgG antibodies which are optimal at this temperature; the human body temperature. A single flow cell detection mode, flow cell 2, was used for binding analysis. Sensograms were analysed with standard BIAcore X control software. Regeneration of the chip surface was completed using 3M $MgCl_2$ at 1 min pulses at 1 µL min^{-1}. The general purpose running buffer, HEPES buffered saline (HBS-EP), was used throughout all experiments.

While weaker D variants are comparatively rare, the most commonly seen human-sourced weaker D variants are weak D (D^{w+}), and the partial D variant, category DVI. Validation of the quantitative detection of these variants was subjected to the availability of human-sourced blood.

Quantification of binding strength for weaker D variants are completely subjective, relying on the analysis of trained personnel. The SPR results were validation with two traditional quantification methods of RBC-antibody interactions using test tubes. First, each blood sample was incubated with anti-D IgG FFMU at 37˚C for 30 mins. Once sensitized, the RBCs were washed vigorously with PBS solution using a Bio-rad Diacent-CW-12mm cell washer. Finally, a drop of AHG was added to facilitate agglutination. If positive, clumps (or agglutinates) of RBCs were visible with varying degrees of binding strength and categorised from 4+ to 1+. If negative, no visible binding had occurred and the sample was thus categorised as 0. Each data point on *each* of the graphs comes

from two separate blood preparations, and each preparation was tested three time, therefore providing six replicates. The average and standard deviations are reported.

4.5 RESULTS & DISCUSSION

A regenerable platform for weaker D variant blood group detection was explored using SPR. The concept is schematically represented in Figure 3. A CM5 sensor chip was functionalised with a monolayer of anti-human IgG prior to injection of anti-D FFMU sensitized RBC concentrated to 10% by volume. If positive, the anti-D FFMU selectively binds to the D antigen sites, allowing the Fc regions of the IgG antibodies covering the RBCs to be detected by the generic anti-human IgG immobilised onto the sensor chip surface. Negative RBCs will not bind and are directly eluted. This distinction for weak D and partial D sensogram is shown in Figure 4. The key question which must be addressed to validate this concept is whether the SPR is sensitive enough to detect and quantify the antibody-antigen interactions of weaker D variants. Each sample was prepared twice, and tested three times during this study to show reproducibility and reliability. Reagent red cells are commercially standardised for the detection of various clinically significant blood groups, including the D antigen. Each set of reagent red cells comes with two positive D blood samples, with varying expression of D antigen sites, and one negative D sample. The three reagent red cells (Abtectcells) were used as a reference compared to the weaker D variants. In total, 3 human-sourced blood samples were tested; 17 weak D variants, 11 partial D category DVI variants, and 6 negative D cells were used as controls.

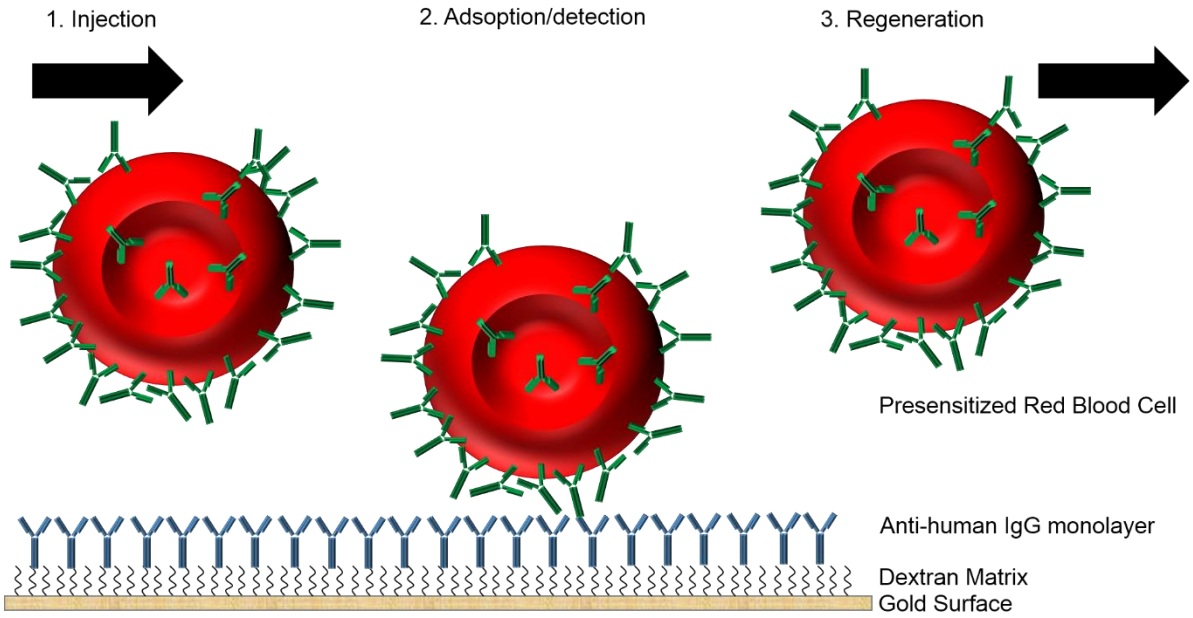

Figure 3. **Schematic representation of the immobilisation of anti-human IgG onto a chip surface for the quantitative detection of positive blood group antigens on red blood cells (RBC) using SPR. (1) A pre-sensitized RBC is injected over the functionalised surface; (2) if positive (shown), the blood group IgG and anti-human IgG will bind during the adsorption phase and detection will occur through SPR; (3) regeneration of the surface using 3M $MgCl_2$ desorbs bound RBCs. Negative cells will be directly eluted.**

Each SPR sensogram from Figure 4 presents four distinct regions of the RBC injection over the sensor surface: (1) a rapid initial rise, corresponding to the introduction of the RBC sample into the SPR detection zone over the functionalised surface. This is caused by the differing refractive index of the RBCs in Celpresol LISS compared to the running buffer, HBS-EP; (2) a pseudo plateau representing the RBC flow over the detection zone at steady state; (3) a rapid decrease region, once the RBC injection ends, illustrating return to the running HBS buffer, and (4) a plateau indicating the final amount of RBC retained over the surface. The difference between the region 4 plateau and the baseline prior to region 1 represents the amount of sensitized RBCs adsorbed upon the functionalised surface, quantified as a binding response (RU). The pseudo plateaux of region 2 varies among samples and represents the reversible adsorption kinetics of RBCs over the surface. Here, the diameter of the analyte probed (RBC, d= 7 µm) is an order of magnitude higher than the thickness (0.3 µm) and two orders or magnitude shorter than the length (L= 1800 µm) of the SPR detection zone. While only 1/20 of the

RBC is detectable by SPR, up to 200 cells can be present at any time in the micro-fluidics capillary, therefore producing a good statistical value. However, these unique conditions of macroscopic cell analysis by SPR explain the unsteady rise/ decrease observed in region 2, which differ from the traditional plateaux observed with small analytes.

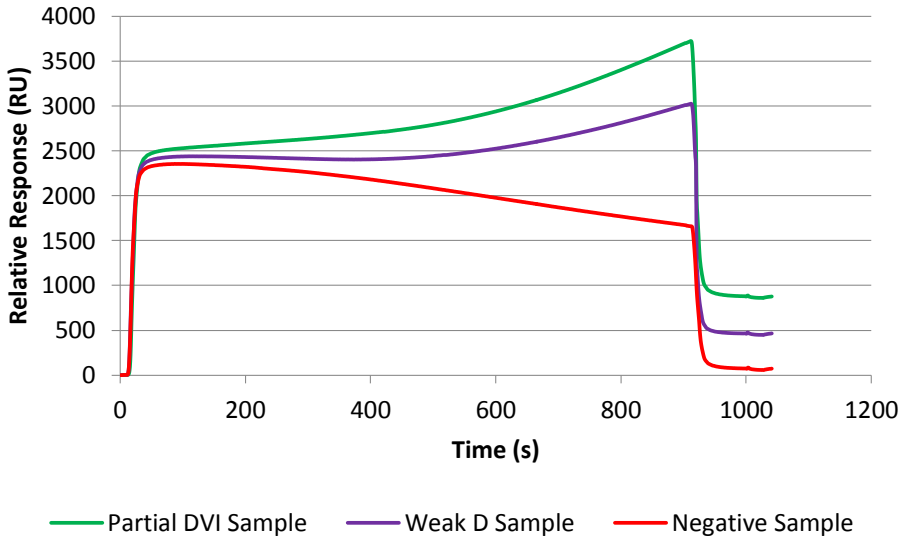

Figure 4. **SPR sensogram presenting the injection of RBC over a IgG antibody functionalised sensor surface, and the difference in relative binding response for a partial DVI sample and a weak D sample compared to that of a negative sample. Pre-sensitized RBCs were washed 4 times prior to being injected at 10% concentration (v/v) in Celpresol LISS at a rate of 1µL/min for 15 min.**

4.5.1 Detection of Weak D Variants

The weak D phenotype is a result of less antigen sites present on the RBC surface (Figure 2b)[26]. As with the D phenotype, the weak D antigen consists of all the D epitopes; however, they are expressed weakly. Also similar to the D phenotype, the estimated number of antigen sites on weak D cells is highly variable within the population, congruent with the findings of this study (Table I).

In traditional blood typing, quantitative analysis for RBC antigen-antibody interaction is observed and rated on a scale from strongest to weakest by 4+ to 1+, respectively (Figure 1). This is achieved through basic visual analysis by trained personnel comparing the size of agglutinates formed in the presence of antibodies; the results are empirical.

As the D antigen sites upon the RBC can be measured quantitatively, this scale also very roughly indicates the approximate number of potential binding sites.

Comparing this scaling system with the results observed from SPR analysis, there was a general trend correlating the strength of binding for each group of samples. RBC incubated with their specific IgG antibody are retained on the anti-human IgG functionalised SPR chip. The SPR detection signal is proportional to the quantity adsorbed and the RU value at the end of the adsorption phase (900s) was measured and plotted for each sample in Figure 5. A large variance between the binding responses for weak D cells was observed with RBC adsorption ranging from 180-580RU (Figure 5). This is consistent with the previous study, where 'normal' D antigens on human-sourced cells were detected between a range of 530-1200RU[24]. We propose that responses below 600RU can be considered weak. Furthermore, all the negative D samples tested showed binding responses below 100 RU, which is also consistent with previous testing. Three reagent cells, two D-positive and one D-negative, were included for comparison. As shown in Figure 5, the adsorption signal of D-positive cells can be very strong compared to that of weak D, but importantly, SPR analysis is sensitive enough to distinguish between all weak D and negative samples; there is no overlapping of binding response.

Weak D samples categorised with 3+ binding strength showed the strongest binding overall among all the samples. The lowest binding responses were observed for weak D 1+ samples, as categorised by the test tube method, with detection as low as 181 RU. There were two exceptions among the 15 samples that did not conform; one 2+ sample and one 1+ sample.

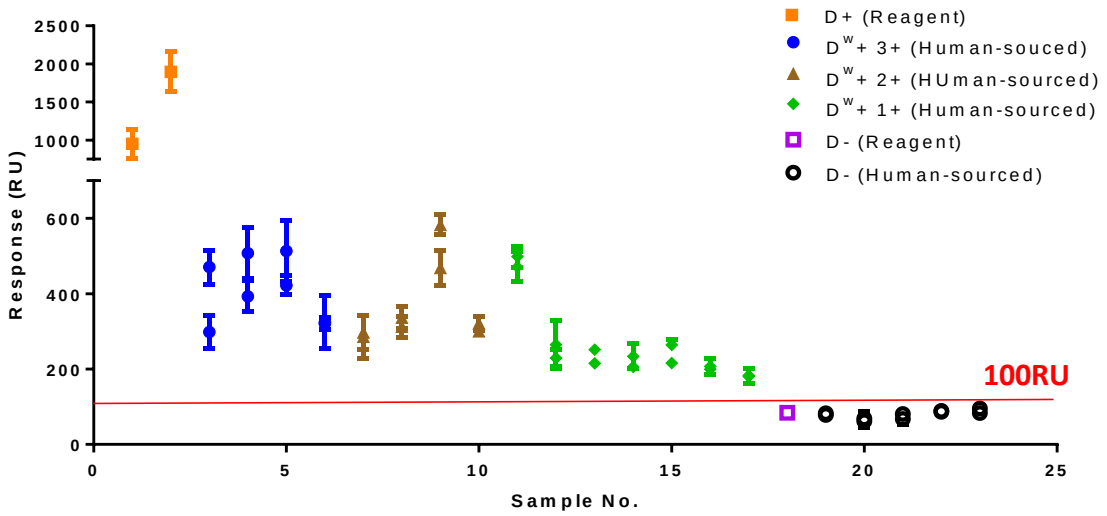

Figure 5. **SPR detection of 15 weak D antigens on human-sourced samples using anti-D IgG FFMU compared to 2 D-positive reagent cells, and 6 D-negative samples (1 reagent, 5 human-sourced). Each sample was prepared twice, and each preparation was tested three times; the average and standard deviation are plotted. *Discontinuous Y-axis was used to better show the detection variance between weak D samples. RU measured at t=900s after injection.**

This variability could be indicative of the weak D genotype as there have been numerous individual types of weak D documented. These are indistinguishable from each other unless the donor's DNA is genotyped, but the estimated number of antigen sites for each genotype exist with a range of variability [26]. Another more likely possibility could be the very subjective nature of visual categorisation for binding strength using traditional methods. Many factors play into the role when analysing the 'strength' of the interactions using the traditional method (Figure 1), a method which is conducted by the naked eye. For example, antibody type used (manufacturer and clone), incubation and detection method, reaction time, and individual technique of the analyst. Simple differences such as how rigorously a test tube is shaken during analysis will affect the size of agglutinates observed; too little can appear stronger, too much and analysis can appear weak. Time of contact is another poorly controlled variable. This is where the benefits of detection via SPR microfluidics lies. Two major advantages of SPR analysis for detection and binding strength quantification are the controlled conditions (time, temperature, shear, concentrations) and the completely objective results.

There are three possible explanations for the high variability of certain results. A first – and by far the most likely – is that it reflects the important RBC antigen density variability among the population (Table I). A second explanation is a possible lack of – or over – sensitivity of the SPR technique. As the SPR is a concentration-dependent technique, any fluctuations in the concentration of a sample could result in an apparent deviance in binding strength. A third explanation for some large variability is due to the SPR microchannel having a limited field of detection from the gold surface (0.3µm). The comparatively large size of RBCs (6-8µm), as well as its biconcave shape, could play a role in the amount of adsorbed mass within the detection field, especially given that binding can theoretically occur at any location on the surface of each RBC. The highly deformable nature of the RBCs travelling through the microfluidic channels (L=1800µm) of the Biacore X for analysis could result in non-uniform shapes and orientation of the RBC when adsorption occurs. However, it was shown in the previous study that the SPR signal provided by the base of the RBC is directly proportional to the RBC concentration, and therefore the RBC orientation and whether the whole cell is in the detection zone is irrelevant.

4.5.2 Detection of Partial D Variants

Partial D variants are D antigens with one or more D epitopes missing from its structure (Figure 2c); however, the estimated number of antigen sites is similar to that of cells with the normal D-antigen, and therefore can vary greatly. The partial antigenic structure can lead to extremely weak binding using conventional IgM antibodies, often seen as false negative. However, detection of partial D variants is possible with the indirect anti-globulin test (IAT) using IgG antibodies and anti-human IgG. Among the partial D variants, there are 9 categories, all of which are rare in the human population. However, some are rarer than others. Category D VI (DVI) is one of the more common partial D variants observed. As testing was constrained by the availability of blood samples, 11 human-sourced DVI blood samples were tested in this study (Figure 6).

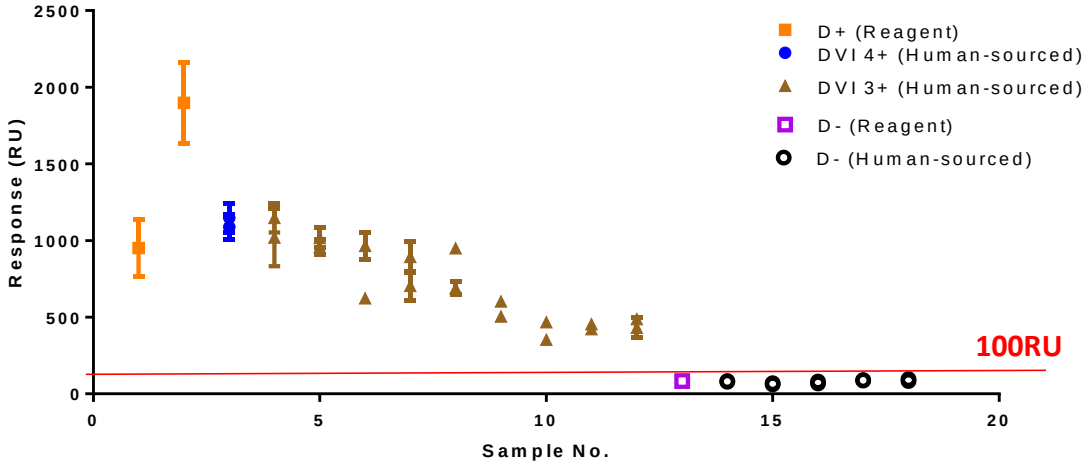

Figure 6. **SPR detection of 10 partial D antigens on human-sourced samples using anti-D IgG FFMU compared to 2 D-positive reagent cells, and 6 D-negative samples (1 reagent, 5 human-sourced). Each sample was prepared twice, and each preparation was tested three times; the average and standard deviations are plotted. RU measured at t=900s after injection.**

There was a clear distinction between positive and negative cells. The general trend of binding response was consistent compared to those cells categorized as 4+ or 3+ using the traditional test tube IAT. 4+ samples showed the strongest binding response, while the responses of 3+ samples were slightly decreased. Also, the responses of 3+ samples of the DVI variants were similar to that of the 3+ sample of weak D variants. The binding responses observed for DVI samples was similar to that of a normal D cell. In fact, the highest DVI sample response was greater than that of the first control for normal D cell, further supporting the correlation between binding response and the number of antigen binding sites.

Similar to previous testing, there was some deviation in binding response among the samples between individual preparations tested for each sample. However, the observed variability between runs for each preparation was noticeably less than for the weak D samples, and even the control samples. As a general observation, cells with higher binding responses showed greater variability than those with lower binding responses. An explanation for this phenomenon could be due to the number of binding sites available in combination, once again, with the shape of the RBCs. A greater number

of binding sites may also increase the possible conformations RBC adsorption to the sensor surface, accounting for the variability observed.

4.5.3 Perspectives

SPR analysis as a quantitative blood typing technique has great potential. While current blood typing techniques are both simpler and faster for normal antigenic expression, detection of weaker blood group variants are more limited and currently still require incubation prior to analysis as they can only be detected using IgG antibodies. Partial D is only one example of such antigen. Others examples include antigens such as Duffy A and B (Fy^a, Fy^b) [25]. Even though SPR analysis is reliant on equipment and expensive consumables, and is confined to a laboratory, the key advantage of this technique is its ability for quantitative detection of blood groups, especially for weaker variants as shown.

Nonetheless, the ability to quantitatively detect both weak D and partial D variants using SPR does hold promise for replacing the current subjective and arbitrary methods of quantifying antibody-antigen interactions for blood groups. Trends can easily be seen when comparing between, and even among, the weak D and partial D VI samples tested in this study. As weak D cells have a decreased number of binding sites, the range of binding observed was much lower than that of the DVI samples and correlated with categorisation using traditional methods. For an accurate comparison of the apparent binding strength and the number of antigen sites on a cell, a correlation study with the known techniques, such as flow cytometry and fluorescence microscopy, could be explored. The concentration-dependent nature of SPR could produce a standardised curve where the number of antigen sites could be accurately discerned from the binding strength observed.

Although the overlap of the estimated number of D sites per cell between weak D and partial DVI variants is substantial (Table I) and would negate the ability for distinction between the two, the practiced method for distinguishing phenotype of D variants in a clinical setting is not through detecting the number of D antigens upon the RBCs. Instead, D variants, in particular partial D variants, are detected using specialised

antibody kits which contain varying types of D antibodies which show a unique binding pattern for each partial D variant. Using the SPR for specific detection of partial D variants could be further explored through the application of these specialised antibody kits. Clinically speaking, however, the type of D antigen, whether it is a normal, weak or partial variant, is not as significant as the actual detection of the presence of a D antigen.

Despite variability between preparations for some samples, especially given the smaller scale of weak D responses, all weak D responses observed were above the 100 RU threshold for D negative samples. This 100 RU threshold provides a good, clear distinction between positive and negative during analysis, particularly when differentiating between weak and negative blood groups.

There were occasional inconsistencies between sample preparations, or between individual tests from the same preparation; this is likely due to the large size and shape of the RBCs which far extends the limited field of detection from the sensor surface (0.3µm). Additionally, the sensor surface is only partially covered within the detection field. These critical factors affect whole cell detection and result in decreased sensitivity [20]. A longer microfluidic channel and SPR detection zone could be beneficial.

4.6 CONCLUSION

We have presented a sensitivity study for blood group detection using surface plasmon resonance (SPR) through functionalisation of a gold sensor chip with anti-human IgG. Anti-human IgG can potentially interact with any IgG sensitized RBC by the binding of the Fc region of IgG antibodies. Using amine coupling, anti-human IgG antibody was covalently immobilised to a gold sensor surface. RBCs are pre-sensitized through incubation with a known IgG antibody (i.e. anti-D IgG). Positive pre-sensitized cells will bind to the anti-human IgG monolayer. The change of mass detected and reported as a response unit (RU) (>100RU). Unbound negative cells are directly eluted (<100RU). The surface is then regenerated in preparation for a new sample.

The variability measured between different red blood cell samples is substantial. However, strong evidence suggests that this variability simply reflects the wide range of antigen concentration found among the population. Some binding interactions are

strong and easily discernible. However, there are weaker variants that can be more difficult to detect. In this study, the binding response and sensitivity of the D variants, weak D and partial D, were explored. Weak D classed cells were detected between a range of 180-580RU. This is due to the lower expression of antigens upon the surface of the cells. Cells with partial D antigens, category D VI, were also positively identified (352-1147RU), showing stronger expression similar to that of normal D antigens. Using SPR, the detection of two classes of weaker D variants was achieved with this fully regenerable platform using SPR, adding another level of sensitivity to previous studies. This opens up great new potential for identifying, quantifying and classifying blood group antibody-antigen interactions using controlled conditions and completely objective results. Furthermore, newer models of SPR biosensors can be automated and multiple samples analysed simultaneously, allowing for a quantitative, multi-antigen blood typing platform.

4.7 ACKNOWLEDGEMENTS

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Chapter 5

DUFFY BLOOD GROUP (FY^a & FY^b) DETECTION USING SURFACE PLASMON RESONANCE

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PREFACE

The most widely known blood groups are ABO and RhD. As the anti-D IgG used in the previous two chapters exhibits extremely potent and strong binding interactions with its D antigen counterpart, this chapter extends the use of surface plasmon resonance for blood group typing to demonstrate the surface plasmon resonance (SPR) platform as potential for multiplex and quantitative detection. The Duffy blood group system exhibits much weaker bioaffinity in laboratory practices. This study focuses on the analysis and detection of the Duffy blood group, Fy^a and Fy^b, using a SPR sensor surface functionalised using anti-human IgG. Both Fy^a and Fy^b blood groups are expressed at a comparably lower density than that of the RhD blood group. With varied success, this chapter explores the potential and limitations of this SPR platform for the detection of the weaker Fy^a and Fy^b blood groups.

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Monash University

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution
Initiation, key ideas, experimental works, analysis of results, writing up	90%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Marie-Isabel Aguilar	Key ideas, paper reviewing and editing	Supervisor
Gil Garnier	Key ideas, paper reviewing and editing	Supervisor

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature	[redacted]	**Date** 15/02/2016
Main Supervisor's Signature		**Date** 15/02/2016

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5.1 ABSTRACT

The most widely known blood groups, ABO and RhD, have been extensively observed as having strong interactions during blood typing. However, not all interactions show the same binding affinity. The Duffy blood group system, where Fy^a and Fy^b antigens are the most clinically significant, are only available with an IgG antibody structure, and display weak binding interactions. While current blood typing techniques are well established, quantifying the binding strength is more limited.

Surface Plasmon Resonance (SPR) could provide avenues for developing more robust detection methods, or can be used as a sensitive quantification technique by itself. Anti-human IgG antibody immobilised upon a SPR sensor surface to detect antigen positive RBCs was achieved previously using the RhD blood group as an example. It was the objective of this study to test SPR for the detection of the weaker antibody-antigen interactions of Duffy blood groups, Fy^a and Fy^b.

This was achieved with varied success. This study showed a threshold of antibody concentration is required for successful detection. Some instances of detection was successful using concentrated commercial anti-Fy^a and anti-Fy^b solution during the incubation stage. However, these results were not reproducible, indicating significant dissociation of the Duffy antigen-antibody complex over time. Attempts to stabilize this antibody-antigen interactions using rapid antibody medium (RAM) and pegylated-RAM (RAMPEG) were also unsuccessful. This indicated that a combination of factors affect the detection of the Duffy antigens using SPR; antibody concentration, antigen expression, antigen structure result in weak and unstable antibody-antigen interactions which are currently unsuitable for SPR detection. Despite these disadvantages, detection of Duffy antigens Fy^a and Fy^b was demonstrated using SPR; however, further exploration is required for robust clinical blood typing.

Keywords: surface plasmon resonance, blood group typing, antibody-antigen interactions, weak variants, IgG antibody

5.2 INTRODUCTION

The success of human blood transfusions heavily relies on accurate and reliable methods for blood typing. The mismatching of incompatible blood types in blood transfusions can lead to immune mediated haemolytic transfusions reactions (HTR) which have side effects ranging from mild to severe, and can even be fatal [1]. The most widely known blood groups are the ABO and RhD systems, where the latter is commonly recognised by its "+" or "-" notation. However, there are currently 36 recognised blood group systems, with greater than 300 blood group antigens [2]. Many of these 300 antigens are clinically significant and can result in HTR if incorrectly typed. Therefore, the detection and identification of the presence of any antibodies and antigens in the blood is crucial prior to transfusion. Antibody-antigen interactions within the ABO and RhD system have been extensively observed as strong, potent reactions during laboratory practice. However, not all interactions show the same binding affinity, especially when IgG antibodies are involved. Serological antibodies for the Duffy blood group system, where the two most clinically significant antigens are Fy^a and Fy^b, are only available with an IgG structure, and display some of the weakest binding interactions among the blood groups tested in the blood banking industry.

Current blood typing methods are well established, most of which rely on the principles of haemagglutination; the formation of red blood cell (RBC) aggregates in the presence of antibodies matching the antigens present on the cell surface. Currently, the most prevalent qualitative method for blood group antigen identification is the column agglutination test (CAT). However, there are very few blood typing methods capable of quantifying the antibody-antigen interactions involved in blood typing.

The current most prevalent method for quantifying the binding strength of antibody-antigen interactions is a rudimentary categorisation of the strength through visual analysis. The 'size' and number of aggregates is arbitrarily valued on a scale from 4+, being the strongest, to 1+, the weakest, while 0 is negative. This quantification is subjective to analysis by trained technicians. It is empirical and not very sensitive.

Two further methods available for quantitative analysis of interactions between RBC antigens and their corresponding antibodies are fluorescence-assisted cell sorting (FACS), and flow cytometry. Fluorescence-conjugation is required for both of these methods, which can affect binding. FACS analysis uses tagged antibodies that emit a brightly fluorescent colour which can be visualised by light detection microscopy at a specific wavelength. Similarly, flow cytometry also relies on fluorescent-labelled antibodies that are attached to blood cells, and measures the light emitted as the cells pass through a laser in single file [3, 4]. Both methodologies also suffer from a time-delayed analysis as interactions are not measured in real-time. Instead, the antibody-antigen complexes react prior to detection of the fluorence using FACS or flow cytometry. These methods are typically used to quantify the number of antigen sites expressed on the surface of the RBCs, and have capabilities for kinetic analysis. However, due to the time-delayed nature required when using fluorescence, interaction analysis is limited.

Surface plasmon resonance (SPR) holds advantages over these methods as it measures real-time interactions and is completely label-free; it can also be very sensitive [5]. This is especially useful for the detection of weak antibody-antigen interactions, such as those observed within the Duffy blood group system.

As SPR is readily capable of monitoring intermolecular binding events in real time and allowing for the interaction analysis of the kinetics between biomolecules, this technique has been widely used for biomolecular detection and analysis [6-19]. SPR Investigations involving whole cell analysis, however, are significantly less common due to cell sizes being considerably larger (8-15µm) than the depth of the SPR's evanescent detection field (~300nm) [14, 20]. Similarly, cells can also be challenging for analysis due to the restrictions born from the close design of the microfluidic systems that are coupled with most commercial SPR instruments. As cells can settle or congregate due to their large size, movement through the microfluidic channels could be impeded, or could even damage the cells, influencing the binding interactions [21]. However, RBCs are unique in that their key physiological function is to transport oxygen and carbon dioxide throughout the body. This ability to navigate through the circulatory system, especially micro-vessels within the capillary network (5-10µm), is reliant on the highly

deformable nature of the RBC membrane structure. This flexibility allows for the suitable use of RBCs with microfluidics.

The use of SPR for blood group antigen [22] and antibody [23-25] detection has been previously reported. One study achieved A and B antigen detection upon whole RBCs with SPR by using a sensor surface functionalised with the corresponding blood group IgM antibody. However, despite successfully demonstrating the selectivity for both A and B antigens, the necessary harsh surface regeneration conditions resulted in a loss of antibody functionality after a single use. This was due to either the inability to fully desorb bound material or the partial removal of the functionalised surface. This poor regeneration reduced the subsequent performance of the biosensor surface.

In our previous studies, an alternate concept using a generic anti-human IgG antibody to detect antigen positive RBCs was explored, using the D antigen as an example [26]. Selected weak and partial D variants, where the expression of the antigen is significantly reduced or structurally incomplete, respectively were also explored [27]. The concept emulated that of the currently used typing method for blood groups where only IgG antibodies are available for detection, the Indirect Anti-globulin Test (IAT) [4]. Anti-human IgG is able to identify human IgG antibodies, allowing for detection by binding to the Fc region of the antibody structure (Figure 1a). By functionalising the sensor surface with anti-human IgG, theoretically, any type of human IgG antibody can be detected. For instance, when a positive-RBC is incubated with a corresponding IgG antibody, this pre-sensitizes the cell with said IgG antibody. When this RBC-IgG antibody complex is injected over the sensor surface, the anti-human IgG is capable of detection through binding of the Fc region of the IgG antibody, and thereby showing the presence of the target antigen (Figure 1b). Additionally, this surface showed full regeneration, with little to no degradation of the functionalised surface following more than 100 regenerations [26, 27].

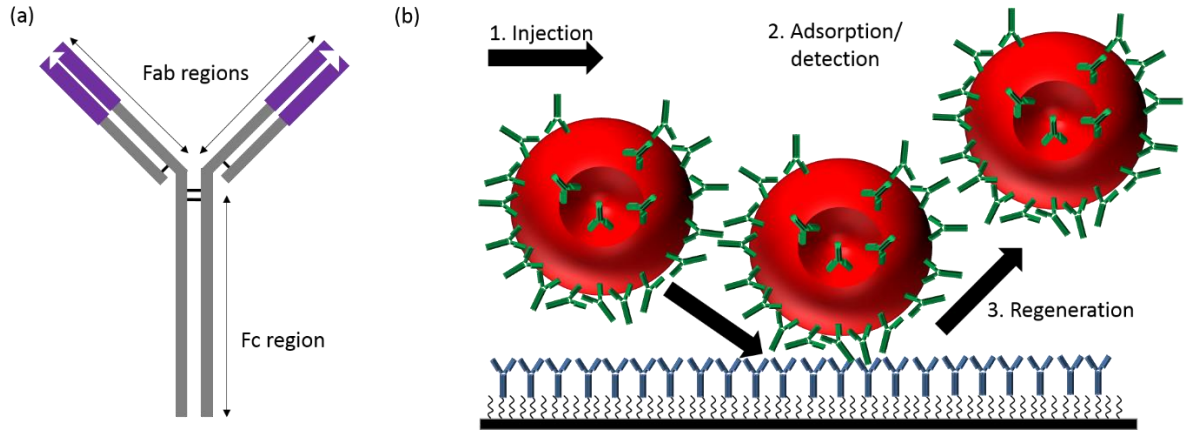

Figure 1: **Representation of (a) the IgG antibody structure, including the Fab regions which are highly variable and responsible for antigen binding, and Fc region which is non-variable and is key to the functionalisation of the SPR sensor surface depicted in (b) where anti-human IgG Fc is immobilised, which specifically binds to the Fc region of any human IgG antibody. (1) Pre-sensitized RBCs are injected over the anti-human IgG immobilised surface; (2) if positive (shown), and anti-human IgG will bind to the Fc region of the blood group IgG antibody for detection; before (3) surface regeneration using 3M $MgCl_2$ desorbs bound RBCs. Negative cells are directly eluted [26].**

Among the D-antigen classes (normal, weak and partial), the expression of the antigen varies greatly, and as the SPR technique is sensitive to concentration, a strong correlation was observed between the estimated number of D antigens expressed and the SPR binding response. The incomplete structure of the partial variants did not appear to affect this correlation. Also, previous studies have shown an almost linear relationship between RBC concentrations with the observed binding response [26].

Blood group tying investigations involving SPR have focused on identification and sensitivity where the antigen, itself, was the main variable. However, not all blood group antigens have the same structure, function or binding affinity to its corresponding antibody. The Duffy system antibody-antigen interactions are notoriously weaker when tested in industry, especially when compared to the RhD blood group. Duffy blood groups express comparatively less antigens than the RhD blood group. Current quantification methods are not very sensitive when testing for Fy^a and Fy^b antigens. This is especially true when the Duffy blood groups are analysed with the traditional

categorisation technique, where binding presents at a strength of 3+, at most. This poses severe limitations when analysing the binding strength of these weak Fy^a and Fy^b interactions. Using SPR could provide avenues of developing more robust detection methods, or can be used as a sensitive quantification technique by itself. Therefore, it is the objective of this study to apply the SPR technique for the detection of the much weaker antibody-antigen interactions of the Duffy blood groups, Fy^a and Fy^b.

5.3 MATERIALS & METHODS

5.3.1 Chemicals and Equipment

Chemicals and sensor chips were purchased from VWR International (Brisbane, Australia) unless otherwise stated. Polyclonal anti-Fy^a and Anti-Fy^b antisera, anti-D IgG For Further Manufacturing Use (FFMU) and polyspecific anti-human globulin (AHG) antibodies were supplied by Quotient EU (Edinburgh, United Kingdom). Abtectcell red blood cells (RBCs), cell preservation solution (Celpresol), Celpresol low-Ionic strength solution (LISS), rapid antibody medium (RAM) and pegylated RAM (RAMPEG) were supplied by CSL Limited (Melbourne, Australia). Anti-human IgG Fc (Clone CBL101) and Millipore centrifugation tubes (100kD cut-off) were purchased from Merck Australia (Melbourne, Australia). The BIAcore X system (GE Healthcare, Uppsala, Sweden) was used for all analyses.

5.3.2 Methods

Through amine coupling, anti-human IgG Fc was immobilised upon the surface of a CM5 sensor chip. Each CM5 sensor chip contains a gold surface upon which a medium molecular weight carboxy methyl dextran layer grafted. The sensor surface was first activated by injecting an equal volumetric mixture of 100mM N-hydroxysuccinimide (NHS) and 400mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) at 5 µL min^{-1} for 7 min. Both NHS and EDC were made with distilled water before use, aliquoted and stored at -15˚C until required. The ligand, Anti-human IgG Fc (0.5 µg mL^{-1}), was dissolved

in 10mM sodium acetate buffer and then injected over the activated surface for 6 min at 5 µL min^{-1}. 1M ethanolamine-HCl, was then injected over the functionalised surface at 5 µL min^{-1} for 7 min to block any unreacted sites. The achieved binding response of anti-human IgG Fc was approximately 10,000 relative units (RU).

Reagent red cells (Abtectcells III 3%) contained the Duffy antigens (Fy^a & Fy^b) for detection. These reagent red cells are human-sourced red cells which have been washed and kept at constant concentration in a cell stabilisation solution for standardisation.

Firstly, a control for the multi-functional capabilities of the sensor surface was tested by injecting unreacted and non-incubated antibodies of Fy^a and Fy^b over the functionalised surface at 5 µL min^{-1} for 1 min. Anti-D IgG FFMU was also injected over the surface at 5 µL min^{-1} for 1 min as a comparison. Furthermore, millipore centrifugation tubes (100kD cut-off) were used to remove supernatant and increase concentration of the antibodies by filtration, also for comparison.

Next, RBCs were incubated with excess polyclonal anti-Fy^a or anti-Fy^b antisera, both at the stock concentration (Neat) and at double the concentration by volume (x2), for 30 min at 37˚C (1:1 by volume) to sensitize the cells. After sensitization, cells were washed 4 times using Celpresol LISS and centrifugation before dilution to 10% concentration in Celpresol LISS for injection into the BIAcore X system.

A constant temperature of 37˚C was maintained during incubation to maximize binding of IgG antibodies which are optimal at this temperature; the human body temperature. A single flow cell detection mode, flow cell 2, was used for binding analysis. The standard BIAcore X control software was used for sensograms analysis. Finally, the functionalised chip surface was regenerated using 3M $MgCl_2$ at 1 min pulses at 1 µL min^{-1}.

5.4 RESULTS & DISCUSSION

5.4.1 Multifunctional Platform for Antibody Analysis

The first step towards using the anti-human IgG Fc functionalised SPR sensor surface was to establish the detection of different types of blood group antibodies without incubation with RBCs. During a single run, anti-D IgG FFMU, anti-Fy^a and anti-Fy^b were all injected over the surface in succession for 1 min, including a regeneration step between each injection.

Following the injection of each antibody, the traditional binding response could be clearly seen, indicating the potential of the anti-human IgG Fc functionalised surface of detecting the presence of any blood group IgG antibody with the Fc region exposed (Figure 2). The SPR sensograms are comprised of four distinct regions depicting the injection of RBCs injection over the sensor surface: (1) a rapid initial rise due to the change of refractive index when RBC samples diluted 10% (v/v) in Celpresol LISS are introduced over the functionalised surface; (2) a pseudo plateau representing the flow of the RBC sample reaching steady state; (3) a region of rapid decline when the RBC injection ends and the use of running HBS buffer is resumed; and (4) a plateau showing the amount of RBC adsorbed upon the functionalised surface. The binding response (RU) is characterised as the difference between the region 4 plateau and the baseline prior to region 1. This represents the amount of sensitized RBCs adsorbed upon the functionalised surface, quantified as a binding response (RU).

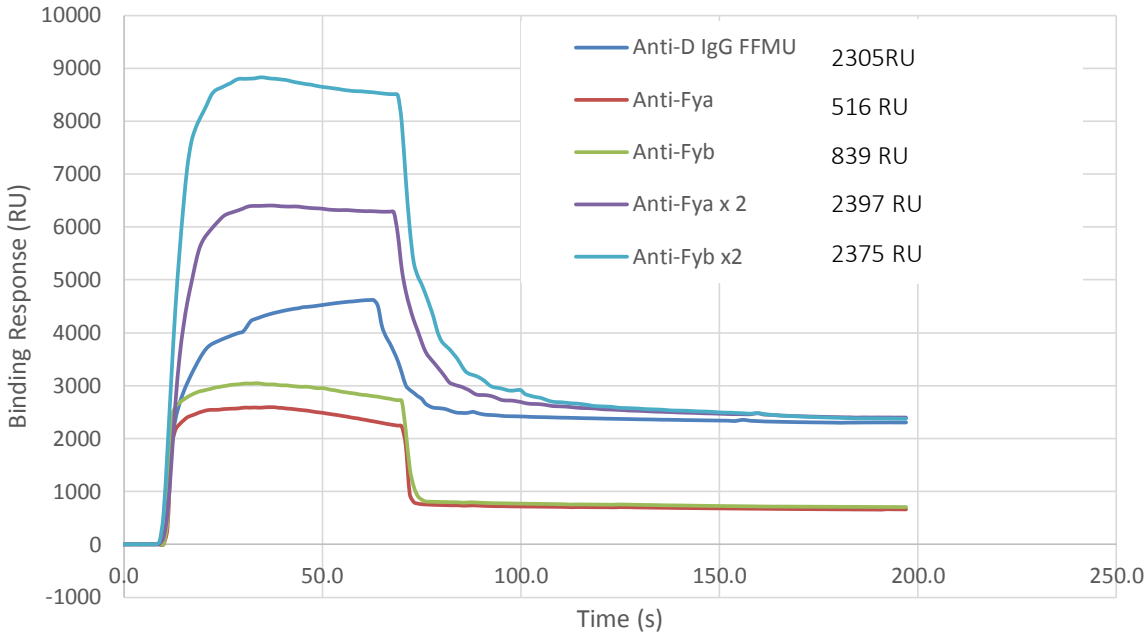

Figure 2: **SPR sensograms presenting the injection of: commercial anti-Fy^a and anti-Fy^b at neat concentration and concentrated (x2) compared with anti-D IgG FFMU, without incubation with RBCs. Injected at rate of 1µL/min for 1 min.**

At this stage prior to incubation there is already an observed difference in the binding response when comparing the anti-D IgG FFMU (2305RU) with the Duffy antibodies (716RU and 839RU for anti-Fy^a and anti-Fy^b, respectively). This is most likely due to different concentration of the antibody content. As an FFMU, the anti-D IgG is an undiluted antibody solution without any additives used to ensure the stabiliation and long-term storage of the final product. Both the anti-Fy^a and anti-Fy^b sera are diluted commercialised solutions, including additives. FFMU products of both Duffy antibodies were unavailable. As SPR is a concentration-dependent technique, the use of a less concentrated anti-sera showed a lower binding response when compared.

Another possible explanation is that the reduced binding activity of the Duffy antibodies could justify the reduced binding response observed. However, the functionalised layer of anti-human IgG only recognises the Fc region of the IgG antibody, and not the Fab region which is responsible for antibody specificity to a certain antigens, and therefore this explanation is less likely.

Subsequently, in order to show that the reduced binding response was due to the antibody concentration samples of both the anti-Fy^a and anti-Fy^b sera were concentrated using Millipore centrifugation tubes (100kD cut-off) to remove supernatant by filtration. IgG antibodies have an approximate molecular weight of 150kDa, while the supernatant includes additives, such as bovine serum albumin (BSA) (~56kDa), with lower molecular weights. The concentration of each antisera commercial product is known as the neat concentration. Through centrifugation and filtration, an estimated half of the supernatant volume was removed, thus doubling the antibody concentration. The resultant increased binding response of both anti-Fy^a (2397RU) and anti-Fy^b (2375RU) confirmed the hypothesis that the difference RU observed in concentration dependent (Figure 2).

5.4.2 SPR Platform for Duffy Antigen Detection

5.4.2.1 At Neat Concentration

Following the success of detecting the RBCs with normal D and weaker D antigen variants using the anti-human IgG Fc functionalised sensor surface with SPR, a similar protocol was explored for the detection of Fy^a and Fy^b antigens. Both the anti-Fy^a and anti-Fy^b sera were incubated separately with each of the three reagent red cells at neat concentration. Incubation sensitizes antigen-positive cells with its corresponding antibody. After incubation, samples were washed thoroughly using Celpresol LISS before preparation of the RBCs at 10% (v/v) dilution, also in Celpresol LISS. All samples failed to show any significant binding response, indicating false negative results (Table I).

Table I: **Binding responses of pre-sensitized reagent RBCs incubated with neat anti-Fy^a and Fy^b over a sensor surface functionalised anti-human IgG Fc. Pre-sensitized RBCs were washed 4 times prior to being injected at 10% concentration (v/v) in Celpresol LISS at a rate of 1µL/min for 15 min.**

	Fy^a		Fy^b	
Cell	**Phenotype**	**Binding Response**	**Phenotype**	**Binding Response**
1	-	60.5RU	+	58.4RU
2	+	73.6RU	-	80.1RU
3	-	69.7RU	+	64.2RU

At this point, there are a number of explanations for the unsuccessful detection of the Fy^a and Fy^b antigens using the current methodology, these include: (1) antibody concentration, (2) antigen expression, (3) antigen structure, and (4) the weak nature of the Fy^a and Fy^b blood group antibody-antigen interactions.

In the previous studies involving RhD testing, the anti-D IgG antibodies used were For Further Manufacturing Use (FFMU), meaning that the antisera was undiluted with no included additives. This implies one of the most potent forms of anti-D antisera. The decreased concentration of the anti-Fy^a and anti-Fy^b sera may have been unable to yield a similarly significant binding response. This could also indicate an antibody concentration-dependent relationship regarding the ability of this functionalised platform to detect sensitized-antigens on the RBC surface.

Another factor could be the number of Duffy antigens expressed on the RBC surface. In comparison to estimated expressions of the normal RhD antigens, the expected expression of antigen sites for Fy^a and Fy^b are much less (Table II). While the expression of Fy^a and Fy^b antigens are reduced, the previous study involving normal D and weaker expressed D variants showed an antigen correlation with the observed binding response [26, 27]. The estimated number of D antigens expressed for both weak D and partial DVI variants is similar to that of Fy^a and Fy^b, showing the capability to detect weakly expressed antigens. Therefore, the combined decreased concentrations of both antibody and antigen could affect the ability for positive detection using SPR, resulting in false negative responses.

Table II: Number of estimated blood group antigen binding sites per red blood cell [28].

Blood Group Antigen	Estimated no. of sites per cell [28]
Normal D	9,900 – 33,300
Weak D	66 – 5,200
Partial D VI	300 – 14,502
Fy^a	~15,000
Fy^b	~7,000

The structures of the Rh antigens and the Duffy antigens are vastly different [1]. The Rh antigens are associated with a non-glycosylated proteins which provides membrane structure and serves in molecular transport. It 12 membrane spanning domains with 6 extracellular loops. Also there is a relationship between the presences of certain Rh antigens – the Rh phenotype - and the estimated number of antigens expressed, which in turn, affects the binding response observed (Figure 3a). In contrast, Duffy antigens are associated with glycoproteins that function as chemokine receptors, binding to pro-inflammatory chemokines present in the blood. It has a large transmembrane glycoprotein structure which spans the RBC membrane seven times (Figure 3b). Furthermore, evidence suggest a junctional protein complex encompassing the Rh antigens and Duffy antigens, among others, with vastly different orientations (Figure 3c). The binding interactions between the antibody and antigen could explain the much weaker observed response due to the different antigen structures and positions. This could be why the Duffy antigens are known to show much weaker binding with current blood group typing techniques.

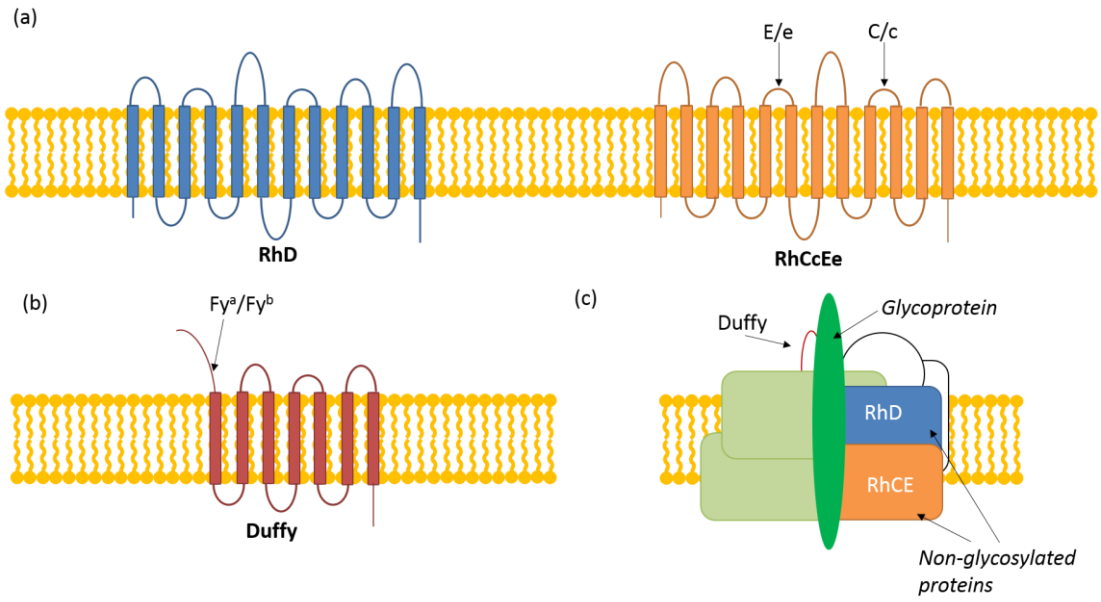

Figure 3. **Representation of the antigen structures for (a) the Rh blood groups, (b) the Duffy blood groups, and (c) a proposed model of a junctional protein complex encompassing both Rh and Duffy blood group antigens, among others. (Redrawn from Daniels & Bromilow, (2007) [1])**

Furthermore, antibody-antigen interactions are known to be reversible [29]. The unique antigen structures could explain why the Duffy blood groups kinetics dissociate differently, at least partially, using this SPR technique. Duffy antibody-antigen interactions are known to show a degree of dissociation after the incubation period [29]. Modern techniques using the IAT for blood group typing with IgG antibodies, which also requires incubation, are typically tested immediately after incubation and washing. For the SPR technique, once removed from the water bath at 37°C, the sensitized blood samples are washed using Celpresol LISS which is refrigerated when not in use, but allowed to reach room temperature before washing. This difference in temperature could aid the dissociation process. However, attempts to wash and maintain cell temperature at 37°C prior to SPR injection also proved unsuccessful. Incubating samples for longer than 30 mins for increased binding time was unsuccessful. This is because antigen-antibody complexes are known to dissociate if the incubation period is prolonged [30].

5.4.2.2 At Increased Concentration

In order to determine if the antibody concentration is a limiting factor SPR sensitivity for the detection of Duffy antigens, the concentration of the antibody sera was doubled by volume through centrifugation. The concentrated (x2) anti-Fy^a and anti-Fy^b antisera were each incubated with one positive-antigen and one negative-antigen reagent red cell.

Initially the binding response of a Fy^a-positive RBC looked promising (Figure 4). A strong binding response was observed (1700RU), indicating a dependence on antibody concentration. However, reproducibility was erratic, subsequent testing of the *same* sample preparation did not achieve the same positive binding response, and appeared as false negative. This indicated a time-dependent factor after incubation, namely dissociation of the antibody-RBC complex, combined with the antibody-concentration factor. Preventing dissociation is critical for robust Duffy group typing.

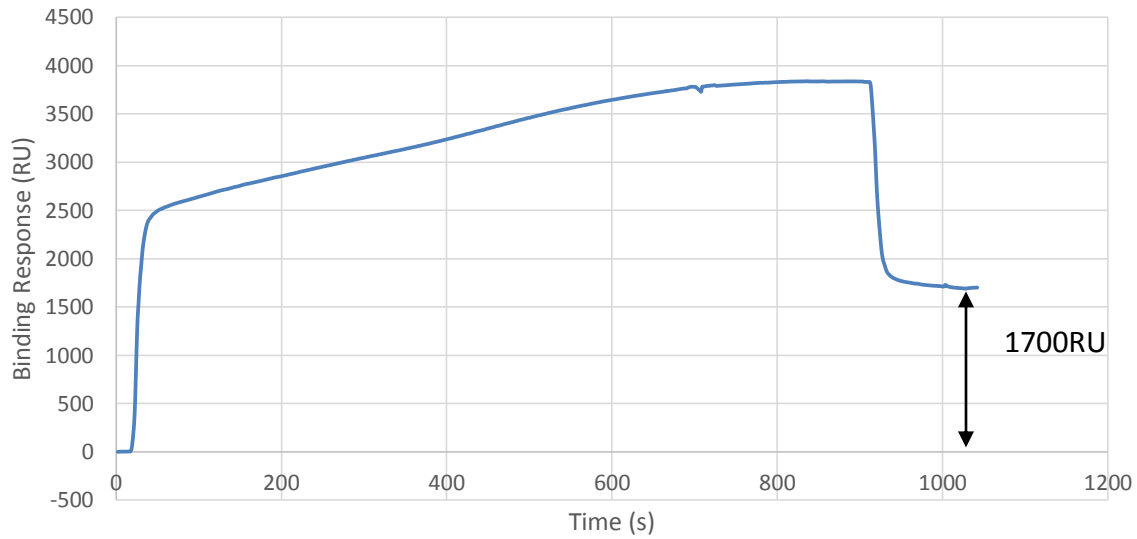

Figure 4. **SPR sensogram presenting the injection of pre-sensitized reagent RBCs incubated with concentrated (x2) anti-Fy^a over a sensor surface functionalised anti-human IgG Fc, and the difference in relative binding response of 1700RU. Pre-sensitized RBCs were washed 4 times prior to being injected at 10% concentration (v/v) in Celpresol LISS at a rate of 1µL/min for 15 min.**

In support of the hypothesis that SPR detection is also reliant on the antibody concentration during incubation, a concentration study using the anti-D IgG FFMU with a D-positive reagent red cell was conducted. As anti-D IgG FFMU is undiluted, and therefore most concentrated and potent, the anti-D IgG FFMU was diluted using 0.9% NaCl solutions in standard serial dilutions of half. Figure 5 depicts a threshold for the antibody concentration required for effective detection using SPR. While steady above a dilution of 1:8, a rapid decline is observed at 1:16, with false-negative results at 1:64. The relatively low dilution factor implied that a higher concentration of antibodies is required for successful detection. These results were reproducible much like the studies previously detecting the D-antigen using SPR, further implicating that the differences in Duffy antigen structure and its interactions with the corresponding antibody as causes for the unreliable detection and apparent false negative results.

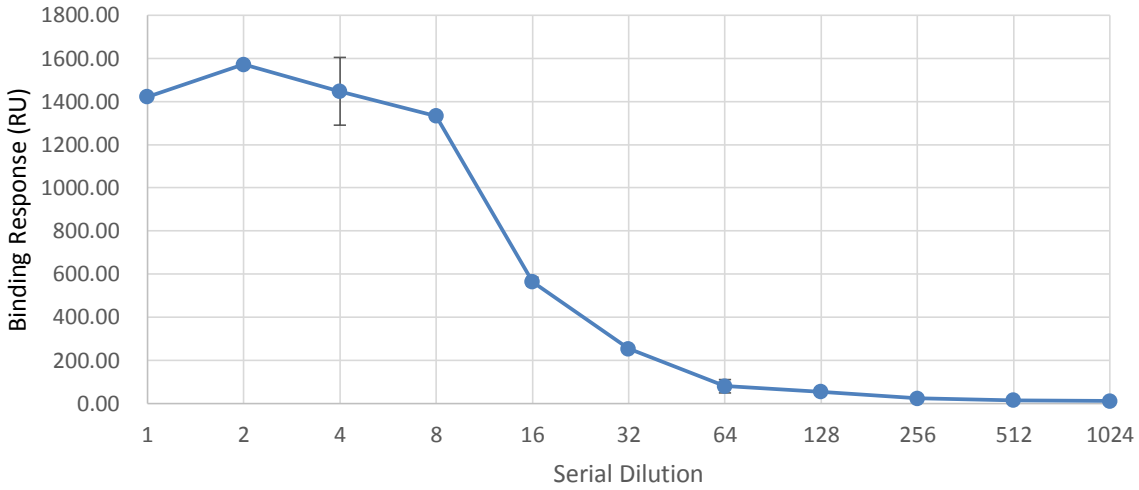

Figure 5. **Binding responses for positive D-antigen RBCs incubated with anti-D IgG FFMU in serial dilutions of 2. Pre-sensitized RBCs were washed 4 times prior to being injected at 10% concentration (v/v) in Celpresol LISS at a rate of 1µL/min for 15 min. (t=900s after injection.)**

5.4.2.3 Using Variable Incubation Conditions

In current blood banking practices, additives are sometimes used to enhance the antibody-antigen interactions of weaker blood group types. At the incubation stage, rapid antibody medium (RAM) or pegylated-RAM (RAMPEG) can be added to increase

the rate of antibody uptake and reduce the incubation time required (from 30 min to 10 min). RAMPEG contains polyethylene glycol to enhance weak interactions.

Varying the incubation conditions was unsuccessful for the reproducible detection of the Duffy antigens, Fy^a and Fy^b. The RAM and RAMPEG additives had no visible effect at stabilising the sensitized RBC and prolonged use caused cell lysis. Table III presents the binding responses; however only the highest value achieved is listed. None of the distinctly positive tests were reproducible despite multiple attempts.

Table III. **Binding responses of pre-sensitized reagent RBCs incubated with concentrated (x2) anti-Fy^a and anti-Fy^b over a sensor surface functionalised anti-human IgG Fc using varying incubation conditions. Pre-sensitized RBCs were washed 4 times prior to being injected at 10% concentration (v/v) in Celpresol LISS at a rate of 1µL/min for 15 min.**

	Fy^a				Fy^b			
Washing using Celpresol LISS at room temperature								
Cell	**Phenotype**	**Additive**	**Incubation Time**	**Binding Response**	**Phenotype**	**Additive**	**Incubation Time**	**Binding Response**
1	-	None	30 min	60.6RU	+	None	30 min	58.0RU
2	+	None	30 min	1700RU	-	None	30 min	63.3RU
1	-	RAM	30 min	89.5RU	+	RAM	30 min	680RU
2	+	RAM	30 min	194.1RU	-	RAM	30 min	90.2RU
1	-	RAMPEG	30 min	75.2RU	+	RAMPEG	30 min	1015RU
2	+	RAMPEG	30 min	137.8RU	-	RAMPEG	30 min	64.9RU
1	-	None	10 min	15.2RU	+	None	10 min	53.4RU
2	+	None	10 min	50.4RU	-	None	10 min	59.6RU
1	-	RAM	10 min	40.2U	+	RAM	10 min	64.3RU
2	+	RAM	10 min	152.2RU	-	RAM	10 min	70.1RU
1	-	RAMPEG	10 min	80.3RU	+	RAMPEG	10 min	90.8RU
2	+	RAMPEG	10 min	152.8RU	-	RAMPEG	10 min	75.6RU

5.5 PERSPECTIVES AND CONCLUSION

When compared to current methods, such as the IAT and paper diagnostics, SPR has the potential for quantitative detection that is far more sensitive. The time and consumables involved in testing are comparable, but whilst SPR requires a more complex and expensive instrument, the advancement of multiplex SPR machines and the capabilities for full regeneration of the sensor surface promise automation and moderate cost testing.

While unsuccessful at achieving the reproducible detection of the Duffy antigen expected of blood typing using SPR with the anti-human IgG functionalised sensor surface, the proof of concept was demonstrated and the principles behind this platform is sound. This is due to weak expression of Fy^a and Fy^b antigens, decrease antibody concentration of antisera, antigen structure and weak stability of the antigen-antibody complex leading to dissociation.

Since SPR is sensitive to antigen expression, the weak expression of Fy^a and Fy^b antigen appears to affect the range of detection achievable using this method. However, SPR detection is also sensitive to antibody concentration during incubation. A concentration study using the anti-D IgG FFMU with a D-positive reagent red cell confirmed that a threshold of 1:8 dilution or above for the antibody concentration was required for effective detection. Also, use of the SPR with whole cells is difficult; largely perceived to be limited by the constraints of the microfluidic channels and detection field. This can be particularly true when attempting to detect antigens on the surface of whole cells with fixed antibodies which are 1000 times smaller.

However, despite these disadvantages, detection of the Fy^a and Fy^b antigens was successful in some instances. While not reproducible, since each sample was tested in triplicate, the positively detected antigens where always achieved during the first injection, where there was the least amount of time between incubation and injection. Subsequent injections of the same sample would consistently appear as false-negative. This indicates that the SPR platform is not the limiting factor, but rather, it is limited by the dissociation of the Duffy antigen-antibody complex. If stability of the complex can be achieved, then reproducible positive detection should be capable using this SPR platform.

The aim of this study was to investigate an anti-human IgG Fc functionalised sensor surface for the detection of the weak interacting Duffy antigens, Fy^a and Fy^b, using surface plasmon resonance. While there were some instances of antigen detection with concentrated commercial anti-Fy^a and anti-Fy^b serum during the incubation stage, efforts to show reproducibility largely failed, showing dissociation of the Duffy antigen-antibody complex over time. Attempts to stabilize this antibody-antigen interactions

using rapid antibody medium (RAM) and pegylated-RAM (RAMPEG) were also unsuccessful. This study showed that a combination of factors affect the successful detection of the Duffy antigens, Fy^a and Fy^b, using this proposed SPR platform. Together, the antibody concentration, antigen expression, antigen structure result in weak and unstable antibody-antigen interactions which are currently, but not indefinitely, unsuitable for detection, once a stronger and stable antigen-antibody complex can be formed.

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Chapter 6

CONCLUSION & PERSPECTIVES

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While well-known ABO and RhD blood groups have been thoroughly explored with paper thus far, there had been no previous investigation of the wide range of other clinically significant blood groups that can also cause haemolytic transfusion reactions if wrongly typed. Furthermore, the effects of contributing factors, such as antigen type, antigen density, antibody structure, antibody serum formulation, binding affinity between antibody-antigen complexes, and properties of the paper and test reagents, were not well understood. As such, there was also very limited analytical techniques capable of quantifying the detection and kinetics of such antibody-antigen interactions, particularly for weakly or partially expressed antigens. Surface plasmon resonance is a widely used bioanalytical technique capable of observing interactions between biomolecules in real-time and label-free.

This thesis has reported two main interrelated avenues of research. The first extended previous research for blood group typing using paper diagnostics, while the second developed a novel platform for blood analysis using surface plasmon resonance (SPR).

6.1 BLOOD GROUP DETECTION USING PAPER DIAGNOSTICS

Blood group typing for other red blood cell antigens such as Rh, Kell, Duffy, and Kidd, had not yet been previously explored using paper diagnostics. The first study focused on the detection of these lesser known, though still clinically significant blood antigens. Other variables tested included reaction time, andreagent concentration. Also the testing methodology was compared i.e. the elution method, which is much like chromatography, versus the filtration-like flow-through method. The importance of antibody type/structure for successful agglutination on paper was confirmed. Some blood group phenotypes showed less agglutination due to weaker antibody-antigen interactions. Most blood groups with antibodies available as IgM, such as C/c, E/e, K and cellano, and Jk^a and Jk^b, and P1, were successful using both elution and flow-through methods. However, other blood groups, especially those with antibodies only available as polyclonal antibodies (IgG) were unsuccessful and require further scrutiny.

6.1.1 PAPER TESTING – ELUTION VS. FLOW-THROUGH

Prior to this research, two paper testing methodologies – an elution and a direct flow-through method – had been previously explored, however a comparison between the techniques had never been conducted. The directionality of the washing buffer was seen to be a key variable for functionality of the detection of blood groups on paper. The elution method operated much like paper chromatography, relying on the microfluidic channels within the paper structure, while the flow-through method behaved more like filtration with testing reagents being washed through the paper.

In comparison, successful results for the elution method were more definitive than the flow-through method, better designed for high throughput testing. Furthermore, the needs for the elution tank and refrigeration for the antibody sera made the elution method unsuitable for in-field testing. Meanwhile, the flow-through method is ideal for point-of-care testing, especially in remote areas or developing countries, as the antibodies can be directly printed upon the paper and stored at room temperature. However, as the hydrophobic barriers often required for definition of the testing zone can have a limited life span after printing, long-term storage and use of the flow-through method requires further engineering development.

6.2 BLOOD GROUP DETECTION USING SPR ANALYSIS

Many biosensing systems rely on surface plasmon resonance (SPR) detection to quantify biomolecular interactions. While SPR has been widely used for characterising antibody-antigen interactions, measuring antibody interactions with whole cells is significantly less common.

Of particular interest is the Duffy blood group system antigens, Fy^a and Fy^b, which have weak antigen expression and only have IgG antibodies commercially available for detection. In an effort to better understand the antibody-antigen interactions for blood group typing using IgG antibodies, the focus of this thesis shifted to using a bioanalytical technique, surface plasmon resonance (SPR). SPR is a label-free diagnostic tool capable of measuring real-time interactions. These measurements could be used to design better, more functional, cheap, simple and rapid blood typing paper diagnostics.

During the following studies, a fully regenerable, multi-functional platform for quantitative blood group typing via SPR detection was achieved by immobilising anti-human IgG antibody to the sensor surface, which binds to the Fc region of human IgG antibodies. The surface becomes an interchangeable platform capable of quantifying the blood group interactions between red blood cells (RBCs) and any IgG antibodies. Complete regeneration of the anti-human IgG surface was also successful, showing negligible degradation of the surface after more than 100 regenerations. This innovation now renders SPR commercially possible for blood typing in particular, for any blood analysis with IgG, in general.

This novel approach was validated with human-sourced whole blood samples to demonstrate an interesting alternative for quantitative blood grouping using SPR analysis. This method is the first and only that: (1) presents a polyvalent platform to analyse the interaction of ANY human IgG with RBC, and (2) that regenerates the SPR chip surface for over 100 tests, significantly dropping analysis costs.

Furthermore, the detection of the important weaker D variants was also explored. A sensitivity study using SPR for blood group detection of the two types of weaker D variants, weak D and partial D, was conducted. Weak D cells are classified as such due to a lower expression of their antigens. Cells with partial D antigens contain an incomplete antigen structure. The detection of two classes of weaker D variants was achieved using this fully regenerable SPR platform, demonstrating its sensitivity capabilities. For the first time, the antibody-antigen interaction between partial and weak antigens has been quantified for blood.

The final study of this thesis focused on the detection of the weaker Duffy blood groups, Fy^a and Fy^b, using the developed SPR platform. Duffy blood group detection is notoriously known to be more difficult in the blood banking industry. The results of this technique varied and highlighted the importance of: (1) antibody concentration during incubation, and (2) stability of the antibody-antigen complex during SPR detection. While some instances showed the successful detection of the Duffy antigens, efforts to show full reproducibility were not as fruitful. This is due to the rapid dissociation of the antibody-antigen complex over time. The combined actions of the antibody

concentration, antigen expression, and antigen structure result in weak and unstable interactions. Though currently unsuitable, the instances of successful detection showed the potential of this SPR platform; however, its current performance does not meet the blood banking criteria.

6.3 PAPER DIAGNOSTICS VS. SPR ANALYSIS

In comparison, when blood group typing using paper diagnostics, the procedure for blood groups with a corresponding IgM antibody is simpler, cheaper and more convenient in terms of portability and disposal. However, detection using IgG antibodies is currently much more difficult and not as reliable. Furthermore, the paper diagnostics lacks a robust quantification method, which is particularly important for weaker interactions. This is where the advantages of SPR analysis lies. SPR is capable of quantitatively detecting the presence of blood group antigens using IgG antibodies, and is sensitive enough to detect weak and partial interactions. This platform is also specific to IgG antibody interactions. Both paper diagnostics and SPR analysis are unsuitable for the detection of Duffy blood group antigens thus far. However, the specific issue is linked with unstable binding of the antibody-RBC antigen complex – not the detection technology.

6.4 PERSPECTIVES

While current blood typing methods are well established, results are subjective and heavily reliant on analysis by trained personnel. Paper diagnostics have great potential as a simple, cheap and robust alternative to current laboratory practices for phenotyping a wide range of clinically significant blood groups. However, techniques for quantifying blood group antibody-antigen interactions are also very limited, including when using paper diagnostics.

The initial development stages of this novel SPR platform provided a means to show label-free quantification of blood group antigens. Further development could allow for a fuller understanding of the different mechanisms and the effects of not only the structure and density of antigens and their corresponding antibodies, but also the additives which are used to enhance blood group antibody-antigen interactions. This

would be invaluable for the development of paper diagnostics which can not only report blood group detection with uniform clarity regardless of these differences, but can also be applied to other areas of biomedicine such as the detection of infectious diseases (e.g. malaria, HIV-1), physiological disorders (e.g. diabetes, liver function), and even cancer, which is the overarching goal of this research.

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APPENDIX

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Appendix I

PUBLICATIONS INCLUDED IN THESIS

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Whui Lyn Then and Gil Garnier*

Paper diagnostics in biomedicine

Abstract: Paper diagnostics are devices made of paper and cellulosic materials to recognize and quantify biomolecules and chemical agents affecting health. There has been a rapid increase in interest for paper-based diagnostic methods in recent years. This is because paper is cheap, widely available, easily engineered, biodegradable, combustible, biocompatible, sterilizable, hydrophilic, and easy to functionalize and process into diagnostic devices. Paper is a very attractive substrate to develop as a low-cost diagnostic platform. Paper can serve four functions in a diagnostic: 1) transport and measurement of sample and analytes, 2) reaction support, 3) separation of reactants from products, and 4) communication of results. When an aqueous analyte, such as blood, saliva, urine or feces is tested using a paper diagnostics analytical device (PAD), the fluid is driven by capillary flow induced by the porous and hydrophilic structure of the cellulose fibers, causing wicking through the paper inter-fiber space. Should hydrophobic barriers be formed onto or within paper, microfluidic systems can be created, allowing for the passive transport of the analyte. This article has four sections. The first presents paper as a composite and highlights its properties and attributes with respect to modern paper diagnostics needs. Functional printing is presented as a technology to manufacture paper diagnostics. The second section analyzes paper diagnostic design. PADs based on one dimension (1D), 2D and 3D flow, the methods of reporting, and the principles of detection are reviewed. The third section investigates applications in health and medicine for paper diagnostics in terms of clinical diagnostics, physiological disorders, and pathogenic diseases. The last section presents an analytical perspective of some of the critical issues. It is the objective of this article to analyze paper as a viable technology for producing low-cost medical analysis and to delimit the range of applications and potential best suited to paper diagnostics.

Keywords: analytical; bioactive paper; biomedicine; paper diagnostics; paper structure; sensitivity.

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List of acronyms

AA	Ascorbic acid
AKD	Alkyl ketene dimer
ALP	Alkaline phosphatase
ASA	Alkenyl succinic anhydride
ASSURED	A ffordable, sensitive, specific, userfriendly, rapid and robust, equipment-free, deliverable
AuNP	Gold nanoparticles
CPAM	Cationic polyacrylamide
ELISA	Enzyme-linked immunosorbent assay
FLASH	Fast lithographic activation of sheets
HBsAg	HepB surface antigens
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV-1	Human immunodeficiency virus type 1
LOD	Limit of detection
MD	Machine direction – long axis of the paper sheet
NCC	Nano-cellulose crystals
o-PADs	Origami paper-based analytical device
PAA	Polyacrylic acid
PAD	Paper-based analytical device
PAE	Polyamide epichlorohydride
PCC	Precipitated calcium carbonate
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
PEO	Polyethylene oxide
PN	Paper network
POC	Point-of-care
RBC	Red blood cell
RCA	Rolling circle amplification
SEM	Scanning electron microscopy
SERS	Surface enhanced raman scattering
SPR	Surface plasmon resonance
TiO2	Titanium dioxide
TLC	Thin layer chromatography
UA	Uric acid
UV	Ultraviolet
□PAD	Microfluidic paper-based analytical device

Introduction

The first step towards treating a disease is accurate and efficient detection. Paper-based analytical devices (PADs) can provide a quick, inexpensive, and simple detection method for biological and chemical analysis, which can easily be employed in medical diagnostics. Currently, applications, such as blood typing and pathogenic detection, require a technical infrastructure operated by qualified staff for accurate interpretation. Paper diagnostics could offer a cheap, simple, and convenient method for the user to directly test and interpret results, such as the pregnancy test and electronic blood glucose tests. The past 5 years has seen the rise of a strong interest in paperbased diagnostic methods. Although PADs hold great promise as a bio-diagnostic platform, there are still many limitations preventing its commercial applicability, as evidenced by the lack of new products on the market after 5 years of intense research and development.

Paper diagnostics are devices made of paper, cellulosic films, cardboard, and fabrics to recognize and quantify biomolecules and chemical agents affecting health. While paper-based analysis has been readily used in biomedicine since the 19th century, its potential in modern medicine has yet to be fully realized (Li et al. 2009a, Pelton 2009, Martinez et al. 2010a). The past 5 years has seen a rapid increase in interest, due to the compelling advantages of using paper-based diagnostic methods. A key advantage is its ubiquitous nature. Paper is a staple in everyday life all over the globe, which is manufactured on a large-scale into a plethora of different structures and properties. Paper is cheap, easily engineered, biodegradable, combustible, biocompatible, sterilizable, hydrophilic, and easy to functionalize and process into diagnostic devices. Paper is a very attractive substrate to develop as a low-cost diagnostic platform (Table 1).

With these attributes, paper can become an ideal platform for point-of-care (POC) diagnostic devices and eliminate or restrict the need for external equipment and specialized technical personnel; this would be ideal in developing countries, and military, emergency, and humanitarian field operations (Pelton 2009). As defined by the World Health Organization, diagnostics in developing countries should be ASSURED i.e., affordable, sensitive, specific, user-friendly, rapid and robust, equipment free, and deliverable (Table 1) (Lee et al. 2010). Additionally, POC diagnostics need to be disposable, portable, sterilized, and rugged (Lee et al. 2010). These requirements help to overcome the unpredictability exhibited during in-the-field testing caused by the surrounding environment (Lee et al. 2010). This includes limited clean water, unreliable electricity, high temperatures, and humidity (Lee et al. 2010). The versatility of paper and its many advantages make it more desirable as a POC platform than other technologies, such as thread and magnetic levitation (Vella et al. 2012). Another requirement of modern paper diagnostics is ease of use and the direct testing and interpretation by users.

Paper can serve four functions in diagnostics: 1) transport and measurement of samples and analytes; 2) reaction support; 3) separation of reactants from products; and 4) communication of results. When an aqueous analyte, such as blood, saliva, urine or feces is tested using a PAD, the fluid is driven by capillary flow induced by the porous and hydrophilic structure of the cellulose fibers, wicking through the paper inter-fiber space. Should hydrophobic barriers be formed onto or within paper, microfluidic PADs (□PADs) can be created (Martinez et al. 2007), allowing for the passive transport of the analyte without any further equipment required (Rivet et al. 2011). This represents a major advantage over traditional microfluidic materials, such as glass, silicone, polydimethylsiloxane (PDMS), and other polymers, which require external pumping (Martinez et al. 2010b, Rivet et al. 2011). Paper microfluidics are achieved by patterning hydrophilic channels that are defined by hydrophobic barriers; the micro-channels allow the testing of small samples with minimal reagents, thus reducing cost (Martinez et al. 2010b, Rivet et al. 2011).

Table 1 The desirable affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, deliverable (ASSURED) properties of paper as a medical diagnostic.

Requirement	Paper is:	
Affordable	Cheap and cost effective	Inexpensive manufacturing process Minimal use of reagents and analytes
Sensitive	White-background	A good contrast medium for colorimetric tests
Specific	Dried reagents	Reagents can be dried and stored within paper fibers
User-friendly	Single use autonomous	Does not require external power sources or equipment
	Minimal step-processes	To eliminate as many unnecessary steps (e.g., washing) as possible
Rapid and robust	Fast acting	Generally faster analysis compared to conventional testing methods
Deliverable	Portable	Easy to manufacture, stack, store, and transport
Other	Disposable	To protect end-users from exposure to bio hazardous wastes
	Particulate filter	Fibers can trap unwanted particulates in loading zone, preventing movement to detection zone

Even though paper has many of the desirable attributes of a diagnostic platform, the current commercial PADs, such as immunochromatographic strips, have limited quantitative capabilities (Rivet et al. 2011). This has generated interest in coupling paper devices with electrical reporting methods, from basic telemedicine (Martinez et al. 2008a) to electrochemical sensing platforms (Dungchai et al. 2009). Another disadvantage is the simplistic nature of the paper device, which has restricted multiple-sample, multiple-analysis or multi-step assays (Rivet et al. 2011).

Despite its recent inception in 2007, the growing interest in paper-based bio-diagnostics has already resulted in several review papers, each detailing the desired properties, patterning methods, and potential applications in health, food, and the environment (Martinez et al. 2007, Pelton 2009, Di Risio and Yan 2010, Martinez et al. 2010a, Ballerini et al. 2012), as well as reviews in microfluidics, in which paper is included (Haeberle and Zengerle 2007, Lee et al. 2010, Rivet et al. 2011). However, few have critically analyzed the potential and limitations of paper as an engineered material to manufacture low-cost biomedical diagnostics.

While medical diagnostics have a single beneficiary, they are used in widely different contexts of applications, each with their specific requirements. Three broad categories can be mapped. The first is generic high throughput diagnostics for routine tests. Cholesterol analysis, basic ABO blood typing, and blood sugar measurement are examples. These generic tests are performed by specialized scientists operating well-automated analytical instruments in dedicated laboratories. Speed of analysis, high throughput, and low cost are the main drivers. The second category of diagnostics regroups the series of patient specific analyses required for a particular diagnostic. Examples are specific antibody identification, complete blood phenotyping for a transfusion, and elemental blood analysis. The main requirements are flexibility and speed. These tests require laboratories with efficient management, a wide analytical expertise, and instrumentation, exclusive to modern medical laboratories. Sample preparation and chemical/biochemical reactions are often required. The last category consists of diagnostics for stand-alone remote testing. These tests are often performed either in the privacy of home or in difficult conditions, either by the patient himself or by untrained personnel in absence of technical or information support. A single specific test is usually performed. Robustness, ease of use, direct quantification/interpretation of results, and speed are four important requirements.

Paper diagnostics cannot pretend to address all medical analytical needs. With the explosion in research development and the strong media interest surrounding paper tests, the medical world and community at large are often left in confusion as to the state of development and potential paper diagnostics offered for medical applications. This article attempts to address these issues and provide an analytic roadmap; it is critical to distinguish between elegant prototypes and research prowess from promising technologies able to impact the medical community. An engineering approach is adopted here, as it best segregates science from development and clearly highlights the strengths and limitations of paper technology in medical diagnostics.

This manuscript consists of four sections. The first presents paper as an engineered composite and highlights its properties and attributes with respect to paper diagnostics needs. Functional printing is introduced as a complementary technology for paper in the manufacturing of paper diagnostics. The second section analyzes paper diagnostic design. PADs based on one dimensional (1D), 2D, and 3D flow are analyzed, followed by a review of the methods of reporting and the principles of detection. The third section reviews high impact applications in health and medicine for paper diagnostics. Applications are studied in terms of clinical diagnostics, physiological disorders, and pathogenic diseases. The last section presents a critical perspective of the current developments, the missing links, and the potential of paper diagnostics. It is the objective of this article to analyze paper as a viable technology for producing low-cost medical analysis and to delimit the range of applications and potential best suited to paper diagnostics.

Paper as diagnostic substrate

Paper is a porous and flexible composite made from cellulosic fibers and functional colloids that can be tailor-made for a plethora of applications (Figure 1). While the near totality of paper diagnostics has been made using filter paper, this substrate represents a negligible fraction of the paper production, and of the range of structures and properties achievable. Furthermore, filter paper is among the most expensive grade of paper. The choice of filter paper has been dictated by the quest for a standard to avoid unknown variables from cellulosic materials engineering. Most filter papers are made of a single type of fiber, mostly cotton, uniformly distributed in all directions. However, bioactive paper engineering relies not only on the fibrous structure, but also on the structure of the void fraction; fibers control the reactants and

biomolecules distribution, while the void fraction dictates liquid transport.

Paper is made by uniformly distributing and draining a suspension of cellulosic fibers onto a moving wire passing over a series of drainage elements, and then into a series of three presses, and finally through a long dryer. Paper is a specific type of non-woven material, manufactured by a wet laid process, which involves four steps: 1) fiber preparation, 2) forming, 3) bonding, and 4) surface treatment. *Fiber preparation* consists of selecting and preparing cellulosic fibers to optimize the properties of the paper, the selection determining whether recycled or virgin fibers are used. Recycled fibers are avoided for bioactive paper and paper diagnostics, due to the high risk of contaminants associated (dirt, oils, polymers, ink and organic residue, microbial growth). For wood-based paper, the fibers can be long softwood or short hardwood fibers. They can also be made by a chemical or by a mechanical pulping process. Chemical processes dissolve most of the extractives (fatty, resin acids, tannin, lignin, and hemicellulose), basically producing almost pure cellulose fibers. Mechanical pulping processes rely on a combination of shear, heat, and plasticization by water to separate fibers from wood by fracturing the middle lamellae; the resulting mechanical fibers are made of cellulose, hemicellulose, and lignin. The fibers are then lightly refined to provide the optimal surface area and bonding ability. Plant fibers from cotton, linen, flax, and hemp are sometimes used for specialty papers. Additives, such as polymers, microparticles (calcium carbonate, clay, kaolin, titanium dioxide), and dyes, are often added to the fibrous suspension to control the strength and the optical properties of paper. A cationic hydrophobic colloid, referred to as internal size, is often adsorbed onto pulp fibers prior to papermaking, to control paper wettability and ensure sharp printability. The typical internal sizes are: alkyl ketene dimers (AKD), alkenyl succinic anhydride (ASA), and rosin. Sizing agents are mostly used in office paper and packaging paper. *Forming* transforms the pulp suspension into paper, a uniform and continuous network of cellulosic fibers and functional colloids. The fiber suspension is impinged onto a moving wire, where it is drained and pressed to give fiber orientation, thickness, and the basic structure of paper. Fiber orientation in paper is affected by the difference of velocity between the moving forming wire and the impinging jet of pulp fibers. *Bonding* links the discontinuous fibers into a continuous non-woven paper network, which is achieved by pressing and drying. The “hydrogen bond” between hydroxyl groups forms a network between the cellulosic surfaces and provides the bonding between fibers. Polymeric strength agents can increase the paper’s wet and dry strength. *Surface treatments* allow paper specific properties. This is achieved by calendering, surface sizing, coating, spraying or other processes. Calendering passes the paper through a series of roll nips of increasing pressure to decrease paper porosity, and increase thickness uniformity and surface smoothness. Surface sizing applies a polymer solution, usually starch with a surface active polymer or latex, onto both surfaces of paper, which creates an interphase when the fluid polymer solution penetrates a few □m within the surfaces of paper. Coating applies a viscous solution of inorganic (filler) and organic (latex) colloids, which smooth the surfaces of paper and provide a surface of controlled microporosity, surface energy, and wettability (Kendel et al. 2008).

The paper properties are in large part defined by the choice of fibers and the papermaking process. Fiber dimension and chemistry depend on the type of lignocellulosic material pulped and the pulping process. The fiber orientation in the X-Y plane and in the paper thickness (Z) is controlled by the process. Process and fiber selection affect the porosity (1/paper density), and size and orientation of pores in paper. This means that the fiber orientation in the machine direction (MD – long axis of the paper sheet) differs from those of the cross machine direction (short dimension of the paper sheet). Basic properties for important grades of paper are presented in Table 2.

Many different structures and interphase morphologies of paper can be achieved using conventional paper technology (Figure 2). Refining and blending fibers, such as nanocellulose crystals or microfibrillated cellulose fibers, can increase the internal surface area, bonding, and density, while reducing internal pore sizes of paper (Figure 2A). Functional colloids, such as gold nanoparticles (AuNP), can be adsorbed either as individual particles (Figure 2A) or as aggregates of controlled size (Figure 2B) (Ngo et al. 2011). By controlling the conditions of the process at the size press and the properties of the solution, polymer layers of different thickness and concentration gradients can be deposited and pressed into both surfaces of the paper (Figure 2C) (Cho and Garnier 2000, Shirazi et al. 2003, Shirazi et al. 2005). The color contrast by scanning electron microscopy (Figure 2C) was achieved using a marker (KI) (Shirazi et al. 2003). The polymer sizing solution can contain a hydrophobic surface active polymer, such as styrene maleic anhydride polymer derivatives, or

Table2 Typeandstructuresofpaper.

Typeof paper	Basisweight (g/m^2)	Structure	Fibers	Additives
Filterpaper	90–200	Randomfiberorientation,variableporosity, andcontrolledporesize(1–20 □m)	Mostlycotton;sometimesbleachkraft softwood	Wetstrengthagent[suchaspolyamide epichlorohydride(PAE)]
Teabags	30	Highporosity,lowdensity,highstrength	Bleachkraftsoftwood	Highconcentrationofwetstrengthagent
Uncoated finepaper	15–80–120	Dense,lowporosity	Blendofbleachchemicalsoftwood(spruce, pine,fir)hardwoodfibers(eucalyptus, maple,aspen),andsometimerecycled fibers	Filler[10–20%precipitatedcalcium carbonate(PCC),clay,ortitaniumdioxide (TiO_2).Retentionaid,internalsize,surface sizestarch,andlatexorpolymer
		Highmachinedirection(MD)fiberorientation		
Coatedfine paper	80–120	Dense,lowporosity	Blendofmechanical,chemical,and recycledfibers.Mostlylongsoftwoodfibers	Sameasabove,pluslatexandPCCfor coating
	48	HighMDfiberorientation		
Newsprint	42	Dense,lowporosity	Blendofmechanical,softwood,and recycledfibers.Resinandfattyacid impuritiesfromwood	Fillerfromrecycledpaper,ultraviolet(UV) brighteners,yellowinginhibitors,retention aids
		HighMDfiberorientation		
Facial tissue	–20perply, –3plyper tissue	Lowdensity/highporosity,excellent formation,moldedorcrepedstructure.1,2or layersperply,1,2,or3plies	Layeredeucalyptus/softwood/fibers structured	Wet(PAE)strengthagents,softener. Sometimessiliconе(polydimethylsiloxane, PDMS)appliedassurfacetreatment
Bathtissue	15–32perply –40singleply	Lowdensity/highporosity,excellent formation,moldedorcrepedstructure.	Layeredeucalyptus/softwood/fibers structured.Recycledfiberssometimesused	Temporarywetstrengthagents, glyoxylatedpolyacrylamide)softener
Towels	50	Moldedorcrepedlowdensityandabsorbent structure	Blendedorlayeredeucalyptus/softwood /fibersstructured.Recycledfibers sometimesused	Wet(PAE)anddrycarboxymethylcellulose strengthagents,softener.Sometimes PDMSappliedassurfacetreatment.
	15–30–30			
Packaging	3	Dense,fibersaligned	Recycledandhighyieldchemicalsoftwood fibers(highligninсontent)	Wetanddrystrengthagents,sizingagents
Linerboard	80–200			
	200–600	Dense,fiberaligned.2or3layerscanbewet pressedintoasinglelayer	Recycledandhighyieldchemicalsoftwood fibers(highligninсontent)	Wetanddrystrengthagents,sizingagents

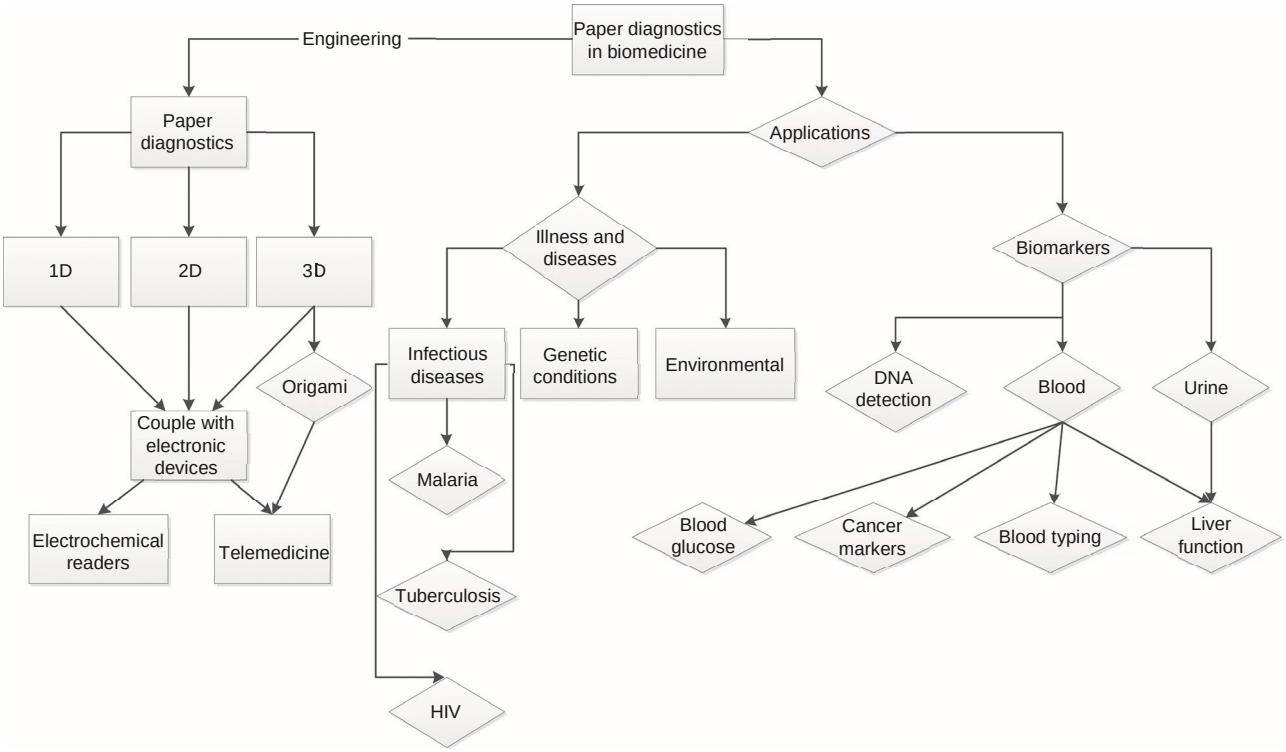

Figure 1 Schematic roadmap of paper and cellulosic diagnostics in biomedicine.

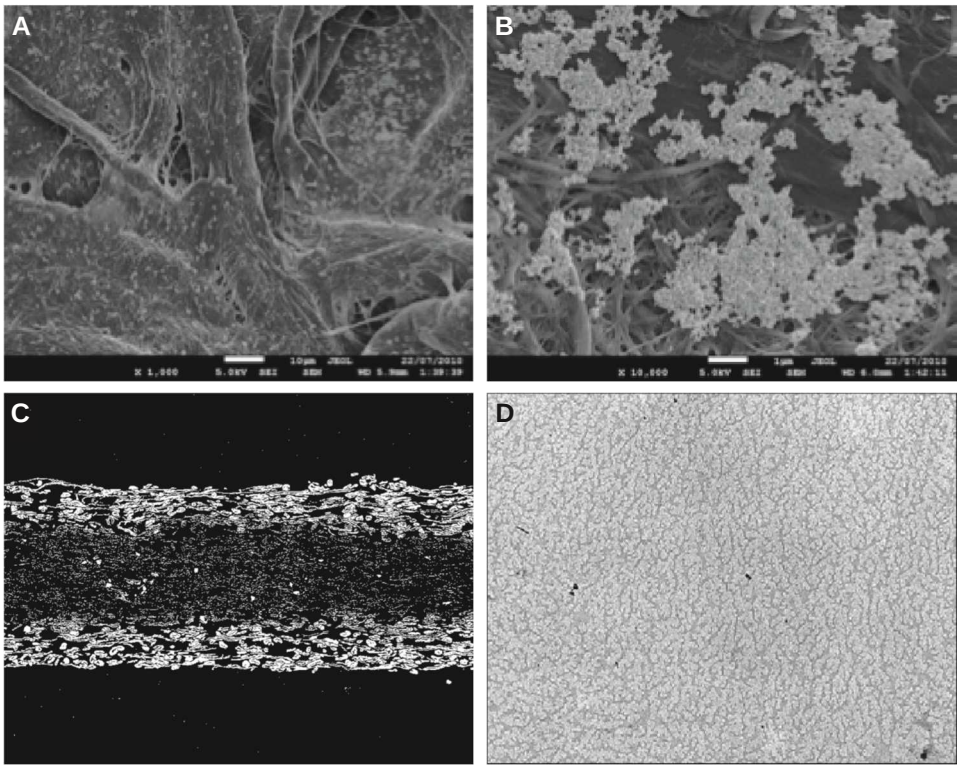

Figure 2 Paper as an advanced material: (A) cellulosic fiber bonding and microfibrillation; (B) retention of aggregated gold nanoparticles on paper; (C) creation of an inter-region on paper: surface sizing a starch-polymer solution; and (D) creation of surface active assembled alternating copolymers.

latex that can migrate to the surface of paper to minimize differences in surface energies. The polymer can assemble into polymer domains, as for styrene-maleic anhydride copolymers (Figure 2D) (Duskova-Smrckova et al. 1999, Garnier et al. 2000). Therefore, controlled paper structures having a complementarity of length scales in the nm, the □m, and the mm ranges can be engineered with the current paper technology.

Functional printing

Printing is a process which transfers controlled patterns of liquids or dye onto a surface: traditionally this has been ink on paper. Ink is made of dyes and particles dispersed in water or an organic solvent, containing additives to control viscosity and surface tension, to give required optical properties. Contact printing, such as flexography, lithography and rotogravure, and non-contact printing, like ink jet or laser jet, allow a printing resolution better than 20 □m. If dye/nanoparticles are replaced by a hydrophobic wax, such as AKD, ASA or wax, hydrophobic barriers can be printed to produce microfluidic systems on paper (Khan et al. 2010a). Biomolecules, such as enzymes, antibody molecules, or cells in an aqueous media form a bio-ink which can be injected in the microfluidic system previously printed on paper. Paper diagnostic prototypes have been entirely manufactured by printing (Li et al. 2010a).

Paper diagnostics design

Optimization of the structures and fabrication methods for microfluidic paper devices has focused on a perceived limitation of the current □PADs: performing multiplex assays. While a simple, single step assay is invaluable in many circumstances, in some instances, multi-test ability is required. Research interest has shifted to the actual design structure of the devices, expanding from single sheets of paper to 2D networks and 3D designs, for creating diagnostics able to test multiple analytes or samples simultaneously. While more complex designs might add functionality where single-step procedures are inadequate, a balance with simplicity is required in PAD design. This section analyzes PAD design.

A simple differentiation between device designs is the directional flow of fluid. For instance, 1D indicates the flow of liquid in a single direction, while 2D describes lateral flow in multiple directions on the horizontal plane (e.g., the spread of liquid from a single corner to multiple detection zones), and 3D designs add a vertical component (Figure 3).

1D paper diagnostics

1D lateral flow paper diagnostics, commonly known as dipstick tests, have been used for decades. These simple dipstick tests were first used to detect urinary glucose levels (Comer 1956, Free et al. 1957). Dipsticks are made of stiff paper and used by dipping one end of the strip into the sample, allowing fluids to be transported passively through the cellulose fibers towards the reagent zone (Haeberle and Zengerle 2007). In the early 1960s, testing evolved to a triple analyte test, adding the ability to detect protein albumin and pH levels; it has since expanded to a 10-type multi-analyte test which can assay for additional biomarkers, such as leukocytes, nitrite, ketones, bilirubin, and urobilirubin. Some assays can also measure and report the sample specific gravity (Fenton et al. 2008).

In the 1980s, the applicability of dipsticks was increased to include immunorecognition. The spotting and immobilization of antibodies on nitrocellulose led to the development of a wider range of PADs, which are now found on the market. Urine analysis tests include the take-home pregnancy tests, testing for human chorionic gonadotropin, and in drug testing, such as the 9-tetrahydrocannobinol agent to detect marijuana users. Immune-based PADs can also test blood analytes for cholesterol levels, diabetes, and pathological diseases, such as hepatitis C and human immunodeficiency virus type 1 (HIV-1), as well as autoimmune screening (Hawkes et al. 1982, Dineva et al. 2005, Binder 2006). In 1989, the need

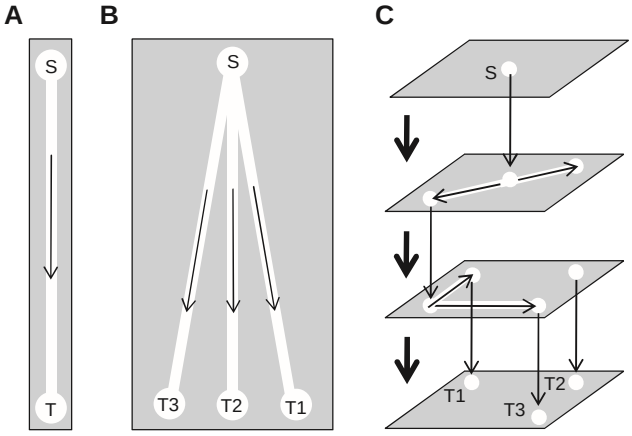

Figure 3 Schematic representation of the different types of paper diagnostics in: (A) 1D; (B) 2D; and (C) 3D design showing separate testing zones (T) connected to a single sample zone (S).

for incubation and wash steps was eliminated with the integration of capillary-driven lateral fluid transport with the dipstick technology. This amplified the total number of captured and detected bioanalytes, thus improving the lower limits of detection (LOD) (Rosenstein and Bloomster 1989, Gordon et al. 1990).

Typically, the reagents are dried and stored in the fleece sections of the assay during fabrication. The fleece sections allowed for different detection zones to be present on a single stick. The fluid solution dissolves the dried reagents, allowing a reaction to occur. Figure 4 illustrates a typical dipstick assay. The 1D flow of liquid through the stick from one end to the other was integrated into a 1D paper diagnostic. While useful, 1D paper diagnostics are limited to single-step tests and lack the ability to perform multiple-step assays, often required for techniques, such as paper-based enzyme-linked immunosorbent assay (ELISA). However, these tests are cheap, reliable, and easy to use.

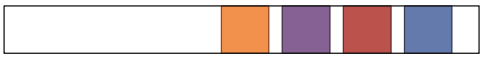

Figure 4 Example of a multiple-analyte 1D paper diagnostic on a dipstick.

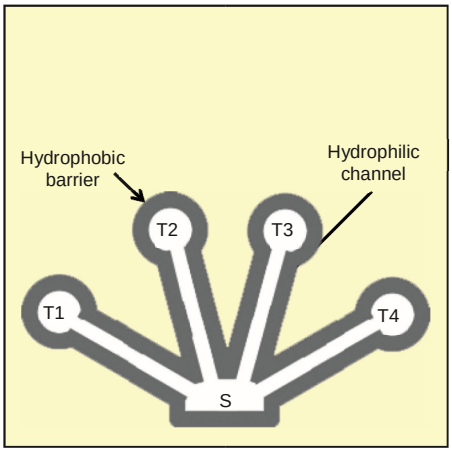

Figure 5 Prototype design of a 2D paper-based microfluidic device that tests multiple analytes simultaneously; showing separate testing zones (T) connected to a single sample zone (S). [Redrawn from Martinez et al. (2007).]

2D paper diagnostics

Simple 2D PADs

The advent of patterned paper to create micro-channel designs has enabled multidirectional flow paper diagnostics. Martinez et al. (2007) patterned a simple device with three detection zones comprising indicators for glucose and protein, as well as a control zone (Figure 5). The "walls", emplaced by hydrophobic boundaries patterned using photolithography, allowed fluid to be directed into the three different zones without cross-contamination, thus adding a spatial advantage against the conventional 1D-□PAD dipsticks.

Previous reviews have summarized patterning methods for diagnostic design. Such methods include: photolithography, plotting, inkjet or plasma etching, cutting, and wax printing (Martinez et al. 2010a). A summary of the patterning techniques analysis is presented in Table 3. The concept is to create hydrophobic barriers onto or within the paper structure and to rely on paper capillarity for liquid flow. While the original techniques created rigid and brittle barriers, technology has nicely progressed, allowing channels as narrow as 250 □m width to be created (Khan et al. 2010a). Common 2D-PAD test designs still lack the ability to perform multi-step assays for more complex applications and remain prone to contamination and fluid evaporation. However, 2D-PADs are very cheap (especially when manufactured by printing), easy to use, versatile, and robust.

Partially and fully enclosed PADs

A major disadvantage of the basic 2D-□PAD design is its exposed nature. Both sides of the paper devices are uncovered, resulting in the evaporation of reagents and samples, and risking contamination from the support beneath. Contact with the support can also result in loss of reagent and sample fluids. Early attempts to avoid such loss and contamination investigated adding samples and solutions onto the device held in mid-air, which is simply impractical (Schilling et al. 2012).

Fenton et al. (2008) proposed a method to avoid imbibing paper with hydrophobic/hydrophilic patterns. Instead, the paper was shaped into one of three desired 2D designs using a computer-controlled x-y knife plotter. Type 1 consisted of a single sheet of paper cut into the desired shape; for type 2, paper was mated with one layer of polyester cover tape before being cut; and for type 3, pre-cut paper was sandwiched between two layers of cover tape. Type 3 was the first example of a fully enclosed □PAD. Partially or fully enclosed PADs are reported to decrease the rates of operator error; however, this may simply be that paper cut into clearly labeled sections leaves no room for error interpretation (Fenton et al.

Table 3 Analysis of microfluidic paper-based analytical device (PAD) fabrication by functional printing.

Paper patterning techniques	
Photolithography	Description: Patterned using chromatography paper soaked in SU-8 photo resist polymer solution before being selectively exposed to ultraviolet (UV) radiation using a patterned mask to shield desired pathways. Shielded regions remain hydrophilic and the unreacted SU-8 is washed away. Unshielded regions become hydrophobic after undergoing polymerization. Advantages: Convenient, useful. Disadvantages: Expensive chemicals and equipment, multiple steps, time consuming, reduced paper flexibility. Examples: Martinez et al. (2007, 2008b)
"FLASH" printing	Description: Fast Lithographic Activation of Sheets (FLASH); Much like photolithography, except the paper is laminated between a transparent film and a black paper sheet. A standard ink-jet printer is then used to print a black ink mask onto the film. After polymerization the black paper and film is removed. Advantages: Faster, customized masks. Disadvantages: Expensive, multiple steps, reduced paper flexibility. Examples: Martinez et al. (2008b).
Etch printing	Description: Completely hydrophobized paper, using a polystyrene toluene solution, is "etched" using a toluene solvent printed on the surface which dissolves the solution to allow for the hydrophilic channels to form. Advantages: Custom designs, faster. Disadvantages: Corrosive/flammable, chemicals. Examples: Abe et al. (2008, 2010).
Polydimethylsiloxane (PDMS) printing	Description: PDMS is dissolved in hexane and printed onto filter paper using a modified x-y plotter. To form the hydrophobic barriers, the PDMS solution penetrates through the paper thickness. Advantages: Enhanced flexibility. Disadvantages: Reduced channel resolution due to "creeping" solution. Examples: Bruzewicz et al. (2008).
Plasma printing	Description: Paper previously hydrophobized using the cellulose reactive compound, alkyl-ketene-dimer (AKD) is patterned using metal masks that are clamped to the paper before being placed in a plasma asher. The AKD hydrocarbon chains are then oxidized by the plasma, leaving the hydrophilic channels. Advantages: Flexible. Disadvantages: Expensive, slow manufacturing rate. Examples: Li et al. (2008, 2010b).
Wax printing	Description: Multiple techniques. The simplest involves patterning both sides of filter paper with a wax crayon before heating it. It was then melted into the substrate to form hydrophobic barriers. Also, it can be extended to inkjet printing for more complicated designs with higher resolution, but at an increased cost. Advantages: Good in resource limited settings. Disadvantages: Low resolution. Examples: Lu et al. (2009), Carrilho et al. (2009a).
Laser cutting	Description: Uses a computer-controlled x-y knife plotter to cut the paper into the desired design with very high detail. Does not utilize imbibing techniques. Advantages: Cheaper fabrication costs, detailed design, clear labeling, can be fully or partially enclosed. Disadvantages: experimental technique, heating. Examples: Fenton et al. (2008).
Inkjet	Description: Office or specialized inkjet printer receives hydrophobic and bioactive ink cartridges. Resolution of 20 m or better determined by the diameter of the ink droplet. Advantages: low cost and flexibility of digital printing, established technology. Disadvantages: interaction ink-paper critical, plugging of nozzles. Examples: Khan et al. (2010a)
Lithography, flexography, silk screening	Description: contact printing techniques. Advantages: fast, cheap, established technology. Disadvantages: require a mold, blanket or negative.

2008). The design is cheaper to fabricate and impervious contaminants.

Olkkonen et al. (2010) presented a partially enclosed device which flexographically printed a polystyrene xylene/toluene ink onto the back of the device, while to external the front was printed with the microfluidic channel design. The hydrophobic back layer provided a protective layer that prevented fluid from escaping

through the back via contact with the underlying support and protected against contamination from the support. This method allows direct roll-to-roll production well suited for high throughput manufacture. The enclosed backing contributed to full penetration of the hydrophobic barrier, while protecting from contaminants and loss of fluids.

More recently, Schilling et al. (2012) investigated printing toner to produce fully enclosed PADs. A thermally bonded thin plastic protective layer was printed onto paper. Similar laser printers and toners are commonly purchased for offices, and have been used extensively in the fabrication of PADs. It is a cheap and convenient method of manufacturing PADs. The toner had no effect on the microfluidic channels, since it does not diffuse into paper, nor does it come off when wetted. Printing four layers of toner could enclose the PADs and resulted in faster wicking rates.

However, the heat required (180°C) during the laser printing method can affect the biological reagents that are affixed prior to printing and enclosed beneath the toner layer (Figure 6). At extreme heats, proteins undergo denaturation, lose their ability to bind to other molecules, and become inactive. Schilling et al. addressed this problem and reported a 90% decrease in enzymatic function using glucose oxidase for testing glucose concentration (Schilling et al. 2012). The decreased function was reported not to affect test sensitivity and to allow detection at concentrations as low as 1 mm. However, this raises serious concerns on optimization and economics, and questions as to why a 90% decrease in enzyme activity did not affect sensitivity. An alternative was explored where a 1 mm diameter hole was designed in the surface to act as a reagent addition port, therefore allowing the addition of reagents after enclosing the PAD, allowing wicking to the reagent-storage zone (Figure 6). This resulted in no loss of reagent or enzyme function, and with the exception of the area of the port, the reagents were protected from the surrounding environment.

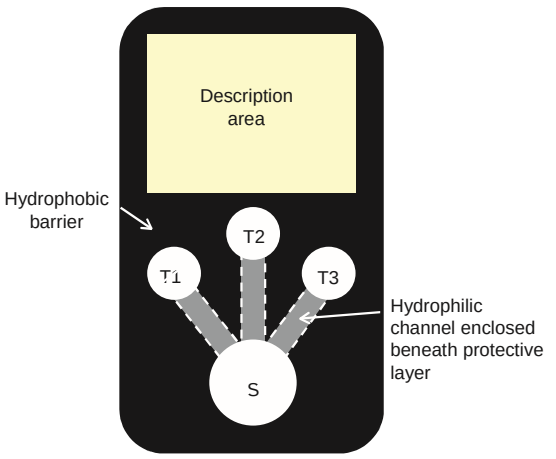

Figure 6 Example of a fully enclosed 2D paper diagnostic; showing separate testing zones (T) connected to a single sample zone (S). (Redrawn from Schilling et al. (2012).

PAD networks

A disadvantage of the "traditional" single sheet PADs is their limitation to single-step processes. A single-step process is ideal for the user, but restricts its applicability, since most laboratory-based diagnostic assays involve multi-step processes. This is the case for multi-step assays, such as ELISA, which improve sensitivity and specificity with signal amplification and washing steps. 2D paper networks (2D-PNs) attempt to emulate multi-step assays, while using a single activation step, thus retaining the simplicity and affordability of the single-step PADs.

Fu et al. (2010) developed a 2D-PN that retains the autonomous nature of PADs, while allowing the complexity of multiple reagents to be delivered sequentially to a detection zone (Figure 7). The first reported 2D-PN used three methods that allowed multiple inlets to simultaneously converge toward a single point (Fu et al. 2010). All methods focused on varying the delivery time of the fluid by: 1) varying the length of paper, 2) varying its width, or 3) creating a dissolvable barrier (trehalose) to slow the liquid in its tracks. Factors, such as paper composition, pore size, and surface chemistry can also affect the fluid flow rate, but were not explored. Paper was patterned using a laser cutter to fabricate the device, much like Fenton et al. (2008), and was supported by doublesided tape on a glass substrate (Fenton et al. 2008, Fu et al. 2010). Absorbent pads were used at the inlets for reagent application, allowing the solutions to wick to the detection zone. Using dye and pH as examples, the 2D-PN design staggered the delivery of each component to a common site. Later studies used the same design for chemical signal amplification (Fu et al. 2010, Fu et al. 2011a). The transport mechanisms of fluid through the paper networks were also explored (Fu et al. 2011a). However, the effects of paper composition, pore size, and surface chemistry remained unaddressed. The added complexity of the 2D-PN design also increases the possibility of errors.

A method using a single fluid source, rather than an individual source for each of the reagents, was explored Fu et al. (2011b). It involved "programming" the device to disconnect each reagent in a particular order. The design

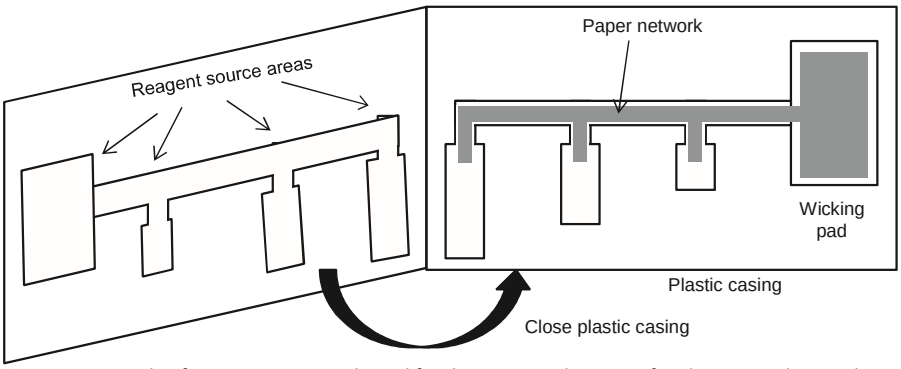

Figure 7 Example of a 2D paper network used for the sequential testing of multi-step analysis with a single activation point. [Redrawn from Fu et al. (2012).]

used a single source well and shaped the 2D-PN by varying the length of each reagent segment. Isolated strips of different lengths were used to show the overall method. The paper strips were housed in a poly (methyl methacrylate) (PMMA) plastic casing to reduce evaporation and provide a support from which to mount the device into the source well. Plastic cartridges and wells were designed to receive a paper cartridge/strip inserted by the user. The paper strips are immersed at different depths into the fluid. The well depletes as fluid wicks paper, thereby disconnecting strips from the source well. This was controlled by: 1) the fluid depletion rate, 2) the immersion depths, and 3) the cross-sectional area of the fluid source. The design also relies on a large and thick paper strip used as a regulator; without it, the lengths of paper varied only slightly. The test is very sensitive to the cross-sectional area of paper. Thin paper strips of small cross-sections are often used, which increases variability due to the high heterogeneity of paper structure at different cross sections. Using these basic principles of managing fluid flow, the 2D-PNs can be programmed to deliver fluids to a common point and disconnect from the source well in a timed, sequential manner. This series of experiments used colored dyes, previously dried onto paper.

3D Paper diagnostics

The desire or need to increase the density of circuitry and testing has led to the development of 3D paper devices with often complex fluidic systems. Improving the multianalyte capabilities of □PADs has been a major focus of development. It has culminated with the advance of multiplex 3D-□PADs prototypes, often consisting of multiple 2D layers. 3D paper diagnostics certainly offer advantages; however, it sacrifices simplicity, cost, and practicality for increased functionality. Other technologies such as threads (Li et al. 2009b, Reches et al. 2010) and traditional microfluidics (Haeberle and Zengerle 2007) devices provide competitive alternatives at this high end market.

The design alternates layers of patterned paper and perforated, double-sided tape stacked upon each other (Figure 8). This allows not only lateral flow, but also vertical flow. The perforated holes were filled with cellulose powder to connect each layer of paper, creating vertical micro-channels through which the analyte solution can wick. This design can create interweaving pathways through the device and allows the analyte to reach multiple detection zones without cross-contamination. Martinez et al. (2008c) designed a four channel device, where each channel underwent eight connections as fluid travelled through the layers at the top to the detection zones at the bottom. The purpose of each layer could vary throughout the device, including fluid distribution, filtration, or combination with other reagents (Martinez et al. 2008c).

Martinez et al. (2010a) later modified the 3D-□PAD design to allow a choice of specific applications. Usually, the function of each test is predetermined during fabrication. However, designing a single platform to test multiple analytes enables a certain level of specificity for each patient. The testing function can be configured at the site, allowing the user to program the device by choosing only the channels needed. This might be useful when

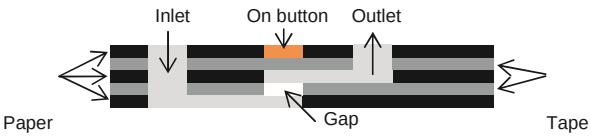

Figure 8 Side-view representation of an example 3D paper diagnostic. [Redrawn from Martinez et al. (2010a).]

a limited sample quantity is available and only specific analytes are desired. Using a single platform allows for mass production of a "universal device" to test a wide range of samples, and eliminates the need to design each test separately. The gaps that were once filled with cellulose powder are left empty, and are instead labeled as buttons. When pushed, the "on" buttons form a bridge in the required channels through which the fluid can travel. When the button is left un-pushed, the bridge is not connected and liquid will only flow to the desired tests. Once a button has been turned "on" it cannot be turned "off" afterwards, as paper deforms inelastically and remains compressed. However, ease of use and reliability tend to decrease exponentially with test complexity. While an elegant study, it is questionable whether the user might be able to identify the needs and modify the test in a time of stress or illness.

Origami

3D-□PADs can be constructed using origami, the art of paper folding. Liu and Crooks (2011) report a method using a single sheet of folded paper to design a 3D-□PAD (Figure 9). A piece of chromatography paper is patterned using a single photolithographic step. The design consists of micro-channels, reservoirs, and a frame. The frame provides the template for the paper folding and ensures correct alignment. When folded in a particular sequence, the micro-channels are matched to allow flow in both

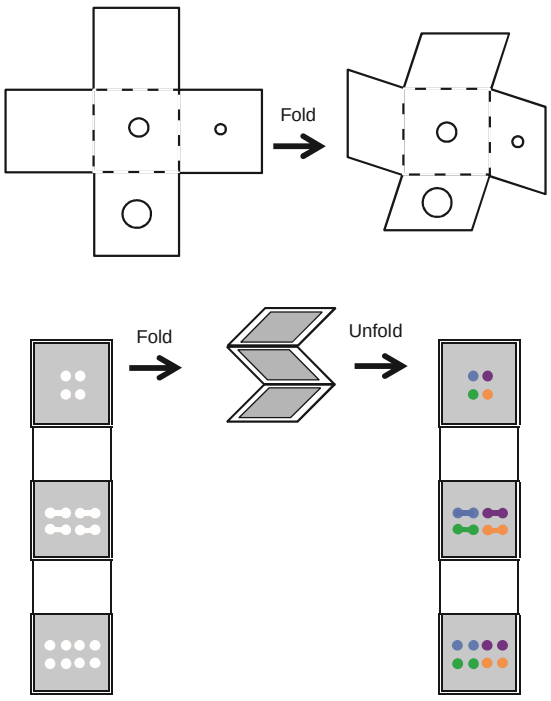

Figure 9 Redrawn examples of origami microfluidic paper diagnostic for multi-step analysis designed by: (A) Govindarajan et al. (2011, 2012); and (B) Liu and Crooks (2011).

the lateral and vertical directions. The four corners are trimmed and fit in an aluminum clamp. Samples and solutions are injected through one of the four holes drilled into the clamp's top plate. An advantage origami-PADs (*o*-PADs) have over the previous designs is the elimination of double-sided tape, which can diffuse into the paper over long periods of time and decrease the hydrophilicity of the micro-channels, along with assembly tools, such as the laser cutter. However, despite these advantages, origami PADs require an aluminum clamp to ensure alignment, which increases the cost of the device and decreases disposability.

Govindarajan et al. (2011, 2012) described a type of 3D-□PAD which utilizes the origami principles (Figure 9) (Govindarajan et al. 2011, Govindarajan et al. 2012). However, it is used functionally rather than to simplify fabrication. The origami serves to sequence the complex processing steps, by folding the layers in succession to prepare collected DNA samples for molecular diagnosis. The design uses stacked layers of cellulose paper and double-sided tape for assembly, but also uses single-sided tape, a repositionable adhesive layer, and card paper to allow for bidirectional folding. Another difference is the way in which the device is constructed. Rather than shaping each layer individually before stacking, a laser cutter is used after stacking instead. The cutting step during fabrication serves as a method for patterning the fluid channels. This is achieved by a combination of fullpass and partial cuts in the device, before peeling away the adhesive layer, leaving fluid channels on a non-wicking substrate.

Detection principles in paper diagnostics

Using PADs for the detection of a disease is a two-fold process that relies on the ability to report a specific recognition event. Both the reporting and recognition methods differ for various applications, and are discussed below.

Methods of reporting

Detecting the presence of an analyte is half the challenge. Accurate and successful diagnosis also requires a reliable and simple method of relaying the result to the user. Currently, most PADs report results visually; some have attempted to incorporate electronics into the reporting process. This section provides an overview of the development and issues of the main technologies.

Colorimetry and visual signals

Most PAD analyses rely on a visual change within the device detection zone to communicate results. Colorimetric analysis is by far the most common reporting technique described in PAD literature. It has also been a common approach in many laboratory-based testing procedures for decades. Colorimetric reporting consists of a visual color change that occurs after the addition of the sample, usually in the presence of the desired target. For example, the ELISA uses an immobilized biosensor on the substrate surface to capture a desired analyte, which induces a color change after binding with a second reporting enzyme, thus showing a positive result.

The appeal for colorimetric analysis on PADs is simplicity and applicability to POC situations. Other visual reporting methods, such as florescence or absorbance, require analytical instrumentation for accurate interpretation. Colorimetric assays in their simplest form can provide a qualitative binary result: whether or not a color change is observed. Paper is an ideal substrate for colorimetric analysis, due to the excellent contrast provided by the white background. Conversely, this white background and the ultraviolet (UV) brighteners present in paper (lignin residue and dyes) can interfere with fluorescence and absorbance reading methods, further supporting the use of colorimetric reporting. Smart phones with applications can also process data directly.

While colorimetric reporting is reliable and easy to implement on PADs, there are, however, a few drawbacks. Accurate interpretation, especially for quantitative analysis, may require trained personnel or analytical instruments. An alternative is telemedicine – using a communication device to interpret or transmit results to a specialized center for analysis before reporting the result back (Martinez et al. 2008a, Martinez et al. 2010a).

The concept of printing symbols or text has been demonstrated for qualitative results to contour userbarriers. The concept behind the “writing” technique is a refinement and simplification of the previous method by Bodenhamer et al. (2000) which described a displacement assay for transparent packaging. A printed pattern of antigens is immobilized on the substrate and saturated by the corresponding antibody with a dye attached. The dye is then released from the surface when interacting with the antigenic target. The resultant disappearance of the printed pattern indicates the presence of the target, in this case a pathogenic antigen, which results from an undesired exposure event.

Reporting with electronics

While stand-alone paper diagnostics are ideal, many reporting methods rely heavily on colorimetric results. This requires interpretation by trained medical personnel. It has been suggested that PADs be combined with mobile phones with camera capabilities for rapid and accurate interpretation. This is a field which has been dubbed telemedicine (Martinez et al. 2008a). It involves the image capture of the PAD, either by a scanner or camera, and then sending it via satellite for expert analysis. The expert would not be required on-site for interpretation, and the delay between when the test was taken and the time it takes for the results to reach the intended party would be minimalized. An alternative is to rely on downloadable applications for direct analysis by intelligent phones.

While colorimetric reporting is the most prevalent method for PAD analysis, electrochemical reporting methods have been explored (Liu and Crooks 2012). Colorimetric detection is adequate, but to discern an accurate result often requires trained personnel for interpretation. The use of telemedicine improves the situation; however it still relies on a person specifically qualified to correctly identify and diagnose the results. Using a metal/ air battery powered biosensing platform could be promising to eliminate the need for a trained professional. The microfluidic paper electrochemical devices method could provide an easily readable result for non-trained users, by adding a user-friendly reporting method to paper devices.

Biorecognition in paper diagnostics

Accurate diagnosis of a disease is not solely reliant on the reporting method, but also the ability to selectively detect the presence of specific biomolecules, also known as biorecognition. The human body has an abundance of distinct analytes which can be used for the diagnosis of not only pathogenic diseases, but also physiological disorders. It all hinges on finding the correct biomolecular target and developing methodologies to detect its presence. Biomolecules, such as nucleic acids, enzyme proteins, and antibodies, can be immobilized onto paper, and can reportedly be dried without denaturation for this purpose. This process allows for storage and use in remote areas, without access to laboratory facilities, while retaining the thermal stability that is often lost with analyte solutions not properly stored at low temperature. This ability to immobilize bimolecular targets upon paper thus provides the other crucial half of the detection process. There are four main methods of biomolecule immobilization on paper: 1) physical, 2) chemical, 3) biochemical couple, and 4) bioactive pigments; Pelton provided a detailed description of these techniques (Pelton 2009). A summary is provided in Table 4.

Perhaps the greatest challenge of achieving biorecognition is choosing the correct target that will detect a specific marker in the body. The job of this

marker is to unequivocally detect the presence of a disease to avoid misdiagnosis of a patient. Whilst the principles of detection have been modeled after current diagnostic methods, the behavior of biomolecules on paper is not always congruent with the behavior exhibited in current laboratory methods.

There is no distinct advantage of a specific biomolecule over another, but is rather a function of the current methods of detection available. For example, utilizing the specificity of antibody-antigen interactions is desired for applications, such as blood typing, while recognition of a specific protein is required when diagnosing malaria. The type of biomolecular target will therefore differ from application to application.

Because of the wide array of types and properties of analytical targets, each must be examined separately to develop a robust detection method for the purpose of each device. The source of the analyte is an important factor in the diagnostic design. Samples can be primarily taken from urine, blood, and saliva for PADs, as they are already present in liquid form, but fecal matter is also a potential analytical source.

Applications in health and medicine

While some □PADs have been designed for a specific purpose, other 2D and 3D platforms have focused on performing parallel or multiplex analysis for general bioassay applications. 3D systems have been explored for paper-based ELISA tests. Both 2D and 3D array formats have been investigated, including an emphasis on converting well-established techniques from plastic to paper. This section reviews and analyzes the important paper test designs and their specific medical applications.

Clinical diagnostics

Although conventional 1D dipstick assays have been used for decades and represent a well-established example of a multiplex assay on paper (Section 3.1), the number of potential applications of PADs in biomedicine has grown rapidly in recent years. The following section outlines general applications, as well as some specific examples for physiological and pathological disorders.

Paper micro-zone plates

Many medical applications rely on paper micro-zone plates (Carrilho et al. 2009b). Conventional micro-zone plates are usually made from plastic and consist of 96 or 384 wells, in which analytes can be deposited. The result is then determined quantitatively via absorbance or fluorescence measurements using a micro-plate reader. Paper micro-zone plates can be functionally similar to its plastic predecessor, but with all the familiar benefits of using a paper substrate: cheap, easy storage and disposal, small volume requirements, etc. Like plastic plates, the paper version can be designed with 96 or 384 detection zones using photolithography. Many paper types were found suitable for the microzone plate format (Carrilho et al. 2009b). The main limiting factor was paper thickness. If the thickness was too great, the entire depth of the paper could not be properly hydrophobized. Paper thickness defines the required volume of samples and the capacity for the hydrophilic zones. It can be improved through plasma oxidation (Carrilho et al. 2009b). In comparison to the plastic micro-zone plates, paper showed a 40-fold increased sensitivity when read using fluorescence. However, under absorbance mode, detection was limited by light scattering caused by paper. The addition of mineral oil or similar liquids matching the refraction index of cellulose allows accurate measurements by absorbance (Carrilho et al. 2009b). The 2D format of the paper micro-zone plates was ideal for telemedicine reporting methods and can perform serial dilutions – a benefit unachievable with the 1D-□PAD design. A unique advantage paper has over plastic is the ability to perform serial concentrations. A sample can be added, and then a known volume of the solution can be

Table 4 Methods of reagent immobilization on a paper substrate (Pelton 2009)

Technique	Description
Physical immobilization	Relies on van der Waals and electrostatic forces. Polymers can be used for bridging
Chemical immobilization	Relies on covalent bonding
Biochemical coupling	Relies on cellulose binding modules or bother biochemical binding agents
Bioactive pigments	Coats colloidal particles that and then printed or coated onto paper

evaporated, thus removing the excess liquid and increasing the sample concentration.

ELISA

The paper micro-zone plate format was incorporated into a 2D paper-based ELISA design (Cheng et al. 2010). ELISA tests typically use microtiter plates or small vials; it is one of the most commonly used assays for disease marker analysis. This paper-based design uses a 96-microzone paper plate printed with a 12×8 grid of circular detection zones (Cheng et al. 2010). This allows for multiple paperELISAs (P-ELISAs) to be run in parallel. Like the micro-zone plates, the P-ELISAs are compatible with existing equipment, such as 8- or 12-channel pipettes and plate readers. The design allows a washing step by leaving the top and bottom faces open to the atmosphere. The buffer is added to the top of the paper and blotted against the bottom. The perpendicular fluid flow removes any unbound reagents through the paper. However, the open bottom surface is impractical, requiring the paper be suspended in mid-air while the reagent solutions are initially added; this prevents the solutions from wicking through the test zones. A colorimetric reporting system is used, enabling the P-ELISA to be coupled with telemedicine if used in remote areas. However, the ability to perform a P-ELISA outside the laboratory environment is dubious.

There are three different methods for ELISAs: 1) indirect, 2) direct, and 3) sandwich ELISA. An indirect P-ELISA was demonstrated to detect rabbit IgG. Indirect P-ELISAs involve five steps (Cheng et al. 2010): 1) immobilization of antigens, 2) blocking of non-specific protein adsorption, 3) labeling the immobilized antigen with enzyme conjugated antibodies, 4) washing of unbound antibodies, and 5) the addition of a substrate solution for the enzyme. The results were proportional to the concentration of the rabbit IgG dilutions; however, the sensitivity decreased 10-fold compared to traditional ELISAs. This could be due to shorter incubation periods for the antibody-antigen interactions, or non-specific interactions between antibodies and the cellulose fibers. Despite this disadvantage, the P-ELISA still has benefits; it requires a shorter completion time, less analytical reagents, and can be quantified using a desktop scanner, thus retaining the appeal of a paper-based device. The test zone concentrations increase as the solution dries, enhancing the binding kinetics involved, however, the rate of evaporation is dependent on the surrounding environment, particularly relative humidity and temperature, and thus could affect the results (Cheng et al. 2010).

The P-ELISA can be integrated with printed electrodes to improve the LOD to a similar sensitivity observed with the colorimetric assays (Li et al. 2010c). Printed electrodes from graphite ink were used for an indirect P-ELISA and the voltage was measured periodically. Using this electrochemical method of detection produced an LOD similar to the conventional ELISA. However, the need for additional equipment and fabrication steps could negate the benefits of a more sensitive LOD.

Liu et al. (2011) also demonstrated an ELISA test using the 3D-□PAD design. The 3D design can test multiple analytes in parallel, which is applicable to ELISA analyses. However, the 3D-□PAD adds another level of complexity to the design. The vertical component allows for the fluid transport within the device to be controlled to fit multistep assay requirements. The difficulty with duplicating conventional ELISAs on paper is the need for multiple distinct working steps. □PADs are mostly designed for a single-step process. The 3D design adds a movable delivery strip to allow multiple reagent delivery and washing steps without cross-contamination. The strip can be manually operated. Reagents are dried and stored on the paper substrate, removing the need for pipetting reagents and buffers. This eliminates the need for operator training. The test takes around 45 min to complete (Liu et al. 2011). The performance of the 3D-ELISA device was demonstrated using the indirect testing of rabbit IgG. The intensity of the colorimetric signals was proportional to the concentration of rabbit IgG, and had a five-fold decreased sensitivity compared to conventional testing, which is an improvement over the P-ELISA. Some clinical uses could accept the reduced sensitivity of paper ELISA; however, whether the sensitivity improvement over the colorimetric techniques justifies the increased cost and complexity is not clear.

Paper for sample preparation and storage

Rather than testing for a particular disease, paper has also been explored as a platform for preparing samples at POC. While paper for sample preparation has been mostly investigated for urine samples, it is applicable to any biofluid (Parker and Cubitt 1999). Parker and Cubitt (1999) developed a dried blood spot (DBS) device where blood samples could be taken, stored, and transported on paper (Parker and Cubitt 1999). The DBS card is an older concept and can collect blood samples using finger or heel pricks, which can then be reconstituted in the laboratory for examination. This is of interest for epidemiological studies in remote regions. Even when kept in conditions varying from cold storage to humid and tropical conditions, the samples were found to remain stable after many years on paper. Additional benefits are

the significantly reduced infection risk, ease of use with minimal training, the elimination of needles and syringes, and storage at ambient temperature, removing refrigerated storage and transport requirements. This method was investigated for the surveillance and detection method of HIV (Parker and Cubitt 1999, Ayele et al. 2007). The interaction between paper and blood is poorly understood, but is critical for improving blood sample aging and preservation.

Sample separation

Not all μPADs report using colorimetry, one of the simplest methods. An alternative strategy is to separate analytes for detection (Carvalhal et al. 2010a). Carvalhal et al. (2010a) reported an electrochemically-coupled device that separates the analytes, uric acid (UA) and ascorbic acid (AA), and amperometrically detects their presence (Carvalhal et al. 2010a,b). Much like a chromatography column, eluent wicks paper by capillary action to dissolve the sample. The solubility is dependent on ionic strength. At an optimal pH lying between the pKa of AA and UA, the AA becomes ionized and more soluble. The type, thickness, and length of the paper control separation efficiency. Electrodes (gold) can be printed on paper for detection. The paper-based device compared to typical high pressure liquid column (HPLC) instrumentation has a slightly longer run time, 16 min compared to 5 min; however, the benefits of paper could outweigh the extended test time. This method could be developed not only for clinical diagnostics, but also for forensics, agriculture, and environment applications.

A potential μPAD application is the separation of red blood cells (RBC) from untreated whole blood, and integration with a colorimetric diagnostic test (Yang et al. 2012). Yang et al. (2012) described a method utilizing RBC agglutination principles using antibody-A,B to form aggregates too large to flow through the porous structure of the substrate (e.g., chromatography paper). However, the plasma constituents can still flow through the pores to adjacent testing terminals functionalized with reagents. Colorimetric detection for various compositional properties of the plasma can be achieved. This principle was demonstrated for measuring glucose concentration in blood. However, this test did not account for the blood group O population.

Blood group typing

The accurate, rapid and reliable blood typing of human blood, particularly during medical emergencies, is important (Hillyer 2007, Daniels and Bromilow 2007, Contreras and Daniels 2010a,b). Mismatched blood typing can lead to a hemolytic transfusion reaction which can be fatal. Traditionally, an individual's blood type is determined by detecting the presence or absence of antigens on the surface of their RBCs, as well as the antibodies present within the blood serum, more specifically the blood plasma. The most commonly used methods for blood typing include the slide test, tube test, micro-plate method, gel column agglutination systems, thin layer chromatography (TLC)-immunostaining, fiber optic-microfluidic device, and spin tube method, etc. (Harmening 1999). The gel column test is the most prevalent in industry, however, it requires centrifugation, and each of these methods requires professionally trained personnel to be reliably analyzed. Developing a low-cost alternative for remote regions or home care which is user-friendly, portable, and equipment free, could provide a great alternative to the blood typing options currently available (Khan et al. 2010b).

In recent years, several studies have explored the possibility of using paper as a blood typing diagnostic tool (Khan et al. 2010b, Al-Tamimi et al. 2011, Li et al. 2012). Much like the traditional blood typing tests, the paper diagnostic also relies on the principles of hemagglutination.

When an aqueous analyte such as blood is tested using paper, the fluid is driven by capillary actions created by the structure of the cellulose fibers, wicking through the inter-fiber space of the paper (Khan et al. 2010b). This is known as capillarity microfluidics and allows for the passive transport of the analyte without any further equipment (Rivet et al. 2011). However, when blood agglutinates due to a specific antibody/antigen interaction, the resultant RBC complexes become too large to adequately transport through the paper structure and retain on paper; in the absence of agglutination, RBCs are easily removed by elution. This contrast in RBC retention or elution allows for the differentiation between when agglutination does occur (specific) and when it does not (non-specific) to directly report typing (Khan et al. 2010b). This has been the primary basis for the investigation of a paper-based diagnostic tool for blood typing.

Khan et al. (2010b) developed a method soaking paper strips in antibody solutions of either A, B or D antibodies. Droplets of blood were then added to the center of the paper strip and the resultant wicking behavior of the blood was observed. A distinct difference was discernible between agglutinated and non-agglutinated blood, the former presenting a chromatographic separation of the RBCs and plasma (Figure 10A). When RBCs agglutinate with the corresponding antibody, very little wicking

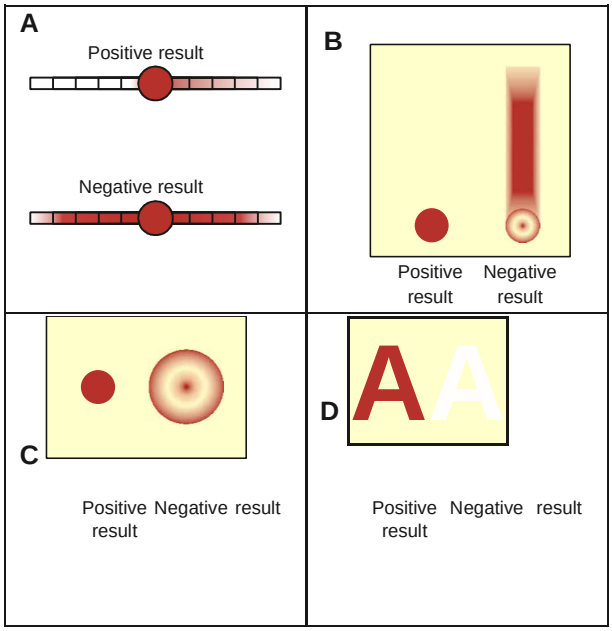

Figure 10 Use of paper for determining blood groups relying on antibody-antigen interactions to show agglutinated and nonagglutinated RBCs: (A) via wicking (Khan et al. 2010a); (B) chromatographically (Al-Tamimi et al. 2011); (C) using a blood spot test (AlTamimi et al. 2011); and (D) using the text-reporting paper substrate method (Li et al. 2012).

is observed, while the plasma does wick along the paper strip. In contrast, non-agglutinated blood forms no separation along the paper strips, but travels uniformly, thus creating a distinction between a positive and negative result.

Al-Tamimi et al. (2011) next developed another paper-based diagnostic for blood typing using the same principles, but presenting two different testing methodologies. The first described an elution-based method using the chromatographic behavior of blood when added to paper (Figure 10B). This was achieved by spotting blood on the surface of the paper where known antibodies had previously been added. A TLC tank was used to elute the blood spot in saline buffer. The capillary action of the paper allowed for the buffer to travel through the paper structure, thus creating a chromatography-like test. Much like Khan et al. (2010b), the fixed agglutinated blood spots showed a positive result, while non-agglutinated elution paths of unbound RBC were negative. Visually, there was a distinct difference between the two results, using both the density of the blood spot and the presence or absence of an elution path to make an informed analysis.

A second method, known as the spot test (Figure 10C), also involved spotting the blood to stock antibodies upon the paper substrate; however they were not eluted in the TLC tank. Instead, saline solution was used to wash the spot directly by pipetting. Separation occurred by filtration through the paper thickness; RBC aggregated by matching antibodies remain on the top of paper, forming an intense red dot, while non-agglutinated cells (non-specific antibody) simply wash through paper and disappear. The results of both tests accurately detected the ABO and RhD blood groups of 100 samples, including four weak AB and four weak RhD samples, thus supporting the use of paper as a robust bio-diagnostic for blood typing. The effect of paper structure on blood typing performance was also investigated in a different study (Su et al. 2012).

Li et al. (2012) recently developed a text-reporting method. The detection principle is an adaptation of AlTamimi's method (Al-Tamimi et al. 2011), relying on filtration through the paper thickness to retain RBC aggregated and coagulated by specific antibody interaction. By patterning the paper substrate with hydrophobic boundaries to surround a hydrophilic channel in the shape of text or symbols, "invisible writing" is present on the paper and dotted with antibodies. The addition of antibodies remains invisible to the naked eye until blood is added, when the shape of the channel is formed, for example an A or a B, and once washed with a saline solution – if a hemagglutination reaction has occurred – the resultant blood type will be directly printed on paper and can be "read" straight from the device (Figure 10D). This test successfully reported the ABO and RhD blood types of 99 samples including weak groups.

DNA extraction and detection

The genetic material of all living organisms is unique. There are well-established DNA detection techniques used for medical diagnosis of pathological diseases, such as the polymerase chain reaction (PCR) and rolling circle amplification (RCA) (Ali et al. 2009). While sensitive, these techniques require complex methodology and elaborate equipment infrastructure. Ali et al. (2009) investigated paper to fabricate a PAD capable of RCA, a technique used for DNA amplification and detection. Despite the wellestablished PCR technique for DNA amplification, RCA is more appealing as a PAD. It can be carried out under isothermal conditions, at room temperature, or 30°C, using a simple biochemical procedure involving Phi29 DNA polymerase. The Phi29 DNA polymerase enzyme can displace short DNA strands to amplify a DNA primer into long single-stranded DNA products. This increases the detection capabilities. Immobilization of a DNA oligonucleotide on strips of paper containing poly(*N*-isopropylacrylamide)

microgels creates a platform for which target DNA could ligate and undergo RCA-mediated amplification, enhancing DNA detection. The advantage of identifying a specific DNA sequence, rather than other biomarkers, is the specificity of the DNA itself. It provides an ultrasensitive diagnosis tool for disease. If achievable, it can be applied to any specific pathogen, including bacteria. However, preliminary results have identified a decreased sensitivity compared with other strategies; methods for improving sensitivity are being explored.

Govindarajan et al. (2011) used 2D-*o*PADs to demonstrate POC cell lysis and bacterial DNA extraction from raw viscous samples of *Escherichia coli*-spiked pig mucin. The sequential folding of 2D surfaces created temporary circuits, which dictated capillary flow without external pumping or power. The ability to detect DNA using 2D-*o*PADs can be used for diagnosis of tuberculosis. However, lysis of the *Mycobacterium tuberculosis* bacteria is difficult and requires further investigation.

Physiological disorders and analytes

The ability to detect a wide variety of physiological biomarkers can be used to develop low-cost bio-diagnostic platforms. Most initial PAD designs used glucose, pH, and protein detection to demonstrate the design's validity for biomedical testing. However, more recently, specific applications of detecting other biomarkers for disorders, such as cancer and liver function, have also been used to show the functionalization of PADs. Whilst the current research has only attempted these specific examples, the use of PADs is not limited to the physiological disorders mentioned below.

Diabetes

One of the most common validation tests used for paper diagnostics is the detection of glucose in urine (Martinez et al. 2007, Fenton et al. 2008, Abe et al. 2008). In fact, dipstick diagnostics to detect glucose were the first functionalization application (Free et al. 1957). While diagnosis and detection of diabetes in patients is well established, paper diagnostics can provide an alternative testing method. The dipstick assay relies on enzymatic reactions of glucose with glucose oxidase to produce gluconic acid and hydrogen peroxide. The hydrogen peroxide would in turn react with orthotolidine which results in a colorimetric change to a deep blue color (Fenton et al. 2008). Later tests involved the addition of red dye and resulted in shades of purple at different intensities to estimate concentration.

Cancer markers

Recently, cancer biomarkers have gained interest as potential analytes for paper diagnostics. An example is a platform for miRNA detection, which does not require the use of equipment for lung cancer diagnosis (Yildiz et al. 2012). miRNA regulates the mRNA function in gene transcription. The miRNA assay detects the formation of duplex and triplex species which show unique and varying optical signals to the naked eye. An miRNA sequence specifically associated with lung cancer is mir21. Analyses showed a linear correlation between concentrations 10 nm to 10 mm. It is selective, displaying a purple color for the duplex and orange color for the triplex. While a good indicative process for lung cancer treatment, further testing is required prior to clinical testing, including an investigation into the sample pretreatment and a sensitivity analysis compared to current standard tests (Yildiz et al. 2012).

Liver function

Paper diagnostics have been considered to monitor liver function (Vella et al. 2012). Overmedication of patients can induce liver toxicity and can also lead to drug resistance, rendering the treatment ineffective. This commonly occurs in developing countries, since tests for monitoring liver function are expensive, and require trained equipment and personnel. Vella et al. (2012) developed a finger-prick paper diagnostic to measure the levels of two enzymatic markers which are indicative of liver function: alkaline phosphatase (ALP) and aspartate aminotransferase. Total serum protein can also be measured. The design comprises a patterned paper-chip, a filter, and selfadhesive laminating sheets. It accomplishes four primary functions: 1) separation of RBC from plasma, 2) distributing the plasma into three regions within the paper, 3) conducting three simultaneous colorimetric assays, and 4) displaying the results for quantitative analysis using telemedicine. The results reported were consistent with the colors seen in tests for all three analytes in artificial blood; however, a sensitivity analysis was not provided. Calibration curves were measured, but failed to take into account the background interference from using whole blood. Additionally, further investigation regarding stability in

warmer climates and selectivity from other potential biomarkers are required.

Pathogenic diseases

Paper diagnostics can immobilize analytes which selectively bind to biomarkers for the detection of pathogenic diseases. Such diseases include: malaria, HIV-1, hepatitis B virus (HBV), and hepatitis C virus (HCV). ELISA paper diagnostics, in particular, have great potential applications. The ability to use paper diagnostics for pathogenic detection is ideally suited for diagnosis in developing countries where equipment, resources, and trained personnel are scarce.

Dipstick assays can detect several pathogenic diseases, and these analyses can also be applied to other paper diagnostic designs (Dineva et al. 2005). Dineva et al. (2005) describe a dipstick test combined with multiplex reverse transcription PCR (RT-PCR) for the detection of HIV-1, HBV, and HBC. No 2D or 3D paper diagnostic prototypes for pathogenic detection have yet been commercialized; however, their applicability in the field has been demonstrated.

Malaria

Malaria is caused by the *Plasmodium* parasite, which is transmitted by infectious mosquito bites, displaying symptoms of fever, vomiting, and/or headaches (World Health Organization 2013). It is endemic in 104 countries, most of which are remote with humid weather conditions.

Successful identification of the disease can be achieved by detection of the *Plasmodium falciparum* histidine rich protein 2 found in malaria (Dineva et al. 2005, Fu et al. 2012). The 2D-PN described by Fu et al. (2012) demonstrated a fully automated system for detection using a sandwich format immunoassay. The 2D-PN diagnostic had two additional processing steps for rinsing and signal amplification using a gold enhancement reagent. The LOD for the amplified assay was similar to that reported for ELISA: 2.9 ± 1.9 ng/ml and 4.0 ng/ml, respectively. This demonstrates the applicability of the automated 2D-PNs in a POC setting for malaria.

HIV-1

The retrovirus, HIV, infects cells within the immune system by destroying or impairing their function (World Health Organization 2013). The immune system is progressively weakened over time, until the patient becomes easily susceptible to infections. While there are antiretroviral drugs which slow down the disease, there is currently no cure for HIV-1. Efficient and accurate detection may not stop the illness, but early detection can help slow progression and patient deterioration, while preventing the spread of the disease.

The dipstick assay uses RT-PCR, which tests for specific nucleic acid sequences. For HIV-1, this means detection and identification of the HIV-1 genome (Dineva et al. 2005). However, the HIV-1 RNA must be prepared before testing. While the instrumentation is relatively inexpensive, it still limits application in remote areas and developing countries where equipment-free diagnostics are preferred.

While the dipstick device relied on signal amplification of nucleic acid hybridization, detection of HIV-1 provides an example for the applicability of the P-ELISA (Cheng et al. 2010). The HIV-1 envelope antigen gp41 successfully detected specific antibodies in human serum by using indirect P-ELISA methodology. Serum samples from HIV-1-positive patients were tested at different concentrations against control samples of human serum without anti-gp41. The colorimetric results indicated decreased intensity signals with serum dilution, but could still distinguish a positive result, thereby showing that a complex mixture, such as human serum, could be analyzed. Additionally, Ayele et al. (2007) described a method to collect and store samples for HIV-1 testing in field conditions.

Hepatitis B and C

Both hepatitis B and C (HBV and HCV, respectively) are viral infections transmitted through contact with blood or other bodily fluids, infecting the liver and causing hepatic diseases. Both vary in severity; however, HCV is known to potentially lead to liver cirrhosis or cancer. A vaccine for HBV is currently available but not for HCV (World Health Organization 2013).

Dineva et al. (2005) described a dipstick method to detect HBV DNA and HCV RNA. However, much like with HIV-1, the procedures and equipment used for preparation are not ideal for POC situations.

Successful HBV analysis showed the applicability of the 3D-ELISA paper device to detect HepB surface antigens (HBsAg) in rabbit serum. However, the protocol varied from the indirect ELISA methodology (Liu et al. 2011). A primary antibody was used combined with an ALP-conjugated secondary antibody to label the HBsAg. The additional steps were achievable thanks to the ease of fabrication and flexibility of the □PADs. Serum samples of HBsAg-

positive hosts were compared to serum controls without HBsAg, and showed that a 10-fold dilution was still discernible, thus supporting its potential for detecting hepatitis B and other infectious diseases.

Perspectives

There are a few critical issues to address relating to the development of paper diagnostics for biomedical applications. These include: 1) test sensitivity, 2) test robustness, 3) single *versus* multiple testing, 4) validity/reproducibility, and 5) simplicity of use.

Test sensitivity

Paper colorimetric tests can directly detect and visually report an analyte down to concentrations of 10^{-6} m (Li et al. 2010b). The detection limit is influenced by the wavelength (color) and intensity of the specific dye chemistry, as well as by the lighting conditions, type of paper, and moisture level/relative humidity. However, a concentration of 10^{-6} m might be insufficient for certain applications, such as early cancer detection, which might be required to detect analytes at a much lower range, possibly concentrations of 10^{-9} m to 10^{-12} m. For such applications, an amplification mechanism is required. Two amplification mechanisms have been tested for paper diagnostics. These include: 1) amplification by an enzyme producing a color product upon detection, such as ELISA, and 2) solid-light interaction principles. Mechanisms based on fluorescent dye are possible, but necessitate special instrumentation; quenching and interaction with paper can bring complications. The ELISA paper test, while possible and tested, requires multiple adsorption/washing steps that are cumbersome, time consuming, and delicate operations for standalone paper tests. ELISA for a paper test would only be realistic when a paper insert/test is used in conjunction with a robotized analytical plate reader apparatus.

An optical detection mechanism offers an alternative, with techniques such as surface plasmon resonance (SPR), surface enhanced Raman scattering (SERS), and similar techniques based on solid-light principles. These techniques usually rely on some light-surface interaction. SPR is widely used in biosensors to quantify the adsorption of molecules at the solid-liquid interface. SPR is the collective oscillation of valence electrons that occurs when light irradiates a surface, typically a metal such as gold or silver. SPR is very sensitive to the refractive index at the metal interphase (zone extending around 50 nm from the surface), thus providing a mechanism to measure adsorption of the small molecules widely used in lab-on chip sensors. SPR of metal nanoparticles, especially gold (AuNP), yields visible color change upon molecule adsorption. Small and well-dispersed AuNPs (diameter of 10 nm–50 nm) show an intense red color with extinction coefficients which are much higher than those of common dyes, due to their localized SPR (Ngo et al. 2012). The surface of AuNPs can be tailored by ligand-functionalization to selectively bind biomarkers. The most general approaches are chemical functionalization and thiol-linking of DNA of AuNPs to detect specific binding of proteins or antibodies. Upon the addition of analytes, functionalized and welldispersed AuNPs are induced into aggregates, which show a significant color shift from red to blue (Figure 11). This is due to interparticle plasmon coupling, which occurs as the surface plasmon of the individual AuNPs combine when their interparticle distance is smaller than their diameter. To achieve an efficient SPR performance on paper, a higher coverage of AgNPs is needed on the paper surface to enhance sensitivity of the nanoparticles-functionalized paper. The paper structure must maintain the adsorption state of nanoparticles (e.g., dispersed or aggregated) upon drying. To carry out ideal colorimetric detection, the activity of nanoparticles must be preserved during storage and remain functional upon rehydration for use. Furthermore, careful control of the size of nanoparticles and the porosity of paper is required, so that the nanoparticles will not be entrapped inside the pores within the paper structure, which would restrict the aggregation or dispersion of the nanoparticles and accessibility of target analyte.

Raman scattering is a light scattering technique in which Raman photons are scattered by interaction with vibrational and rotational transitions in a molecule. However, its sensitivity is limited because the Raman signal is very weak. The development of SERS has increased dramatically since Fleischmann et al. (1974)

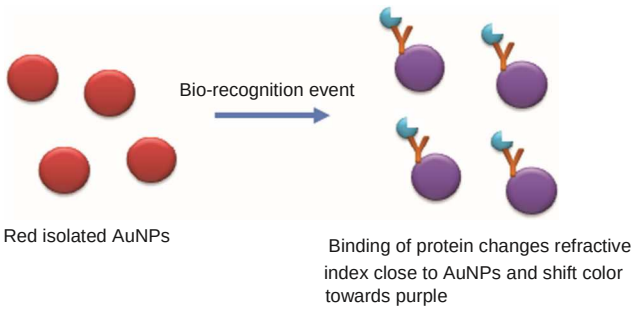

Figure 11 Colorimetric detection of biological targets by gold nanoparticles (AuNPs). [A5] [redrawn from (Nath 2009).]

published their discovery on enhanced Raman signals from pyridine on a rough silver electrode. This discovery of SERS has transformed Raman spectroscopy, from a structural analytical tool, to a sensitive single-molecule detection and nanoscale probe (Freeman et al. 1994). Intense SERS enhancement is often present at the point of contact between two or more metal nanoparticles (Michaels et al. 2000). As the metal nanoparticles are contacted to form aggregates, their transition dipoles couple to each other and the enhanced fields of each nanoparticle coherently interfere at their contact point. When molecules are adsorbed in this contact point – often called the hot spot – their Raman signals can be significantly enhanced (10^{14}– 10^{15}) (Toderas et al. 2007). Aggregates of nanoparticles have more efficient SERS properties than individual nanoparticles, because larger enhancements can be achieved at particle junctions of the aggregates.

Since the aggregation of nanoparticles plays an important role in enhancing the SERS signal, their adsorption and aggregation state within the paper structure is critical. Stability of nanoparticles on paper substrates must be preserved, so that they can be stored for long periods between measurements. Reproducibility of their aggregation state is another important factor to achieve accurate SERS results. Since the spot size of Raman's laser beam is approximately 1 □m, an ideal SERS active substrate needs to have uniform distribution of nanoparticles on a submicrometer scale in order to achieve a high degree of reproducibility. This is a critical issue to address when paper is used as a SERS substrate, since it is a challenge to achieve uniform distribution of nanoparticles on paper, due to its high roughness and porosity. In addition, because of the high sensitivity of SERS, the paper substrates must be free of additives or fillers, to avoid any background or fluorescence interference during the analysis. Figure 12 illustrates the different length scales involved in SERS analysis with AuNP-treated paper.

SERS using AuNPs is an exceptional technique for quantifying the adsorption of target molecules on substrates, and allowing different orientations and interactions of the molecules with the substrates to be determined (Wu and Fang 2003). Single molecule detection is achievable via SERS and this concept has been used to design protein and nucleic acid biosensors (Bizzarri and Cannistraro 2007). A highly SERS-active substrate is the most important factor in producing efficient SERS applications. Previously, aqueous metal colloids were employed in most SERS techniques, but this limits the application of SERS, since specimens analyzed must be water-soluble. Filter paper coated with AuNPs offers a much simpler method and the compounds examined do not have to be water-soluble. Ngo et al. investigated the effect of AuNPtreated paper as a generic platform for SERS detection and quantification of analytes (Ngo et al. 2012, 2013a,b). The effects of AuNP surface coverage, aggregation size, and distribution on paper on the efficiency of SERS were quantified (Ngo et al. 2012, 2013a). Methods of producing AuNP paper for SERS application were optimized. Not all analytes are SERS-sensitive; a better understanding of the SERS efficiency as a function of the analyte molecular structure is needed.

Test robustness

The proper life time of paper diagnostics is a major issue. This requires both the microfluidics and the bioanalytical detection system to be stable for at least 6 months, or better, 1 year or more, to ensure proper time for commercialization and distribution. A critical requirement is for the adsorbed biomolecules to be stable and to remain bioactive over paper. Enzymes, antibodies, and antigens are of special interest for biorecognition in diagnostics. To be practical, paper diagnostics must be stored dry until use.

Khan and Garnier measured the stability and the kinetics of enzyme immobilized on paper with and without polymers used as retention aids (Khan and Garnier 2013). The stability of ALP and horseradish

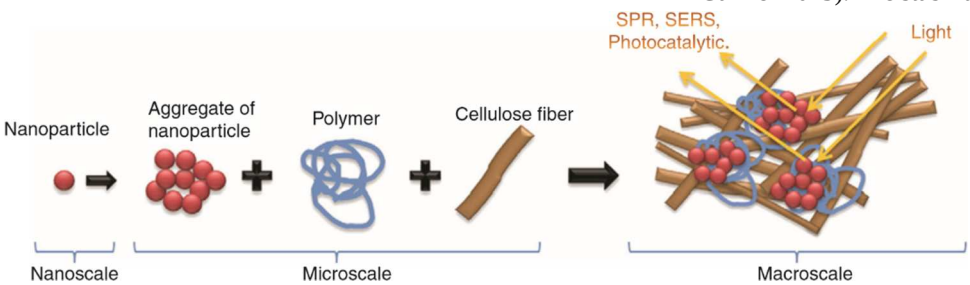

Figure 12 Different length scales of the constituents of gold nanoparticle (AuNP)-treated paper for surface enhanced Raman scattering (SERS) application (Ngo et al. 2011).

peroxidase adsorbed on paper was found to be two to three orders of magnitude higher than in solution, but also two to three orders of magnitude slower (Khan et al. 2010c). Khan et al. (2010c,d) also modeled the thermal deactivation of ALP on paper to predict enzyme activity. Heat deactivates and denatures enzymes by modifying their conformation, due to increased thermal movement and decreased solvent stabilization (Khan et al. 2010c,d). The idea that immobilization of an enzyme on paper prevents aggregation and retards the conformation disorder, by stabilizing the secondary and tertiary structures of the enzyme, was studied. The effects of aging time and temperature on the relative activity of ALP enzymatic papers, with and without polymer treatment, are illustrated in Figure 13. The enzymatic activity quickly dropped within the initial hours of thermal treatment and then gradually decreased at a slower rate. The enzyme deactivation was faster at higher temperatures. The deactivation rates of ALP enzymatic papers treated with polymers were much faster. Zhang and Rochefort (2010, 2011) and Rochefort et al. (2008) investigated enzyme microencapsulation to protect biomolecules. Enzymes microencapsulated in cross-linked PEI and coated on paper using standard technology displayed a marked improvement in stability without losing selectivity (Zhang and Rochefort 2010, 2011, Rochefort et al. 2008). A better understanding on the aging of biomolecules, such as antibodies and antigens adsorbed or immobilized on paper, is required.

Test design and application

An important issue is the validity of the test and understanding how design affects the detection threshold and its effect on diagnostics and treatment. A consequence of increasing sensitivity can be the increase in the occurrence of false positive tests. This can be achieved by optimizing the antibody conditions to react with an antigen. A lack of selectivity can also create false positives tests. The opposite is a false negative test, which can result either from too low sensitivity, i.e., level of detection, or from the lack of recognition of the analytical biomolecule. The lack of recognition can be due to poor binding specificity, but also from weak or partial antibody/antigen. Examples are the weak D and partial D antigen on the RBC recognition in blood typing. For blood typing tests, false positive and false negative results can lead to a fatal hemolytic transfusion reaction. A false negative can result in the transfusion of a donor with blood A, B, or D to a recipient of blood O-; conversely, a false positive can cause a O- recipient to be incorrectly identified as A, B, or D, resulting in the same crisis upon improper transfusion. For certain applications,

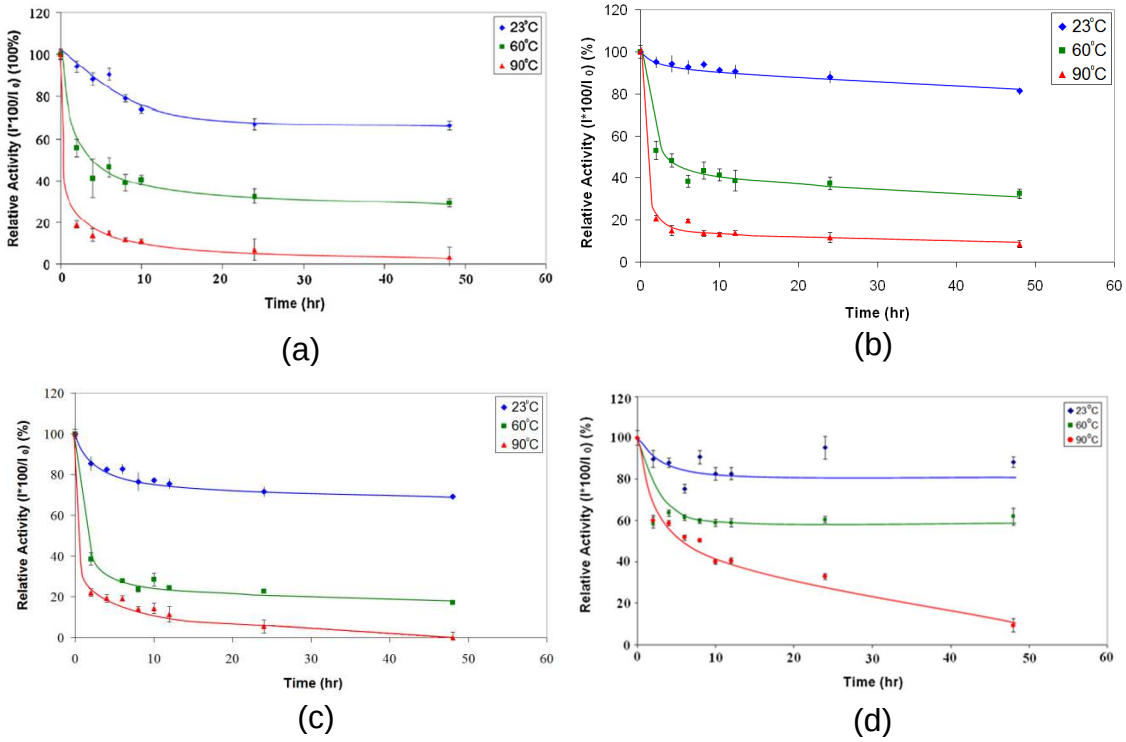

Figure 13 Effect of time and temperature on the relative activity of enzymatic papers. Alkaline phosphatase (ALP) stabilized on paper using: (A) ALP paper without polymer; (B) ALP cationic polyacrylamide (CPAM); (C) polyacrylic acid (PAA); and (D) polyethylene oxide (PEO) papers. (Khan et al. 2010c,d)

such as blood typing of primary and secondary groups, total accuracy and the absence of any false positive/negative results are imperative. For other applications, in which a paper test is used as the first detection line, reproducibility/sensitivity can be traded for simplicity and low cost, allowing wider and more frequent public use. Unfortunately, very few studies have analyzed the issue of false positive/negative results in diagnostics and their potential effects.

Simple detection on/off binary tests identify the presence or absence of a critical analyte. The main advantage of these tests is their simplicity and ease of use and interpretation. The best example is the pregnancy test; the patient is either pregnant or not. Of interest is the ability to control the critical analyte concentration at which a "positive" is communicated. For example, cholesterol and prostate specific antigen are becoming health issues only over a certain concentration. A binary test only reporting concentrations above these critical thresholds would be very useful for home testing.

Significant research effort has been dedicated for multiple paper test use, as it had been identified as a major weakness. But is that so? The higher the test complexity, the higher the price and the lower the robustness, ease of use, and interpretation. At the limit, these tests become non-competitive relative to the traditional tests and methods they were designed to replace in the first instance. Research progress has demonstrated that paper tests can be designed to provide alternatives to most diagnostics needed. However, paper diagnostics will make sense and be successful only should they provide a significantly cost saving and simplicity over their targeted replacement. Research prowess and high level publication is a facet of paper diagnostics; public acceptance and commercialization is another. These should not be confused.

Conclusion

The development of paper diagnostics analytical devices (PADs) for medical applications has exploded in recent years. This is because paper is cheap, widely available, easily engineered, disposable, sterilizable, hydrophilic, and easy to functionalize and process into diagnostic devices. Paper is a very attractive substrate to develop a generic low-cost diagnostic platform. Paper can serve four functions in a diagnostic: 1) transport and measurement of samples and analytes, 2) reaction support, 3) separation of reactants from products, and 4) communication of results. A plethora of manuscripts present new concepts of rapid/instantaneous medical analytical tests and analyzing the performance of PADs. Many ingenious and sometimes complex 1D, 2D, and even 3D flow prototype diagnostics have been developed. Printing has emerged as the manufacturing technique of choice for PADs, first by printing microfluidic systems on paper and then the reagents and biomolecules. From literature, paper diagnostics have become ubiquitous for medical applications; but surprisingly, very few commercial applications exist.

This article has attempted to provide a roadmap, by reviewing the different types of paper tests, their principles, and medical applications and has analyzed their performances and limitations. Paper tests for medical applications must meet two requirements: very low cost and ease of use. The challenge is to ensure the selectivity and sensitivity of the diagnostics, while maintaining the simplicity and affordability that paper diagnostics attracts. The need to create more complex PADs to suit multi-step processes has often hindered the ability for many designs to be applicable in the field and commercialized. Additionally, each biomedical application differs in its own right, making it impracticable to design a single PAD to suit multiple diseases and disorders. Development is needed to reconcile the potential of very low cost, meaningful, and robust paper tests for specific medical analyses with increased sensitivity, flexibility, and reliability of the concept.

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ORIGINAL PAPER

The detection of blood group phenotypes using paper diagnostics

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Vox Sanguinis

Background and Objectives Paper biodiagnostics for blood typing are novel, cheap, fast and easy to use. Agglutinated red blood cells cannot travel through the porous structure of paper, indicating a positive antibody–antigen interaction has occurred. Conversely, non-agglutinated blood can disperse and wick through the paper structure with the ease to indicate a negative result. This principle has been demonstrated to detect blood group phenotypes: ABO and RhD. However, typing for red blood cell antigens such as Rh, Kell, Duffy and Kidd has not yet been explored on paper.

Materials and Methods Two paper testing methods – an elution and a direct flow-through method – were investigated to detect red blood cell antigens excluding the ABO system and RhD. Antigens explored include the following: C, c, E, e, K, k, Fy^a, Fy^b, Jk^a, Jk^b, M, N, S and s, P1, Le^a and Le^b. The variables tested include the following: reaction time and reagent concentration. The importance of antibody type/structure for successful agglutination on paper was confirmed.

Results Some blood group phenotypes showed less agglutination due to weaker antibody–antigen interactions. Most blood groups with antibodies available as IgM, such as C, c, E, e, K and k, and Jk^a and Jk^b, and P1, were successful using both methods. However, other blood groups, especially those with antibodies only available as polyclonal antibodies, were unsuccessful and require further scrutiny.

Conclusion Paper can be used as an alternative blood grouping diagnostic tool for selected blood group phenotypes.

Key words: blood groups, blood typing, haemagglutination, IgG, IgM, paper diagnostics.

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Introduction

Although ABO and RhD are the most important blood group systems in transfusion medicine, many other blood group antibodies can cause serious or fatal haemolytic transfusion reactions (HTRs) or haemolytic disease of the foetus and newborn (HDFN). Current blood typing techniques rely on antibody–antigen interactions to cause red blood cell agglutination for positive identification. Techniques, such as gel columns, are well established. Common methods for phenotyping blood groups are the tube test and column agglutination test (CAT), which require centrifugation only available under laboratory conditions, unsuitable for in-field and remote testing [1]. Currently, CATs are more widely used, relying on gels or glass beads to visualize agglutination. However, the process requires trained personnel, is more expensive, lengthy and time-consuming to operate. Low-cost and equipment-free methods, such as the glass slide test, can be insensitive and are unsuitable for antigen identification, particularly for neonatal samples [2]. This is especially true when testing for the wide array of clinically significant blood group phenotypes.

For many years, blood groups were the best genetic markers and have played a key role in the mapping of

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the human genome [3]. Blood group antigens are found on the surface of red blood cells (RBCs). These dictate an individual's blood type. Blood group antibodies are present in the blood plasma and generally result from an immune response triggered by prior exposure, such as previous transfusions or during pregnancy, to the corresponding antigen not located on that individual's RBC surface (alloantibody). There are now 35 blood group systems recognized, with more than 300 blood group antigens, many of which are clinically significant, requiring detection and the identification of antibodies to be performed prior to blood transfusions [3, 4]. The discovery of the ABO blood groups made blood transfusions safe; the disclosure of the RhD antigens led to the understanding and prevention of HDFN [2, 5, 6]. Each of the blood group phenotype systems contains antigens that are unique and have a different frequency depending on the patient's ethnicity [7]. The blood groups and their corresponding alloantibody examined in this study are summarized in Table 1. Aside from these blood groups, some alloantibodies, such as Anti-M, N, P1 and Lewis antibodies, are seldom active at body temperature (37°C) and are generally not considered clinically significant [2]. The mismatching of a blood group antigen in the presence of a clinically significant antibody could cause immediate, delayed or severe HTRs or HDFN, necessitating tests to identify or confirm the presence of these antigens. Prior to blood transfusion, the accurate and fast identification of the blood group phenotypes is essential for patients who possess an alloantibody [8].

Table 1 Blood groups and the corresponding antibodies used for blood group phenotyping on paper. The antibody structure and clone is also included

Blood system	Blood type	Reagent type	Clone
Rh	C	IgM	P3x25513G8
	E	IgM	DEM1
	c	IgM	H48
	e	IgM	MS-36 and P3GD512
Kell	K	Polyclonal	Polyclonal (human source)
	K	IgM	MS-56
	k	IgM	Lk1
Kidd	Jk^a	IgM	P3HT7
	Jk^b	IgM	P3·143
Duffy	Fy^a	Polyclonal	Polyclonal (human source)
	Fy^b	Polyclonal	Polyclonal (human source)
MNS	M	IgM	LM1
	N	IgM	LN3
	S	IgM	P3S13JS123
	s	Polyclonal	Polyclonal (human source)
P	P1	IgM	650
Lewis	Le^a	IgM	LEA2
	Le^b	IgM	LEB2

Further development in low-cost minor blood typing methods is necessary, especially for testing in developing countries and the battlefield. Novel paper diagnostic for human blood typing through the haemagglutination of RBCs was developed [9–17]. Paper can be pretreated with a blood typing antibody reagent. When a blood sample is subsequently introduced onto the antibody pretreated paper, if positive, a haemagglutination reaction will occur between the RBCs and corresponding antibody. These blood agglutinates are formed within the porous structure of paper and cannot be removed when washed with solutions such as phosphate-buffered saline (PBS) or 0·9% NaCl saline. Inversely, during negative reactions, the RBCs do not agglutinate and the cells are easily washed away. This phenomenon is the foundation of paper substrates for blood typing which was used to design diagnostics for ABO and RhD blood testing [12].

In this study, paper diagnostics were engineered to identify blood groups using haemagglutination, focusing on common clinically significant blood group phenotypes. The method for phenotyping these blood groups proved not to be as simplistic as the ABO and RhD groups; time-dependent reactions became evident [10, 17]. The influences of antibody types, reaction time, washing conditions and antisera concentration on the identification of blood group phenotype were investigated.

Two separate testing methods were developed using paper, each using different principles regarding the interactions of the analytes, samples and washing method. One method follows the principles of chromatography, allowing the washing solution to elute unbound blood cells laterally along the paper structure (wicking). This method was used for ABO and RhD groups [11]. The second uses the flow-through method [12, 18, 19], which washes through the paper, rather than along, utilizing a filtration mechanism. While each method has been successful when testing ABO and RhD blood types, this is the first report testing paper diagnostics for the detection of blood group phenotypes other than ABO and RhD using both methods.

Materials and methods

Materials and equipment

Professional Kleenex paper towel from Kimberly-Clark, Australia, was employed as paper. Antisera blood typing antibodies were purchased from ALBA bioscience, Edinburgh, United Kingdom, and used as received (Table 1). Washing solutions 0·9% (w/v) NaCl saline and PBS were prepared using MilliQ water, analytical grade NaCl (Uni-

var) and PBS tablets (Sigma-Aldrich, Castle Hill, Australia), respectively. Alkyl ketene dimer (AKD) from BASF was used for paper hydrophobilization. Analytical grade n-heptane from Sigma-Aldrich was used to formulate ink-jet solution to print text on paper. EDTA blood samples were sourced from Australian Red Cross Blood Service (ARCBS), stored at 4°C and used within 7 days of collection.

Micropipettes (Eppendorf research®, 2·5–50 ll) were used to introduce the antibodies, blood samples and saline solutions on paper. Millipore centrifugation tubes (50 kD cut-off) from Merck, Australia, were used to remove supernatant and increase concentration of the antibodies by filtration.

Method

Two methods were tested: the first was the elution methodology for high-throughput diagnostics in laboratory settings [11] and the second flow-through method used the direct-reporting method suited for remote and emergent situations [12]. These methods differ by the direction of liquid transport within paper during the washing step. The elution method relies on the paper structure to wick saline solution laterally and separate by chromatography along paper, while direct-reporting washes via a flow-through filtration-like method.

Elution

A known antibody reagent is dotted along the bottom of paper (10 ll) before the addition of mixed EDTA blood sample (3 ll) [11]. After a reaction period, ranging from 30 to 120 s, the substrate was hung in a vertical-standing elution tank containing 0·9% NaCl saline solution at a depth of 1 cm. Following an elution period of 5 min, the substrate was removed and dried in the laboratory (T = 22 – 1°C; relative humidity = 30–50%) (Fig. 1). Qualitative evaluation of results was conducted visually. Agglutination showed a distinct bloodspot, while non-agglutinated RBCs were defined by the absence of a blood spot and by a clear elution path on paper. Quantitative analysis was achieved using IMAGEJ software (National Institute of Health, Bethesda, MD, USA) to measure colour densities of the blood spot (BS) and elution pathway (EP).

Kleenex paper towel was selected for performance, attainability and cost. Compared with other paper, such as Whatman filter, the results achieved were clearer and more reliable [11, 16].

Flow-Through Direct reporting

Agglutinated cells can form patterns to resemble text or signs. This can be achieved by hydrophobic barriers printed onto the paper prior to the addition of cells. The hydrophobic barrier guides where antisera will be absorbed into the paper. This strong hydrophobic–hydrophilic contrast can be used to border the RBC–antibody interactions on paper, forming shapes or 'text' that can be easily read when fabricating a user-friendly blood group device [15]. A reconstructed Canon ink-jet printer (Pixma iP3600) was used to print channels onto paper with an AKD–heptane solution (Fig. 2).

Aliquots of 2·5 ll of antibody solutions were introduced into the corresponding patterns. After the antibody solution was dried, 2·5 ll of blood sample was introduced

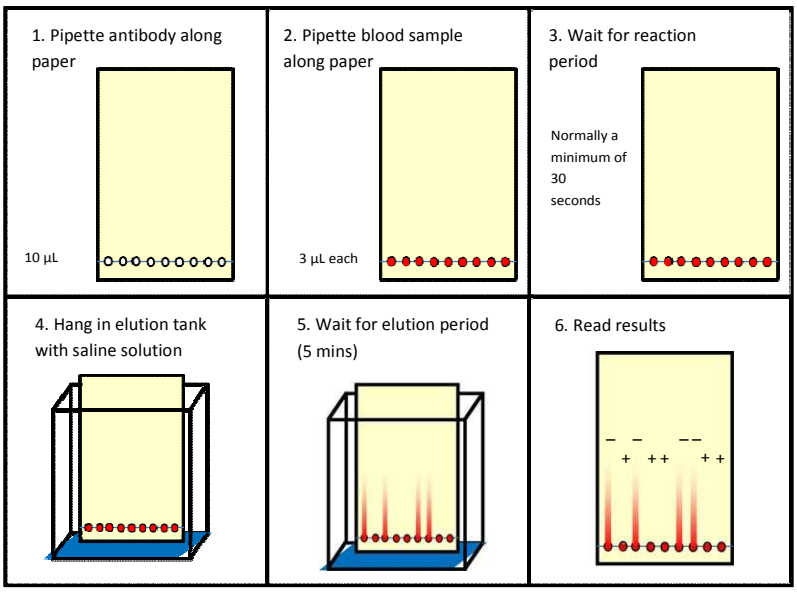

Fig. 1 Methodology for blood group phenotyping using paper via elution.

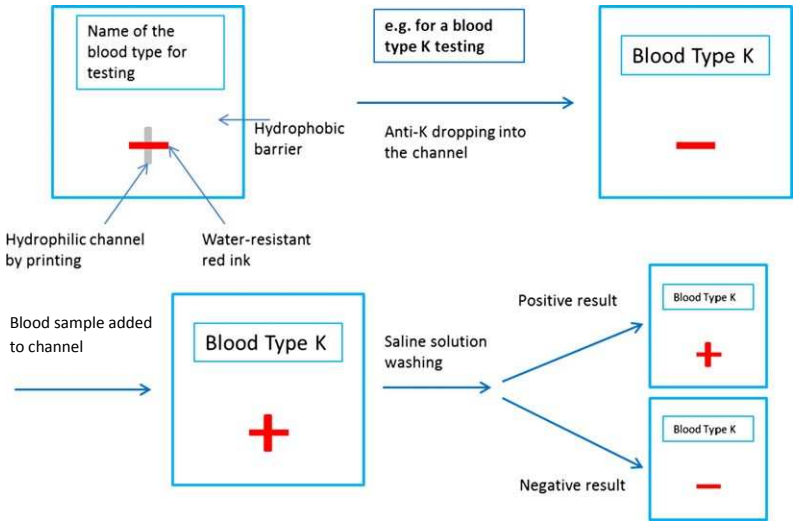

Fig. 2 Fabrication, testing procedures and result reporting of paper diagnostics for antigen detection using example: blood group K.

into the vertical channel. A reaction period was allowed for the antibodies to interact with the RBCs inside the channels. Washing was performed with two 30 ll aliquots of saline solution introduced into the patterns to wash out non-agglutinated RBCs. The blood types were then read directly. Quantitative analysis used IMAGEJ to measure channel density.

Analysis criteria

Successful testing should easily distinguish positive from negative, while preventing false-positive and false-negative results. For the elution method, a positive result should report a well-defined blood spot (BS) with no RBCs in the elution pathway (EP); conversely, a negative result would have no BS, but show distinct wicking in the EP. The direct-reporting method prints a positive '+' sign, while a negative in the absence of RBCs leaves a negative '-' sign. Two hundred and thirty-six different patient samples were tested. However, not all samples were fully phenotyped. The number of samples for each antigen tested was restrained by blood availability, outlined in Table 2.

To compare the strength of each blood group's antibody–antigen reactions and the testing conditions, the colour densities of the resulting BS were measured using IMAGEJ. Distinct patterns emerged when comparing positive and negative results. Higher density values indicated a positive result (usually >100), while lower values indicated negative samples (<40). Densities between this range (40–100) could be classified as weaker reactions. This distinction was used for the text-reporting method.

However, to analyse the elution method results, the BS alone was insufficient to indicate the interactions of certain blood groups. In such instances, the BS optical densities bordered the weak reaction threshold. In some cases, the BS for negative and positive looked too similar for accurate determination by the naked eye. It was the presence/absence of an EP that confirmed the result. Therefore, in addition to BS density, an area of the EP 2 cm above the BS was measured. Positive results contained little or no RBCs in EP with low density values. Conversely, negative results showed high densities representing unbound RBCs travelling through paper after washing. This difference was translated into a fraction, the optical density ratio (ODR), comparing density of BS to EP. A higher ODR value (>6·0) indicated positive, while low values (<3·0) indicated negative, with weak reactions between indicated in between (3·0–6·0),

$$\text{ODR} = \frac{\text{BS}}{\text{EP}}.$$

Results

A series of antibody–antigen reaction tests were performed using both procedures to detect blood group phenotypes (Figs 1 and 2). As each antibody–antigen pair behaves differently when tested, each needed to be analysed individually. This differs from the major blood groups (A, B, O and RhD), which react strongly and clearly for most patients; exceptions include the weak subgroup variants [10, 11, 16]. It was found that several factors impacted the interaction strengths observed for each antigen tested. These factors are as follows: (1) reagent type, (2) reaction time, (3) antibody concentration and (4) washing conditions. The washing conditions were found to affect the results of the text-reporting method, but not the elution method. This is due to the effects of directional flow during washing. Table 2 and Fig. 3 depict the

Table 2 Blood group phenotyping using the elution (E) and text-reporting (TR) methods on paper. Blood spot (BS) and elution pathway (EP) are represented as density. Optical density ratio (ODR) compares density EP:BS. Positive is denoted by high density and ODR, negative has lower densities and ODR

Antigen		C		E		c		e		K		K		k	
Antibody type		IgM		IgM		IgM		IgM		IgM		Polyclonal		IgM	
No. of samples		76		67		54		8		11		53		25	
Reaction time	(s)	30		30		E:120, TR:180		E:60, TR:180		E: 60, TR:120		120		120	
Buffer		NaCl		NaCl		PBS		PBS		NaCl		NaCl		NaCl	
		+	–	+	–	+	–	+	–	+	–	+	–	+	–
No. of samples		31	45	17	50	40	14	41	8	2	9	12	41	23	2
Elution method	BS	123 – 9·1	51 – 9·4	118 – 5·1	42 – 7·9	105 – 4·3	44·0 – 2·4	107 – 4·63	38 – 4·1	82 – 3·6	41 – 7·7	97 – 24	58 – 7·0	97 – 6·0	64 – 1·7
	EP	8·2 – 3·1	32 – 6·8	8·5 – 3·0	49 – 11	5·8 – 1·0	32 – 6·5	6·1 – 0·48	54 – 6·5	13 – 8·8	27 – 4·3	16 – 9·4	34 – 7·1	16 – 3·1	44 – 6·0
	ODR	17 – 2·7	2·9 – 0·55	16 – 3·6	1·0 – 1·4	12 – 3·7	1·8 – 0·39	18 – 1·5	0·72 – 0·12	8·4 – 5·5	2·0 – 0·46	0·18 – 0. 18	0·59 – 0·13	6·4 – 1·5	1·5 – 0·25
Text method		152 – 7·4	28 – 3·8	145 – 7·1	30 – 4·9	154 – 5·7	30 – 3·7	135 – 9·2	30 – 4·2	106 – 5·0	34 – 4·0	46 – 4·1	35 – 7·1	131 – 8·3	31 – 5·0

Antigen		Fy^a		Fy^b		Jk^a		Jk^b		P1		Le^a		Le^b	
Antibody type		Polyclonal		Polyclonal		IgM		IgM		IgM		IgM		IgM	
No. of samples		12		8		30		25		40		46		23	
Reaction time	(s)	120		30		120		120		30		180		180	
Buffer		NaCl		NaCl		NaCl		NaCl		NaCl		PBS		PBS	
		+	–	+	–	+	–	+	–	+	–	+	–	+	–
No. of samples		9	3	7	1	22	8	15	10	26	14	13	33	11	12
Elution method	BS	60 – 13	60 – 23	37 – 5·6	40	106 – 8·7	48 – 4·0	100 – 7·5	67 – 8·3	128 – 16	69 – 10	N/A	N/A	N/A	N/A
	EP	39 – 15	38 – 13	33 – 5·8	35	14 – 3·6	40 – 4·2	15 – 3·6	34 – 9·1	14 – 8·8	35 – 15	N/A	N/A	N/A	N/A
	ODR	1·6 – 0·17	2·0 – 0·028	1·1 – 0·19	1·1	8·3 – 2·7	1·2 – 0·13	6·8 – 1·8	2·9 – 1·2	12 – 1·9	1·7 – 0·63	N/A	N/A	N/A	N/A
Text method		36 – 11	27 – 4·0	N/A	N/A	95 – 6·2	37 – 4·8	122 – 9·8	34 – 6·2	140 – 5·7	31 – 5·5	68 – 3·1	35 – 6·2	116 – 5·9	37 – 6·2

results achieved for both the elution and text-reporting method for the blood groups investigated (Table 1). However, testing for Duffy groups, Fy^a and Fy^b, MNS groups, and Lewis groups, Le^a and Le^b, was unsuccessful.

Testing conditions

The main variables investigated for optimization include the following: (1) reagent type, (2) reaction time, (3) antibody concentration and (4) washing conditions.

Reagent type

The effect of reagent type was investigated. Certain antibodies are only commercially available as polyclonal (human source), not immunoglobulin M (IgM). IgM antibody reagents are generally monoclonal, containing a pentameric structure, with ten binding sites. IgG antibodies are monomers, containing only two binding sites. While IgM antibodies show strong agglutination, IgG antibodies generally only sensitize cells without agglutination. This is due to electrostatic repulsion between RBCs. IgM antibodies provide over twice the bridging distance, overcoming the electrostatic double-layer surrounding RBCs. IgM antibodies are expected to exhibit better binding capabilities compared with IgG antibodies, allowing direct agglutination rather than only sensitization to occur. The identification of certain blood groups by direct agglutination using paper is therefore constrained by the commercial availability of IgM antisera.

Reaction time

The reaction time is the time allowed for the antibody sera and RBCs to react. Haemagglutination between ABO and RhD antigens and antibodies can be achieved within 30 s. However, some minor blood group antigens take longer

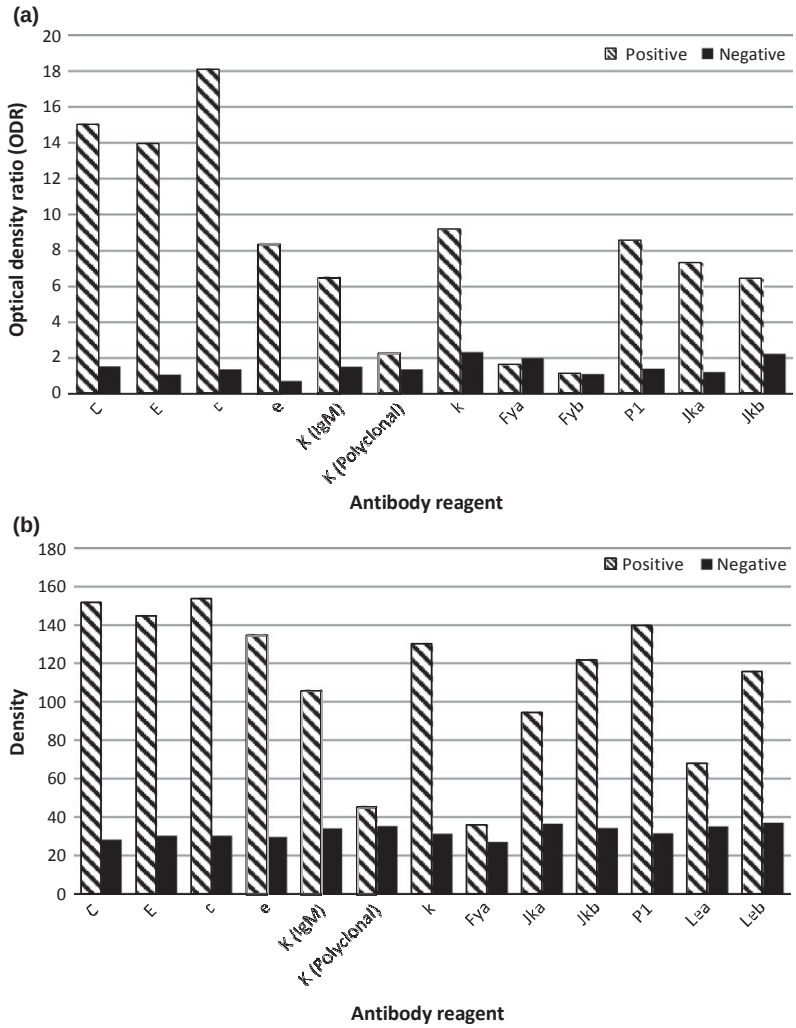

Fig. 3 Blood group phenotyping using (a) elution and (b) text-reporting methods on paper. Blood spot (BS) and elution pathway (EP) are represented as density. Extent of coagulation is represented as optical density ratio (ODR) comparing density EP:BS. Positive is denoted by high density and ODR, negative has lower densities and ODR. (Fy^b was not tested using the text-reporting method.)

than 30 s to bind with their antibodies to show strong haemagglutination for clear identification. Thus, the time the RBC antigens had to bind was extended and compared. Reaction times 30, 60, 120 and 180 s were tested, with optimum conditions reported in Table 2. Reaction times beyond 180 s were not explored as longer reaction times could increase the potential of false positives.

Antibody concentration

The effect of antibody concentration was explored. Antibody concentration was increased by filtering the reagent using Millipore centrifugation tubes. These tubes retain biomolecules 50 kDa or larger and remove supernatant. Increased concentration allows faster collision rates and resulted in stronger agglutination for positive tests. However, this raises the possibility of provoking the prozone effect where agglutination does not occur due to an excess of antibody or antigen.

Washing conditions

Varying the washing buffer was investigated to determine the effect of pH, ionic strength and specificity has on the elution of RBCs and clarity of results. Using the text-reporting method, washing using PBS yielded clearer results, while the effects were nominal for the elution method.

Discussion

Each blood group typing reaction behaved differently when tested, and therefore, each required individual analysis.

Rh blood group system

Apart from the ABO blood groups, the antibodies against the Rh blood group system are the most common clinically significant antibodies. Of these, D is the most immunogenic antigen. The additional antigens investigated within the Rh system which are important when screening patient or donor blood are C, c, E, and e. Testing using paper for these four antigens was successful using the commercial antibody reagent at a standard reaction time of 30 s. The Rh antisera used were all monoclonal IgM (Table 1).

Using the elution method, the C and E antigens were strongly detected (ODR pos: 17 – 2·7, neg: 2·9 – 0·55; pos: 16 – 3·6, neg: 1·0 – 1·4, respectively) (Fig. 3a), only needing 30-s reaction time. However, c and e antigens showed a slightly less clear BS with higher density in the EP. The clarity of tests for c and e improved with longer reaction times of 120 s (ODR pos: 12 – 3·7, neg: 1·8 – 0·39; pos: 18 – 1·5, neg: 0·72 – 0·12, respectively). Extending the RBC–antibody contact time to 2 min or more showed better agglutination with less unbound RBCs able to migrate through the paper structure.

A maximum reaction time of 180 s was selected as longer reaction times could increase false positives. For the elution method for c and e, a 180-s reaction time provided less distinct results with negative ODRs of 1·8 – 0·39 and 3·0, respectively. While above the threshold, a lower ODR for negative results provides a clearer result (Fig. 4).

The text-reporting method was similar. C and E had clear and distinct densities (pos: 152 – 7·4, neg:

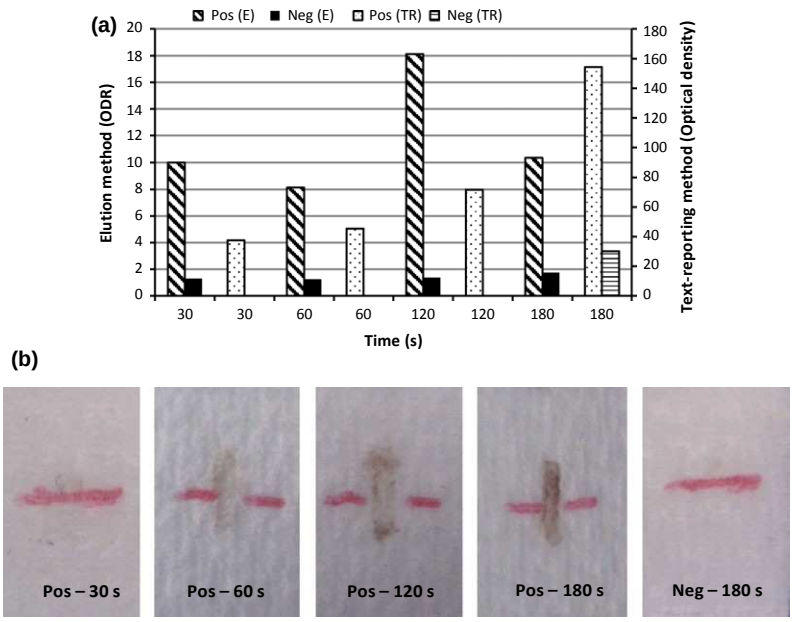

Fig. 4 (a) Effect of time on the reaction period tested with c antigen, comparing elution method (E) and text-reporting method (TR). ODR of E is compared to density of TR. (b) Effect of time on the reaction period tested with c antigen using text-reporting.

28 – 3·8; pos: 145 – 7·1, neg: 30 – 4·9, respectively). When compared to the BS data of the elution method, the text-reporting method showed a clearer distinction between positive and negative. The c and e tests similarly improved with a longer reaction time of 180 s (pos: 154 – 5·8, neg: 30 – 3·7; pos: 135 – 9·2, neg: 29 – 4·2, respectively) (Fig. 4).

Kell blood group system

Two clinically significant blood group antigens from the Kell system were explored: K and k. Two types of K reagents commercially available were tested: monoclonal IgM and polyclonal (Table 1). The anti-K polyclonal reagents manufactured from human plasma are expected to be composed of IgG antibodies because the immune response against the Kell system produces predominantly IgG antibodies [2]. The efficiency of both reagents was tested with both paper testing methods.

The elution method reported ODR pos: 8·4 – 5·5, neg: 2·0 – 0·46; pos: 2·3 – 0·11, neg: 1·4 – 0·39, for IgM and polyclonal, respectively. The text-reporting method had densities of pos: 106 – 4·9, neg: 34 – 4·0; pos: 46 – 4·1, neg: 35 – 7·1, for IgM and polyclonal, respectively. Interactions between IgM antibodies and the RBCs are strong, and there is no overlap between a positive and a negative test. While both paper methods were accurate with the anti-K IgM antibody, they both failed with polyclonal anti-K (Fig. 5). A clear distinction between positive and negative is fundamental for blood group testing, and only IgM antibodies have systematically achieved such distinction.

In the Saline tube test, IgM antibodies can directly agglutinate antigen-positive red cells, whereas IgG antibodies require anti-human globulin (AHG) to effect agglutination. For both paper testing methods, agglutination between IgG antibodies and RBC antigens without AHG led to false-negative results. As in saline tube testing, this is explained by the greater size of the IgM pentamer able to bridge red cells for electrostatic stabilization. To achieve bridging, the critical length of the antibody must be greater than the electrical layer surrounding the RBC. While IgM antibodies can bridge red cells, where IgG monomers cannot, this allows IgM antibodies to agglutinate red cells in saline solution.

Washing buffer solution affected the clarity of results for the text-reporting method. Using PBS improved the density compared with 0·9% NaCl saline. This is likely due to the directional flow when washing. Only nominal effects were observed for the elution method.

Testing with polyclonal anti-K antisera was used to determine the effect of increasing antibody concentration. Surprisingly, increasing the concentration of the commercial polyclonal antibodies by removing excess serum enhanced reaction clarity. Antibody concentration was increased by filtering the serum to retain biomolecules 50 kDa or larger.

Increasing antibody concentration improved the stoichiometric ratio between antibody and antigen (or RBCs). A higher stoichiometry ratio allows for more colloidal interaction; however, it is costly. This provides a counterpoint to reaction kinetics, as a longer reaction time decreases rapidity and could increase potential false positives.

This worked unexpectedly well, enhancing the clarity for polyclonal anti-K when concentration was doubled (pos: 6·8, neg: 1·4 – 0·46 with a reaction time of 60 s), with optimum at 120 s (pos: 7·8, neg: 0·61 – 0·18) (Fig. 6). However, the same method applied to other polyclonal reagents was unsuccessful. Polyclonal anti-K antisera, while assumed to be predominantly IgG antibodies, could contain residual IgM from the original source. Removing the excess supernatant probably increased the IgM concentration in addition to the IgG, thus promoting agglutination. To test this hypothesis, a simple immediate spin tube test and an indirect antiglobulin test (IAT) were

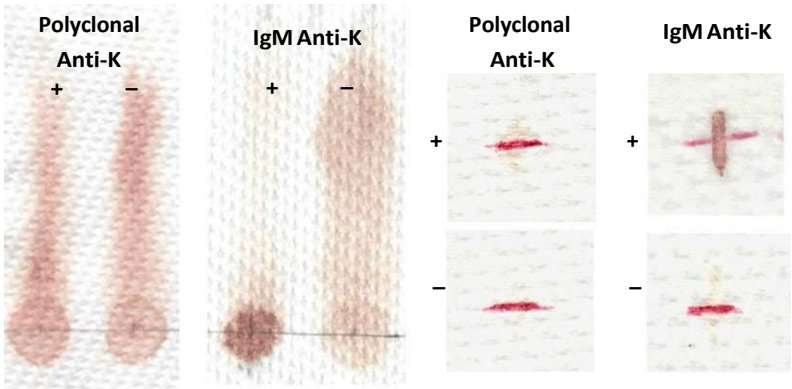

Fig. 5 Comparison of antisera (polyclonal or IgM) effecting identification of testing results for K antigen.

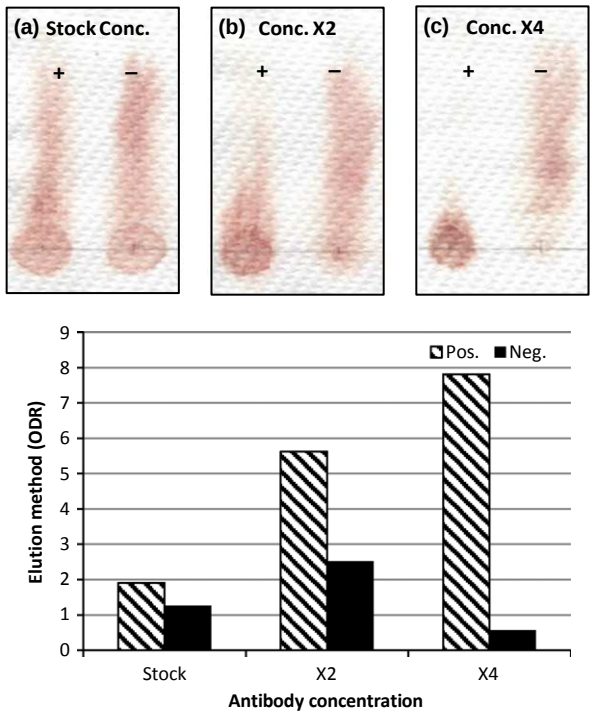

Fig. 6 Effect of anti-K polyclonal concentration on efficacy; (a) at stock solution, (b) double-stock concentration by volume and (c) quadruple stock concentration by volume.

performed with the original polyclonal reagent, against K positive cells. Surprisingly, cell agglutination was observed with both tests, albeit significantly weaker in the immediate spin test. This result indicates that the polyclonal reagent used was predominantly IgG antibodies with some residual IgM antibodies present.

Drawbacks of increasing concentration are as follows: antibody sera are expensive, and not all polyclonal reagents have an IgM component.

Testing for blood group k typing using a monoclonal IgM antibody was straightforward. Much like the Rh groups, results for both the elution and text-reporting methods were clear. For the elution method, positive and negative ODR was 6·4 – 1·5 and 1·5 – 0·25, respectively. Density results for text-reporting method were 131 – 8·3 and 31 – 5·0 for positive and negative, respectively (Table 2 and Fig. 3).

Duffy blood group system

Unlike the anti-K antibody reagent, Duffy antibodies, anti-Fy^a and anti-Fy^b, are not available as IgM. Polyclonal Duffy antibody reagents are predominantly IgG [1]. No optimization techniques could achieve clear results as positives reported as (false) negatives. When reaction time was varied, despite an increased density for positive results, tests for negatives showed an equal increase in density. Attempts to duplicate the improved results seen when concentrating the polyclonal anti-K were unsuccessful for both anti-Fy^a and anti-Fy^b. All results were negative, showing the inability of IgG antibodies to form RBC agglutinates retainable within paper. The antibody structure (IgM vs. IgG) is crucial for successful identification.

Kidd blood group system

The Kidd blood groups studied are Jk^a and Jk^b. The corresponding typing reagents are available as monoclonal IgM antisera and generally showed a clear distinction between positive and negative results. However, the BS achieved was not as defined as desired, requiring optimization. Extending reaction time improved results. Reaction time of 120 s was determined for group Jk^a (ODR pos: 8·3 – 2·7, neg: 1·2 – 0·13; pos: 95 – 6·2, neg: 37 – 4·8; elution and text-reporting methods, respectively) and Jk^b (ODR pos: 6·8 – 1·8, neg: 2·9 – 1·2; pos: 122 – 10, neg: 34 – 6·2; elution and text-reporting methods, respectively). Both elution and text-reporting results were accurate.

MNS blood group system

Paper testing for the M, N, S and s antigens was unsuccessful for both methods. The M and N blood groups often reported falsely as ‘weak’ positives. Numerical data are available as Supplementary Material.

P blood group system

P1 antigen was tested using a monoclonal IgM antibody. Like the C and E antigens, no special optimization was required. The results were clear and easy to distinguish between positive and negative results for both elution and text-reporting (pos: 12 – 1·9, neg: 1·7 – 0·63 ODR; pos: 140 – 5·7, neg: 31 – 5·5, respectively) (Fig. 3a,b).

Lewis blood group system

Testing the Lewis blood system revealed the importance of the antibody solution composition. Unlike other blood groups, testing with anti-Le^a and anti-Le^b showed anomalous patterns using the elution method. Rather than displaying a clear BS or EP, the blood dispersed irregularly (Fig. 7). This is likely due to potentiators, such as dextran, often added to formulation. Potentiators, consisting of polymers, are often used to enhance the extent of RBC agglutination for low potency antibodies. Potentiators can

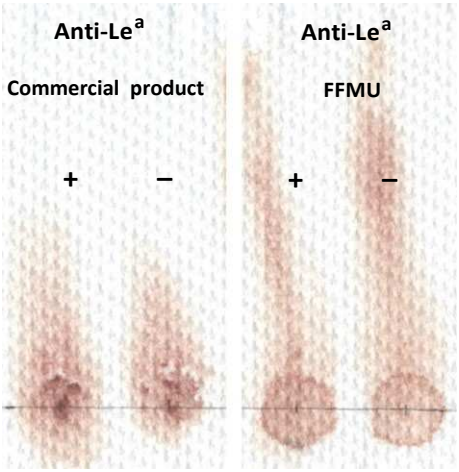

Fig. 7 Dispersion difference between the commercial product and FFMU antibody Le^a.

become problematic for paper tests should they non-specifically retain individual RBC on paper. Instead of absorbing into paper, the antibody solution created a film above the surface, affecting elution and causing irregular dispersion. There was no distinction between positive and negative. The irregular dispersion pattern in paper caused by potentiators was confirmed using 'For Further Manufacturing Use' (FFMU) anti-Le^a, the raw material without potentiators. However, the FFMU anti-Le^a was still unsuccessful for distinguishing positive or negative, due to the lack of potentiators to affect agglutination.

Unlike the elution method, the flow-through method distinguished both positive and negative samples. This is due to directionality and the strength of washing, unaffected by potentiators. The perpendicular washing allowed the saline solution to flow through paper and filtrate, rather than eluting RBCs. Instead, unbound cells could flow through paper. Colour density for Le^a and Le^b using the text-reporting method was pos: 68 – 3·1, neg: 35 – 6·2; and pos: 116 – 5·9, neg: 37 – 6·2, respectively.

Perspective

More than half of the clinically significant blood group phenotypes explored within this study were successfully optimized; the exceptions being Fy^a, Fy^b, S and s. This demonstrates the potential for paper biodiagnostics as a viable alternative for blood group phenotyping. Both testing methods are unique and designed for varying purposes. Successful results for the elution method were more definitive than the flow-through method, better designed for high-throughput testing. Meanwhile, the flow-through method is ideal for point-of-care testing, especially in remote areas or developing countries. Although in-field testing is unlikely to achieve standard laboratory conditions for temperature and humidity, paper blood typing diagnostics would still provide a cheap, fast and easy to use alternative to current conventional blood grouping methods.

Conclusion

Two paper methods – an elution and a flow-through direct-reporting method – were investigated to determine the blood group phenotype of red blood cells. Clinically significant antigens tested include C, c, E, e, K, k, Fy^a, Fy^b, Jk^a, Jk^b, S and s antigens. The M, N, P1, Le^a and Le^b antigens were also tested. As each group behaved differently, optimization for each antigen was required. This optimization was achieved by controlling the main variables: antibody–antigen reaction time, antibody concentration and changing the washing buffer solution. Antibody class is of the utmost importance on paper. Most antigens with antibodies available as IgM monoclonal antisera were successful with both paper methods (Rh, K (IgM), k, Kidd, P1), though to varying degrees of clarity. Testing with polyclonal antisera was unsuccessful (K and Duffy). Unexpectedly, increasing the antibody concentration of polyclonal anti-K showed a discernible difference between positive and negative results; this was due to increasing the concentration of IgM component. Results were not replicated using polyclonal Duffy antibodies (IgG). Increasing reaction time between antibody and antigens (RBC) showed increased clarity with both methods, while changing washing solution improved testing using the flow-through method. The formulation of the antibody solution is also an important variable for paper testing; any additive able to non-specifically bind RBC to paper affects test sensitivity; this was observed with the Lewis antibodies.

While successful detection was achieved with most IgM antibodies, except Lewis, M, N and S, using both paper assays, polyclonal antibodies, consisting predominantly of IgG, resulted in inconsistent results.

Acknowledgements

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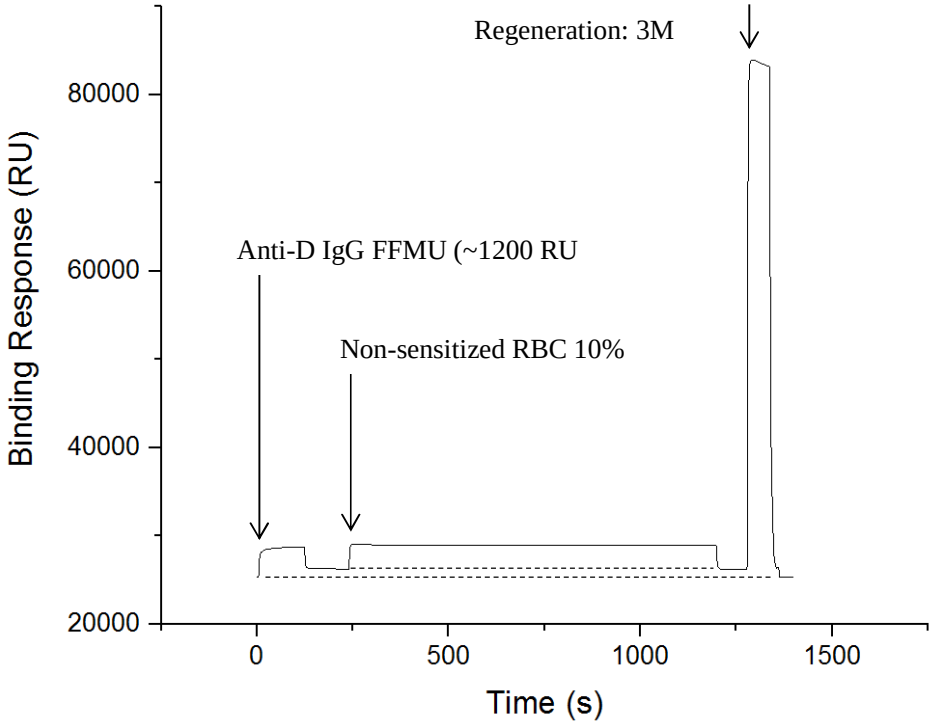
Supplementary Materials

Figure S1. Sensogram of non-incubated positive D-antigen red blood cell over a anti-human IgG functionalised surface using pre-injected anti-D IgG FFMU. Example of surface



regeneration sensor using 3M $MgCl_2$ with little to no degradation of the functionalised surface is also depicted.

Table S

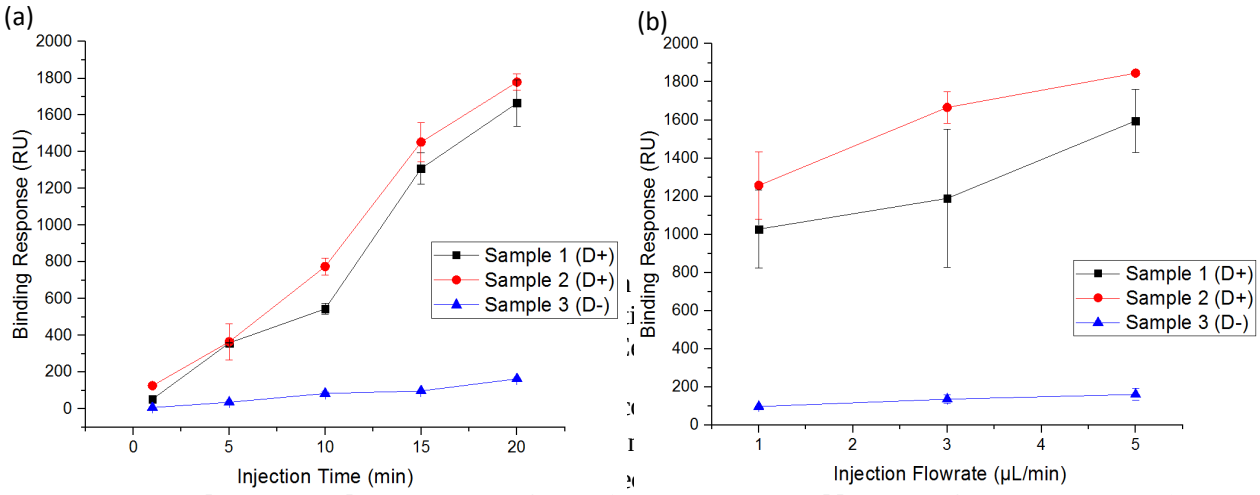
1. Series of reproducibility tests using reagent red cells. Each cell was tested three times on two separately prepared sensor chip surfaces. Each sample was washed 4 times in Celpresol LISS, and diluted to 10% (v/v) prior to injection for 15 mins at 1µL/min.

	Binding Response (RU)			
Control Conditions	**Immobilized surface anti-human IgG**	**Sample 1 (Ave.)**	**Sample 2 (Ave.)**	**Sample 3 (Ave.)**
Functionalized CM5 chip preparation 1	10,562	1309	1453	97.9
Functionalized CM5 chip preparation 2	14,929	2038	2372	79.0

Table S2. Series of control tests to ensure no non-specific binding has occurred.

	Binding Response (RU)		
Control Conditions	**Sample 1**	**Sample 2**	**Sample 3**
Brand new non-functionalized CM5 chip with sensitised incubated cells	18.0	<0	13.2
Functionalized CM5 chip with non-sensitized cells in Abtectcell stabilization solution	30.4	10.2	1.45
Functionalized CM5 chip with non-sensitized cells in Celpresol LISS [Washed 4 times]	<0	<0	<0
Functionalized CM5 chip with non-sensitized incubated cells in Celpresol LISS [Washed 4 times]	<0	<0	<0

Optimization



area exposed to the microfluidic channel within the SPR. When the injection time was prolonged beyond 15 mins, the rate of increase slowed compared to that of injection times below 15 mins (Figure S2a), and while the positive detection had stronger binding responses at injection times longer than 15 mins, so did the negative samples (>100 RU). The higher binding response for negative samples was undesirable as it did not meet the cutoff requirements established at the beginning of this experiment (<100RU), therefore injection times longer than 20 mins were not explored. Also, a longer injection time is unfavourable in terms of blood group detection since current methods take only minutes for a complete phenotype to be determined. The advantage of using SPR lies in its ability to quantitatively determine binding strength, which is impossible using the current techniques, such as the CAT or tube test. Therefore an optimum injection time of 15 mins was selected for this study.

Effect of Flow rate

The effect of injection flow rate was examined as a higher flow rate in SPR analysis is known to improve binding response. Flow rates of 1µL/min, 2 µL/min and 5µL/min were examined (Figure S2b). Increasing the flow rate showed increased binding for all samples. A faster flow rate requires a larger sample volume to be in contact with the sensor surface, possibly resulting in greater binding. However, the binding responses of the negative samples once again played a key role in the decision to test at lower flow rates as the higher flow rates also showed an increased binding response for sample 3, going above the desired threshold. This could simply be the result of requiring a larger sample volume for testing, which would still contain minute concentrations of free unbound anti-D IgG. Despite rigorous washing, it is impossible to remove all free unbound anti-D IgG, which still bind preferentially due to its smaller size.

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Quantitative blood group typing using surface plasmon resonance

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abstract

The accurate and reliable typing of blood groups is essential prior to blood transfusion. While current blood typing methods are well established, results are subjective and heavily reliant on analysis by trained personnel. Techniques for quantifying blood group antibody–antigen interactions are also very limited. Many biosensing systems rely on surface plasmon resonance (SPR) detection to quantify biomolecular interactions. While SPR has been widely used for characterizing antibody–antigen interactions, measuring antibody interactions with whole cells is significantly less common. Previous studies utilized SPR for blood group antigen detection, however, showed poor regeneration causing loss of functionality after a single use. In this study, a fully regenerable, multi-functional platform for quantitative blood group typing via SPR detection is achieved by immobilizing anti-human IgG antibody to the sensor surface, which binds to the Fc region of human IgG antibodies. The surface becomes an interchangeable platform capable of quantifying the blood group interactions between red blood cells (RBCs) and IgG antibodies. As with indirect antiglobulin tests (IAT), which use IgG antibodies for detection, IgG antibodies are initially incubated with RBCs. This facilitates binding to the immobilized monolayer and allows for quantitative blood group detection. Using the D-antigen as an example, a clear distinction between positive (4500 RU) and negative (o100 RU) RBCs is achieved using anti-D IgG. Complete regeneration of the anti-human IgG surface is also successful, showing negligible degradation of the surface after more than 100 regenerations. This novel approach is validated with human-sourced whole blood samples to demonstrate an interesting alternative for quantitative blood grouping using SPR analysis.

1. Introduction

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Accurate and reliable typing of blood groups is of the utmost importance prior to blood transfusion. The mismatching of incompatible blood types could lead to a potentially fatal hemolytic transfusion reaction (Daniels and Bromilow, 2007). The most widely known blood groups are ABO and RhD, however there are actually 35 blood group systems with over 300 identified antigens, many of which are clinically significant. During blood typing, two types of antibodies are used: 1) pentameric IgM, and 2) monomeric IgG. Based on hemagglutination principles, antigens upon the surface of a red blood cell (RBC) will bind in the presence of corresponding antibodies. IgM antibodies bind to multiple RBCs, facilitating agglutination and positive identification. However, not all blood group antibodies are available with an IgM structure, necessitating the use of IgG antibodies instead. RBCs binding with IgG antibodies do not agglutinate and require the use of an additional agglutination reagent, anti-human IgG, to indicate positive antigen identification. This is known as the indirect antiglobulin test (IAT) (Harmening, 1999).

While there are many well established blood typing methods available, such as the column agglutination test (CAT), current methods for quantifying blood group antibody–antigen interactions are very limited. This is particularly important when identifying weak interactions between weak subgroup variants, which are often difficult to determine via traditional testing using simple visual analysis, and can easily be overlooked or misinterpreted (Issitt and Anstee, 1998). There are currently only two methods available for quantitative analysis: flow cytometry and fluorescence microscopy. Flow cytometry uses time delayed measurements, while the conjugation required for fluorescence microscopy may influence activity. This is where the advantages of surface plasmon resonance (SPR) lie.

SPR is a widely used technique for the detection and analysis of interactions between biomolecules, including antibodies and antigens. It is a label-free optical technique that can monitor intermolecular binding events in real time.

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SPR relies on a polarized laser to measure the change of adsorbed mass on a metal chip surface by monitoring the change in refractive index (RI), and reporting the change as a binding response unit (RU) (Krupin et al., 2014). However, SPR investigations of whole cell interactions are significantly less common (Stojanović et al., 2014). This is because detectors are often coupled with a microfluidic system with a diameter similar to the average cell size which ranges from 10 to15 mm. Therefore, most large cells are unsuited for use with microfluidic systems as cells are more likely to settle or congregate (Gutiérrez-Gallego et al., 2009). However, unlike most cells, RBCs are highly deformable in nature to allow for easy vascular transport, which bares similarities to microfluidics.

A few previous studies have investigated SPR for blood group antigen detection (Quinn et al., 1997) and antibody detection (Kimura et al., 2005; Yurugi et al., 2007). Using RBCs, Quinn et al. (1997) demonstrated the detection of A and B antigens with SPR by immobilizing the corresponding blood group antibodies (IgM). RBCs were then passed over the surface with positive cells captured for detection. However, while successful, this method showed poor regeneration despite trying multiple regeneration buffers at different pH. Furthermore, the harsh regeneration conditions resulted in a loss of antibody/biosensor functionality after a single use due to the inability to fully desorb bound material.

In this study, a novel concept is explored. In contrast to the use of specific IgM antibodies, by immobilizing an alternative antibody, anti-human IgG, the chip surface becomes an interchangeable platform capable of quantifying the blood group typing interactions between RBCs and antibodies (IgG). Anti-human IgG is able to recognize and bind to the Fc region of human IgG antibodies for detection. Much like the IAT, blood group IgG antibodies incubated with RBCs. The cells become sensitized with IgG and are able to bind to the immobilised anti-human IgG. This test has the potential to quantitatively detect any blood group with a corresponding IgG antibody. The D antigen, more commonly recognized as the 'þ' of group bloods, was detected as an example using antiD IgG. It is the objective of this study to develop this novel concept of functionalizing a SPR sensor chip with anti-human IgG to detect RBCs which have been presensitized with anti-D IgG. In the first part, the method is optimized for sensitivity and selectivity. The second shows validation using human-sourced blood samples.

2. Materials and methods

2.1. Chemicals and equipment

All chemicals and sensor chips were purchased from VWR International (Brisbane, Australia) unless otherwise stated. Anti-D IgG For Further Manufacturing Use (FFMU) antibodies were supplied by Quotient EU (Edinburgh, United Kingdom). Abtectcell red blood cells (RBCs), Celpresol and Celpresol Low-Ionic Strength Solution (LISS) were supplied by CSL Limited (Melbourne, Australia). Antihuman IgG Fc (Clone CBL101) was purchased from Merck Australia (Melbourne, Australia). Analytical grade phosphate buffered saline (PBS) was purchased from Sigma-Aldrich (Sydney, Australia). EDTA blood samples were sourced from the Australian Red Cross Blood Service (ARCBS) (Melbourne, Australia), stored at 4°C and used within 7 days of collection. The BIAcore X system (GE Healthcare, Uppsala, Sweden) was used for all analyses.

2.2. Methods

Anti-human IgG Fc was immobilised upon a CM5 SPR chip surface using amine coupling. The CM5 chip consists of a gold surface grafted with a medium molecular weight carboxy methyl dextran layer. Firstly, the surface was activated by mixing and injecting equal volumes of 100 mM N-hydroxysuccinimide (NHS) and 400 mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) at 5 mL/min for 7 min. Both were made with distilled water before use, aliquoted and stored at 15 ˚C until required. Then 5 mL of anti-human IgG Fc (0.5 mg/mL) ligand was dissolved in 10 mM sodium acetate buffer before injection over the activated surface for 6 min at 5 mL/min. Unreacted sites were then blocked by injecting 1 M ethanolamine-HCl at 5 mL/min for 7 min.

Reagent red cells (Abtectcells III 3%) and human-sourced red cells were used during these experiments. Reagent red cells are washed human red cells that are kept in a preservation solution at a constant concentration for standardization. Human-sourced red cells are collected as whole blood from donors, including plasma, and stabilized with Ethylenediaminetetraacetic acid (EDTA); these cells have not been standardized.

Prior to injection, RBCs were incubated with excess anti-D IgG FFMU for 30 min at 37 ˚C (1:1 by volume), then washed 4 times with Celpresol LISS and centrifugation before dilution to a known concentration in Celpresol LISS for injection into the BIAcore X system. Magnesium chloride was used to regenerate the chip surface with 1 min pulses at 1 mL/min.

Three variables were explored for optimization including: experimental buffer, washing, and RBC concentration. Temperature was kept constant at 37 °C to maximize binding of IgG antibodies which are optimal at said temperature. A single flow cell detection mode, flow cell 2, was used for binding analysis. Sensograms were analyzed with standard BIAcore X control software. The different buffers investigated include: PBS, Celpresol, and Celpresol LISS.

3. Results and discussion

Using anti-D IgG FFMU antibodies and reagent red cells as a model, a regenerable platform for blood group detection was explored and optimized using SPR. The concept consists of producing a monolayer of anti-human IgG capable of interacting with any IgG sensitized RBC through anti-IgG/anti-human IgG interaction. If positive, the IgG antibody will specifically bind onto the RBC antigen site. These IgG covered RBCs can then be retained with the generic anti-human IgG grafted on the SPR chip through binding of the Fc region of the IgG antibody structure; negative unbound RBCs will be directly eluted (Figure 1). Several questions must be addressed to validate this concept. Firstly, what is the sensitivity and reproducibility of the technique; secondly, how well does the SPR surface regenerate; thirdly, how quantitative can the technique be; fourthly, how dependent on surface blocking/interaction is the technique; and finally, whether or not the SPR can be used to

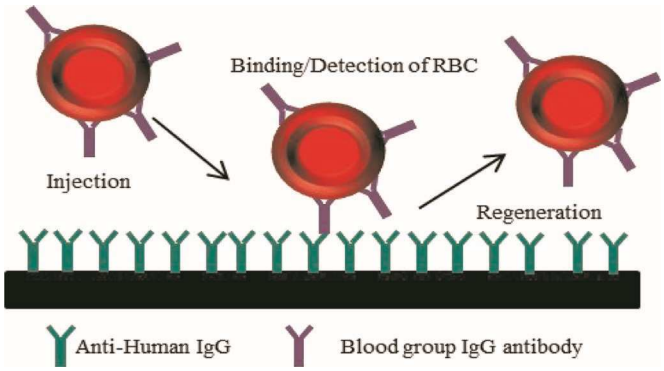

Fig. 1. Diagrammatic representation of immobilizing anti-human IgG upon a chip surface for the quantitative blood group typing using SPR.

quantify the antibody–antigen interaction binding and kinetics. Furthermore, the validity of using SPR for detection was also tested using clinically derived EDTA blood samples. Throughout this study, each sample was prepared twice, tested three times, and upon two different sensor chips to show reproducibility and reliability.

3.1. Functionalization

Anti-human IgG Fc (Clone:CBL101) antibodies were immobilized onto the CM5 sensor chip surface using EDC/NHS amine coupling. The maximum detection limit is defined by the amount of anti-human IgG immobilized as evidenced by the differeing binding responses observed between sensor chip preparations (Supplementary Table S1). The achieved binding response was approximately 10,500 relative units (RU). In general, 100 RU corresponds to an adsorption of 100 pg/mm^2 (Stenberg et al., 1991). RU is measured from a change in refractive index which corresponds to a variation in mass adsorbed onto the chip surface. It does not determine specificity of blood group type but rather detects the presence of RBC antigen–antibody interaction. The CBL101 clone showed strong binding with full regeneration using single 1 min pulses of 3 M $MgCl_2$. Little to no degradation of the anti-human IgG monolayer was observed between each sample run, however, a prolonged use of the same surface showed a slight but steady decrease in the baseline, usually occurring after more than 100 regenerations.

A series of controls were tested to ensure that no non-specific binding was occurring (Supplementary Table S2). These controls included the injection of: 1) sensitized RBCs and 2) anti-D IgG FFMU over a non-functionalised sensor chip (r20 RU), indicating the necessity of the functionalization process as neither RBCs nor anti-D IgG was detected without it; 3) non-sensitized RBCs over a functionalised sensor chip prepared in the abtectcell stabilization buffer (r30 RU) and 4) prepared in Celpresol LISS (o0 RU), demonstrating the importance of the sensitization process, and that no non-specific binding has been promoted in either the abtectcell stabilization buffer, in which the reagent RBCs are packaged, and Celpresol LISS by the sensitization process. Surface blocking to prevent non-specific interactions is critical to ensure sensitivity and selectivity, thus avoiding false positive or negative readings.

3.2. RhD blood group detection

Reagent red cells (Abtectcells) are commercial red blood cells which have been standardised for the detection of blood groups, including the D antigen, as well as a wide range of other clinically significant blood groups. Each comes with two positive D antigen samples and one negative sample. These cells were first used as a model to develop the methodology and evaluate the selectivity and sensitivity when using SPR for RBC antigen detection. The use of reagent RBCs prevents the tremendous variability expected from human-sourced EDTA blood samples. Three types of reagent RBCs were sequentially injected over the antibody-treated SPR chip and the response signal (RU) was measured as a function of time, forming a sensogram of the RBC injection (Fig. 2). Reagent RBCs were used to show the successful and selective detection of the D antigen (Fig. 2). The sensograms display the distinct binding differences between positive and negative RBCs against anti-D IgG FFMU. However, due to the large size of RBCs, significant dispersion was observed, as evidenced by the irregular curves (Fig. 2), making it unsuitable for conventional kinetic analysis. Efforts to minimize dispersion were ineffective.

Blood group typing using IgG antibodies requires incubation of RBCs with the desired antibody at 37 ˚C for 30 mins. This ‘sensitization’ procedure is used for blood grouping due to the

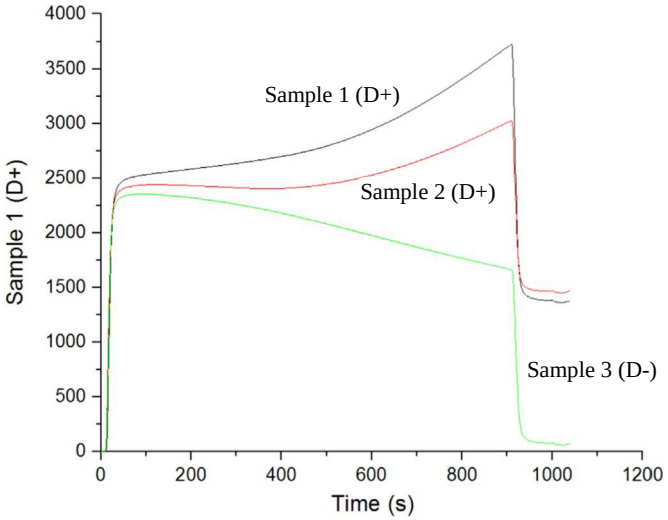

Fig. 2. SPR sensogram: injection of RBC at 10% concentration for 15 min at 1 mL/ min. Cells were presensitized and washed 4 times in Celpresol LISS prior to injection.

electrostatic repulsion between RBCs, allowing the small-sized IgG specific antibodies to bind to the RBC antigens by increasing kinetic collision and allowing ample time for binding to occur; no binding occurs with antibodies non-specific to the RBC tested. Therefore, incubation of cells with the desired IgG antibody is required prior to injection. Attempts to bind an anti-D IgG to the surface anti-human IgG, and subsequently injecting RBCs over the surface was unsuccessful (Supplementary Fig. S1). Sensograms showed very little binding, indicating a false negative. Most likely, the large size of the RBCs combined with their momentum as they flow over the sensor surface hindered binding of the antibody to the RBC antigen. The antibody–antigen interactions are either too weak, or the distance of bioaffinity is too short.

Prior sensitization of the RBCs with the anti-D IgG allowed for binding to the immobilized anti-human IgG, and showed distinct differences between positive (1500 RU) and negative blood samples (50 RU) (Fig. 2). This is due to the binding of anti-D IgG to the D antigen, making the site more readily available for detection by the anti-human IgG. It is believed that the D antigen, in particular, is integrated within the RBC membrane, making access to its binding site even more difficult without pre-sensitization (Reid et al., 2012).

Full regeneration of the sensor surface was achieved using 3 M $MgCl_2$, allowing for any bound material to be completely removed with little to no degradation of the sensor surface (Supplementary Fig. S1). This is due to the very high ionic strength of the regeneration solution, screening all electrostatic interactions and leaving only van der Waals attraction forces. This observation suggests that electrostatic interactions play a dominant role in the antibody–antigen interactions of RBCs. After over 100 regenerations, the sensor still accurately detected positive and negative blood samples with consistent binding strength. The ability to fully regenerate the sensor surface presents a potential multi-antibody detection technique, particularly with newer models of SPR biosensors, which can be automated and have multiple samples analyzed simultaneously.

3.3. Optimization

The initial detection study showed the potential of the SPR technique for blood typing, especially using IgG antibodies. However, non-standardised blood samples are expected to be weaker. To ensure the best possible binding strength is observed, the effect of critical variables were tested and optimized. Reagent red cells were also used during the optimization process, where the experimental buffer, washing, and RBC concentration were varied to determine the best conditions to define standard testing (Fig. 3). Optimized injection time and flowrate were chosen as 15 min and 1 mL/min, respectively. Effects of injection time and flowrate are discussed in the supplementary material section. Using the D antigen as an example, the targeted response for positive detection was greater than 1000 RU, while no more than 100 RU for negative samples was observed (Fig. 2). This difference of 900 RU between positive and negative samples ensures good sensitivity and clear identification. One limitation to achieving a standard response for positive and negative antigen interactions is that the binding response between blood samples differs. Differences could even be seen between the two reagent cells which are standardised for detection. Therefore, the key objective was to achieve distinct responses between positive and negative samples. The binding response of positive samples could vary quite vastly, while negative responses should be maintained below 100 RU.

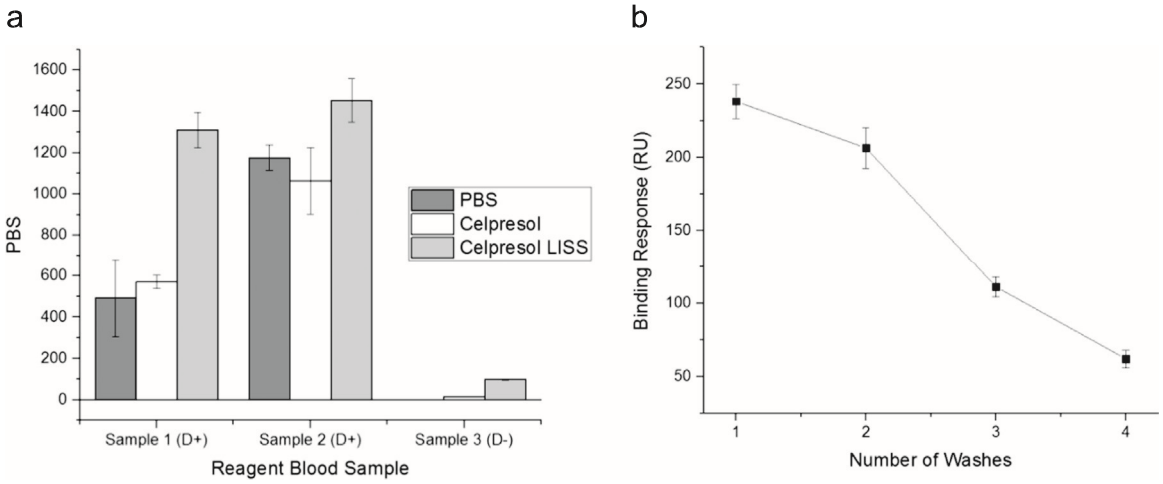

Fig. 3. Detection of the D antigen using different a) washing buffers, b) varying degrees of washing for sample 3 (D-). With the exception of the variable tested, each set of experiments were: washed 4 times in Celpresol LISS, and injected for 15 mins at 1 mL/min.

Binding efficiency during SPR analysis is dependent on factors such as experimental buffer, washing, and RBC concentration. Due to the higher activity rate of IgG antibodies, a temperature of 37 °C was used at each stage. The greater mass of the anti-human IgG ligand to the sensor surface therefore increased adsorption of the sensitized RBCs when injected.

3.3.1. Effect of washing buffer and washes

The effect of the washing buffer was shown to be one of the most important factors to effect binding during optimization (Fig. 3a) due to buffer pH and ionic strength. Three buffers were tested and compared: PBS, Celpresol (pH 7.05–7.25), and Celpresol Low Ionic Strength Solution (LISS) (pH 6.7–6.9). When tested using PBS, the binding response for positive D samples was weak, particularly using the Sample 1 DþRBC. Two other experimental buffers were explored, both specifically designed for testing with RBCs; Celpresol and Celpresol LISS. Celpresol is a cell preservation solution used for long term storage of RBCs. It contains nutrients required to keep cells stable. Celpresol LISS is a similar solution; however, it has a low ionic strength to enhance antibody interactions. D antigen detection using Celpresol had similar results to PBS. Conversely, Celpresol LISS showed markedly higher improvements in binding response for positive D RBCs. While the negative D samples showed a slightly increased RU compared to PBS and Celpresol, the difference compared to the positive D samples was significant enough for blood group determination. This is likely due to the Celpresol LISS changing the isoelectric point of the RBCs and allowing less repulsion between cells and the dextran matrix on the sensor surface. Celpresol LISS performed significantly better as the washing buffer compared to PBS and Celpresol. This is especially true for Sample 1, where the positive D antigen detection binding response was greater than double that of the latter two solutions.

The contrasting binding strength observed between both positive D antigen RBCs is likely due to donor variability. However, further testing showed that the Rh phenotype of a RBC could play a role in the binding efficiency of the D antigen (Section 3.4).

The number of times each sample was washed prior to dilution to the desired concentration played a key role in accuracy when comparing negative samples. A reduced number of washes resulted in higher, non-specific binding responses for the negative sample 3. This is likely due to free, unbound antibodies within the supernatant binding to the functionalised sensor surface. Antihuman IgG binds non-selectively to human IgG antibodies; therefore rigorous washing is necessary to ensure all unbound antibody molecules are removed. Four washing steps were subsequently used to minimize binding of any free unbound IgG antibodies in the supernatant. This is standard practice for current methods using IgG antibodies, including IAT. While still distinctly lower than the binding responses seen for positive samples, more washes ensured less binding of free unbound anti-D IgG, and higher sensitivity (Fig. 3b).

3.3.2. Effect of RBC concentration

Similar to previous studies, testing using varying RBC concentration showed a linear correlation, indicating its potential as a quantitative study for blood group detection (Fig. 4). An optimum RBC concentration of 10% was chosen. Lower concentrations resulted in lower binding, while higher concentrations push the limitations of the SPR instrument's microfluidic structure. Whole blood contains approximately 40% RBCs and is quite viscous; high viscosity increases the likelihood of false positives and must be avoided to minimize potential clogging within the microfluidic channels, therefore a lower concentration range was tested.

3.4. Validation

Finally, 50 human-sourced EDTA samples were tested to verify the efficiency of SPR for blood group detection (Table 1 and Fig. 5). In addition to not being optimized for blood group testing, unlike the reagent red cells, there are other factors affecting the binding

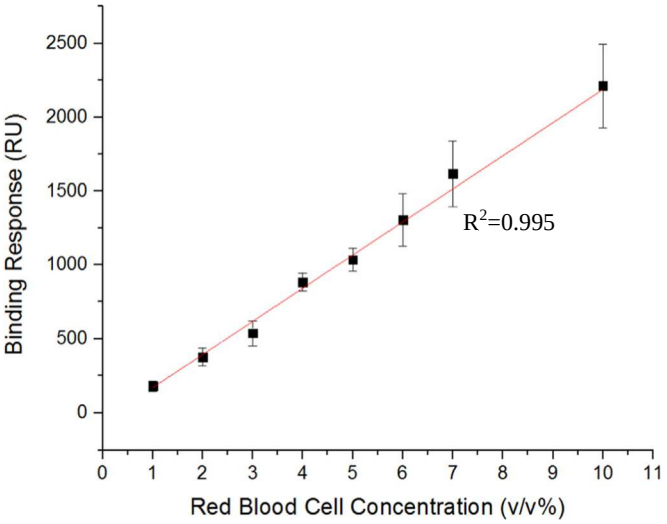

Fig. 4. Detection of the D antigen using different RBC concentrations of reagent sample 1 (Dþ), washed 4 times in Celpresol LISS. Injected at 1 mL/min for 15 mins.

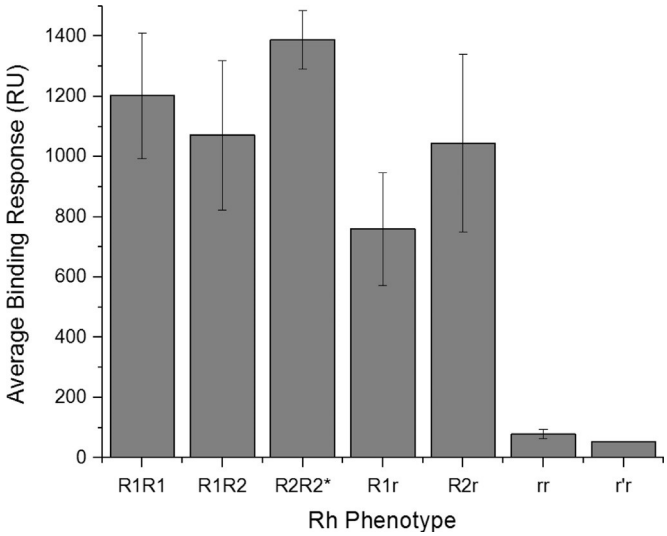

Fig. 5. Effect of Rh Phenotype on binding response when testing for the D antigen using anti-D IgG FFMU. *Except for the R2R2 phenotype, all testing was completed using human-sourced EDTA samples. Data from R2R2 phenotype testing using reagent RBCs was included only as a reference to its stronger binding response.

Table 1
Effect of Rh Phenotype on binding response when testing for the D antigen using anti-D IgG FFMU. Average binding responses observed for each Rh phenotype shows strong correlation to the number of estimated D binding sites. *Except for the R2R2 phenotype, all testing was completed using human-sourced EDTA samples. Data from R2R2 phenotype testing using reagent RBCs was included only as a reference to its stronger binding response.

Rh Rh No. of antigens	Ave. Std. dev response (RU)		Estimated D binding sites	phenotype per cell (Daniels, 2008)
R1R1	DCEce	7	1202	2209	14,500–22,800
R1R2	DCe	4	1071	2248	23,000–31,000
R2R2*	DEc	6	1388	998	15,800–33,300
R1r	DCce	16	760	187	9,900–14,600
R2r	DEce	5	1045	296	12,000–19,700
rr	ce	17	78	15	N/A
r′r	Cce	1	53	N/A	N/A

efficacy, which show a strong correlation with a patient or donor's genetics. There was a wide spectrum of positive D antigen detection. Binding responses ranged from as low as 530 RU to as high as 1200 RU. This variance in binding strength is a recognizable trend in D antigen detection practice. Interestingly, the binding strength observed for each positive D antigen correlated with the Rh phenotype of that sample. Each Rh phenotype is a category defined by the presence or absence of antigens from the Rh blood group family. These antigens include: D, C, E, c, e (Daniels, 2008). Table 1 describes each Rh phenotype and the average binding response observed. Testing was limited by the constraints of the blood samples available at the time of testing, as such collation of particular phenotypes was more difficult due to scarcity, i.e. the R2R2 phenotype.

Among the three reagent red cells, the Rh phenotype of each sample is R1R1 (1), R2R2 (2) and rr (3). During conventional blood typing, the R2R2 phenotype binds the strongest. This was supported during optimization where sample 2 always showed a higher binding response.The order of binding strength during testing from strongest to weakest is as follows: R1R1, R1R2, R2r, and lastly R1r. R1r is the most commonly observed Rh phenotype in the general Melbourne population but has the weakest binding capacity. This is why a large number of the human-sourced samples tested appeared not to meet the initial 'standard' criteria for this study. R2R2 phenotypes only comprise approximately 2% of the population, and are therefore rare during random sampling. Data from the reagent red cells was included as reference to show the strong binding capabilities of the R2R2 phenotype (Table 1 and Fig. 5). However, due to the standardized nature of reagent cells, it is unsuitable for accurate comparison with the results of the human-sourced EDTA samples tested.

Patient variability offers three possible explanations for the differences observed: 1) number of D antigen sites on the cell surface 2) Rh antigen conformation upon the cell surface, or 3) interference from other biological material, for example, within the plasma.

The most likely explanation for the differing binding strength between Rh phenotypes is the number of D antigen binding sites (Table 1). There is a strong correlation between average binding responses observed to the number of estimated D binding sites. Additionally, the large standard deviation observed for each Rh phenotype could be explained by the large range, and often overlapping, estimation of D antigen binding sites.

Another possible explanation is the conformation of Rh antigens within the RBC membrane. The structure of each antigen is predicted to be closely related, hence affecting the binding ability of anti-D to the D antigen (Daniels, 2008).

Since reagent RBC samples are standardised, there is a consistency observed between different batches of reagent RBCs. However, human-sourced RBCs are not standardized, therefore, it is possible that the variation measured could be caused by interference from components in the plasma, cell concentration, condition of cells, etc.

Despite the large variability, binding responses for both D negative antigen Rh phenotypes, rr and r′r, consistently remained below 100 RU. Since the lowest observed binding response for a D positive human-sourced sample was 530 RU, there is a distinct difference between positive and negative D antigen detection, indicating the potential to use SPR as a diagnostic tool for quantitative blood group typing.

3.5. Limitations and perspectives

Despite the potential of SPR analysis as a quantitative blood typing technique, current blood typing diagnostics are both simpler and faster, which is more desirable during emergent situations. SPR biodiagnostics are also reliant on expensive equipment and consumables, such as the sensor chip, and are

confined to a laboratory environment. Emerging technologies, such as the paper diagnostics for blood typing, are less reliant on equipment and trained personnel (Khan et al., 2010; Li et al., 2012; Then et al., 2015). The one key advantage of SPR analysis is the ability to quantitatively detect blood groups, which could be especially useful when detecting weaker variants.

RBC detection using SPR is dependent on many factors. However, one of the main concerns is the large size of the RBCs compared to the sensor surface. Large responses that were expected due to the RBCs' large size were not observed. While the RBCs are deformable in nature, the evanescent field of detection using SPR only extends to a depth of 0.3 mm from the sensor surface. The size of RBCs far extends this limit, and therefore only partially cover the sensor surface. While detection is possible within the field, the partial coverage resulted in decreased sensitivity. RBC size also hinders the ability to monitor binding kinetics, which is of particular interest since there are no studies that have been able to accurately quantify the kinetic mechanisms of blood group detection. However, as prior incubation is necessary for detection, kinetic analyses would be between the anti-human IgG Fc and anti-D IgG FFMU, not the RBC.

4. Conclusion

This study presents a new methodology for quantifying blood typing interactions using surface plasmon resonance (SPR). An anti-human IgG antibody capable of interacting with any IgG sensitized RBC through anti-IgG/anti-human IgG binding is covalently immobilized to the sensor surface. If positive, the IgG antibody will specifically bind to the RBC antigen sites, which are then retained by the generic anti-human IgG monolayer through binding of the Fc region of the IgG antibody. Negative unbound RBCs are directly eluted. The SPR chip surface can potentially be used as an interchangeable platform to quantify the interactions between RBCs and any IgG antibody.

Using the RhD blood group as an example, a clear distinction between positive (4500 RU) and negative (o100 RU) D antigen RBCs is achieved with anti-D IgG. Complete regeneration of the anti-human IgG surface is also successful, showing little to no degradation of the immobilized surface after more than 100 regenerations. The technique is also validated using whole human blood samples. An interesting correlation between the binding response and Rh phenotype is reported showing the sensitivity of the technique; it is likely due to the different concentration of D antigen binding sites on the RBC surface.

This novel approach to blood typing using SPR analysis presents an interesting alternative for quantitative blood grouping. This platform has potential as a multi-functional, fully regenerable method for quantifying antibody–antigen interactions for blood group typing using SPR.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.05.053.

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Appendix II

Related Co-Authored Publications

that were Not Included in Thesis

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Paper Microfluidics: Applications and Perspectives

Gil Garnier* and Whui Lyn Then

BioProcessing Research Institute of Australia (BioPRIA)
Australian Pulp and Paper Institute (APPI),
Department of Chemical Engineering, Monash University, Clayton, VIC 3800, Australia.

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CONTENTS

KEYWORDS

Paper microfluidics, biosensors, immunoassays, biodiagnostics, capillarity, cellulose fibers, wicking.

1 INTRODUCTION

The explosion of interest and development in paper microfluidics has so far been linked with a single application: integration in diagnostics for medical testing devices. Paper bio- diagnostics has the potential to revolutionize preventive health care in two ways. First, by providing developing nations and remote locations with the cheap, simple and robust test they have not been able to afford or have access to; second, by developing a new generation of convenient, non-invasive and low cost tests for home and point of care testing. The latter category aims at the easy and early detection of illnesses or monitoring a condition; early cancer detection, cholesterol and hemoglobin levels are three examples. Paper diagnostics are to be used directly by the patient or by untrained workers- sometimes in stress conditions. Faster, more reliable and accurate tests relying on modern instrumental analytical chemistry are already available. This market vision is essential to define the position and boundaries of paper diagnostics and their microfluidics component, in the spectral of biomedical analysis. Paper test must be: 1) very cheap, 2) very easy to use, 3) robust and reliable and 4) easily disposable. The paper-functional printing material-process combination offers one of the lowest cost and flexible manufacturing platform; it is the keystone of the paper microfluidics/diagnostic success. From medical applications, paper microfluidics and diagnostics can then be adapted to more cost sensitive applications such as veterinary health test, food quality, and environmental monitoring.

A few excellent reviews of bioactive paper are already available. Pelton, from McMaster University, presented an authoritative and comprehensive analysis on bioactive paper as a low cost platform for diagnostics from a paper [1] and a health [2] perspective. Shen et al., from APPI- Monash University, published a perspective on paper-based microfluidics [3], patterned paper and alternative materials as substrates for microfluidics diagnostics [4]. Liana et al., CSIRO Australia, reviewed advances in paper-based sensors [5]. Whitesides, Martinez et al., from Harvard University, analysed microfluidic paper-based diagnostics [6] and electroanalytical devices [7] for the developing world. Then et al., also from APPI- Monash University [8] reviewed biodiagnostics for medical applications.

This review analyses the recent progress and development perspectives of paper microfluidics mainly as component of a low cost, easy to use and disposable paper biodiagnostic. The usefulness of each development reviewed will be examined in terms of 4 issues: 1) Is it cheap? 2) Is it simple to use and practical? 3) Is it robust and sensitive? 4) Is it disposable or recyclable?

Paper microfluidics are usually small and disposable systems to transport an aqueous solution by relying on the natural capillary forces of paper. Paper can serves six main functions: 1) to transport and measure samples and reactants, 2) to safely store biosamples and reagents, 3) to provide a substrate/surface for a reaction, 4) to separate, fractionate or concentrate a component, 4) to communicate the presence or concentration of an analyte, 5) to provide a permanent record of the analysis performed, 6) to safely capture and provide a media for the disposal of biosamples. Paper can be used as a substrate for patterning hydrophilic fluidic channels using printing technology. In the majority of applications, paper microfluidics are an integral component of a system used for fractionating or analyzing an analyte for health or environmental applications; very seldom are microfluidics system used independently. Most of the reports on microfluidics paper have been for low cost biodiagnostics or instantaneous, rapid or stand-alone tests. Microfluidics systems are an integral parts of most paper diagnostics. Paper biosensor, bioassays, immunoassay and diagnostics are used as synonyms for paper biosensors. These fluidic paper devices can be directly integrated with a colorimetric, chemiluminescence or an electrochemiluminescence detection mode to form a biosensor. The attraction toward

paper microfluidics stems from their pump free fluid delivery mechanism based on wicking, their very low cost, and their integration in a functional system (diagnostics) easy to use and dispose of. The biocompatibility of paper with important biomolecules such as enzymes, antibodies, RNA and DNA is another advantage of paper which enables stable paper biodiagnostics. Microfluidics systems can be printed on paper by various processes. And better, complete paper based biosensors are now designed to be manufactured completely by functional printing as part of an efficient roll-to-roll process which involves the sequential printing of the microfluidic and then the chemical and biochemical reagent systems. This review aims at presenting a critical review of the current state of paper micofluidics systems and a perspective on their integration and applications into functional devices- current and future.

The 2007 article: “Patterned paper as a platform for inexpensive, low volume, portable bioassays “ from Whitesides et al. at Harvard has revolutionized the field of low cost diagnostics [9]. The team of Whitesides demonstrated the modern concept of paper microfluidics by printing hydrophobic barriers into paper; they also deposited enzymes and reagents within the paper microfluidics system to highlight the potentials of integrating microfluidics into diagnostics. In the 6 years that have followed, hundreds of studies have been published and dozens of patents filed. Research groups in most countries are now investigating paper-based diagnostics integrating microfluidics systems; surprisingly, very few of these studies have come from traditional pulp and paper research teams. Paper microfluidics studies can be distinguished into two broad categories: microfluidics and biosensors development. This review paper analyses the recent development of paper microfluidics in the context of their application. In the first part, the mechanism for liquid transport in porous media in general, and paper in particular are reviewed; this includes wicking, wetting, diffusion and evaporation. Mechanisms of analyte/colloid separation by paper are then analyzed. In the second part of the study, the types of paper microfluidics and their specific applications are reviewed. The third part of the study presents a perspective of paper microfluidics applications, mainly as low cost test/biosensor. Alternative to paper diagnostics are also presented. It is the objective of this review to highlight the potential, limitations and future directions and development needs for paper microfluidics application.

2 TRANSPORT MECHANISMS

2.1 Why Paper Microfluidics?

As independent systems, paper microfluidics are not particularly efficient, polyvalent nor rapid systems to transport fluids. It is their combination of advantages for very particular applications – chemical and biochemical analysis relevant to rapid and simple to use diagnostics – that justifies their inherent low performance. Paper offers five main properties for diagnostics: 1) very cheap, widely available and easy to process material; 2) the capillarity wicking of aqueous solutions along the cellulosic fibers of paper provides a driving force to transport fluids without the need for external system/energy; 3) the porosity and biocompatibility of cellulose/paper allows reagents and biomolecules to be stored in the paper device; 4) paper combined to printing offer a very flexible and cheap material/process combination to manufacture microfluidics and diagnostics; 5) paper is combustible, biodegradable and recyclable, offering many easy option of disposal after use [1, 6].

2.2 Mechanisms of liquids transport in paper

When a droplet of liquid contacts with paper, the competition of 4 main phenomena dictates its transport and distribution toward an equilibrium condition. First, the droplet wets the surface of paper; second, paper absorbs and wicks the droplet by capillary action, third evaporation proceeds and fourth, diffusion occurs. The time frame of theses phenomena varies with wetting occurring in seconds, absorption/wicking in seconds-minutes, evaporation and liquid diffusion in minutes-hours.

2.2.1 Wicking

Liquid transports in paper mostly by wicking through the inter-fibre spaces. The capillary driving force is caused by the difference in surface energy between the fluid and the solid. The resistances to movement are friction and viscous dissipation. Wicking can be simplistically described by the liquid flow in a capillary. The Lucas-Washburn equation states that a liquid of viscosity η and surface tension γ will flow in a capillary of radius r and length at a velocity V defined by [10]:

$$V = \frac{\gamma r \cos\theta_E}{\eta l} \qquad (1)$$

here θ_E is the equilibrium contact angle formed by the liquid in the capillary. Liquid velocity can be increased by augmenting the driving force: increase surface tension of liquid (γ), improve wettability of fibers (lower θ_E), or by decreasing the resistance: bigger and shorter pores, decreasing the fluid viscosity.

Equation (1) is often expressed as:

$$l = \left[\frac{r\gamma t \cos\theta_E}{2}\right]^{\frac{1}{2}} \qquad (2)$$

which better reflects the square root relationship between wicking distance and time. Wicking occurs only in dry paper- not in wet paper- as the driving force disappears once the pores are filled with a liquid. Wicking proceeds as long as there is a driving force: dry paper (cos θ_E) and a liquid reservoir. Wicking is counter acted by viscous dissipation and sometimes gravity- for vertical samples. While the choice of liquid is dictated by the application, paper surface treatment (cos θ_E) and the selection of paper (bigger and shorter pores) can improve wicking. Surfactants are detrimental (decrease γ**).**

2.2.2 Wetting

The contact angle of a static droplet resting at equilibrium on a surface with its vapour is expressed by the Young Equation:

$$\gamma_{SV} = \gamma_{LV}\cos\theta_E + \gamma_{SL} \qquad (3)$$

where γ_{SV}, γ_{LV}, γ_{SL} are respectively the solid-vapor, liquid vapour (surface tension) and solid-liquid interfacial energies. Equation 3 simply states that the equilibrium contact angle formed by a liquid droplet on a surface is dictated by the balance of three interfacial forces. While the choice of liquid is often set by the application, wetting can be favoured by surface treatment to increase the surface energy (γ_{SV}) and the use of surfactants to decease surface tension (γ_{LV}). For dynamic wetting, the instantaneous contact angle of a droplet wetting a smooth surface can be described by the Hoffman-Tanner equation [11, 12]:

$$\theta_E^{\,3} - \theta_{(t)}^{\,3} \propto \mathrm{Ca} \qquad (4)$$

Where Ca is the capillary number defined as:

$$\mathrm{Ca} = \frac{\mu \mathrm{u}}{\gamma} \qquad (5)$$

and $\theta_{(t)}$ is the instantaneous contact angle formed by a fluid of viscosity μ and surface tension γ, moving at a velocity u, and θ_E is the equilibrium contact angle. Eqn.(4) states that the surface forces expressed by a function of the difference of the droplet equilibrium contact angle minus its instantaneous angle are balanced by viscous forces. A low equilibrium contact angle and fluid viscosity will promote wetting velocity.

Moderassi et al. investigated the effect of chemical and physical heterogeneity on the mechanism of wetting and absorption of water droplets over sized papers[13]. Wetting and absorption rates were calculated from the contact angle, volume, and contact line of the droplets on paper. Absorption was found to start only after the drop had wetted the surface to a certain extent. There was a time delay before absorption occurred. By the end of this delay, a pseudo-equilibrium contact angle was reached, a metastable contact angle function of chemical composition of the surface. Wetting on a partially hydrophobized porous surface follows a power law model with wetting rates slower than in hydrodynamic wetting by a factor H, a function of surface roughness. Surface roughness also affects the pseudo-equilibrium contact angle, as by entrapping air, it renders the surface more hydrophobic. The wetting dynamics was found to be independent of the chemical heterogeneity of the surface. The initial rate of wetting and wicking of water on paper are equivalent; however, at later stages, wetting slows down as decreases the driving force (θ_t^3 - θ_E^3) while wicking proceeds at constant rate [13].

The typical evolution of contact angle, baseline and volume of a water droplet (5-13 μL) deposited on sized paper is shown as a function of time in Figure 1 [13]. The effect of paper surface roughness on the dynamics of wetting and absorption of water droplets on sized paper (ASA) calendered at two different intensities to create two levels of surface roughness at constant surface chemistry is shown in Figure 2 [13].

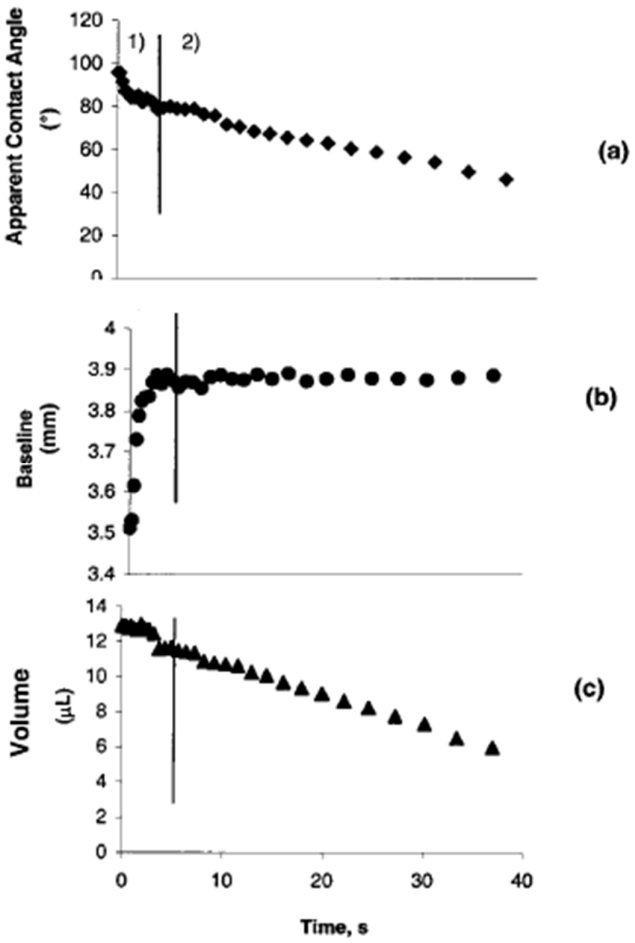

Figure 1: Dynamics of a water droplet on sized paper highlighting 2 regimes:1) governed by wetting; 2) dominated by absorption into paper. Evaporation was kept negligible. Fig 3 from ref [13] by Modaressi et al.

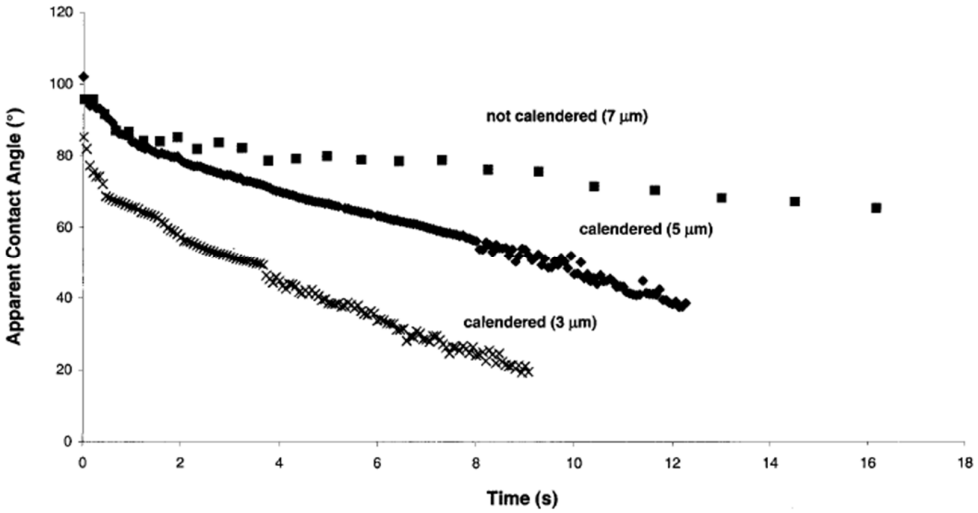

Figure 2: Effect of paper roughness on the apparent contact angle of water droplet. The average paper roughness achieved by calendaring is indicated. Figure 2 by Modaressi et al. [13].

2.2.3 Diffusion

According to Fick's law, the Diffusion flux (J) is proportional to the negative gradient of concentration(c):

$$J = D\,\nabla c \quad \text{with} \quad J_i = -D\,\frac{\partial c}{\partial x_i} \quad (6)$$

with the diffusivity (D) as proportionality constant. Diffusion is mostly used to describe the transport of gas systems. For an analyte in a liquid, diffusion is driven by a difference in concentration and also promoted by molecular movement. Diffusion becomes a transport mechanism for a solute once paper has been filled by the liquid and the driving force for liquid transport has vanished; it is however, significantly slower than wetting and wicking.

Nilsson et al. [14] investigated experimentally the diffusion of water vapour through pulp and papers. Two pulps and eleven grades of paper were studied. The diffusivities measured ranged from 2.1×10^{-8} m^2/s to 5.4×10^{-6} m^2/s. Effective water vapour diffusivity (D_{eff}) correlated with the density of the paper sheet (Figure 3), but was independent of relative humidity (Figure 4). The profile of relative humidity across a stack of eight paper sheets is nearly linear, whereas the profile of the moisture content is non-linear (Figure 5) [14]. This means that if the relative humidity (water vapour partial pressure) in the gas phase is considered as the driving force, the effective vapour diffusivity remains nearly constant.

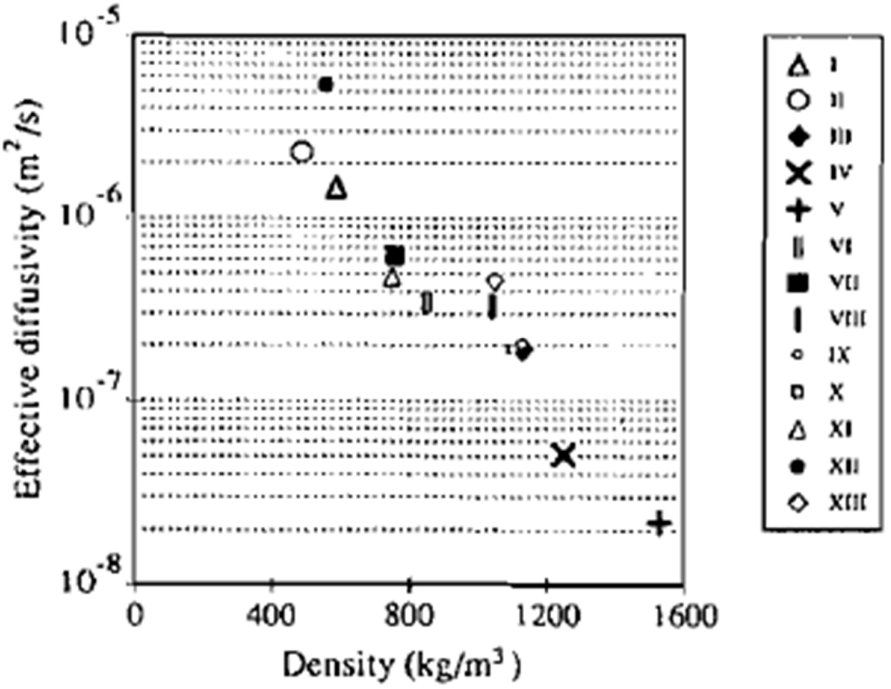

Figure 3: Effective vapour diffusivity as a function of paper sheet density. Figure 5 from Nilsson et al [14].

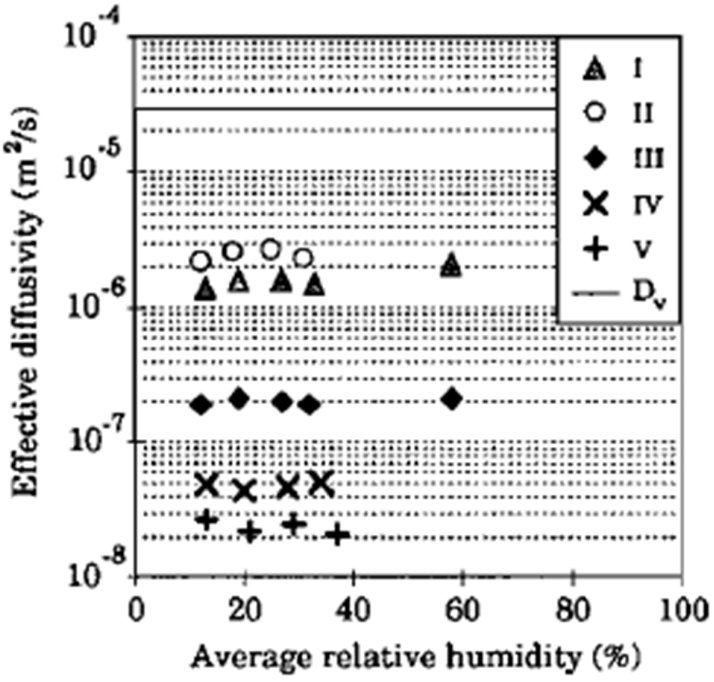

Figure 4: Effect of Relative humidity across paper sheet on vapour effective diffusivity (D_{eff}). Figure 8 from Nilsson et al [14].

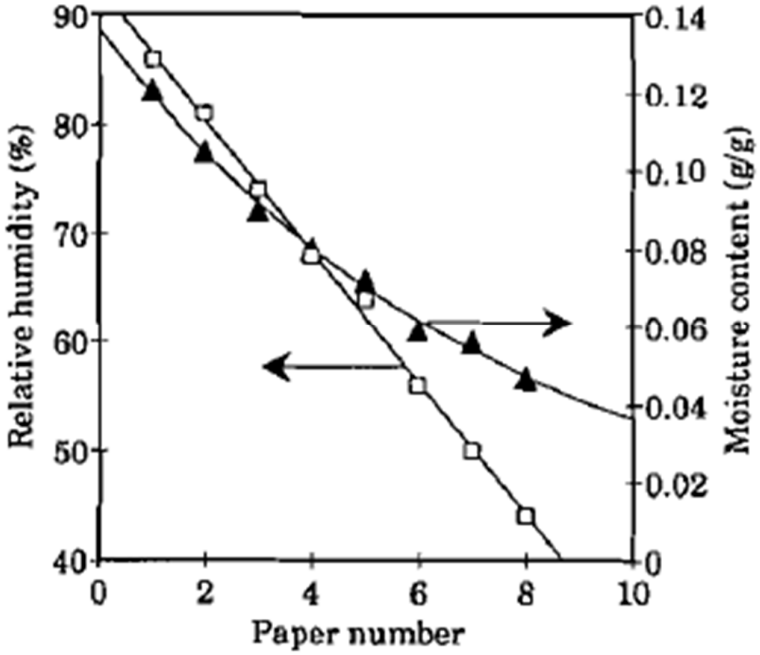

Figure 5: Moisture content and relative humidity profiles across eight sheets of paper. Figure 9 from Nilsson et al [14].

2.2.4 Evaporation

Evaporation provides a driving force for the transport of liquid in paper. Evaporation proceeds by heat and mass transfer from a combination of conduction, convection and radiation. The rate of evaporation of a given liquid from a surface is a function of the wetted surface area, temperature, relative humidity and the affinity liquid-surface; that is in absence of radiation and convection.

There are two modes of evaporation of liquid droplets on surfaces: one at constant contact area and one of constant contact angle [15]. The constant contact area mode is the dominating evaporation mode (variable contact angle). Erbil et al. analysed sessile droplet from surface under the constant angle mode [15]. Hu and Larson [16] investigated the evaporation of sessile droplets with a pinned contact line on a surface experimentally, analytically and by computation using the finite element method. The difference in water vapour concentration $(1\text{-}H)c_v$ drives the evaporation of water into air according to the diffusion equation (7):

$$\frac{\partial c}{\partial t} = D\,\Delta c \qquad (7)$$

Where c is the local water vapour mass concentration, D is the vapour diffusivity, H is the relative humidity of the ambient air and c_v is the vapour saturation concentration. At the air-liquid surface, the local evaporation flux J (r, t) is expressed by:

$$J\,(r,t) = D\,\nabla c \qquad (8)$$

Hu et al. [16] approximated an expression for the droplet evaporation rate [m (t)] at any wetting contact angle ($0^o < \Theta < 90^o$) as:

$$-m\,(t) = \pi RD\,(1-H)c_v\,(\,0.27\,\theta^2 + 1.30) \qquad (9)$$

At a given contact angle the evaporation rate is proportional to the contact line radius (R), the vapour concentration difference $(1\text{-}H)c_v$ and the diffusivity D and depends weakly on the contact angle Θ. Figure 6 compares theoretical model with experimental results for the evaporation dynamics of a sessile water droplet on glass.

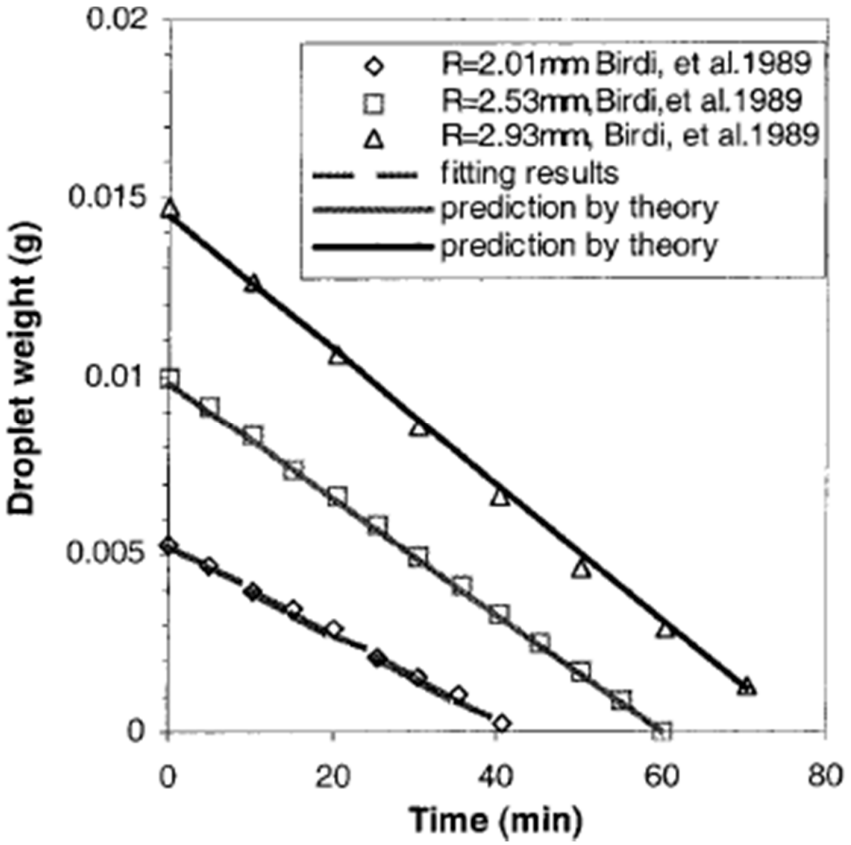

Figure 6: Evaporation of sessile water droplets on glass. Comparison experiments-theory of the time-dependent weight of water droplets of radii 2.01, 2.53 and 2.93 mm on glass at T =22°C. Figure 14 from Hu and Larson [16].

The additional effects of convection and radiation on free water evaporation were studied on lakes by Kohler et al. [17]; Uno provides evaporation formulas considering convection and temperature [18].

Swerin and Claesson investigated the evaporation of very small water droplets on paper and model surfaces in the context of inkjet printing [19]. Initial drying rates of 0.4 to 0.5 pL/ms second were measured at 23°C and 45% relative humidity; typical 6-pL droplets evaporated in around 1 s [19]. The major role of the surface is to increase surface area of the droplet as decreases the equilibrium contact angle between water and the surface.

2.2.5 Liquid transport in porous combined materials and imperfect surfaces

Shen et al. [20] stylized the effect of groove geometry on wicking. Khan et al. [21] investigated the dynamic of wicking and wetting of droplets impinging a narrow V-groove on a quartz surface. The variables of interest were the impinging droplet velocity and the groove width; the surface energetic and droplet size were kept constant. Wetting and wicking velocities were measured by high speed image analysis. (Figure 7) The maximum wetting and wicking velocities were found to be equal (Figure 8). The wetting velocity reaches a maximum instant after it touches the surface and decreases exponentially until the resting contact angle is reached, at which instant wetting stops. Groove wicking velocity has a similar behaviour. At steady state, wicking proceeds at constant velocity (0.05 m/s). The maximum wetting and wicking velocity both increase with the droplet impinging velocity.

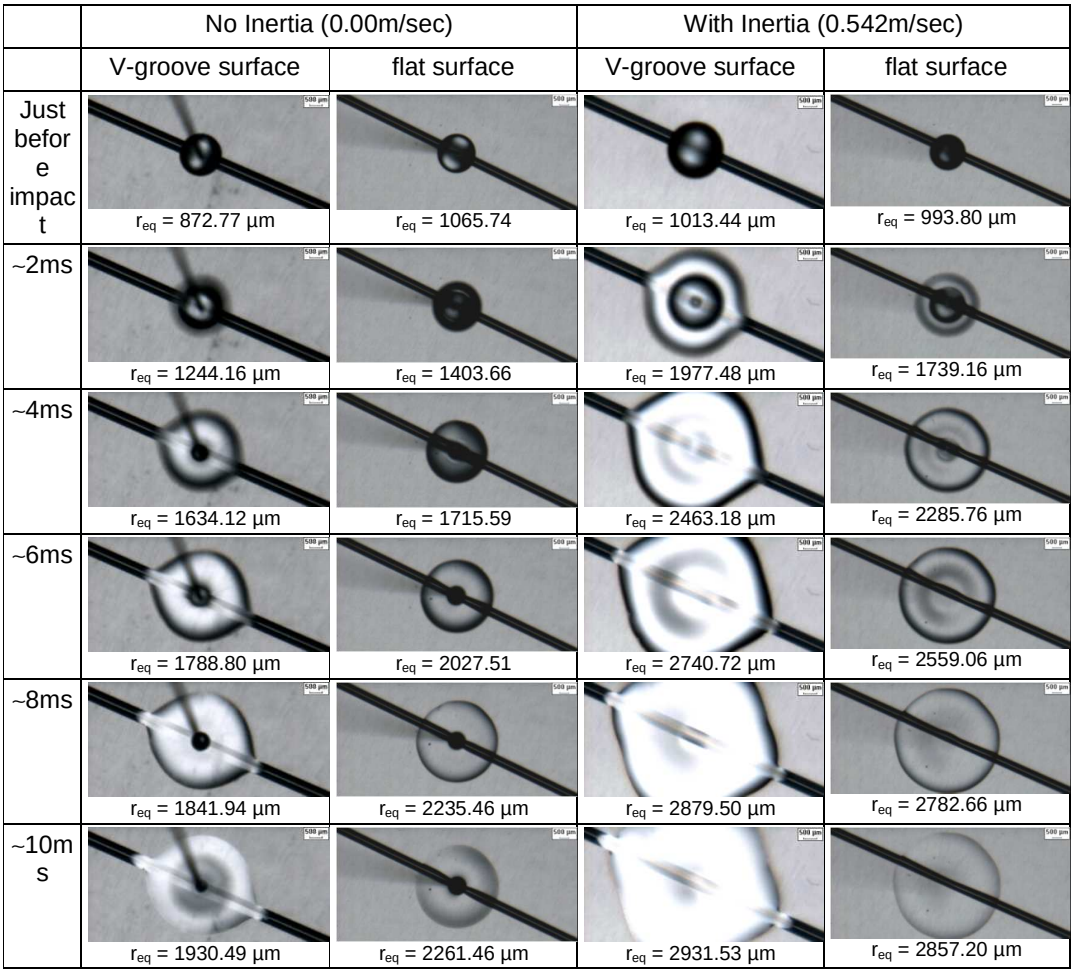

Figure 7: Dynamics of a water droplet impinging, with and without initial velocity, a flat quartz surface or a V-groove (w_0 = 0.5mm; quartz surface) on a quartz surface; images were taken at different magnifications; the inclined straight lines represent high contrast shadow of V-groove against white background. Khan [21].

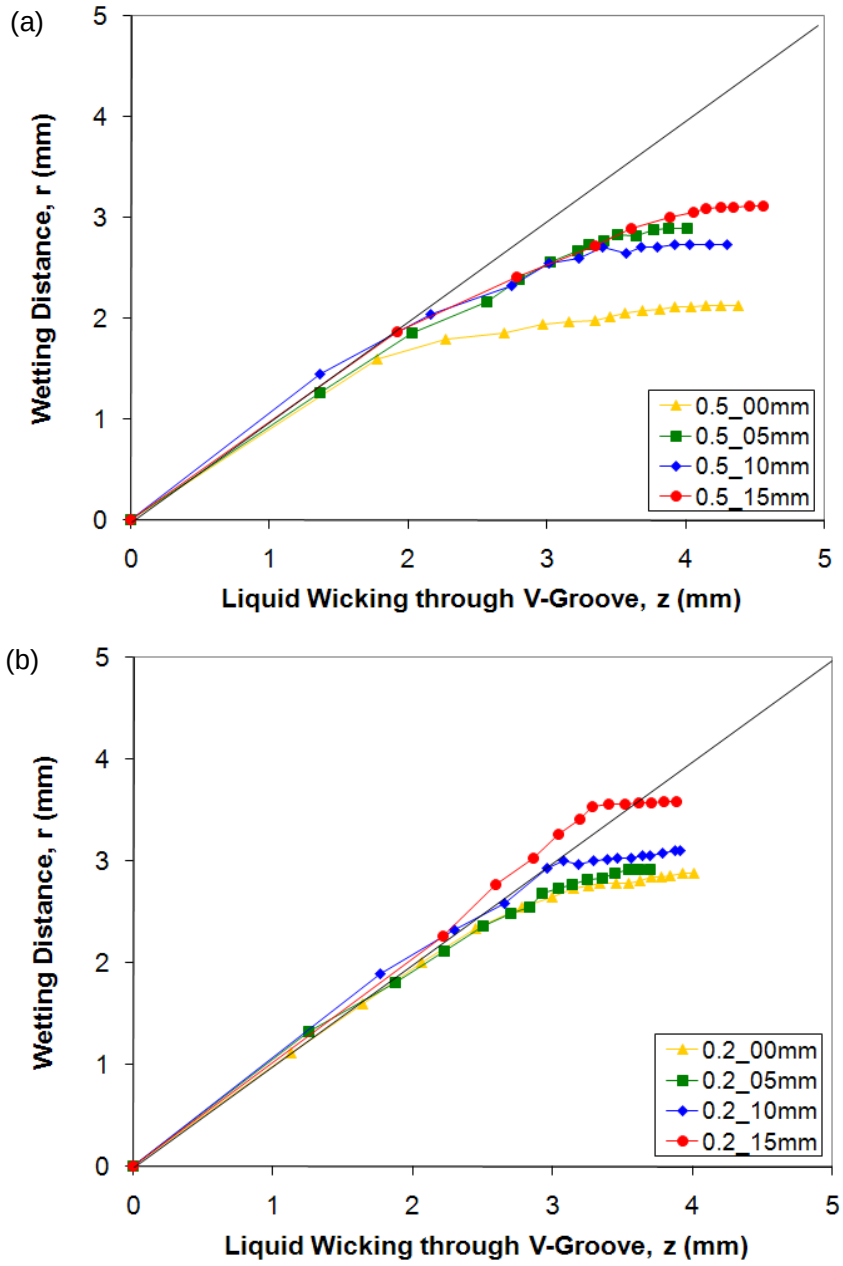

Figure 8: Comparison of average liquid wetting and wicking distance on V-groove geometry: (a) w_0 = 0.5mm, (b) w_0 = 0.2mm. Khan [21].

Solid dispersed in a drying drop will migrate to the edge of the drop and form a solid ring [22] (Figure 9). In a classic manuscript, Deegan et al. investigated capillary flow as the cause of ring stains from dried liquid droplets [23]. This is the mechanism of the so-called coffee stain: when a spilled drop of coffee dries on a solid surface, it leaves a ring-like deposit around the perimeter. The coffee droplet, initially dispersed over the entire drop, becomes concentrated into a fraction of it, forming a ring at the drop perimeter. The characteristic pattern of colloid/dye deposition was ascribed to a capillary flow in which pinning of the contact line of the drying drop ensures that liquid evaporating from the edge is replenished by liquid from the interior. The full mathematical characterization of the coffee ring formation by an evaporating drop is

provided in ref [22]. Deegan et al. predict the flow velocity, the rate of growth of the ring and the distribution of solute within the drop (Figure 10).

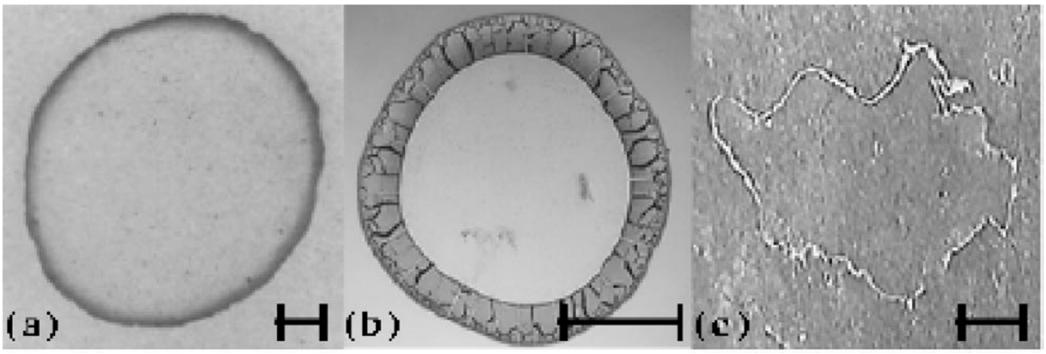

Figure 9: Pictures of Coffee stain (a), dried colloid (b) and salt deposits (c); the bar represents 1cm. Figure 9 from Deegan et al [22].

Plant scientists describe transpiration as the motion of water from the soil through a vascular plant and into air. Transpiration has long been linked to “passive wicking”; however wicking cannot transport water across dozens of meter to nourish the highest trees. Wheeler and Stroock [24] recently described this mechanism by a cohesion-tension mechanism theory by which the loss of water by evaporation reduces the pressure of the liquid water within the leaf relative to atmospheric pressure. This reduced pressure pulls liquid water out of the soil and up the xylem to hydrate the tree (Figure 11). The absolute pressure within the capillaries of the xylem is negative such that the liquid is under tension. Quantitatively, the difference in pressure generated in the capillaries of plants to drive flow can be two orders of magnitude higher than those generated by wicking [24].

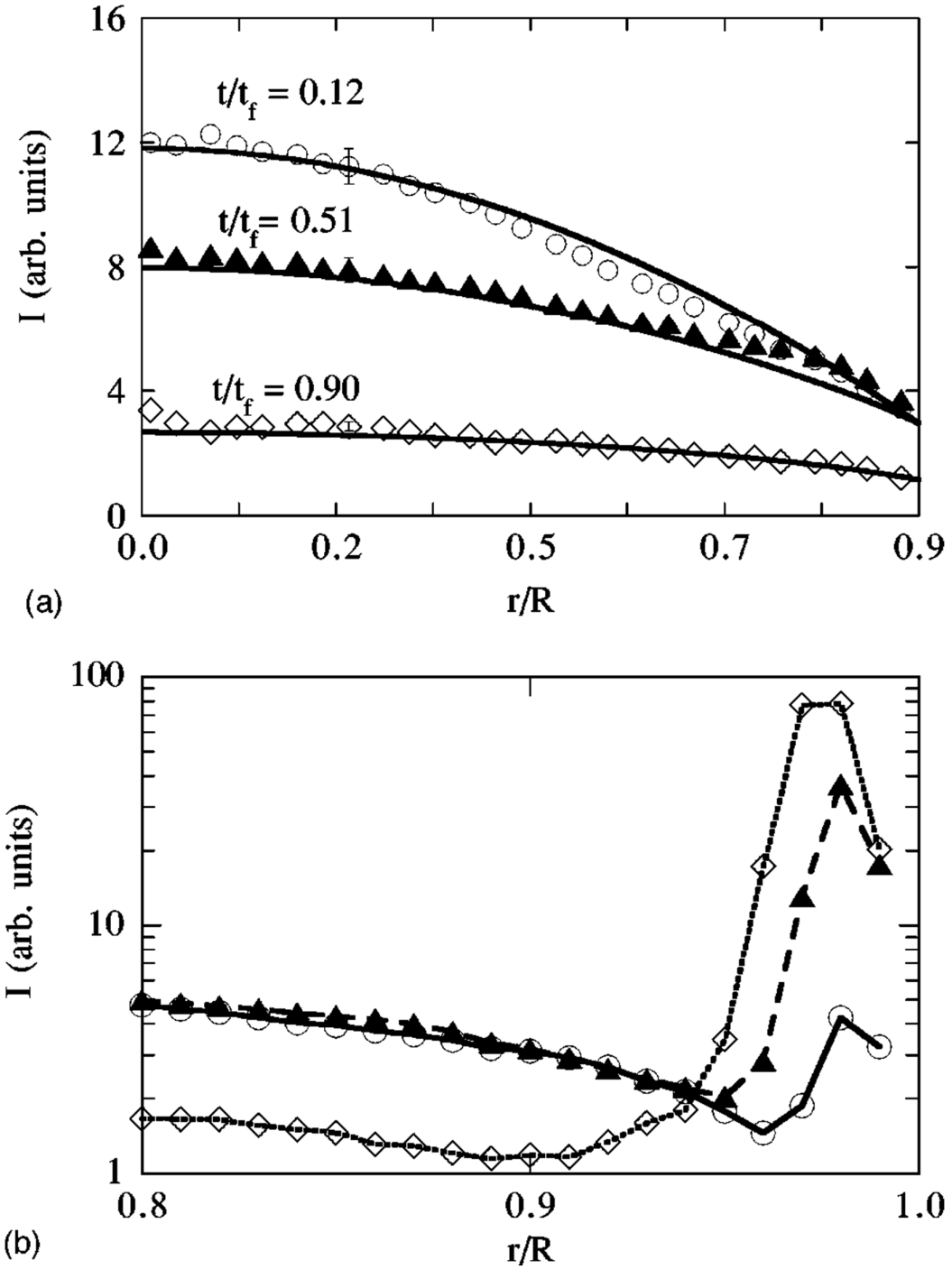

Figure 10: Distribution of colloids as a function of time in an evaporating droplet. The temporal and special coordinates are normalised as t/tf and r/R, respectively. Fluorescent latex was dispersed in evaporating drops and the intensity is expressed as a function of time and distance. The solid lines of a) represents the theoretical model. Figure 10 from Deegan et al [22].

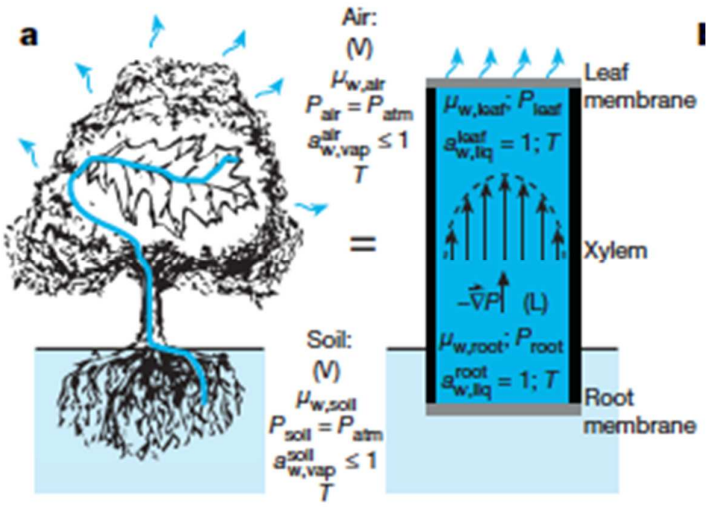

Figure 11: Schematic representation of the transport of water through the tree and transpiration of water at negative pressure. Figure 11 from Wheeler and Stroock [24].

2.2.6 Water transport in paper.

Schilling et al. [25] measured capillary wicking in fully enclosed paper channels (Figure 12). The channels were 8 cm long and 2 mm wide. The channels were enclosed with zero (0 toner), four (4 toner), or six (6 toner) layers of toner (Figure 12 B−E) Images of the wicking experiments were taken after dipping the bottom of the device into a reservoir of aqueous blue dye for 5 and 25 min. The experiments were carried out at two levels of relative humidity: 53% and 100%. Up to 5 min, all channels wicking were almost identical results. After 25 min, large differences in the distance that each fluid wicked appeared, showing the effect of evaporation. Wicking distance was plotted as a function of time and square root of time [25] (Figure 12F).

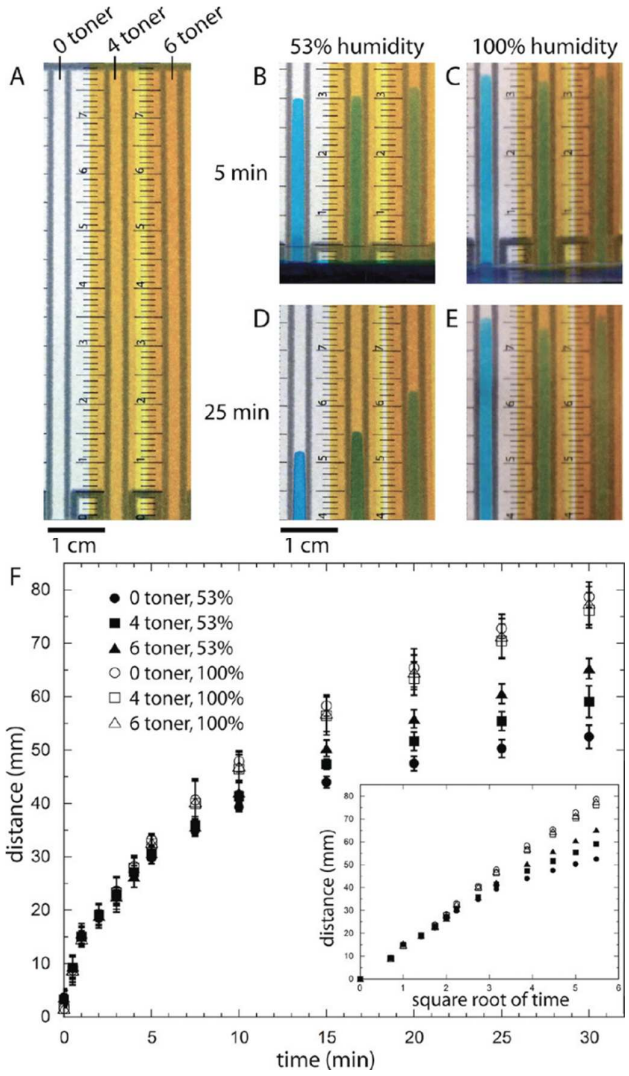

Figure 12: Capillary wicking in channels fully enclosed by toner coating. A) experimental representation of paper coated with various layers of toner coting and different humidity; F) wicking dynamics as a function of thickness of coating and relative humidity. Figure from Schilling et al. from ref [25].

2.3 Separation of solutions in paper

Paper can not only transport liquid, it can also separate the components from analyte mixtures. A few separation mechanisms are possible: filtration/size exclusion, chromatography /selective adsorption, ion-exchange and electrophoresis. Research and development on paper for separation culminated in the 50's and 60's [26].

2.3.1 Paper filtration- size exclusion

Paper is commonly used in air filter for domestic and industrial applications and liquid filters: oil filters for vehicle and water filters. Filter papers are extensively used for laboratory application and Whatman paper filter #1 has become by default the basesheet for paper microfluidics and biosensors prototypes. Few studies have quantified the role of the paper basesheet structure and filter paper pore size on filtration and sample separation. Su et al. investigated the effect of paper filter pore size, basesheet basis weight and type of fibers on the efficiency to separate blood

aggregates from stable red blood cells (RBC) in blood typing paper sensors [27]. Filtration and size exclusion was the basis of separation to segregate red blood cells (RBC) agglutinated by specific antigen from the stable red blood cells in paper based blood typing biosensors [28, 29].

2.3.2 Paper Chromatography and paper Thin Layer Chromatography

Paper chromatography have first been studied by Runge et al in the period 1850-1910 [26]. Zweig et al. reviewed the advances of paper and thin layer chromatography mostly for biological research and separation of inorganic compounds [30]; a complete list of reference is provided. A comprehensive manual on paper chromatography is also offered [26]. For optimum chromatographic separation three rules are suggested. First, to keep solvent composition and temperature constant; second, to move solvent at slow rate (2-3 cm/h), third, the choice of solvent should be one in which the components to be soluble have a small but definite solubility. If adsorption and ion exchange are neglected, the movement of a substance in paper chromatogram is a function of its solubility in the developing solvent [26]. In their book, Block et al. provided many practical considerations to optimize 1D and 2D paper chromatographic separation [26] and presented many applications and paper chromatography separation systems, including amino acids and peptides.

The resolution of mixture of solute on filter paper depends on surface adsorption, ion exchange and partition between solvents [26]. However, the predominant factor is the partition between two immiscible phases. Excellent amino acid separation was achieved with solvents that were only partially miscible with water. The theory of chromatography is often based on an analogy with distillation with fractionating columns. A chromatographic column is divided into successive layers of thickness such as the solution issuing from each is in equilibrium; the thickness of the layer is termed the height equivalent to one theoretical plate (H.E.T.P.) [26].

2.3.3 Paper Electrophoresis.

Konig published the first report on paper electrophoresis in 1937 [31]. Electrophoresis is the movement of charged particles in solution under the influence of an external electrical field [32]. Paper electrophoresis typically employs filter paper strips soaked in buffer solutions. Paper electrophoresis is the technique used for the separation of small charged molecules, especially amino acids and small proteins. Continuous flow electrophoresis has been found very useful in blood separation application. Block et al. also published a comprehensive manual of paper electrophoresis in 1958 [26].

3 PAPER MICROFLUIDICS AND DIAGNOSTIC DESIGN

Paper microfluidics aim at transporting, measuring, mixing, reacting, separating and storing liquid solutions. Paper systems rely on the porous paper structure to achieve these functions. The most important role of paper is capillary wicking which provides the driving force for liquid transportation without the need of external system or energy. The initial development phase of paper microfluidics has concentrated on the development of hydrophilic channels defined within hydrophobic barriers formed on paper [3, 9, 33-35]. The development of paper microfluidics has then been driven by low cost paper diagnostic application for easy and rapid analysis.

Paper diagnostic design currently focuses on the perceived limitation of performing multiplex assays using micro paper pads (µPADs), optimizing methods for both structure and fabrication. In many circumstances, single step assays are invaluable, however the simplicity is unsuitable for tests requiring multiple step processes. The design research has hence expanded from single paper sheets to 2D networks and 3D designs, which have demonstrated a degree of multi-test ability. However, this

increases the complexity of the design, necessitating a balance between simplicity and functionality. This section analyses the different types of PAD design.

Device designs can be simply differentiated by the directional flow of fluid. For instance, when the flow of liquid is in a single direction it is cited as 1D. 2D describes lateral flow in multiple directions on the horizontal plane (eg. the spread of liquid from a single corner to multiple detection zones) and 3D designs add a vertical component (Figure 13).

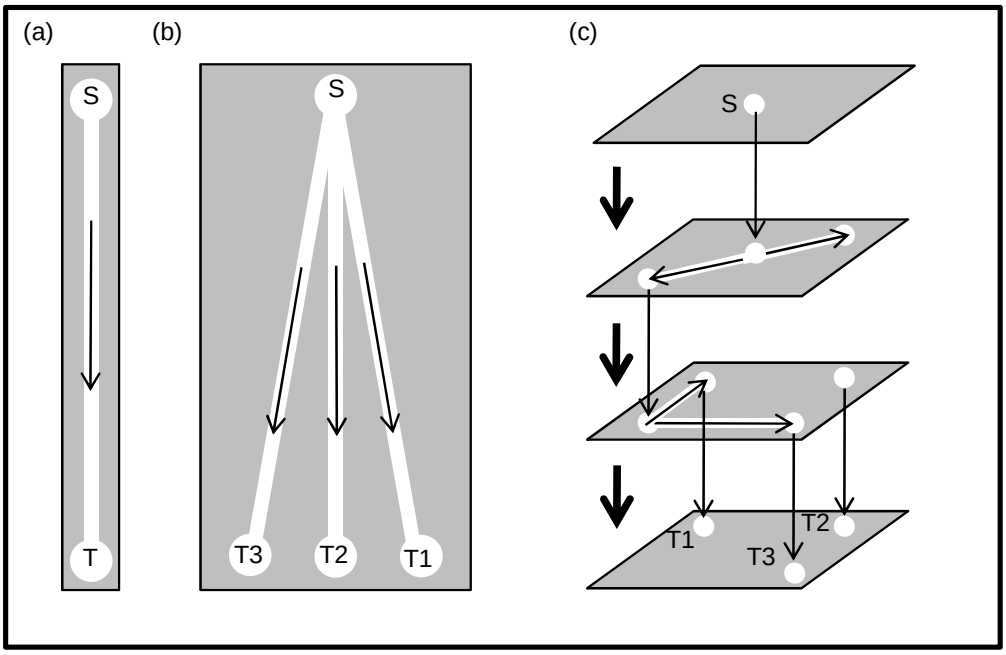

Figure 13: Schematic representation of the different types of paper diagnostics in (a) 1D (b) 2D and (c) 3D design.

3.1 1D Paper Diagnostics

Dipstick assay tests are the commonly known 1D lateral flow paper diagnostics that have been used for decades, and were first used to detect glucose levels in urine [36, 37]. The dipstick test is used by dipping one end of the stiff paper strip into the liquid sample to be analyzed (Figure 14). The fluids are then passively transported to a reagent zone through the cellulose fibres[38]. Testing evolved during the early 1960's, creating a triple analyte test with the ability to also detect protein albumin and pH levels of urine. Since then, dipstick tests have expanded to a 10-type multi-analyate with the ability to detect a wide range of biomarkers, namely leukocytes, nitrite, ketones, bilirubin and urobilirubin [39]. Some assays are also able to measure and report the specific gravity of the sample [39].

Further development during the 1980's led to the added ability of immunorecognition using dipsticks. Antibodies were spotted and immobilized on nitrocellulose creating a wider market range for PADs. Take-home drug and pregnancy tests are a prime example of urinalysis tests available. Other applications include immuno-based PADs that test blood analytes for cholesterol levels, diabetes, autoimmune screening and pathological diseases such as hepatitis C and human immunodeficiency virus type 1 (HIV-1) [40-42]. In 1989, dipstick technology integrated capillary-drivel lateral fluid transport, thereby eliminating the need for incubation and wash steps for certain

applications. The result was an improved lower limit of detection (LOD), achieved by amplifying the total number of captured and detected bioanalytes [43, 44].

Typical dipstick assays have the analytes dried and stored in the fleece sections of the assay during fabrication. This allows for different detection zones to be present on a single stick. The dried reagents are dissolved by the fluid sample solutions, hence allowing the reaction to occur. A typical dipstick assay is illustrated in Figure 14. The flow of liquid from one end to the other of the stick is one dimensional, thereby forming a 1D paper diagnostic. These tests are cheap, reliable and easy to use. Despite the success of 1D paper diagnostics, they are limited to single step tests and are unable to perform multiple-step diagnostic assays which are often required for techniques as enzyme-linked immunosorbent assay (ELISA).

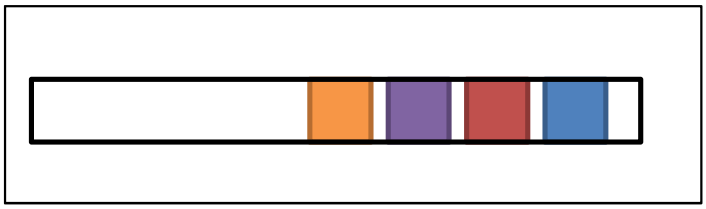

Figure 14: An example of a multiple-analyte 1D paper diagnostic on a dipstick.

3.2 2D Paper Diagnostics

3.2.1 Simple 2D PADs

Multidirectional flow in paper diagnostics is enabled by the ability to pattern paper with microchannel designs. In 2007, Martinez et al. [9] patterned 3 detection zones onto paper, creating a simple analytical device (Figure 15). It had the ability to detect glucose and protein, as well as a control zone. Hydrophobic boundaries were printed using photolithography, forming channel 'walls' which directed fluid into the 3 separate zones without cross-contamination. This added a spatial advantage compared to the conventional 1D- μPAD.

Patterning methods for diagnostic design have been summarized in previous reviews. Such methods include: photolithography, plotting, inkjet or plasma etching, cutting and wax printing [6]. A summary of the patterning techniques analysis is presented in Table I. Shen [3] highlighted 10 processes by which microfluidic channels have been patterned on paper. Of those, there are 6 printing processes: 1) photolithography, 2) ink jet printing, 3) inkjet etching, 4) wax printing, 5) flexo printing and 6) screen printing; and 3 radiation/paper processes: 1) plasma treatment, 2) paper cutting and 3) laser abrasion. The design concept of 2D-μPADs is to create hydrophobic barriers onto or within the paper structure, relying on the capillarity of the substrate for liquid flow. Original techniques created rigid and brittle barriers, however, technology has progressed to allow channels as narrow as 250μm width to be created [45]. Multi-step assays are still unable to be performed using common 2D-PAD designs. They are also prone to contamination and fluid evaporation. Despite this, 2D-PADs are very cheap, easy to use, versatile and robust.

Of interest is the work of Citterio et al. [33-35], from Keio University, Japan, with inkjet printing. Well-defined 3D hydrophilic microfluidic patterns were created on paper by inkjet etching. Filter paper was soaked in a polystyrene solution, dried and flow channels and sensing area were etched by inkjet printing toluene [33, 34]. A direct inkjet patterning method for microfluidic paper devices was also developed by Citterio by printing a hydrophobic UV-curable ink [35]. Shen et al. developed microfluidic

systems by inkjet printing a reactive hydrophobic ink made of sizing agent (AKD) [45, 46]. Microfluidic systems were also achieved by plasma treating heavily sized paper; the pattern was defined by the shape of a mask [47].

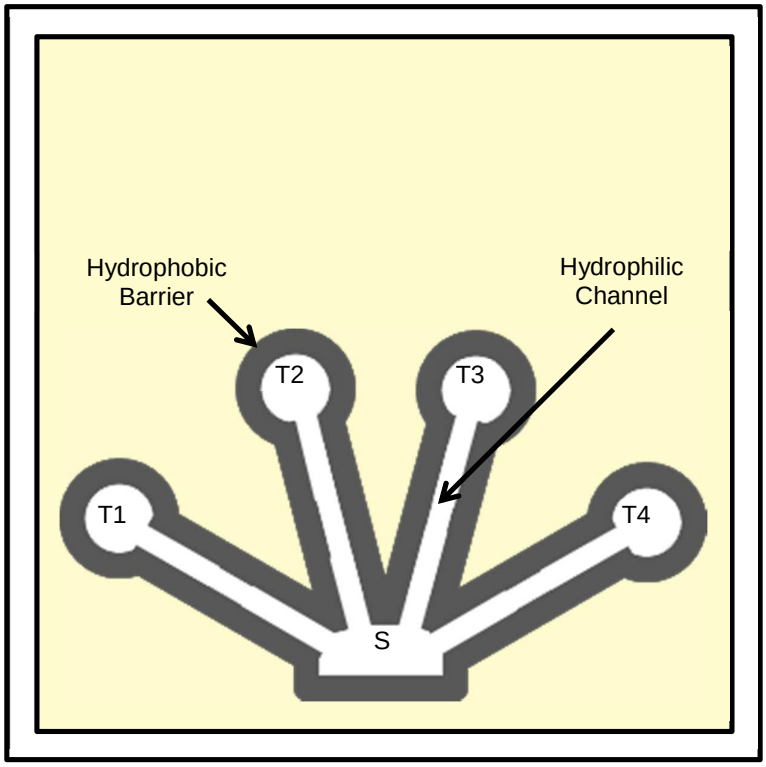

Figure 15: Prototype design of a 2D paper-based microfluidic device that tests multiple analytes simultaneously; showing separate testing zones (T) connected to a single sample zone (S). Redrawn from Martinez et al. (2008) [9].

Table I: Analysis of µPAD fabrication by functional printing

Paper Patterning Techniques	
Photolithography	**Description:** Patterned using chromatography paper soaked in SU-8 photo resist polymer solution before being selectively exposed to ultraviolet (UV) radiation using a patterned mask to shield desired pathways. Shielded regions remain hydrophilic and the unreacted SU-8 is washed away. Unshielded regions become hydrophobic after undergoing polymerization. **Advantages:** Convenient, useful; **Disadvantages:** Expensive chemicals and equipment, multiple steps, time consuming, reduced paper flexibility; **Examples:** Martinez et al. (2007)[9], (2008)[48]
"FLASH" printing	**Description:** Fast Lithographic Activation of Sheets (FLASH); Much like photolithography, except the paper is laminated between a transparent film and a black paper sheet. A standard ink-jet printer is then used to print a black ink mask onto the film. After polymerization the black paper and film is removed. **Advantages:** Faster, customized masks; **Disadvantages:** Expensive, multiple steps, reduced paper flexibility; **Examples:** Martinez et al. (2008)[48]
Etch Printing	**Description:** Completely hydrophobised paper, using a polystyrene toluene solution, is "etched" using a toluene solvent printed on the surface which dissolves the solution to allow for the hydrophilic channels to form. **Advantages:** Custom designs, faster; **Disadvantages:** Corrosive/flammable, chemicals; **Examples:** Abe et al. (2008)[34] (2010)[33]
PDMS Printing	**Description:** PDMS is dissolved in hexane and printed onto filter paper using a modified x-y plotter. To form the hydrophobic barriers, the PDMS solution penetrates through the paper thickness. **Advantages:** Enhanced flexibility; **Disadvantages:** Reduced channel resolution due to "creeping" solution; **Examples:** Bruzewicz et al (2008)[49]
Plasma Printing	**Description:** Paper previously hydrophobised using the cellulose reactive compound, alkyl-ketene-dimer (AKD) is patterned using metal masks that are clamped to the paper before being placed in a plasma asher. The AKD hydrocarbon chains are then oxidised by the plasma, leaving the hydrophilic channels. **Advantages:** Flexible; **Disadvantages:** Expensive, slow manufacturing rate; **Examples:** Li et al. (2008)[47], (2010)[50], (2010)[45],
Wax Printing	**Description:** Multiple techniques. The simplest involves pattering both sides of filter paper with a wax crayon before heating it. He was then melts into the substrate to form hydrophobic barriers. Also can be extended to inkjet printing for more complicated designs with higher resolution, but at an increased cost. **Advantages:** Good in resource limited settings; **Disadvantages:** Low resolution; **Examples:** Lu et al (2009)[51], Carrilho et al. (2009)[52]
Laser Cutting	**Description:** Uses a computer-controlled x-y knife plotter to cut the paper into the desired design with very high detail. Does not utilize imbibing techniques. **Advantages:** Cheaper fabrication costs, detailed design, clear labelling, can be fully or partially enclosed; **Disadvantages:** experimental technique, heating; **Examples:** Fenton et al. (2008)[39]
Inkjet	**Description:** Office or specialized inkjet printer receive hydrophobic and bioactive ink cartidges. Resolution of 20 µm or better determined by the diameter of the ink droplet. **Advantages**: low cost and flexibility of digital printing, established technology. **Disadvantages**: interaction ink-paper critical, plugging of nozzles. **Examples:** Li (2010)[45].
Lithography, Flexography, Silk Screening	**Description:** contact printing techniques. Advantages: fast, cheap, established technology. **Disadvantages**: require a mold, blanket or negative

3.2.2 Partially and Fully Enclosed PADs

The basic 2D-μPAD design is usually exposed, thus leading to the possibility of contamination and evaporation of reagents and sample. This is due to both sides of the device being uncovered. Also, contact with the support can potentially result in loss of reagent and sample fluids. Early investigations attempted to avoid such loss and contamination by adding samples and solutions while the device was held in mid-air. This is simply impractical for testing purposes [25].

A method proposed by Fenton et al. (2008) [39] avoided the imbibing of hydrophobic/hydrophilic patterns on paper. Paper was alternatively shaped into 2D designs using a computer-controlled x-y knife plotter. Three designs were proposed: 1) a single sheet of paper cut into the desired shape; 2) paper was mated with one layer of polyester cover tap before being cut; and 3) pre-cut paper was sandwiched between two layers of cover tape. The latter was the first example of a fully enclosed μPAD. Decreased rates of operator error were reported for partially or fully enclosed PADs; however, this may due to less room to error interpretation when paper is cut into clearly labeled sections [39]. This design is reportedly cheaper to fabricate and is impervious to external contaminants.

A partially enclosed device was reported by Olkkonen et al. (2010) [53] The design involved flexographically printing polystyrene xylene/toluene ink onto the back of the device, while the microfluidic channel design was printed on the front. A protective layer was provided by the hydrophobic back layer, preventing fluid from escaping via contact with the underlying support. It also protects against contamination from the support. Due to the direct roll-to-roll production, this method is well suited for high throughput manufacture. Full penetration of the hydrophobic barrier was achieved by the enclosed back, protecting from contaminants and loss of fluids.

Schilling et al. (2012) [25] recently investigated using printing toner to produce fully enclosed μPADs. A protective layer was printed by thermally bonding a thin plastic onto the paper. The laser printers and toner are similar to commonly purchased office printers, which have been extensively used for μPAD fabrication. This method is cheap and convenient for μPAD manufacture. Since the toner reportedly did not diffuse into paper, it does not affect the microfluidic channels, nor does it come off when wetted. A fully enclosed μPAD can be achieved with four printed layers and faster wicking rates were reported.

Since the biological reagents are affixed to the paper prior to printing so to be enclosed beneath the layer of toner, the heat of 180℃ required for this laser printing method could potentially have a negative effect of the functionality of the reagents. Proteins undergo denaturation at extreme heats, losing the ability to bind to other molecules and thus becoming inactive. Schilling et al. reported an enzymatic function decrease of 90% decrease when using glucose oxidase as an example [25]. A negligible effect to the sensitivity was reported when testing for glucose concentration, demonstrating a limit of detection (LOD) of 1mM. However, the 90% decreased in enzyme activity raises concerns regarding the optimization and economics of this method. Schilling also reported an alternative method where the surface was designed with a 1mm diameter hole. The hole was to act as a reagent addition port, allowing reagent delivery after enclosing the μPAD. Wicking would transport the reagent to the storage zone (Figure 16). With the exception of the port area, no loss of reagent or enzyme function was reported as the reagents were now protected from the surrounding environment.

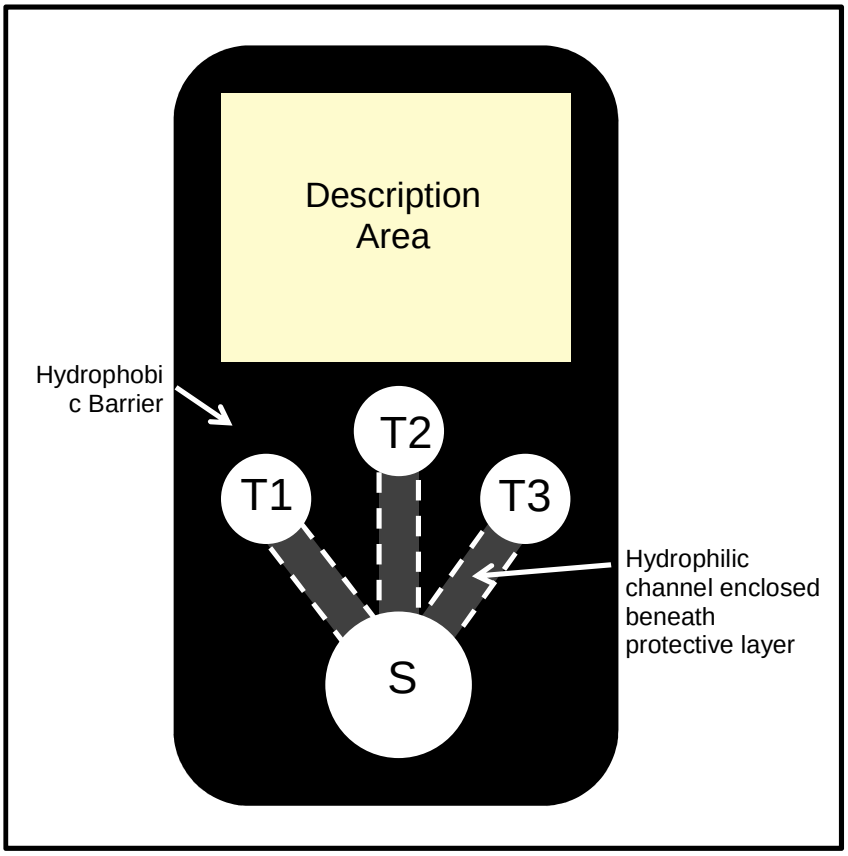

Figure 16: Example of a fully enclosed 2D paper diagnostic; showing separate testing zones (T) connected to a single sample zone (S). (Redrawn from Schilling et al. (2012) [25]).

3.2.3 PAD Networks

'Traditional' single sheet µPADs are limited to single-step processes. While ideal for the end-user, single-step designs restrict the applicability as most laboratory-based diagnostic assays are actually multi-step processes. Assays, such as the ELISA, have signal amplification and washing steps to improve sensitivity and specificity. Multi-step PADs were attempted using two-dimensional paper networks (2D-PNs). While using a single activation step to retain simplicity and affordability of the single-step µPADs, the 2D-PNs expanded to involve multiple processes within the network.

A 2D-PN was developed by Fu et al. (2010) [54]. The design retains the autonomous nature of µPADs with a single activation step, but allows multiple reagents to be delivered sequentially to the detection zone (Figure 17) [54, 55]. The first design reported three methods that allowed the simultaneous conversion of multiple inlets to a single point [54]. The focus was varying the delivery time of the fluid. This was achieved by 1) varying paper length, 2) varying paper width or 3) the implementation of a dissolvable barrier, consisting of trehalose, with the function to slow the liquid in its tracks. Paper composition, pore size and surface chemistry were other contributing factions that could potentially affect fluid flow rate. However, these were not explored. PAD fabrication involved using a laser cutter, before being supported on a glass substrate using double-sided tape [39, 54]. The inlets held absorbent pads for the application of reagents, relying on wicking for transportation to the detection zone. The

2D-PN design used dye and pH as examples to show the staggered delivery of individual components to the convergence site. The same design was later used to demonstrate chemical signal amplification[54, 56].

While the transport mechanisms of fluid through the paper networks were explored, the effects of paper composition, pore size and surface chemistry were not addressed[57]. Despite the 2D-PN design allowing multiple-step processes, the added complexity also increases the possibility of errors.

Alternatively, a method using a single fluid source was explored, removing the individual sources for each of the reagents [58]. The device was 'programmed' to sequentially disconnect each reagent. A single source well was used, while each reagent section had a varying length of paper in the 2D-PN. A plastic casing of poly (methyl methacrylate) (PMMA) was used to house the plastic strips. The purpose was to reduce evoparation, as well as provice a support to mount the device into the source well. The casing and well design allows a paper cartridge/strip to be inserted by the user. Paper strips are immersed into the fluid well at different depths, depleting as fluid wicks into paper. As the level of fluid in the source well depletes, it 'disconnects' strips. Disconnection is controlled by 1) the rate of fluid depletion, 2) the depth of the paper strips and 3) the cross-sectional area of the fluid source. Disconnection also relies on a large and thick regulator strip made of paper; without it, the lengths of paper varied only slightly. Fluid flow is managed using basic principles of wicking, allowing the 2D-PNs to be programmed. This series of experiments used colored dyes, previously dried onto paper.

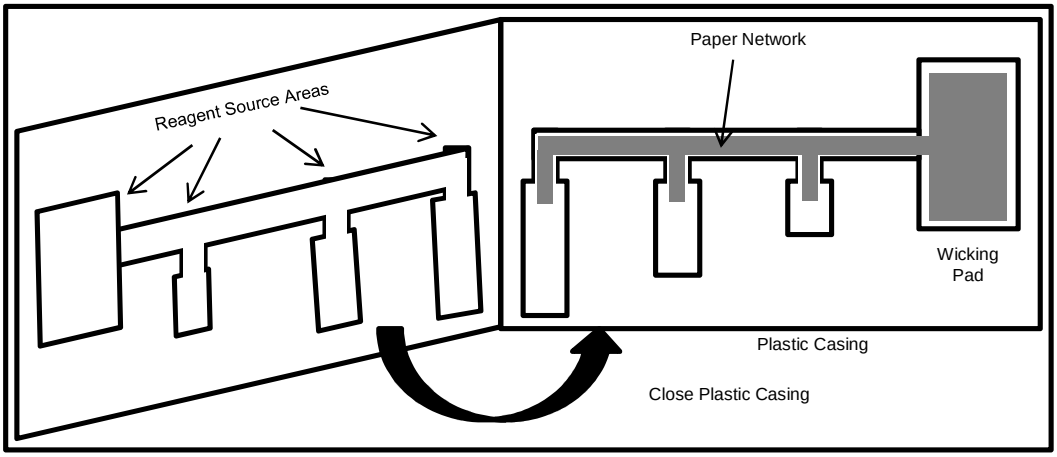

Figure 17: Example of a 2D paper network used for the sequential testing of multi-step analysis with a single activation point. (Redrawn from Fu et al. (2012) [55]).

3.2.4 Microfluidics to control liquid flow

Certain applications require mixing, reacting or sequencial addition of reactants. This has been achieved with paper microfluidic systems. Shen et al. designed valves and reactors on paper to control the addition of fluids, their retention time and extent of reaction [3, 35, 46, 59].

3.3 3D Paper Diagnostics

3D paper devices developed from the desire to increase the number of microfluidic channels available, allowing for the design of more complex systems. This in turn would lead to the ability to test more analytes simultaneously, and thus create a multiplex 3D-μPAD. Several prototypes have been reported, each consisting of

multiple 2D paper layers. While 3D-μPADs offer certain advantages, the increased functionality sacrifices the design's simplicity, cost and practicality.

The first 3D-μPAD design alternated layers of patterned paper and perforated double-side tape, each stacked upon the other. The fluid was then allowed to flow vertically through the device in addition to the lateral flow shown in 1D and 2D devices. In order to connect each layer, the perforated holes were filled with cellulose powder, thus providing the 'vertical micro-channels'. Interweaving pathways could be created to allow the analyte to reach several detection zones while avoiding cross contamination. An initial design by Martinez et al. (2008)[60] demonstrated a 4 channels device. Each channel had 8 connecting channels that allowed fluid to travel from the top layer of the device to the bottom, where the detection zones were located. Each layer could hold a varying purpose, including fluid distribution, filtration or combination with other reagents (Figure 18).

The fabrication process usually predetermines the function a device test. A modified 3D-μPAD design demonstrated a single device which could be used for specific applications[61]. Multiple analytes could be configured on-site using a single device, enabling some specificity depending on the patient's need by choosing the channels required and 'activating' each specific test. Activation is achieved by leaving certain perforations in the tape without cellulose powder. These are labeled 'on-buttons'. One needs only press to the button down to connect it with the desired channel, and thus turn the test 'on'. Unpushed buttons would remain disconnected, only allowing flow to the required tests. This could prove particularly useful when the sample quantity is limited. The single platform could be hailed as a 'universal device' to test a wide range of samples, eliminating the need for separate designs for each test.

However, once a button is pressed, it cannot be turned 'off'. The paper compresses when pushed and deforms inelastically. The simplicity, ease of use and reliability decrease with test complexity, especially in times of stress or illness. These tests are getting too complex to be used directly by the patient.

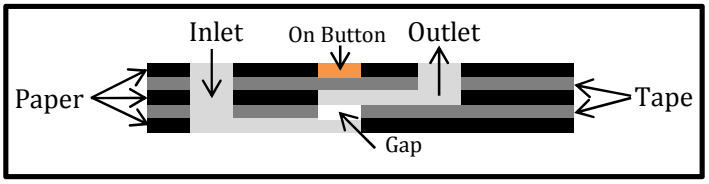

Figure 18: Side-view representation of an example 3D paper diagnostic. Redrawn from Martinez et al. (2010) [61].

3.3.1 Origami

Origami is the art of paper folding. 3D-μPADs by Liu and Crooks (2011)[62] report a design method using a single sheet of folded paper (Figure 19). Chromatography paper is patterned with micro-channels using a single photolithographic step. A metal frame then provides the template for the paper folding to ensure the alignment is correct. A particular sequence is followed during the folding process, allowing micro-channels to be matched, and thus flow in both lateral and vertical directions. This design also calls for an aluminum clamp, where the four corners of the folded paper are trimmed in order to fit. The clamp holds four holes drilled on top, access ports allowing samples and solutions to be injected into the device. Origami-PADs (*o*-PADs) allow for the elimination of double-sided tape, which reportedly diffuse into the paper

after long time periods, thus decreasing the micro-channels' hydrophilicity. It also removes required assembly tools such as laser cutters. Despite these advantages, origami PADs still require an aluminum clamp to ensure proper alignment. This increases cost of the device, complexity of use and decreases disposability.

Another 3D-μPAD which utilizes the origami principles was reported by Govindarajan et al. (2012) (Figure 19) [63, 64]. However, the origami steps do not simplify fabrication, but are instead integrated as part of the function. By folding the layers in a certain sequence, the origami steps allows for complex processing. This was demonstrated to prepare collected DNA samples for molecular diagnosis. Stacked lays of cellulose paper and double-sided tape were used to assemble the device. Additionally, single-sided tape, a repositionable adhesive layer and paper card were used and folded. During fabrication, a lazed cutter is used after stacking to pattern the fluidic channels. When the adhesive layer is peeled away, full and partial cuts on the device leave fluidic channels on the substrate.

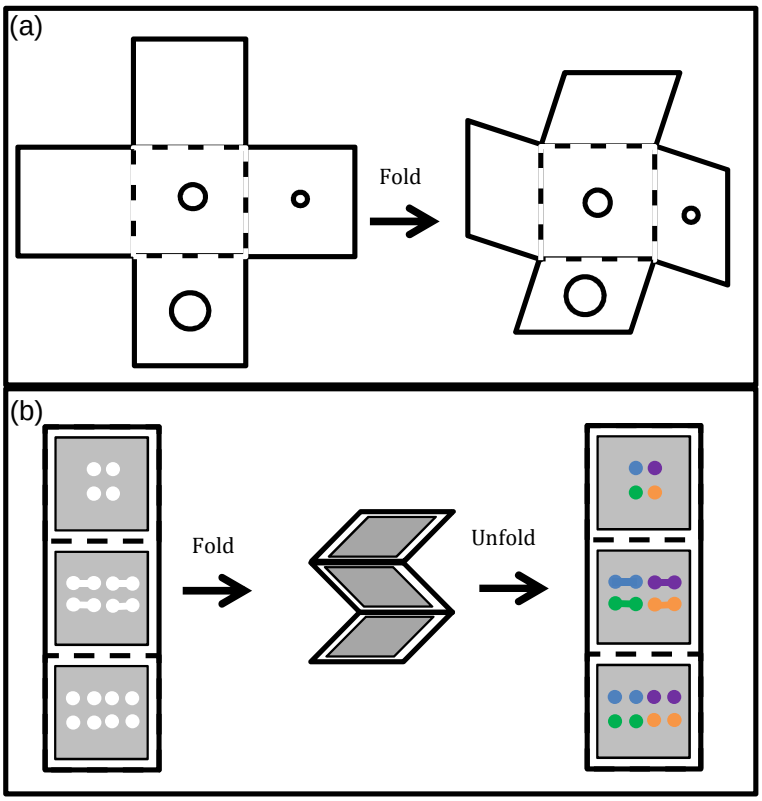

Figure 19: Redrawn examples of origami microfluidic paper diagnostic for multi-step analysis designed by a) Govindarajan et al. (2012) [63, 64]. b) Liu and Crooks (2011) [62].

3.4 Detection Principles in Paper Diagnostics

The detection of diseases using PADs is a two-fold process that relies on the ability to report a specific recognition event. Various applications require different reporting and recognition methods. This field is currently undergoing a very fast development as witnessed by the proliferation of publication and patents. Such methods are discussed below.

3.4.1 Methods of Reporting

Half the challenge of accurate and successful diagnosis is detecting the presence of an analyte. It also requires a reliable and simple method of relaying the information to the end-user. Most PADs visually report results. Additionally, some devices have attempted to integrate electronic reporting processes. A brief overview of the development and issues of the main technologies is provided in this section.

3.4.1.1 Colorimetry and Visual Signals

The visual change within the detection zone of a device is the most used reporting method for PAD analysis. The most common technique described in the literature is colorimetric reporting. A visual color change occurs in the presence of a desired target once a sample has been tested. A prime example is the ELISA, where a biosensor is immobilized onto the surface of a substrate. Once the desired analyte is captured, a second reporting enzyme is bound and induces a color change, announcing its presence.

Colorimetric analysis on PADs is simple. Also, it is applicable to point of care (POC) situations. Analytical instrumentation for accurate interpretation is often required for other visual reporting methods, such as florescence or absorbance. In its simplest form, a qualitative binary result can be provided by colorimetric analysis, i.e. whether or not a color change is observed. The white background makes paper an ideal substrate for colorimetric analysis because it provides an excellent contrast. However, it can also interfere with fluorescence and absorbance reading methods, further supporting the use of colorimetric reporting. Lignin residues, dyes and UV brighteners sometimes used in paper can interfere with fluorescence measurements.

However, colorimetric analysis does have some disadvantages. Interpretation may require trained personnel or analytical instruments, especially for quantitative analysis. In the modern age, telemedicine can be used to communicate, interpret and/or transmit results for analysis [6, 65].

Alternatively, another concept of printing symbols or text has been demonstrated for qualitative results to contour user-barriers. The “writing” technique was first reported by Bodenhamer [66], describing a displacement assay for transparent packaging. Immobilized antigens were printed in a pattern upon the substrate and saturated by a corresponding dye-coupled antibody. When interacting with the antigenic target, the dye is then released resulting in disappearance of the printed pattern, and thus indicating the presence of the target. In this case the target was a pathogenic antigen, resulting from undesired exposure. Li et al. printed directly the results of blood typing analysis by controlling blood-antigen coagulation in microfluidic letter patterns formed on paper [67].

3.4.1.2 Reporting with Electronics

Stand-alone paper diagnostics are ideal. However, many reporting methods rely heavily on colorimetric results which require some interpretation by trained medical personnel. A combination of mobile phones with camera capabilities, and µPADs could allow for rapid and accurate interpretation of results. This is known as telemedicine [65]. The image of a PAD can be taken using a scanner or a camera, before sending the image via satellite to an expert for analysis. This would eliminate the need for an on-site expert during interpretation. The test interpretation would then only take as long as the time needed for the results to reach the intended expert. Another possibility would be to rely on smart phone applications for direct analysis.

Electrochemical reporting methods have also been explored [68]. Electrochemical analysis provides simplicity, reduced costs and portability to current analytical methods. Integration with paper-based microfluidic devices could be an ideal solution for analyses that utilize electrochemistry reporting methods [69]. An example is reported by Carvalhal et al. (2010) [69] describing a paper-based separation device that electrochemically detects the presence of ascorbic acids (AA) and uric acids (UA). Patterned strips of paper can be printed with thin-layer electrodes for coupling to an instrument. The paper acts like a chromatography column, allowing for separation. The electrode can detect the presence of electro-active compounds when the solvent dissolves the AA or UA samples, allowing for quantitative concentrations to be detected chromo-ampero-graphically. Liu and Crooks (2011) [68] also reported a battery-powered electrochemical sensing platform that electro-chromically displays results. Nie et al. (2010) [70] described electrochemical paper-based microfluidic devices (μPEDs), fabricated by screen printing electrodes using conducting inks (e.g. carbon, or Ag/AgCl). Furthermore, research also combined μPEDs with commercial electrochemical readers, such as glucometers, for analyzing numerous biomarkers in blood or urine [71]. Such biomarkers include glucose, cholesterol, lactate and alcohol. Paper-based electrochemical ELISA devices were also explored to detect rabbit IgG [72].

Another electro-based detection method which has been gaining interest in recent years is electrochemiluminesence (ECL) [73-76]. Microfluidic paper devices can be coupled with a chemiluminescence reaction, which is initiated and controlled by the application of an electrochemical potential. ECL is reported to combine the advantages of luminescence and electrochemistry, but also added selectivity to the device. Delaney et al. (2011) [73] report using an ECL reagent, Tris(2,2'-bipyridyl)ruthenium(II),($Ru(bpy)_3^{2+}$) to detect co-reactants, such as nicotinamide adenine dinucleotide (NADH). The reaction between ECL and its co-reactant generates light upon oxidization. Mobile phone cameras are then employed as luminescence detectors for ECL emissions. The device is still reported to maintain simplicity and low-costs as the fluidic channel detection zones are printed with ECL using an inkjet printer, similar to other reported methods of μPAD fabrication. Yu et al. (2011) [74] successfully demonstrated the ability to detect UA in artificial urine using electrochemiluminescence. Subsequently, a paper-based ELISA using chemiluminescence detection was explored using biomarkers such as ∝-fetoprotein, cancer antigens and carcinoembryonic antigens as examples [75].

3.4.2 Biorecognition in Paper Diagnostics

Accurate diagnosis can rely on the ability to detect the presence of specific biomolecules selectively. This is known as biorecognition. There is an abundance of distinct analytes within the human body. Each could potentially be used for the diagnosis of disease, both pathological and physiological. When the correct biomolecular target is identified, a detection method can subsequently be developed. Nucleic acids, enzyme proteins and antibodies are prime examples of biomolecules which can be immobilized on paper. They can reportedly be dried onto paper or the substrate's surface without denaturation, which allows for storage and use in remote areas lacking access to laboratory facilities.

However, to achieve biorecognition, the correct target is required to detect a specific marker in the body. The greatest challenge is utilizing this ability to unequivocally detect the presence or absence of a disease. Current diagnostic methods often provide the basic outline of detection principles. However, biomolecular behavior on paper sometimes varies from the behaviour exhibited in solution, when using established laboratory techniques.

Examples of selective biorecognition on paper devices include applications for diseases such as diabetes, malaria, HIV-1 and cancer; and non-disease related analyses such as liver function and blood typing. Each example detects a different type of biomarker. Diabetes-related devices are able to detect and quantify the concentration of glucose in blood or urine [9, 34, 39]. A cancer diagnostic has been reported with the ability to detect a specific miRNA sequence indicative of lung cancer [77]. Alternatively, other biomarkers could include proteins such as alkaline phosphatase (ALP) and aspartate aminotransferase (AST) enzymes which are used for liver function analysis [78]; or *Plasmodium falciparum* histidine rich protein 2 (*Pf*HRP2) used to detect a malaria infection [42, 55]. The specificity of antigen-antibody interactions can also be utilized for biodiagnostics on paper. Prime examples are its use in blood typing [28, 29, 67], HIV-1 detection [79], and Hepatitis B &C detection [80].

The potential of biorecognition on paper is limitless. Success is not dictated by the use of one specific type of biomolecule over another, but is a result of the detection methods available. As can be seen, each application is different, using samples from a range of sources (e.g. urine, blood and saliva), and thus requires separate examination and methodologies for the paper analytical device (PAD) to succeed.

4 PERSPECTIVES

Four important aspects of paper microfluidics remain to be analyzed in this review. The first is the effect of paper structure on microfluidics; the second are alternatives to paper for low cost microfluidic systems able to be integrated in a diagnostic; the third is an overview of the manufacturing process; the last is an outlook of challenges and future directions of paper microfluidics.

4.1 Paper structure

Very few studies have analyzed the effect paper structure has on microfluidics performance. Paper structure represents the 3 dimensional distribution of fibers within the paper matrix; its negative is the structure of the void space. Two observations are of importance. The first is that only the porous void fraction of paper, bordered and driven by the hydrophilic fibers, transports liquids in paper by wicking. The second is that wicking of a liquid in paper only occurs in dry paper; the driving force disappears once a liquid has filled the paper's pores. Critical variables are the diameter, orientation and length of pores, which are defined by fiber orientation and spatial distribution.

Paper offers a three dimensional (3D) flow in the design of microfluidics as liquid can be transported and fractionated not only in the plane of the surface (X-Y), but also through the thickness of paper (Z direction). The fibers type and spatial distribution dictate the size, connectivity and directionality of the capillaries through which a liquid transports and the analytes distribute. Of special interest are the type of fibers, paper density and thickness (basis weight) and the fibers orientation. The important papermaking process variables to engineer the basesheet for microfluidics paper include the jet to wire speed differential, the choice of chemical additives (filler, sizing agents), calendering and surface treatments such as surface sizing and coating.

X-ray microtomography is a powerful technique to characterize the 3D microstructure of porous media; for paper, it allows the visualization and digitalization of fibers and pores. Paper thickness-porosity profiles, microtomograph segmentations and 3D visualization can then be derived. Bloch et al. have combined X-ray synchrotron microtomography with transport modeling of a fluid within the digitalized paper

structure [81-83]. Flow rate, filtration size cut-off and separation efficiency can then be simulated. The model derived can also be used to design the optimal paper structure for a specific application.

4.2 Alternatives to paper microfluidics

Paper combined with functional printing provides a very cheap and flexible material/process combination for manufacturing microfluidic devices to transport liquids. Currently, the primary application of these microfluidics is in low cost diagnostics for medical applications. There are at least three material/process alternatives to the paper technology that can serve this function. These are: 1) threads, 2) hydrophilic and porous polymeric films and 3) grooves on hydrophilic surfaces.

Multifilament cotton and cellulosic threads have been investigated by a few groups including Shen [84-86], Whitesides [87] and Juncker [88] as alternative to paper for low cost microfluidic systems and biodiagnostics. Capillarity drives the flow of a fluid along the thread. While thread and paper are chemically similar, many properties of the material differ. The main differences include the length of the fiber, the interfiber bonding and the porous channel structure [86]. Shen et al. also devised mechanisms to control the liquid flow in thread based systems [86]. Figure 20 illustrates the effect of thread structure (with one, two and three threads) on the capillarity flow rate [86] for vertical devices. The fluid penetration length varies with the square of time according to equation 2, until gravity and evaporation become significant phenomena. Similar to paper, thread is supported by a very established, robust, widely available and low cost technology that can be used to manufacture diagnostic devices.

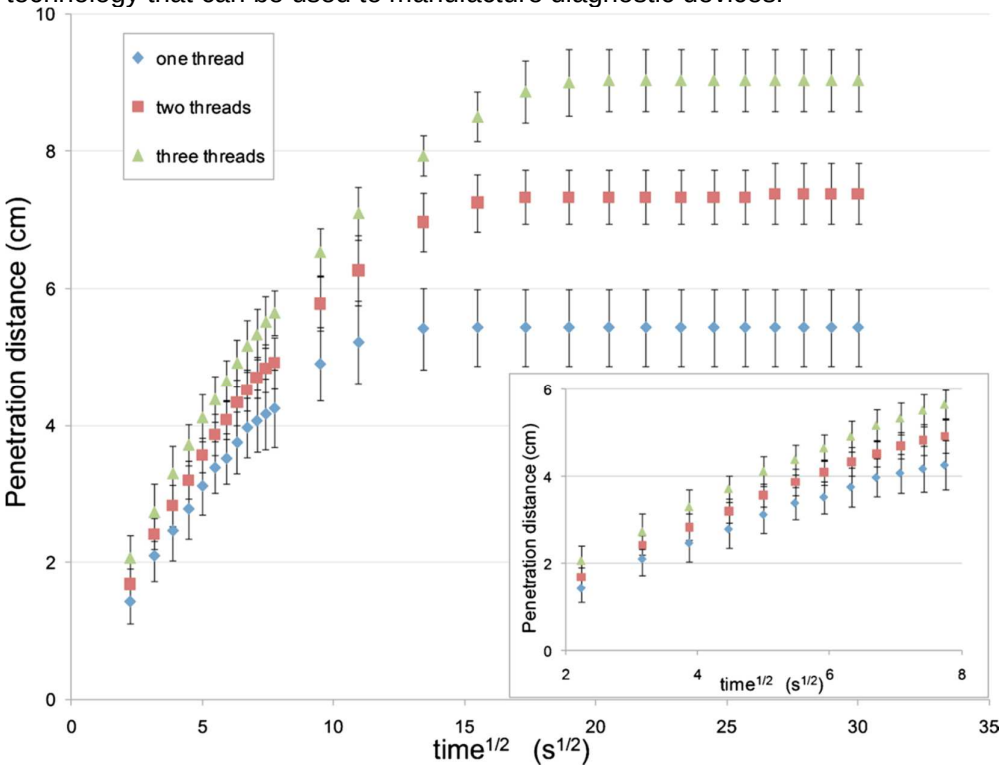

Figure 20: Effect of the Thread structure on the capillary flow of an aqueous dye solution penetrating vertically suspended threads with an end immersed in a reservoir solution. Figure 3 from Shen et al [86].

Hydrophilic and porous polymeric films represent another alternative to paper, which can however use the same process technology. Cellulosic materials, and especially nitrocellulose films, have long been used in low cost diagnostics. Yager et al [89], from the University of Washington in Seattle, reviewed the evolution of nitrocellulose as a material to engineer bioassays [89]. Biomolecules (RNA-DNA complexes) were immobilized on porous nitrocellulose membranes in the 1960's [89, 90]. After 25 years of development, porous nitrocellulose membrane has become a prominent material for lateral flow (LF) diagnostics; it provides liquid capillary flow and preserves the functionality of immobilized biomolecules [89]. Nitrocellulose has a unique compatibility with three of the most important classes of biomolecules: proteins (enzyme, antibody), DNA and RNA [89, 90]. Yager et al. have developed complex and elegant diagnostics including dissolvable fluidic time delays for programming multisteps assays with nitrocellulose membranes [91].

Shen et al. have investigated V-grooves on quartz surfaces as microfluidic systems for fluid delivery and diagnostic applications [92]. For a given (hydrophilic) surface, the wicking velocity of the liquid increases as decreases the angle of the grove but increases with the grove width.

4.3 Manufacturing paper microfluidics and diagnostic devices.

The combination of roll-to-roll process combined with paper and polymeric films as materials offer a very efficient, low cost and flexible process/material combination to manufacture paper microfluidics and paper diagnostics on a large scale. The manufacturing process consists of a sequence of unwinders, printers, IR/UV dryers and rewinders. An example is the large-scale production of paper tests to measure the level of glucose in blood; this can be a 2D pad involving a microfluidic system and an enzyme for the detection. A roll of paper is unwinded and fed through a first inkjet printer to pattern the microfluidic system (AKD solution or UV sensitive hydrophobic ink) and then into a UV/IR dryer to cure the hydrophobic barriers. Next, patterns of the enzyme/reactants are printed on paper within the detection zones of the microfluidic system using a second inkjet printer. The roll of microfluidic/enzyme paper diagnostic is then sandwiched between two films of polymer wrapping, fused and cut, using the standard equipment for diaper manufacturing. Such a manufacturing line operating at a velocity of 800 m/min with a paper roll 1m wide, and producing diagnostics 10cm x 10cm, will process 100 million diagnostics/day. Assuming the international market for diabetes type II to be 300 million (5% population) needing a daily test, the world's needs can be supplied with only 3 such functional printing manufacturing lines. Being first to market and having proper intellectual property then become critical for product protection.

4.4 Challenges and Future Directions

Two types of paper microfluidics development are needed to make paper diagnostics a commercial reality. The first is a simplification of the microfluidic/diagnostic systems to further decrease manufacturing cost and enable commercial viability of products for price sensitive markets (such as water quality testing). The second is an increase in the performance of the paper diagnostics for specialized health applications that might require separation of the sample components on paper, more sensitive mechanisms of detection and easier and better mechanisms for the communication of results.

Implementation of new technology often first proceeds with the high value products and then rolls down toward commodities, as a preferred technology is selected, processes are optimized and manufacturing price decreases. Following paper test for human health diagnostics, applications in veterinary health diagnostics, food quality

(antibiotics in milk, proscribed pesticide on fruits, hormones in meat, fish freshness) and environmental diagnostics (heavy metals in water, bacteria in restaurants kitchens) are expected. The level of microfluidics sophistication is foreseen to decrease in this order as the product value is projected to decrease. When large volumes of the liquid sample to test are available, a simple dipstick suffices; simpler modes of diagnostics analysis with a basic yes/no binary logic are needed. To ensure paper diagnostics provide a reliable analysis, a first challenge is to control the threshold analyte concentration at which a positive is reported. A second challenge is to better control false positive and false positive tests and to err on the side of cautious for health safety.

The very low cost of paper microfluidic systems offers opportunities for routine indicators for personal care devices and packaging [93]. Microfluidic systems can indicate wetness in diapers, or be printed as integrity indicator on paper packaging.

Developing paper as tool for advanced and sensitive diagnostic has a few challenges. A first one is the sensor ability to analyse complex samples with separation abilities integrated into paper. A second challenge is to design and develop label-free analytical platforms. A third challenge is to significantly decrease the detection limit beyond nanomolar or part per billion (PPB) [94]. The last few years have witnessed the integration of microfluidic paper analytical devices with optical and electrically active nanomaterials [94]. Ngo et al. reviewed applications and issues with nanoparticles treated paper [95]. Ngo et al. also investigated the effect of gold nanoparticles (AuNP) treated paper on the signal enhancement to detect very low concentrations of chemical and biochemical analytes using Surface Enhanced Raman Spectroscopy (SERS) technology [96-99]. The effect of AuNP concentration, aggregation state and paper 3D distribution on the detection selectivity, sensitivity and reproducibility of SERS analysis were studied. However the use of nanoparticles on paper for signal enhancement drastically increases the cost of the diagnostics and requires the use of analytical instrumentation.

5 CONCLUSION

Paper microfluidics are not the most efficient or useful systems to transport liquids when taken as independent systems; neither are bioactive papers particularly sensitive or accurate for medical diagnostics. However, combining microfluidics with bioactive paper enables the creation of invaluable diagnostics for health care applications. This is the result of the very low price, great design flexibility, and simplicity of use given by paper-based diagnostics manufactured by functional printing and roll-to-roll process. Paper can serves many complementary functions for a diagnostic. The capillarity wicking along the cellulosic fibers of paper provides a driving force to transport fluids without the need for external system/energy. The porosity and biocompatibility of paper enable reagents and biomolecules to be stored. Paper offers the separation of analyte constituents by filtration and chromatography. Finally, paper is a combustible, biodegradable and recyclable material offering many easy options of disposal after use. Surprisingly, very little attention has been given to optimizing paper structure for microfluidics and diagnostics application. This offers a great opportunity to further push the boundaries of microfluidic paper and engineer new generations of low cost diagnostics.

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PAPER IN FOREFRONT

Paper-based device for rapid typing of secondary human blood groups

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Abstract We report the use of bioactive paper for typing of secondary human blood groups. Our recent work on using bioactive paper for human blood typing has led to the discovery of a new method for identifying haemagglutination of red blood cells. The primary human blood groups, i.e., ABO and RhD groups, have been successfully typed with this method. Clinically, however, many secondary blood groups can also cause fatal blood transfusion accidents,despite the fact that the haemagglutination reactions of secondary blood groups are generally weaker than those of the primary blood groups. We describe the design of a user-friendly sensor for rapid typing of secondary blood groups using bioactive paper. We also present mechanistic insights into interactions between secondary blood group antibodies and red blood cells obtained using confocal microscopy. Haemagglutination patterns under different conditions are revealed for optimization of the assay conditions.

Keywords Bioactive paper, Blood typing, Secondary blood groups, Confocal microscopy, Haemagglutination

Introduction

Blood groups were discovered at the beginning of the twentieth century, and for many years they have been considered the best human genetic markers since they carry a significant amount of information for mapping the human genome. To date, 30 blood group systems, including 328 authenticated blood groups, have been classified [1, 2]. The discovery of the ABO blood groups by Landsteiner made blood transfusion feasible. The later discovery of the RhD antigens led to the understanding and subsequent prevention of haemolytic disease of the newborn (HDN) [3–5]. Although the ABO and RhD groups are the most important systems in transfusion medicine, many other blood group antibodies are also capable of causing haemolytic transfusion reactions or HDN. These blood groups, known as minor or secondary blood groups, are also of great clinical and biological importance in blood transfusion and transplantation [5, 6]. Each of these blood groups contains unique subtype antigens and has a different weight of distribution in the human population [7]. Furthermore, secondary blood groups do not follow the second part of Landsteiner's law—"If an agglutinogen is absent in the red cells of a blood, the corresponding agglutinin must be present in the plasma"—which is true only of the ABO groups. Although antibodies A and B are naturally present in human blood serum corresponding to the antigens which they lack, the Rh and secondary antibodies in serum are generated only as a result of an immunization response triggered by transfused red blood cells (RBCs) that carry secondary antigens, or by fetal RBCs leaking into the maternal circulation during pregnancy or during birth [5]. Table 1 summarizes the common secondary blood groups and their subtype antigens. Among these blood groups, some antibodies, such as M, N, Lewis and Lutheran system antibodies, are inactive below 37 °C and are therefore not considered clinically important [5]. Others, however, are as clinically significant as primary blood groups. The mismatching of these blood groups may cause immediate and severe haemolytic transfusion reactions and HDN.

Accurate and rapid identification of human secondary blood groups is important for blood banking and medical procedures under either laboratory or field conditions [8]. Routine minor blood grouping methods rely primarily on the haemagglutination reactions between antigens and antibodies that are performed either manually or by automatic means

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Table 1 Common secondary blood group systems

Secondary blood group systems	Antigens
Rh	D*, C, c, E, e
Kell	K, k
P	P_1
Kidd	Jk^a, Jk^b
MNS	M, N, S, s
Lewis	Le^a, Le^b
Lutheran	Lu^a, Lu^b

* Group D is one of the blood groups in the Rh system, but it is not considered as a secondary blood group

[9, 10]. Currently, the commonly used method for secondary blood grouping is based on a gel card test procedure, which requires concentrated RBCs and centrifugation [9]. Because there are a large number of antigens in secondary blood groups, a full identification of the antigens in those groups using this procedure is expensive and requires central laboratory conditions. Other methods, such as slide techniques, although of low cost and equipment-free, are insensitive and therefore are not recommended for initial or definitive antigen determinations, particularly when dealing with neonatal samples [5]. Investigations into novel techniques for rapid and low-cost secondary blood group typing diagnostics are therefore necessary and significant, especially for use in lessindustrialized areas, for home care, and for local and temporary blood banking in disaster-response missions.

Recent research on the use of bioactive paper for human blood typing has established a new method for identifying haemagglutination of RBCs [11–16] which relies on using fibre networks in paper with controlled pore sizes to filter out and retain the agglutinated RBC lumps. When a blood sample is introduced onto a piece of paper pretreated with the corresponding grouping antibody, haemagglutination will occur, leading to the formation of agglutinated RBC lumps inside the fibre network [17]. The agglutinated lumps of RBCs are captured by the fibre network and cannot be removed by chromatographic elution with phosphate-buffered saline (PBS) or saline solution, leaving a clearly visible bloodstain on the paper. Conversely, if a blood sample is introduced onto paper treated with non-corresponding grouping antibodies, haemagglutination will not occur and free RBCs can be easily washed out of the fibre network, leaving no discernible stain. This phenomenon forms the foundation of using paper to make low-cost, rapid and user-friendly devices for ABO and RhD blood typing assays [16].

The antigens of some secondary blood groups are found to be less antigenic than the primary blood groups [18]. Those antigens show weaker interactions with their corresponding antibodies, resulting in increased difficulty in their identification in blood grouping assays. Different antibodies [e.g. immunoglobulin G (IgG) instead of immunoglobulin M (IgM)] available to certain secondary RBC group antigens present further difficulties in blood grouping assays. Although the capabilities of IgG and IgM antibodies in the typing of secondary blood groups have been well understood, RBC responses to bonding with those antibodies are not. In this study, we investigate the secondary blood group antibody–RBC interactions in the fibre network of paper for the purpose of designing highly efficient paper-based secondary blood grouping devices. The confocal microscopy method we developed for paper-based blood grouping assays [17] was used to obtain mechanistic details of RBC behaviour in the interactions with secondary blood group antibodies. Our results elucidate (1) the differences in antibody-specific RBC haemagglutination caused by primary and secondary blood group antibodies, (2) the responses of RBCs to bonding with IgG and IgM antibodies, (3) the time-dependent antibody– antigen interactions in secondary blood grouping, and (4) a concept of secondary blood group assay result reporting using symbols. The protocols and conditions of assaying have also been established and are discussed. The outcome of this work provides microscopic details for the engineering of low-cost, sensitive, specific and rapid paper-based blood grouping devices for secondary human blood groups.

Experimental

Materials and appliances

The paper substrate used in this study was Kleenex paper towel (Kimberly-Clark, Australia). Alkyl ketene dimer (AKD; wax 88 Konz) as a paper hydrophobilization reagent was obtained from BASF. AR grade n-heptane was obtained from Sigma-Aldrich (Australia); it was used to formulate an inkjet-printable solution for patterning text on paper through the formation of hydrophobic borders. Blood samples were sourced from Red Cross Australia (Sydney). They were stored at 4 °C and used within 7 days of collection. All antibodies were purchased from Alba Bioscience (Edinburgh, UK). The 0.9 % (w/v) NaCl saline solution and the PBS were prepared with AR grade NaCl (Univar) and phosphate (SigmaAldrich), using Milli-Q water. Fluorescein isothiocyanate (FITC; isomer I, product number F7250, from SigmaAldrich) was used for labelling RBCs [17, 19, 20]. Anhydrous dimethyl sulfoxide (Merck, Australia) was used to dissolve the FITC. Anhydrous D-glucose was provided by Ajax Finechem (Australia).

A reconstructed Canon inkjet printer (Pixma iP3600) was used to print the AKD–n-heptane solution onto a Kleenex paper sheet to form the required patterns defined by the hydrophilic–hydrophobic contrast. A series of micropipettes (Eppendorf Research®, 2.5–50 μL) were used to transfer antibodies, blood samples and saline solutions onto the paper device.

A Nikon Ai1Rsi confocal microscope in the Melbourne Centre for Nanofabrication was used to obtain the confocal micrographs. The objective lens used for imaging was a×60 oil immersion lens.

Symbol patterning onto paper

In our previous work for ABO and RhD blood group tests [16], the fabrication of a paper-based blood grouping device involved patterning letters and symbols as hydrophilic sites for RBC and antibody interactions, surrounded by hydrophobic borders, onto paper. It was done with inkjet printing of AKD onto paper to selectively hydrophobize it. The hydrophobic–hydrophilic contrast allows unambiguously legible text patterns of agglutinated blood to be displayed. Following the international convention, the blood types of the ABO group are reported directly with letters, whereas Rh and other secondary blood groups are reported with the symbol "+" or "-" to indicate either their presence or their absence in RBCs. In this work, for patterning paper for secondary blood grouping, we used "+" and "-" to indicate either a positive or a negative assay result. The symbol consists of a vertical hydrophilic channel and an overlapping horizontal bar printed with water-insoluble ink, as shown in Fig. 1.

An assay for identifying multiple secondary blood groups was performed by introducing 2.5 μL of the corresponding antibodies onto the vertical section of the symbols, and allowing the antibodies to dry under ambient conditions. Two and half microlitres of a blood sample was then introduced onto the vertical channel of all the symbols; a predetermined reaction time was allowed for the antibodies to interact with the RBCs. Subsequently, two 30-μL aliquots of saline solution were introduced onto the vertical section of the cross symbol to wash out the non-agglutinated RBCs.

After washing, the results can be directly read from the device—"+" indicating positive and "-" indicating negative.

For confocal imaging, RBCs were labelled with FITC using the method reported by Li et al. [17], Hauck et al. [19] and Hudetz et al. [20]. The whole blood sample was first centrifuged at 1,300g relative centrifugal force for 3 min to separate the RBCs from the plasma. The plasma layer was then removed and the RBC layer was washed once with physical saline solution (PSS). FITC was dissolved in dimethyl sulfoxide at a high concentration (40 mg/mL), and was then diluted with CellStab solution to 0.8 mg/mL. This FITC solution and a D-glucose solution in PBS were then added to the RBCs until the concentrations of FITC and D-glucose reached 0.5 mg/mL and 0.4 mg/mL, respectively. This RBC suspension was then incubated in the dark for 2 h to allow FITC to attach onto the cell surface. After incubation, the RBC suspension was washed 13 times with PSS to remove unattached FITC and the RBCs were then resuspended at a haematocrit of around 35 % for further use.

Kleenex paper towel was cut into 10 mm×10 mm pieces for use as the test substrate. Ten microlitres of antibody solution was dropped onto paper pieces and allowed to dry, and then 6 μL of labelled RBCs was dropped onto them. A preset reaction time was allowed. Then, 20 μL of PSS was pipetted onto the sample and the sample was immediately placed onto a glass slide for confocal imaging.

Results and discussion

Following the testing procedure illustrated in Fig. 1, a number of secondary blood groups can be assayed on a paper strip; the typical assay results of one blood sample are shown in Fig. 2 by putting the assay papers together. Gel card tests of the same blood sample performed in the laboratory of Red Cross Australia were used to compare the results with the results obtained using our paper-based assays.

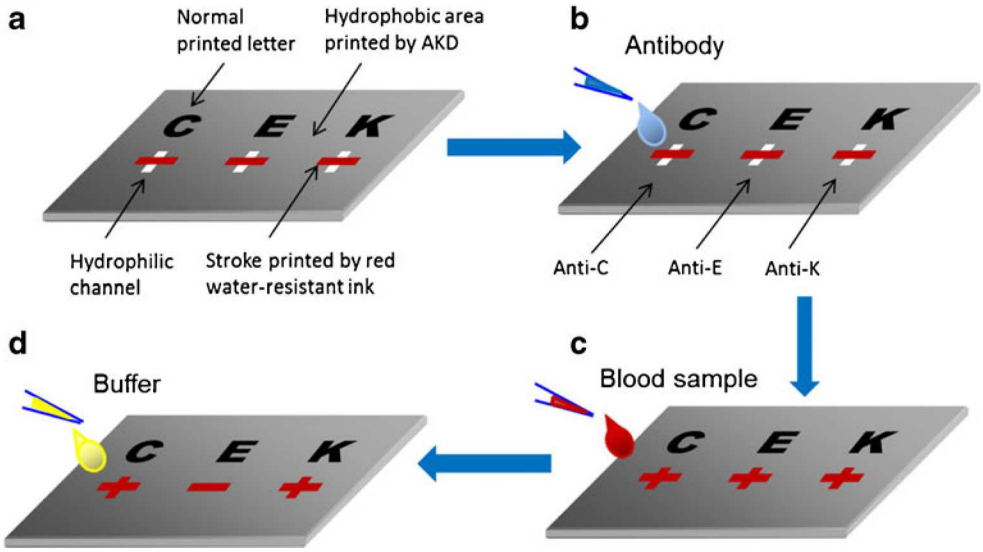

Fig. 1 Design, fabrication, testing procedures and result reporting of paper diagnostics for secondary blood group typing. AKD alkyl ketene dimer

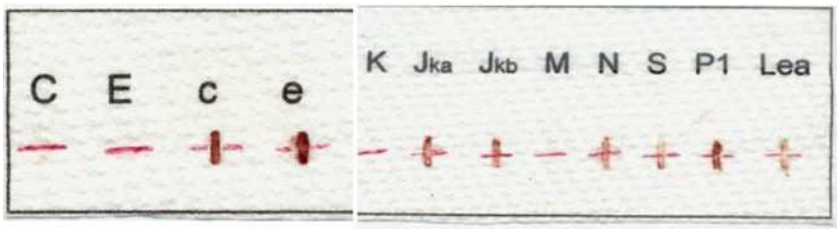

Fig. 2 Paper-based blood group assay designed for identifying 12 antigens in six secondary blood group systems. The blood grouping assay was performed under the same conditions: the reaction time was 30 s and the washing of free red blood cells (RBCs) was done using 0.9 % NaCl physical saline solution. Reference assays using mainstream technology (gel card) showed that the secondary blood group systems of this blood sample were C(−), E(−), c(+), e(+), K(−), Jka(+), Jkb(+), M(+), N(+), S(+), P1(+), and Lea(+)

blood sample performed in the laboratory of Red Cross Australia were used to compare the results with the results obtained using our paper-based assays.

Two observations can be made. First, with the exception of blood group M, the secondary blood grouping results reported by our paper-based assays matched the gel card results. Second, like the gel card tests, where the clarity of the results of secondary blood groups is less consistent than that of primary blood groups, on paper-based assays the colour intensity of antigen positive RBCs differs significantly despite all tests being conducted under the same conditions. These results suggest that paper-based sensors are capable of performing assays for secondary blood grouping, and the reaction conditions of secondary blood group antigens and antibodies differ. To further understand the antigen–antibody interactions of secondary blood groups, and to optimize the assay conditions, we focused on the influence of the following factors on RBC agglutination: the antibody–antigen reaction time, antibody types, and washing conditions. Microscopic information obtained with confocal microscopy was compared with information obtained from the visual assays to study the interactions of RBCs in paper.

The effect of the interaction period

The bonding of ABO and RhD antigens with their corresponding antibodies normally occurs instantaneously [16]; therefore, haemagglutination can be visually identified within 30 s [17]. This reaction time, however, is not sufficient for some secondary blood group antigens as they are less antigenic. As a result, the time required for secondary blood group agglutinations to be visually identifiable ranges from 30 s to 3 min, depending on the antibody–antigen pair. For example, blood groups C and E have strong interactions with their corresponding antibodies within 30 s, whereas blood groups c and e would show falsenegative results if the same interaction time of 30 s were allowed. We found that longer interaction times (i.e. 2–3 min) must be allowed for secondary blood groups to improve the clarity of the results (Fig. 3). However, a prolonged reaction time can lead to false-positive results, as excessive exposure of a blood sample to the atmosphere causes aggregation of RBCs. Ourstudy showed thata reaction time up to 3minis appropriate and adequate for the unambiguous identification of the weaker haemagglutinations.

Confocal microscopy was used to gain a microscopic understanding of haemagglutination processes of RBCs carrying secondary blood group antigens. For comparison, haemagglutination of RBCs by corresponding primary and secondary blood group antibodies was investigated to show the differences in the strength of RBC agglutination. Figure 4 shows four confocal microscopy images taken with a×60 oil immersion lens. RBCs carrying D(+) (primary) and E(+) (secondary) antigens showed rapid agglutination within 30 s after contact with their corresponding antibodies. RBCs carrying primary blood group D antigens interacted strongly with their corresponding antibodies, leading to strong deformation of RBCs, which formed large lumps; individual RBCs in the lump could not be identified (Fig. 4a). RBCs carrying secondary blood group E antigens showed weaker agglutination than the RBCs carrying group D antigens. Although E(+) RBCs also formed lumps on reacting with the E antibody, cell

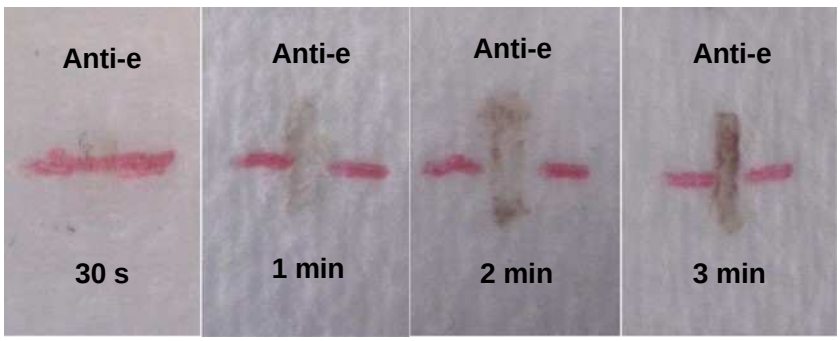

Fig. 3 Increasing degrees of haemagglutination of secondary blood group e in antibody-treated paper as a function of time

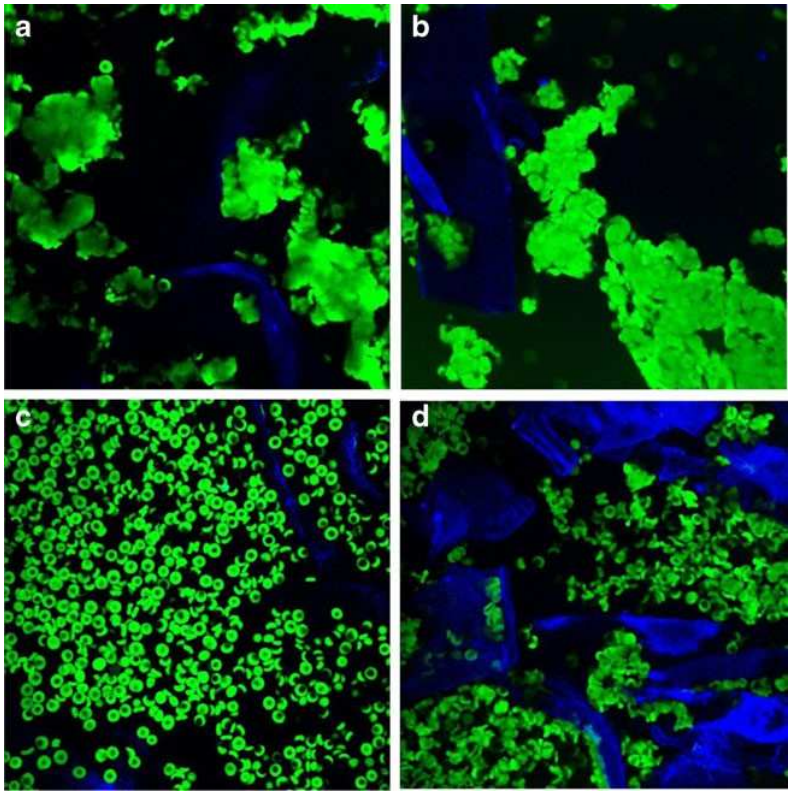

Fig. 4 Haemagglutination behaviour of three blood groups investigated by confocal imaging (×60 oil immersion lens): a RBCs of primary blood group D(+) agglutinated by D antibodies within 30 s; b RBCs of secondary blood group E(+) agglutinated by E antibodies within 30 s; c RBCs of secondary blood group e(+) did not show any sign of agglutination after reacting with e antibody for 30s; d blood group e(+) RBCs showed clear agglutination when reacting with e antibody for 3 min

deformation was less severe than that of the D(+) RBCs (Fig. 4b). The e(+) RBCs showed even weaker agglutination; no agglutination was observable for 30 s of reaction time (Fig. 4c). However, when 3 min of reaction time was allowed, the e(+) RBCs clearly showed agglutination, even though the degree of agglutination was much weaker than for RBCs of the D(+) and E(+) groups (Fig. 4d). These results correlate with the visual assays performed on paper (Fig. 3). More importantly, the confocal microscopy results confirmed the time-dependent haemagglutination reaction of some secondary blood groups. Table 2 summarizes the reaction time required by different secondary blood groups.

The effect of antibody structure

Although some commercial antibodies for secondary blood grouping can easily identify the corresponding secondary blood group antigens through RBC haemagglutination, others cannot. This is partially due to the structure of antibodies; the IgM and IgG antibodies show a strong performance contrast in causing haemagglutination of RBCs. The commercial products of IgM are monoclonal immunoglobulin which has a pentameric form; each monomer has two binding sites, and IgM therefore has ten sites in total. IgG antibodies, however, are monomers with only two binding sites in total. The molecular dimension of an IgM molecule is also greater than that of an IgG molecule, being 30 nm and 14 nm, respectively [5]. Consequently, the distancebetween the two binding sites ofan IgG molecule is too small to allow it to simultaneously bind with antigens on two RBCs, thus producing sufficiently large agglutinated RBC lumps that can be

Table 2 Agglutination reaction time required by each secondary blood group for the Alba Bioscience antibodies specified in this study

	Blood groups										
	C	c	E	e	K	k	Jk^a	Jk^b	P_1	S	s
Required reaction period	30 s	3 min	30 s	3 min	2 min	30 s	2 min	2 min	30 s	2 min	2 min

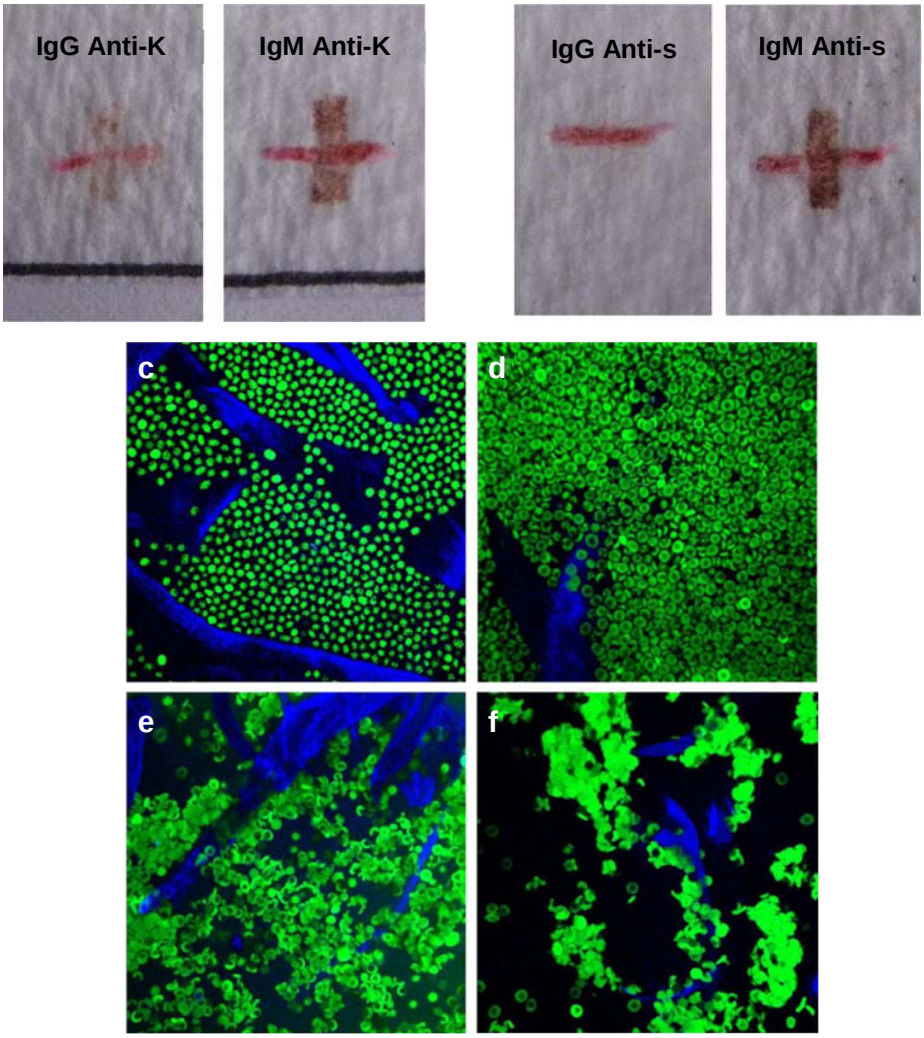

Fig. 4 Haemagglutination behaviour of three blood groups investigated by confocal imaging (×60 oil immersion lens): a RBCs of primary blood group D(+) agglutinated by D antibodies within 30 s; b RBCs of secondary blood group E(+) agglutinated by E antibodies within 30 s; c RBCs of secondary blood group e(+) did not show any sign of agglutination after reacting with e antibody for 30s; d blood group e(+) RBCs showed clear agglutination

immobilized in the porous structure of paper. In contrast, agglutination reactions between IgG and RBC antigens without anti-human globulin are either extremely weak or do agglutination reactions between IgG and RBC antigens not occur at all, leading to false-negative results. The example

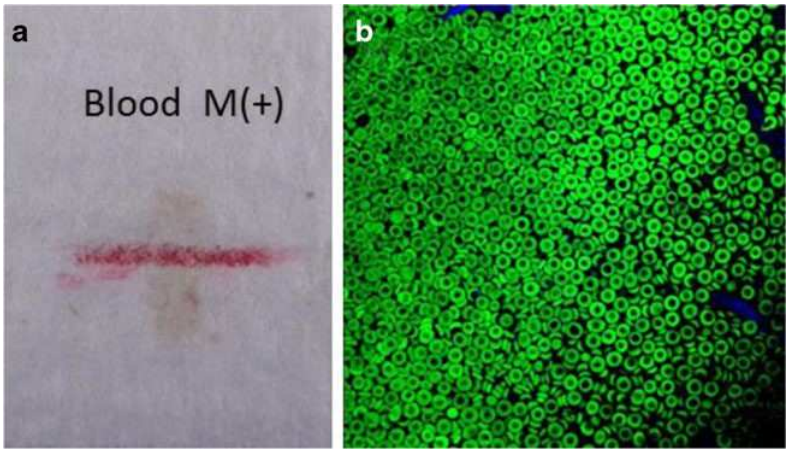

Fig. 6 a Visual assay of an M(+) blood sample on paper and b confocal image of the same sample. A commercial anti-M IgM obtained from human sera was used for the assay and a reaction time of 3 min was allowed

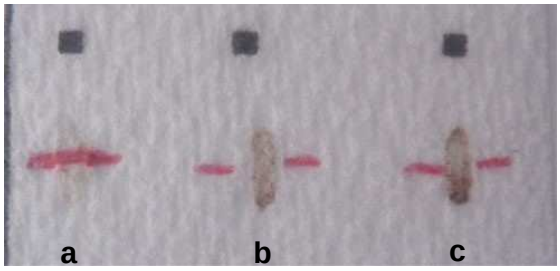

Fig. 7 Identification for agglutinated RBCs of blood group c washed with distilled water (a), 0.9 % NaCl (b), and phosphate-buffered saline (c). An RBC–antibody interaction time of 2 min was allowed

of K antigen in the Kell system is used to illustrate the different performance of antibodies with different structures. Both IgM and IgG antibodies corresponding to K antigen are commercially available. Figure 5a shows a distinct difference in RBC agglutination patterns: whereas IgM causes strong agglutination of the K-positive sample, IgG shows little or no effect. Another example of detecting s antigen (of the MNS system, Table 1) using IgM and IgG antibodies also shows results very similar to those of the K antigen in the Kell system, confirming that the different structures of antibodies can lead to extremely different agglutination behaviours (Fig. 5b).

Confocal microscopy was used to capture the agglutination patterns of the K(+) and s(+) RBCs by IgM and IgG antibodies. Figure 5c and d show agglutination patterns of K(+) and s(+) RBCs after they had being introduced onto papers treated with anti-K IgG and anti-s IgG, respectively. In both cases, no discernible RBC agglutination could be observed. An interesting observation was that K(+) RBCs show strong deformation and crenation when mixed with anti-K IgG. This is because the commercial anti-K IgG reagent was directly obtained and prepared from a donor's plasma. The impurity of the antibody solution might have caused the RBC deformation. After a drop of 10 μL CellStab solution had been added to the paper, the RBCs regained their natural biconcave disc shape (result not presented), but agglutination of RBCs never occurred. In contrast, when K(+) and s(+) RBCs were introduced onto papers treated with anti-K IgM (Fig. 5e) and anti-s IgM (Fig. 5f), agglutination of RBCs was clearly observed under the confocal microscope. The confocal microscopy results also show that anti-s IgM causes a stronger degree of agglutination than anti-K IgM (Fig. 5e, f); these results concur with the visual assay on the paper surface (Fig. 5a, b). They provided a clear understanding of the effects of antibody structures on the efficiency of RBC agglutination; such effects were further confirmed on a microscopic level.

In another study, antibody–antigen interactions of secondary blood group M were investigated using confocal microscopy. The motivation of this investigation was to understand why a false-negative result was observed for this blood group in our screening test (Fig. 2). A repeat of the assay on paper showed no RBC agglutination, even though a reaction time of 3 min was allowed (Fig. 6a). The confocal image showed that M(+) RBCs could not be agglutinated by anti-M IgM (Fig. 6b). Although anti-M is IgM antiserum, it is considered as a naturally occurring antibody in the human body, rather than an antibody generated by immunoreactions [21]. This type of antibody is a cold-reacting antibody which is not active at 37 °C, and therefore is regarded as clinically insignificant [5, 21]. In general, the detection of anti-M can be disregarded for transfusion purposes.

The effect of washing conditions

The selection of the washing solution affects the result of secondary blood grouping assays. Firstly, water cannot be used to remove the non-agglutinated blood because of the osmotic effect. Under osmotic pressure, water penetrates into the RBC through its membrane, causing RBC haemolysis by bursting. The agglutinated RBCs therefore can also be removed from the paper (Fig. 7, test a). Secondly, the pH is also an important factor in washing. Under normal circumstances, a washing solution with a pH of about 7 is acceptable because RBCs carry a negative charge and at pH 7–7.5 most antibody molecules have weak positive charges. This enhances the attraction between the RBCs and antibody molecules during the first increases the dissociation of the antigen–antibody complexes. This influence of pH levels on the assay result was found to be much more important for secondary blood groups than for primary blood groups; this is due to the weaker antibody–

Table 3 Secondary blood group typing data obtained using paper devices

	Blood type										
	C	E	c	e	P_1	K	k	Jk^a	Jk^b	S	s
Antibody type	IgM	IgM	IgM	IgM	IgM	IgM	IgM	IgM	IgM	IgM	IgM
Reaction period	30 s	30 s	3 min	3 min	30 s	2 min	30 s	2 min	2 min	2 min	2 min
Number of samples	127	127	127	127	79	79	19	30	30	11	11
Matching with the laboratory results (%)	100	100	100	100	100	100	100	100	100	100	100

antigen interactions of secondary blood groups. Figure 7 (tests b and c) shows the washing of the agglutinated RBCs from the paper. Our results show that for the best results of secondary blood grouping assays, PBS should be used for washing.

Five clinically important secondary blood group systems, including 11 blood groups, have been tested under their optimum conditions. Each blood group involved from 11 to more than 100 samples and all test results using the bioactive-paper devices matched the results obtained in a pathology laboratory using gel card technology. Details of the samples tested are presented in Table 3. The accuracy of this paper diagnostic assay for the blood samples studied was 100 %.

Conclusion

We have shown in this study that paper-based diagnostics are capable of testing various clinically important secondary blood types. Compared with the typing of primary blood groups, the major challenges in secondary blood group assaying were identified to be the antibody–RBC interaction time, the antibody types, and the pH of the washing buffer. Visual observation of the agglutination pattern on paper was used to evaluate the degree of agglutination under the conditions of the investigation, and confocal microscopy was used to obtain microscopic information on the agglutination patterns in a fibre network at a cellular level. The microscopic and macroscopic results presented in this work establish a detailed understanding of secondary blood grouping in paper. First, RBCs carrying some secondary blood group antigens require a longer time to react with their corresponding antibodies in order to form a sufficient level of agglutination for unambiguous visual identification on paper. Second, the types of antibodies are important in secondarybloodgrouptyping. Thisworkconfirmed,ata cellularlevel, that whereas IgM antibodies are able to cause direct agglutination of RBCs, IgG antibodies are unable to do this. In that case an indirect method must be used. Third, the pH of the washing buffer must be maintained at neutral, or at a slightly basic level, to prevent the undesirable dissociation of the agglutinated RBC lumps during washing.

This work is the first research on using paper-based diagnostics for secondary blood group typing. Of the 11 clinically important secondary blood groups we investigated in this study (Table 3), the results obtained by paper-based devices showed a 100 % match with the mainstream gel card technology. The design concept of the paper assay allows both professionals and non-professionals to easily understand and perform the assay.

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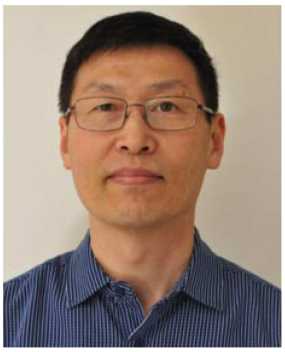

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