MONASH University

On the Delivery of Blood Stage Malaria DNA Vaccine Using Magnetic Nanoparticles

A thesis submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

By

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March 2013

DEDICATIONS

This thesis is dedicated to my beloved husband

Sami

for his constant love, sacrifice, and continuous support.

This thesis is also dedicated

to my lovely two children …

Monash University

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Declaration for a thesis based or partially based on conjointly published or unpublished work

General Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other equivalent institution and affirms that to the best of my knowledge and belief, the 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This thesis includes original papers published in peer reviewed journals and unpublished publications. The core theme of the thesis is the design of magnetic gene vectors for delivery blood stage malaria DNA vaccine and their use for *in vitro* and *in vivo* applications. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Departments of Chemical Engineering, Microbiology and Immunology/Monash university under the supervision of Dr. Cordelia Selomulya and Prof. Ross Coppel.

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

Fatin M. Nawwab AL-Deen

March 2013

Abstract

Biomedical nanotechnology is revolutionizing the approach to many infectious diseases by providing rapid and simple therapeutic and diagnostic agents. About half of the world's population is at risk of infection with malaria, while treatment and control have become more difficult. Traditional protein-based malaria vaccines elicit only antibody-mediated (humoral) immune responses and are often expensive because they rely on costly manufacturing methods that are not usually available in countries where the disease is endemic. Thus, there is an urgent need to develop a rapid, effective and inexpensive vaccine that might be affordable in low-resource areas. DNA vaccines have emerged as a potential new strategy against malaria because of their low manufacturing cost and ability to induce both humoral and cellular immune responses against antigens encoded by recombinant DNA. However, the efficiency of delivery of the DNA vaccines is often observed to be low. The use of superparamagnetic nanoparticles (SPIONs) vectors to deliver the malaria gene via magnetofection could help improve the efficacy of gene delivery with a low dose and site specific *in vivo* applications.

Here, magnetofection was used to enhance the delivery of malaria DNA vaccine encoding *Plasmodium yoelii* merozoite surface protein $MSP1_{19}$ (VR1020-$PyMSP1_{19}$) that plays a critical role in Plasmodium immunity. The plasmid DNA (pDNA) containing membrane associated 19-kDa carboxyl-terminal fragment of merozoite surface protein1 ($PyMSP1_{19}$) was conjugated with polyethyleneimine (PEI) - coated superparamagnetic nanoparticles (SPIONs) with different molar ratios of PEI nitrogen to DNA phosphate (N/P). The effects of SPIONs/PEI complexation pH on the properties of the resulting particles are reported, including their ability to condense DNA and express gene in eukaryotic cells *in vitro*. SPIONs/PEI complexes under acidic pH conditions showed a better binding capability with VR1020-$PyMSP1_{19}$ than those at neutral conditions, despite the negligible differences in size and surface charge of complexes. The transfection efficiency of magnetic nanoparticles as a carrier for malaria DNA vaccines *in vitro* as indicated via $PyMSP1_{19}$ expression was significantly enhanced under the application of an external magnetic field, while the cytotoxicity was comparable to the benchmark non-viral reagent (Lipofectamine 2000).

Subsequently, magnetofection also showed higher serum antibody titers against $PyMSP1_{19}$ with intraperitoneal and intramuscular injections via *in vivo* mouse study, compared to subcutaneous and intradermal injections. Robust IgG2a and IgG1 responses were observed for intraperitoneal administration, which could be due to the physiology of peritoneum as a major reservoir of macrophages and dendritic cells. Heterologous DNA prime followed by a single protein boost with recombinant $EcPyMSP1_{19}$ protein vaccination regime was also tested. The regime showed enhanced IgG2a, IgG1, and IgG2b responses, indicating that the induction of appropriate memory immunity that can be elicited by proteins on recall.

The assembly order of different quaternary magnetic gene vector configurations comprising SPIONs, PEI, hyaluronic acid (HA), and pDNA (VR1020-$PyMSP1_{19}$) was also investigated in this thesis. The impacts of different cell media on the particle stability in terms of complex size, surface charge, stability, and ability to bind and release DNA were studied. Generally, all vectors showed a relatively small size in water, whereas a higher degree of aggregation was observed immediately after transferring to high-ionic strength media such as 150 mM NaCl buffer and RPMI 1640 culture media. However, the pre-addition of HA to DNA prior adding them to SPIONs/PEI complexes effectively reduced the extent of aggregation in serum-free RPMI, particularly at high HA : PEI % charge ratio. This study demonstrated that structurally well-defined magnetic gene carriers could be designed to improve malaria DNA vaccine delivery systems, for *in vitro* and *in vivo* applications.

Non-viral polymeric DNA vaccine carriers are likely to be more immunogenic if they can be efficiently taken up by potent antigen-presenting cells (APCs) such as dendritic cells (DCs).

Efficient DC targeting vaccines require high efficiency for binding and up-take of the vaccine by cells, followed by DC activation and maturation. Since the pre-addition of HA to DNA prior to SPION/PEI configuration vectors, in particular, displayed the desired degree of stability in terms of narrow size distributions and high stability in RPMI cell media, these vectors was used to transfect dendritic cells that were problematic in order to transfect through HA receptor-mediated endocytosis. The effects of magnetic fields on the transfection and maturation of DCs *in vitro* by these vectors were investigated using different molecular weights of the HA and % charge ratios of HA: PEI. Among the vectors

tested, complexes containing high molecular weight of HA with % high charge ratio of HA : PEI under an external magnetic field yielded a better DC transfection/ maturation than others. This phenomenon was attributed to gene complex stability and transfection efficiency, possibly due to long molecular chains and the higher mucoadhesive properties of high molecular weight HA that enhanced HA ligands accessibility to the DC cell receptors and promoted the multivalent binding to the receptors. Insights gained should improve the design of more effective DNA vaccine delivery systems.

List of Publications

Journal Publications:

1- **Al-Deen, F.N.**, Ho, J., Selomulya, C., Ma, C. and Coppel, R. (2011). Superparamagnetic Nanoparticles for Effective Delivery of Malaria DNA Vaccine. **Langmuir** 27 (7): 3703-3712. **(Chapter 3).**

2- **Al-Deen, F.N.**, Ma, C., Xiang, S. D., Selomulya, C., Plebanski, M., and Coppel, R (2013). On the efficacy of malaria DNA vaccination with magnetic gene vectors. **Controlled Release** 168 (1)**:** 10-17. **(Chapter 4).**

3- **Al-Deen, F.N.**, Selomulya, C. and Williams, T. (2013). On designing stable magnetic vectors as carriers for malaria DNA vaccine. **Colloids and Surfaces B: Biointerfaces** 102 (0): 492-503. **(Chapter 5).**

4- **Al-Deen, F.N**, Selomulya, C., Kong, Y., Xiang, S. D., Ma, C., Plebanski, M., and Coppel, R. Design of magnetic polyplexes taken up efficiently by dendritic cell for enhanced DNA vaccine delivery. **Submitted. (Chapter 6).**

5- Ho, J., **F. N. Al-Deen**, Al-Abboodi, A., Selomulya, C., Xiang, S. D., Plebanski, M., and Forde, G. M. (2011). N, N'-Carbonyldiimidazole-mediated functionalization of superparamagnetic nanoparticles as vaccine carrier. **Colloids and Surfaces B-Biointerfaces** 83 (1): 83-90.

Refereed conference paper:

1. Al-Deen, F. M. N., Ho, J., Selomulya, C., Ma, C., Coppel, R. L., 2010, PEI-coated superparamagnetic iron oxide for delivering malaria DNA vaccine encoding merozoite surface protein MSP_1. Proceedings of the ***Chemeca 2010 conference***, 26-29/09/2010, Engineers Australia, Australia, pp. 865-873, ISBN: 978 085 825 9713. (Oral presentation).

Conference Presentations:

2. PEI-coated superparamagnetic iron oxide for delivering malaria DNA vaccine encoding merozoite surface protein$1_{19.}$ Published in the proceeding of the ***ICONN2010 Conference on Nanoscience and Nanotechnology***, Sydney, Australia, February 2010. (Poster presentation).
3. PEI coated magnetic nanoparticles for malaria DNA vaccine delivery. Published in the proceeding of the ***VIIN Victorian Infection and immunity network symposium,*** Melbourne, Australia, June 2010. (Poster presentation).
4. Dendritic cell- targeting by application magnetic polyplexes as malaria DNA vaccine carriers. Published in the proceeding of the ***Malaria in Melbourne 2011 (MiM2011) conference***, Royal Melbourne Hospital, Melbourne, Australia, October 2011. (Poster presentation).
5. Design of Dendritic cell-targeting magnetic polyplexes for enhanced Malaria DNA vaccine delivery. Published in the proceeding of the ***1st Annual Monash University Chemical Engineering Conference***, Melbourne, Australia September 2011, and the ***14^{th} Asia Pacific Confederation of Chemical Engineering Congress APCChE 2012***, Singapore, February 2012. (Oral presentation).
6. Effects of different administration routes on the *in vivo* immune response for malaria DNA vaccination with magnetic gene vectors. Published in the proceeding of the ***1st International Conference on BioNano Innovation (ICBNI)***, Brisbane, Australia, July 2012. (Oral presentation).

7. Magnetofection: Enhancing *in vivo* immune response for malaria DNA vaccination with magnetic gene vectors using different administration routes. Published in the proceeding of the ***2nd Annual Monash University Chemical Engineering Conference***, Melbourne, Australia, September 2012. (Oral presentation).

Awards:

8. Functional magnetic nanoparticles for effective DNA vaccine delivery. Published in the proceeding of the ***13th Asia Pacific Confederation of Chemical Engineering Congress 2010***, Taipei, February 2012. **The best poster paper award in Biochemical Engineering and Systems Biology session**.

Acknowledgements

At the beginning of the end I owe thanks to so many people, forgive me those whom I may have forgotten to mention. It is always your love, help, guidance, and friendship that pushes me forward and helps me to grow. I wish you all the best in the future.

I would like to deeply express my gratitude to my principal supervisor Assoc. Prof. Cordelia Selomulya for her continued encouragement, great guidance, stimulating suggestions and extraordinary patience through the establishment and completion of my PhD studies. To me, she embodies all the best characteristics of a successful scientist and has been a source of inspiration and support – both academic and moral.

I would like to express heartfelt appreciation to my associate supervisor Prof. Ross Coppel for his continuous support, and guidance. His contribution was invaluable, which set me in the right direction towards accomplishing my PhD. I would also like to thank him for providing me opportunities to use many of the laboratory facilities in his lab and discuss with his research group at the Department of Microbiology, Monash University.

I also would like to express my sincere appreciation to Mr. Charles Ma at the Department of Microbiology, for his different experimental training, which involved the use of laboratory animals, enlightening discussions and constant encouragement. I appreciated his willingness to discuss any research problems and his endless support. He acted as an elder brother and provided substantial help in both my research and life. Thank you so much for everything you have given me.

I am also very grateful to Prof. Magdalena Plebanski and Dr. Sue Xiang (Department of Immunology, Alfred Hospital/ Monash University) for their collaborative assistance, enlightening discussions, and for the always prompt feedback throughout the years. Thanks also to Mrs. Ying Ying Kong for her help with experimental setup in the Immunology Department.

I would like to thank Dr. Tim Williams, the TEM manager at Monash Centre for Electron Microscopy (MCEM) for his assistance in TEM imaging and valuable discussions.

I extend my very very warmest thanks to Mrs. Shirley McNamara and her lovely team at South Oakleigh Secondary College for their beautiful spirit and taking such a good care of my son that I am amazed at the transformation in him since he has been in their wonderful care. You really have transformed his life and for that I will be eternally grateful.

My sincere thanks go to Mrs. Lilyanne Price, who helped me a lot in getting this opportunity to study at the Chemical Engineering Department/Monash University. I am also grateful to all staff and postgraduate students in the Chemical Engineering, Microbiology, and Immunology Departments for creating a friendly atmosphere and making my time enjoyable.

To my parents, without your love and support I would not be the person I am today. Dad, you pushed me to be a better person and I can only hope that I can surpass what you have accomplished in your life. Mom, you are my rock and I owe you everything.

Finally I would like to thank my dear husband Sami, the source of my inspiration and my wonderful children. Thanks for being so supportive and patient during all these years.

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List of Abbreviations

SPIONs	Superparamagnetic nanoparticles
PEI	Polyethylenimine polymer
Plasmid VR1020-PyMSP1$_{19}$	Malaria DNA vaccine encoding *Plasmodium yoelii* merozoite surface protein MSP1$_{19}$
E. coli	*Escherichia coli*
DNA	Deoxyribonucleic acid
RPMI	Roswell Park Memorial Institute Tissue Culture Medium
Nd -Fe- B	Neodymium iron boron magnet
pDNA	Plasmid DNA
N/P ratios	Ratio of nitrogen containing groups of the PEI polymer to phosphate groups of the nucleic acid
SPIONs/PEI-A	SPIONs/PEI complexes under acidic condition pH4.0
SPIONs/PEI-N	SPIONs/PEI complexes under neutral condition pH7.0
COS-7 cells	African green monkey kidney cells
ELISA	Enzyme linked immunosorbant assay
PBS	Phosphate buffered saline
RBCs	Red blood cell(s)
i.p. route	Intraperitoneal route of immunization
i.m. route	Intramuscular route of immunization

s.c. route	Subcutaneous route of immunization
i.d. route	Intradermal route of immunization
i.v. route	Intravenous route of immunization
IgG	Immunoglobulin G
IgG1	Immunoglobulin G1 subclass
IgG2a	Immunoglobulin G2a subclass
IgG2b	Immunoglobulin G2b subclass
IgG3	Immunoglobulin G3 subclass
OD	Optical density
EcPyMSP1$_{19}$	EcPyMSP1$_{19}$ recombinant protein expressed in *Escherichia coli*
FBS	Fetal bovine serum
HA	Hyaluronic acid
LMW HA	Low molecular weight hyaluronic acid
HMW HA	High molecular weight hyaluronic acid
DNase	Deoxyribonuclease enzyme
APCs	Antigen-presenting cells
DCs	Dendritic cells
GM-CSF	Granulocyte and macrophage colony stimulating factor
CD11c	Transmembrane protein found at high levels on most dendritic cells

CD86	A protein expressed on antigen-presenting cells that provide costimulatory signals necessary for T cell activation and survival
YFP	Yellow fluorescent protein
MHC I	Major histocompatibility complex Class I molecules, are found on every nucleated cell of the body
MHC II	Major histocompatibility complex Class II molecules, are found only on antigen-presenting cells and lymphocytes
CD44	Cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration and it is a receptor for hyaluronic acid

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CHAPTER 1

Introduction

1.1 Background

Malaria is one of the most prevalent and devastating of all human parasitic diseases, exacting a heavy toll of death and illness particularly in children and pregnant women in the developing countries. According to the World Health Organization report 2011 (WHO 2011), there were approximately 216 million cases of malaria worldwide with an estimated 655 000 deaths in 2010 alone. It is estimated that more than 40% of the world's population are at risk from malaria, most residing in the sub-Saharan Africa (Hay et al. 2010) (Figure 1.1). Most of malaria deaths occur among children living in Africa and the rest occur in Asia and Latin America (Snow et al. 2005; Pimentel et al. 2007). There are four different types of malaria that infect humans: *Plasmodium falciparum* – the most common form of malaria, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale* **.**

Plasmodium falciparum is known to be the most serious form of the Plasmodium parasite and it often can be fatal in the absence of proper treatment. Considerable progress has been made in the fight against malaria and different methods have been used to protect individuals living in endemic areas, or to prevent the spread of the disease, these include prophylactic drugs, mosquito eradication, and insecticide-treated bed nets. However, the prophylactic drugs are expensive especially for most people living in these endemic areas. In addition, treatment and control have become more difficult with the spread of drug-resistant strains of parasites (Nuwaha 2001) and insecticide-resistant strains of mosquitoes that carry the parasites (Ito et al. 2002). Therefore, there is an urgent need for an affordable and effective vaccine for malaria that can promote the fight against this deadly disease.

Figure 1.1: World Malaria risk areas, 2010. Malaria transmission is concentrated in tropical and sub-tropical regions in Africa, South America and Asia (WHO 2010).

1.2 Challenge

The use of a malaria vaccine could be one of the most cost-effective interventions to reduce the transmission of infection and the burden of the malaria disease. A number of distinct vaccine platforms have been evaluated, including synthetic peptides and viral vectored vaccine platforms aiming toward an effective vaccine against malaria (Li et al. 2007; Bruder et al. 2010; Bruder et al. 2010). Among different types of vaccines, DNA vaccines emerged as a scalable new approach for prevention and treatment of malaria and other diseases because of their ability to elicit both humoral and cellular immune responses (Liu 2011), whereas vaccination with traditional protein-based vaccines elicit only antibody mediated (humoral) immune responses and often requires promoter injections (Berzofsky et al. 2001; Robinson 2007).

Moreover, traditional vaccines are expensive and take a longer time to produce, while rapid and large-scale DNA vaccine production is available at considerably lower costs. DNA

vaccines are also fairly stable at room temperature for ease of storage and transportation especially in poorer countries without advanced facilities, in contrast to a number of live attenuated vaccines whose storage and global delivery are complicated as these vaccines require to be kept in cold chain when transporting them to remote areas (Liu 2011). In addition, using a recombinant DNA technology, DNA vaccines are constructed to encode several antigens or proteins that can be delivered to the host in a single dose.

The field of genetic vaccines has been limited by its deficiency in safe and effective delivery systems, although various methods have been used in the gene delivery challenges. Viruses represent highly evolved natural vectors for the transfer of foreign genetic material to cells (Kay et al. 2001). Because of the safety concerns regarding viral gene vectors, non-viral gene delivery systems have become the subject of intense examination for the last 15 years. However, many different modalities of non-viral gene delivery have proven to be inefficient for the rapid and specific accumulation of active gene vectors on the target cells. For this reason, a novel means of physical targeting, exploiting magnetic forces acting on the nucleic acid vectors which are associated with magnetic particles, in order to mediate the quick contact of vectors with target cells named magnetofection has recently been used for gene delivery *in vitro* and *in vivo* (Dobson 2006).

In magnetofection, magnetic nanoparticles need appropriate surface fabrication to connect with gene complexes and also increase their stability in solution. The stability of magnetic nanoparticles in biological fluid can be improved by modifying their surface using several materials including both inorganic and polymeric materials in order to increase repulsive forces between particles, thus balancing magnetic and van der Waals attractive forces (Wu et al. 2008). A highly positively charged agent such as polyethyleneimine (PEI) cationic polymer has advantages over other polycations in that it readily associates with negatively charged DNA accompanied by intrinsic endosomolytic activity (Lungwitz et al. 2005). PEI has been suggested as a promising transport vehicle for a non-viral gene delivery system because of its capability to destabilize endosomal membrane by the so-called proton sponge effect resulting in endosomal disruption and DNA release from endosomes (Boussif et al. 1995; Behr 1997).

1.3 Research motivation

The asexual intraerythrocytic stage of the malaria life cycle is totally responsible for all clinical pathologies associated with the disease, while the number of injected parasites is low in the initial stage of infection. For these reasons, developing an effective asexual blood stage vaccine provides a good opportunity to activate the immune response and eliminate the parasite

One target for growth-inhibitory antibody is the membrane associated 19-kDa COOH terminal fragment of merozoite surface protein ($MSP1_{19}$) that plays a crucial role in *Plasmodium* immunity, it is the first and best characterized of many proteins on the merozoite surface that are being targeted for vaccine development (Diggs et al. 1993; Good et al. 1998; Thera and Plowe 2012). MSP1 is found in all *Plasmodium* species and it is the most abundant protein. It is synthesized as a high molecular mass precursor (approximately 200 kDa) during the merozoite maturation stage inside red blood cells RBCs and deposited on the surface of infected cells. Antibodies against $MSP1_{19}$ interrupt important proteolytic steps during the process of RBCs invasion which produce only the small carboxyl–terminal 19kDa domain $MSP1_{19}$ on the surface of infected RBCs (Blackman et al. 1990). This domain remains membrane bound and it anchors to the merozoite surface via a glycosylphosphatidylinositol (GPI) (Gilson et al. 2006) (Figure 1.2). $MSP1_{19}$ contains epidermal growth factors (EGF) like domain that forms a tightly packed, disc-like structure and contains protective epitopes (Morgan et al. 1999).

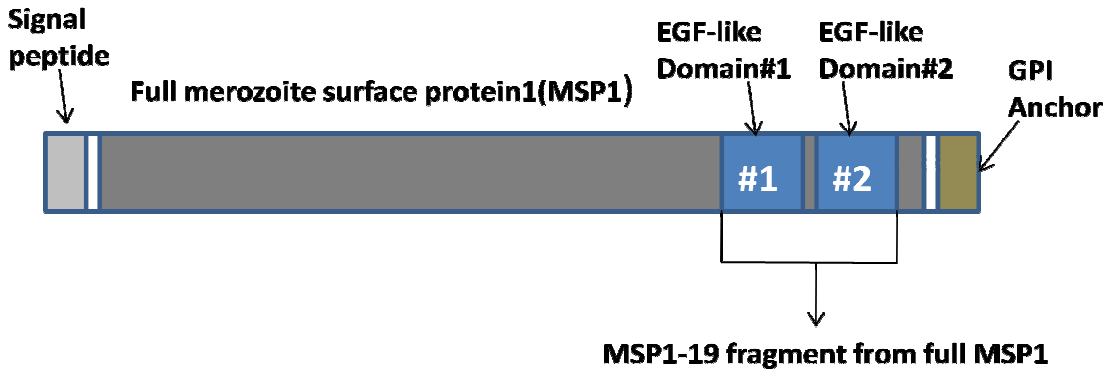

Figure 1.2: Schematic figure of derived $MSP1_{19}$. $MSP1_{19}$ is a final product of proteolytic cleaving of MSP1 during schizogony and merozoite maturation. This small carboxyl–terminal 19kDa domain ($MSP1_{19}$) remains membrane bound and it anchors to the merozoite surface via a glycosylphosphatidylinositol (GPI). $MSP1_{19}$ contains epidermal growth factors (EGF) like domain that forms a tightly packed, disc-like structure and contains protective epitopes.

1.4 The research aims and thesis outline

The main aim of this project is to employ the principle of magnetic targeting of the gene vector to improve the delivery of the malaria DNA vaccine. A promising candidate as a carrier for the malaria DNA vaccine is biocompatible magnetite (Fe_3O_4) superparamagnetic iron oxide nanoparticles (SPIONs) with suitable size and appropriate surface chemistry. Magnetic nanoparticles conjugated with the DNA vaccines are expected to provide a better delivery of the malaria vaccine by applying an external magnetic field during gene transfection *in vitro* and *in vivo.*

1.4.1 Specific research aims

➢ To utilize superparamagnetic iron oxide nanoparticles (SPIONs) coated with polyethylenimine (PEI), specifically by studying the effects of SPIONs/PEI complexation pH (acidic and neutral) conditions on resulting size, surface charge, DNA binding, and gene transfection efficiency of magnetic gene vectors in COS-7 cell lines *in vitro*.

➢ To investigate the *in vivo* immunogenicity of malaria DNA vaccination delivered using SPIONs/PEI/DNA complexes via different routes of immunization.

- To generate stable magnetic gene vectors with dendritic-cells (DC) targeting functionality in different cell media. This was done by investigating the effects of assembly order for configurations comprising SPIONs, PEI, hyaluronic acid (HA), and DNA, on vector size, surface charge, stability, ability to bind and release DNA, DNase I-sensitivity, and resistance to polyanion-mediated dissociation.

- To understand the effects of combining low and high molecular weights of HA at different charge ratios for SPIONs/PEI/DNA-HA complexes on stability, cytotoxicity, and dendritic cells (DCs) transfection / maturation efficiency *in vitro.*

1.4.2 Thesis outline

The thesis is organized into 7 sections:

Chapter 2: Literature review

This chapter presents a comprehensive review of DNA vaccines, the malaria DNA vaccine and DNA delivery strategies, focusing on magnetic nanoparticles applications as a gene carrier, synthesis of magnetic particles, surface modification of magnetic particles with different materials in general and polymers in specific, and surface modification of these particles with polyethyleneimine for gene delivery.

Chapter 3: Superparamagnetic Nanoparticles for Effective Delivery of Malaria DNA Vaccine

This chapter describes the effects of SPIONs/PEI complexation pH (acidic and neutral conditions) on the resulting SPIONs/PEI/DNA vector size, surface charge, ability to condense DNA and gene transfection efficiency in COS-7 cell lines *in vitro*.

Chapter 4: Effects of administration routes on *in vivo* immune responses for malaria DNA vaccination with magnetic gene vectors

This chapter demonstrates the impact of the magnetofection technique on the immunogenicity of malaria DNA vaccination condensed in SPIONs/PEI/DNA complexes via different routes of administration, in homologous (DNA vaccination alone) as well as heterologous (DNA prime-protein boost) regimens.

Chapter 5: On designing stable magnetic vectors as carriers for malaria DNA vaccine

This chapter shows that the assembly of different magnetic gene vector configurations consisting of SPIONs, PEI, HA, and DNA as carriers for malaria DNA vaccine affected the vector size, surface charge, stability, and ability to bind and release DNA in different cell media.

Chapter 6: Design of magnetic polyplexes taken up efficiently by dendritic cell for enhanced DNA vaccine delivery

This chapter reports a novel quaternary system of SPIONs/PEI/DNA-HA malaria gene complexes to target and transfect dendritic cell DC. Specifically studies were conducted based on using different molecular weights of HA with different % charge ratios of HA: PEI to understand their effects on stability, cytotoxicity, and DC transfection / maturation efficiency of the complexes.

Chapter 7: Conclusions and Recommendations

The conclusion based on the major findings from this PhD project is presented, with a recommendation for further investigations in the field of malaria gene delivery using the magnetofection technique.

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CHAPTER 2

Literature Review

2.1 DNA vaccine

The first scientific investigations of vaccination were explored over 200 years ago by Edward Jenner (1798), when he showed that previous cowpox infection protected people from smallpox infection. Although in two centuries of the development and widespread use of vaccines against a great variety of infectious agents has been achieved throughout the world, there are only 27 human diseases avoidable by vaccination (Gurunathan et al. 2000; Nguyen et al. 2009). Vaccines against such dangerous pathogens including tuberculosis, malaria, human immunodeficiency virus (HIV) are either ineffective or unavailable (Hilleman 2000). One of the main obstructions for successful vaccination against the previous infectious agents is the requirement to activate cellular immune response for protection. Thus, stimulating cell-mediated immune response which suppresses and clear intracellular pathogens are a highly desirable process. Though all currently licensed vaccines are capable to induce a humoral immune response, only vaccines derived from live attenuated organisms are able to efficiently stimulate cellular immunity. It should also be noted that many of the live attenuated vaccines are prohibited by practical restrictions such as manufacturing and safety concerns (Gurunathan, Klinman et al. 2000).

The observation that the gene expression of naked DNA encoding several different reporter genes via direct intramuscular injection in the mice last decade by Wolf and coworkers (1990) offered an attractive alternative to the traditional vaccine strategy and generated considerable argument in the vaccine community (Gurunathan, Klinman et al. 2000). Therefore, the demonstration over the last decade about DNA vaccine's ability to elicit a protective antibody and cell-mediated immune responses in a wide variety of preclinical

animal models for viral, bacterial, and parasitic diseases has caused considerable enthusiasm in the vaccine community. In addition, the major advantage of the DNA vaccines at the immunological level is their capability to induce antigen-specific $CD8^+$ T-cell including CTLs, which is an important mechanism of protection against intracellular pathogens (Gurunathan et al. 2000).

Gene-based vaccination might provide several important benefits over the traditional vaccine:

- In a typical DNA vaccination protocol, the antigen is not given to individuals but DNA encodes the antigen in order to produce the antigen of interest inside the body's own cells. After immunization potent antigen-presenting cells (APCs) such as dendritic cells (DCs) usually uptake the DNA molecules and transcribe the gene. Fragments of the expressed protein are presented by major histocompatibility complexes to stimulate immune response toward both antibody and cellular immune responses (Forde 2005).

- DNA vaccines can be easily manufactured with low cost and are easily stored under a vast array of conditions either dried or in a solution. This eliminates the need for the "cold chain" to maintain the stability of the vaccine during distribution. In addition, the ability to rapidly and cheaply produce a DNA vaccine from bacteria using a relatively simple procedure, makes DNA vaccine an attractive approach in vaccine development (Forde 2005; Listner et al. 2006).

2.1.1 DNA delivery strategies

The conventional viral gene delivery system has made great progress in comparison to non-viral gene delivery system because of its extremely high transfection efficiency and capacity for treating a wide range of diseases (Voigt et al. 2008). However, the use of viral vectors has raised serious safety concerns in the field of gene therapy due to immunological complications, insertional mutagenesis, narrow cargo capacity, and also large-scale production difficulty with high manufacturing costs (Voigt, Izsvak et al. 2008). Furthermore, the viral gene delivery system lacks specific targeting on the cell membrane

which may cause local inflammation at the site of the injection, and even rapid removal from the blood stream by the immune system. All of these problems and risks prevent gene therapy using viral vectors (Patil et al. 2005).

Another method to introduce therapeutic genes to the target site is by systemic circulation or direct injection of naked DNA into local tissues (Horn et al. 1995). In spite of the simplicity of this method, very few copies of the negatively charged DNA can pass through similarly charged cell membranes because of electrostatic repulsion. Naked DNA is also being degraded by nuclease and substantially removed on the first-pass of the liver (Pouton and Seymour 2001). Therefore, these all might reduce the intracellular uptake of naked DNA (Pathak et al. 2008).

Many delivery systems have been developed to overcome the barriers associated with the delivery of genetic materials. At present, a non-viral delivery system is being increasingly used because of its engineering simplicity (He et al. 2010). The use of non-viral gene vectors allows the design of multivalent carriers which can integrate many functional components with different tasks. Therefore, it can be said that the best gene vector should raise the efficiency and undermine the drawbacks of virus vectors.

Non-viral gene delivery systems include physical methods i.e., microinjection, electroporation, magnetofection, hydrodynamic pressure techniques, and particle bombardment (Luo and Saltzman 2000; Mehier-Humbert and Guy 2005), and chemical methods which can be classified into two major types depending on the nature of synthetic materials: I) Polymeric delivery systems and II) Liposomal delivery systems (Patil, Rhodes et al. 2005).

There are abundant physical techniques to introduce genes inside the cells such as mechanical and electrical methods (von der Leyen et al. 1999). For example, Stechschulte and colleagues (2001) injected naked plasmid DNA under pressure using a 1⁄2-inch 33-gauge needle with a 30° bevel on a 10-ml gas tight syringe into the corneal stroma of albino mice. Another technique for introducing naked DNA into the cell is microinjection in which the genetic material is inserted directly into the cell nucleus by microneedles (McAllister et al. 2000). The direct insertion of genes into the nucleus makes this technique conceptually simple and appealing in its approach in the gene delivery field however this method has several drawbacks. Microinjection means that only one cell at a time can be

delivered by DNA, thus this method becomes impractical for manipulating large numbers of cells especially for *in vivo* gene delivery applications. Other drawbacks are slow and low success rate of DNA expression since only a small number of cells can express the protein (Luo and Saltzman 2000). As a result, these deficiencies make the microinjection technique inapplicable for various biochemical assays.

Another technique for delivering genes is particle bombardment which is also known as a biolistic particle delivery system. In this method, DNA is coated onto the surface of gold microparticles and fired at the surface of tissue using high gaseous pressure (Pouton and Seymour 2001). The bombardment technique is popular in the DNA vaccination field. However, it is restricted to a local expression of delivering DNA in the dermis, muscle, or mucosal tissue (Luo and Saltzman 2000). Electroporation is also another effective method for DNA vaccination against infectious agents (e.g. Hepatitis B virus, human immunodeficiency virus-1) (Prud'homme et al. 2006). By using high-voltage electrical current to insert DNA inside the target cells through temporarily increase of cell membrane permeability, thus allowing the direct access of DNA molecules, and the extracellular degradation pathway can be avoided (Liu et al. 2006). Indeed, *in vivo* electroporation dramatically increase the gene expression compared to the injection of free DNA. However, this technique also induces a high level of cell mortality due to the high-voltage exposure which could limit its clinical and therapeutic utility (Luo and Saltzman 2000).

Chemical methods offer the advantages of simplicity, ease of production, and potential safety over viral and non-viral gene delivery system via physical methods (Lalani and Misra 2011). The chemical structure of these vectors decides the efficiency of gene expression. Therefore, the characteristics of the non-viral vectors can be manipulated successfully to achieve high gene delivery by different processes, such as:

a) Neutralizing the negatively charged DNA molecules to avoid charge repulsion with anionic cell membrane and condensing the DNA molecules in a compact structure for appropriate transport through the plasma membrane and then to the nucleus of the target cell (Vijayanathan et al. 2002).
b) Protect the DNA molecules against enzymatic degradation from both extracellular and intracellular nuclease degradation (Wong et al. 2007).

c) Facilitate DNA escape from the endosomes into the cytoplasm (Godbey et al. 1999).
d) Enhance DNA stability upon storage and reconstitution, preserve DNA integrity and stability after *in vivo* administration (del Barrio et al. 2003).

The development of a nano-carrier-based vaccine formulation has notably become one of the newest areas of vaccine research. Different studies verified that non-viral carrier systems in a nanometer size were successfully used as a transfection agent to deliver nucleic acids for both *in vitro* and *in vivo* applications (Vijayanathan, Thomas et al. 2002). A particulate delivery system for vaccines can be prepared with a wide variety of materials including lipids, proteins, polymers, latex, polystyrene, gold , and silica (Xiang et al. 2010).

Recently magnetofection has been successfully used for gene delivery as a feasible technique in the wide range of cells, especially for hard transfected cells such as primary cells (endothelial cell) which have been practically identified as resistant cell types to DNA transfection (Krotz et al. 2003; Chapman et al. 2008).

Different studies have demonstrated the dramatic increase in the cell transfection accompanied by efficient gene expression through making gene vectors susceptible to magnetic force (Plank et al. 2003). With this technique, gene vectors are associated with superparamagnetic nanoparticles (SPIONs) in order to mediate rapid contact with the target cells (Barcena et al. 2009; O'Grady 2009). The size and surface coating of superparamagnetic nanoparticles can be tailored according to the specific biological demand and the linkage between gene vector and the nanoparticles can be designed accordingly. However, the main concern of this technique is that the magnetic particles might show undesirable effects as foreign particles in the bloodstream, rendering the particles endocytosis by the body's major defense system the reticuloendothelial system (RES) which can result in their removal from blood circulation (Berry and Curtis 2003). In particular, the mechanism of interaction between the magnetic nanoparticle and the blood components depends strongly on the coating material for magnetic nanoparticles. Without appropriate coatings, these particles after injection are rapidly covered (opsonization) by macromolecules such as plasma proteins and glycoprotein in the blood circulation and removed by the immune system (Berry and Curtis 2003).

Size is a key parameter in clearance of magnetic particles. Following systemic administration larger particles with diameters greater than 200 nm usually accumulate in organs with high phagocytic activity, such as liver and spleen as a result of mechanical filtration, and finally they are removed by the phagocytic cells (Gupta and Gupta 2005; Colombo et al. 2012). In contrast particles below 10–15 nm can be rapidly removed through extravasation and renal clearance (Figure 2.1). The optimal particle size for gene or drug delivery treatments ranges between 10 to 100 nm, since in this size range these particles are small enough to evade RES of the body and at the same time able to penetrate through very small capillaries within the body tissues, thereby offering the most effective distribution in certain tissues (Gupta and Wells 2004)

The process of magnetic particle capture and recognition by the immune system is a big concern for *in vivo* application which can be modulated by size and surface modification to ensure particles biocompatibility and stability to the RES (Gaur et al. 2000).

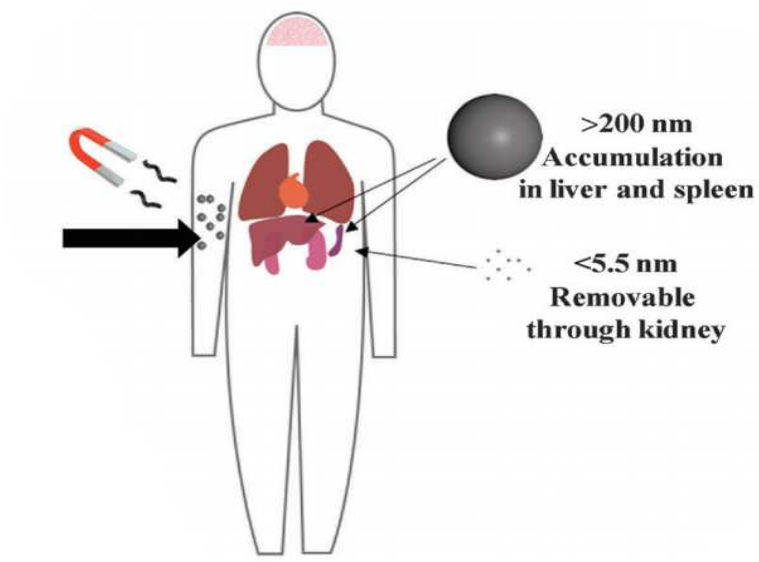

Figure 2.1: Large magnetic particle (>200 nm) is usually sequestered by the spleen and livers a result of mechanical filtration and are eventually removed by the cells of the phagocyte system, while very small particles (<10 nm) can be cleared through the kidneys. The optimal particle size for drug and gene delivery treatments ranges between 10 to 100 nm, as these will have the longest blood circulation time (Colombo, Carregal-Romero et al. 2012).

2.1.2 Malaria DNA vaccine

The main goal of the malaria vaccine research is to develop a vaccine that prevents the most naive recipients from developing any clinical symptoms of disease after exposure to *P. falciparum*, and to prevent infected people from developing severe disease and possible death by limiting the effects of blood stage infection. There are two major approaches for malaria vaccine development: the first is for a vaccine designed to prevent any clinical symptoms of malaria through stimulating cytotoxic T cells to destroy all the infected cells in the liver before the parasite has a chance to multiply (Tuteja 2002). The second approach is for a vaccine designed to prevent morbidity and mortality rate by preventing parasite replication and invading red blood cells after the parasite leave the liver, reducing cytoadherence, and/or inhibiting the effects of toxic materials released by the parasite (Doolan and Hoffman 2001).

Vaccination with DNA is a recent technology possessing promised benefits over traditional vaccines (killed or attenuated pathogens) because of their demonstrated ability to induce preferentially CD8+ T cell immune responses which have been difficult to induce by the more traditional vaccines (Liu 2011). The first promising study for malaria vaccine was during the 1970s, when Nussenzweig et al. (1967) reported that immunization with radiation-attenuated *Plasmodium berghei* could provide protection against challenge with fully infectious sporozoites to about 67% in the injected mice compared with control. Many of the basic works carried out on rodent malaria models provided an important information to irradiated sporozoite vaccine-induced protection and also led to an increase the number of candidate vaccines (Nussenzweig and Nussenzweig 1989; Hoffman et al. 1996; Hoffman and Miller 1996). Though there is an enormously large number of malaria research projects, a significant number of these studies target the elimination of parasites before they reach the blood stage (Matuschewski 2006). Since more mortality and morbidity are associated with *P. falciparum*, developing an effective vaccine against this parasite might lead to eradicate this disease in a lot of malaria endemic areas. RTS,S is the world's most advanced malaria vaccine candidate against *P. falciparum* (Heppner et al. 2005). It is a yeast-expressed subunit recombinant vaccine, in which *P. falciparum* surface protein of circumsporozoite (CS) has been fused to the pre-S2 region of the hepatitis B virus (HBV)

surface antigen as a matrix carrier and the hybrid protein expressed in transformed *Saccharomyces cerevisiae* yeast cells (Regules et al. 2011). This recombinant is injected along with the potent AS02 lipid emulsion adjuvant (Ballou 2009). To date, the majority of clinical trials of this vaccine has used a formulation known as RTS,S/AS02, which has shown evidence of safety in all age groups and protective efficacy against the CS protein. The history of the development of the AS02 adjuvant system combined with malaria vaccine antigen RTS,S has been reviewed in detail by Garçon et al. (2003). Recently, a new formulation known as RTS,S/AS01, in which the lipid emulsion vehicle was replaced by a liposomal one and this system has been described as the most developed and clinically validated malaria vaccine formulation which has the ability to induce higher levels of antibody and Th1 cell-mediated responses against the *P. falciparum* circumsporozoite protein CSP (Mettens et al. 2008).

DNA immunization against the malaria parasite has been extensively studied especially when the sequence of the *P. falciparum* genome was published in 2002, providing the foundation for new drug and vaccine targets (Gardner et al. 2002).

The early studies of DNA vaccine were established by studying the immunogenicity of four different *P. falciparum* pre-erythrocytic stage DNA vaccines in mice (Hedstrom et al. 1998) and rhesus monkeys (Wang et al. 1998). In early 1994, Sedegah et al. observed that the immunization of mice with plasmid DNA encoding a pre-erythrocytic stage *Plasmodium yoelii* antigen PyCSP stimulate antigen-specific $CD8^+$cytotoxic T lymphocyte (CTL) and antibody responses that confer protection against sporozoite challenge (Sedegah et al. 1994). The pre-erythrocytic stage vaccine would be expected to considerably reduce the early blood stage parasite burden and consequently the amount of the following asexual stage magnification (Doolan and Hoffman 2001). Because of the complicated life cycle of the parasite (Figure 2.2) and many candidate vaccines have shown poor immunogenicity with ineffective capabilities to stimulate immune response against this lethal disease, therefore other stage vaccine are required to provide 'sustainable' protection against malaria.

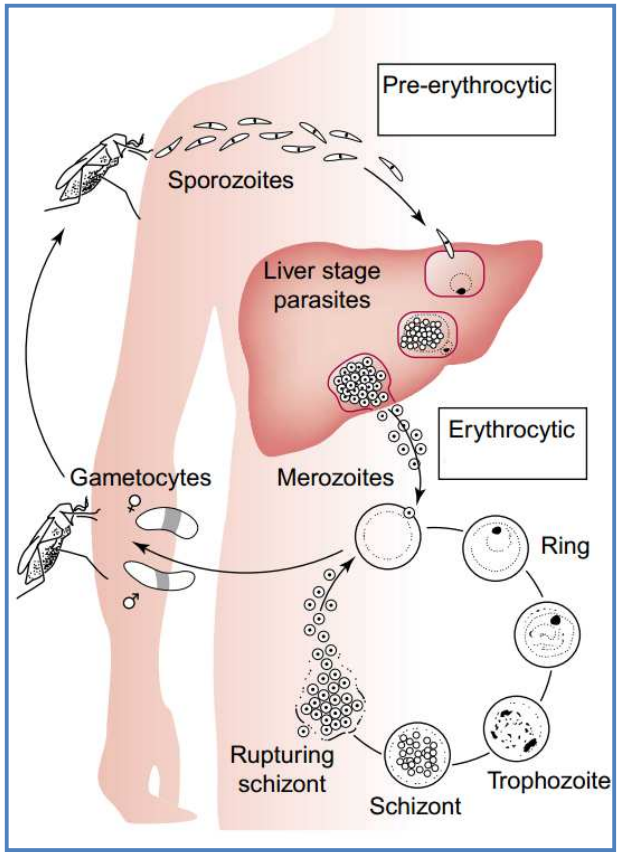

Figure 2.2: Life cycle of *Plasmodium falciparum* (Kumar et al. 2002).

Blood-stage malaria vaccines aim to diminish the asexual parasite burden through inhibition of merozoite invasion by antibody immune response or infected erythrocytes by cytoadhesion (Matuschewski 2006). Among different types of vaccines, DNA vaccine has emerged as a potentially practical approach for the prevention and treatment of malaria infection because of its ability to elicit both humoral and cellular immune responses (Liu 2011). Different strategies have been followed in the quest of developing the DNA malaria vaccine, including the inhibition of the parasite replication by targeting a number of surface proteins of merozoites (Tuteja 2002). Different antigens expressed on the surface of invasive merozoite of *Plasmodium sp* have been revealed to be targets of inhibitory antibody function, therefore a number of studies have been done on the immunity and vaccine approaches of these antigens (Diggs, Ballou et al. 1993; Richie and Saul 2002; Sachs and Malaney 2002).

Of particular interest among the potential vaccine candidates, membrane-associated 19-kD COOH-terminal fragment of merozoite surface protein MSP1 molecule has been the focus for malaria DNA vaccine development. $MSP1_{19}$ is one of the leading malaria blood-stage vaccine candidates (Holder and Freeman 1984; Diggs, Ballou et al. 1993; Good, Kaslow et al. 1998).

MSP1 molecule is present in most species of malaria parasite as a major component on the merozoite surface which is carried into red blood cells RBCs during merozoites invasion (Hodder et al. 1996). Several *in vitro* and *in vivo* studies have revealed that $MSP1_{19}$ is the target of protective immune responses against asexual blood-stages of malaria parasites (Daly and Long 1995; Kumar et al. 1995; Tian et al. 1997) and the antibodies against $MSP1_{19}$ are thought to act through direct inhibition of merozoite invasion into the RBCs (O'Donnell et al. 2001).

High levels of specific antibodies against $MSP1_{19}$ are required for the development of current DNA malaria vaccine candidates which could prevent the secondary processing of $MSP1_{19}$ precursor molecule and formation of $MSP1_{19}$. Antibody response against $MSP1_{19}$ hampers merozoites dispersion through forming immune clusters of merozoites (ICM) (Lyon et al. 1989), and also stimulate monocyte-dependent antibody-mediated protection mechanism against the parasite (Bouharountayoun and Druilhe 1992). Consequently, all these mechanisms can effectively inhibit merozoite invasion of RBCs (O'Donnell, de Koning-Ward et al. 2001).

Along with the development of a non-viral gene delivery system, scientists have used different materials for gene delivery. For ideal gene delivery systems, the gene vector should satisfy the requirements of successful intracellular transport, safety of the host, resistance to the enzymatic degradation and specific targeting ability into *in vivo* applications. Various non-viral vectors have been utilized to deliver genes in different kinds of cell lines *in vitro*, including lipid-based and polymer-coated gene vectors (Park et al. 2006). Inoculation of nanoparticle- or cationic lipid-formulated plasmid DNA has enhanced antibody responses and protection from malaria and other diseases (Sakai et al. 2003; Helson et al. 2008; Boyoglu et al. 2009). Using non-viral gene delivery system for the malaria vaccine, Vaxfectin^{TM} (a cationic lipid based formulation) has been shown to enhance a robust *in vitro* T cell and antibody response against DNA vaccines encoding *P.*

falciparum antigen MSP1 (Sedegah et al. 2010). In addition, this system also improved the immunogenicity and protective efficacy of plasmid DNA vaccine encoding the *P. yoelii* circumsporozoite antigen PyCSP, either as a stand-alone malaria vaccine, or as a priming component of a heterologous prime-boost regimen with recombinant attenuated vaccine *in vivo* (Sedegah et al. 2006). High levels of gene expression *in vitro* were also generated with biodegradable poly (lactic-co-glycolic acid) (PLGA) microparticles of a malaria DNA vaccine encoded the *P. yoelii* merozoite surface protein 4/5 (PyMSP4/5) (Liu et al. 2009), presumably due to narrower particle size distribution (0.8–1.9 µm) and high DNA encapsulation efficiencies (82%–96%). The same group subsequently reported high levels of gene expression and moderate cytotoxicity in COS-7 cells using a similar carrier but this time with plasmid DNA encoding $PyMSP1_{19}$ formulated via ultrasonic atomization technique (Liu et al. 2010).

More recently, covalently conjugated merozoite surface protein MSP1 of *P. falciparum* to polymer-coated quantum dot CdSe/ZnS nanoparticles (QDs) used as a vaccine delivery system was found to have 2–3 log higher antibody titres compared to those obtained with MSP1 administered with conventional adjuvants such as Montanide ISA51 and complete Freund's adjuvant CFA (Pusic et al. 2011). In addition, intravenous administration of newly designed an anionic ternary complex of polyethylenimine and γ-polyglutamic acid with plasmid encoding MSP1 of *P. yoelii* (pVAX-MSP1/PEI/γ-PGA) recorded a significantly higher level of immune responses and partial protection from lethal *P. yoelii* challenge infection as compared to the naked DNA (Shuaibu et al. 2011). The same group later examined the immunostimulatory effect of DNA/PEI/γ-PGA nanoparticle coated plasmid encoding *P. yoelii* MSP1-C-terminus in C57BL/6 mice using three different routes of administration; intravenous (i.v.), intraperitoneal (i.p.) and subcutaneous (s.c) (Cherif et al. 2011). After priming and boosting twice at 3-week intervals then challenging 2 weeks, 100%, 100% and 50% mean of survival was observed in immunized mice with coated DNA vaccine by i.p., i.v. and s.c., respectively. Furthermore, coated DNA showed significant immunogenicity and elicited protective levels of antigen specific immunoglobuline IgG and its subclass with an increased proportion of CD4+ and CD8+ T cells, INF-γ and IL-12 levels in the serum and the cultured splenocyte supernatant.

2.2 Bioapplications of magnetic particles

The concept of superparamagnetism of magnetic materials in the nanometer - size range was considered by Frenkel and Dorfman in (1930). They first speculated that a particle of magnetic material, below a size < 15 nm, would comprise a single magnetic domain, i.e., a particle that is in a state of uniform magnetization at any field, and their speculation was confirmed by subsequent studies. A particle of the magnetic material at the nano-scale is known to exhibit superparamagnetism, or the lack of magnetic memory, i.e. they are only magnetized in the presence of a magnetic field. This feature is a desirable for bioapplications because the unfavorable effect caused by permanent magnetic-force-induced aggregation to biomolecules, e.g. conformational changes, is avoided. As well, these particles maintains well disperse stability under aqueous conditions and do not retain magnetism upon removal of the magnetic field (Schmidt 2001). The covering of these nanoparticles with appropriate coating materials would increase their hydrophilicity and long time stability inside water-based solution, forming ferrofluid. Magnetic fluids as a result of their composition have distinct combinations of fluidity and ability to interact with a magnetic field (Khalafalla and Reimers 1980; Hamley 2003). For that reason, these ferrofluids can be used for different bioapplications including *in vitro* and *in vivo.*

Bioapplications of superparamagnetic nanoparticles include *in vitro* applications such as cell labeling and magnetic separation (Kularatne et al. 2002), cell separation (Prestvik et al. 1997), magnetically enhanced nucleic acid delivery (Scherer et al. 2002), while *in vivo* applications include drug and gene delivery (Lubbe et al. 1996; Lubbe et al. 2001; Scherer, Anton et al. 2002), hyperthermia (Nielsen et al. 2001), MRI contrast agents (Bulte et al. 2001; Gade et al. 2008; Ramaswamy et al. 2009) and tissue repair (Bulte, Douglas et al. 2001). In order to apply magnetic fluids in any *in vitro* and *in vivo* applications, the stability of these colloids is of the utmost importance. Biocompatibility, size distribution, toxicity, surface properties, and various other parameters of the particles should be considered in advance as well.

Magnetic nanoparticles have large surface area to volume ratio, therefore due to hydrophobic interactions in the absence of coating materials these particles tend to interact with each other producing a large agglomeration. These clusters then exhibit ferromagnetic

behavior (Hamley 2003; Gupta and Gupta 2005). For the *in vivo* applications, the opsonization and phagocytosis of these large aggregates by the reticuloendothelial system (RES) are the main hindrance for particle uptake by target cells (Neu et al. 2005). The agglomeration of the magnetic nanoparticles could further induce by strong magnetic dipole–dipole attractions between them leading to the blockage of capillaries within the body tissues (Chollet et al. 2002).

For long term stability with long blood circulation time of magnetic nanoparticles, often a very high requirement of appropriate surface coating is desired. The coated particle core provides nanocontainer with magnetic properties, while the coating material offer (i) protection against core agglomeration, (ii) chemical attachment for different molecules, and (iii) resistance against non-specific protein adsorption inside blood circulation. Some coating materials such as a surfactant molecule or a polymer (anionic, cationic) are usually added at the time of magnetic particle preparation to prevent aggregation of the nanoscale particle (Gupta and Wells 2004). However, most of these polymers adhere to surfaces in a substrate-specific manner (Mendenhall et al. 1996).

2.2.1 Magnetofection for gene delivery

Many viral and non-viral gene vectors have been developed to introduce genes into the target cells, which is the main requirement in the gene therapy field. The efficiency of any delivery system is mainly determined by the ability of gene vectors to overcome various biological barriers that hamper the cellular entry of therapeutic gene which may be broadly classed as being of an extracellular and the intracellular nature (Figure 2.3). The gene vectors should satisfy the requirement of superior transfection efficiency, resistance to biological degradation, and excellent cell targeting.

The gene delivery process is mainly determined by the manner in which the gene moves to and makes contact with the target cell, which is the limiting step under normal cell culture condition.

The efficacy of the any gene delivery system is restricted by three major aspects:

(1) Slow accumulation and low concentration of gene vectors at the target cell culture (Luo and Saltzman 2000).

(2) Vector intracellular pathway and nuclear uptake. The glycocalyxe structure of some types of target cell membrane may further hinder the interaction between gene vector and endocytosis receptors on the target cells (Arcasoy et al. 1997).

(3) Rapid degradation by nuclease especially *in vivo* gene transfection (Barry et al. 1999) and limited nuclear entry in non-dividing cell (Dean et al. 2005).

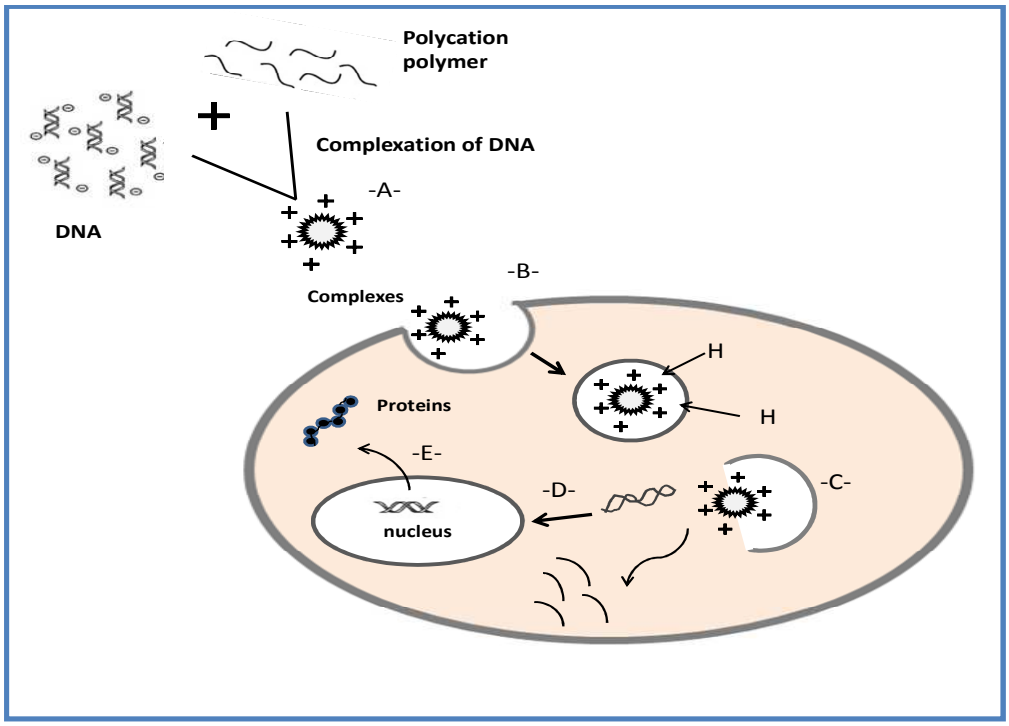

Figure 2.3: Design requirements for gene delivery systems include the ability to (A) package gene molecules with compact structure; (B) gain cell entry; (C) escape from the endosomal/lysosomal pathways (D) transport through the cytoplasm and into the nucleus; (E) facilitate gene expression.

To overcome these deficiencies, a new technology termed “magnetofection” has been developed (Scherer, Anton et al. 2002; Mykhaylyk et al. 2007). Magnetofection basically depends on the concept of magnetic drug delivery that was developed by Widder, Senyi and colleagues in the late 1970s (Senyei et al. 1978; Widder and Senyei 1983). Magnetic particles have proven to be a feasible method to elevate any gene delivery vector up to several hundred times and reduce the incubation time required to achieve high transfection/transduction efficiency (Scherer, Anton et al. 2002). Magnetofection is

universally applicable to viral and non-viral vectors, because it is extremely rapid and specific technique for gene transfection with low dose *in vitro* and site specific *in vivo* applications (Plank et al. 2003; Dobson 2006).

This technique is based on the coupling of DNA molecules with a non-viral transfection reagent or virus vector to superparamagnetic magnetic nanoparticles SPIONs (in some case microparticles), and the complexes introduce into the target cell culture with a rare earth magnet such as neodymium iron boron (Nd-Fe-B) magnets deposit underneath the cell culture (Figure 2.4). This technique enhances the sedimentation of the gene complexes to come close to the cellular level in the culture media, consequently accelerating the cellular uptake of gene and transfection kinetics (Scherer, Anton et al. 2002).

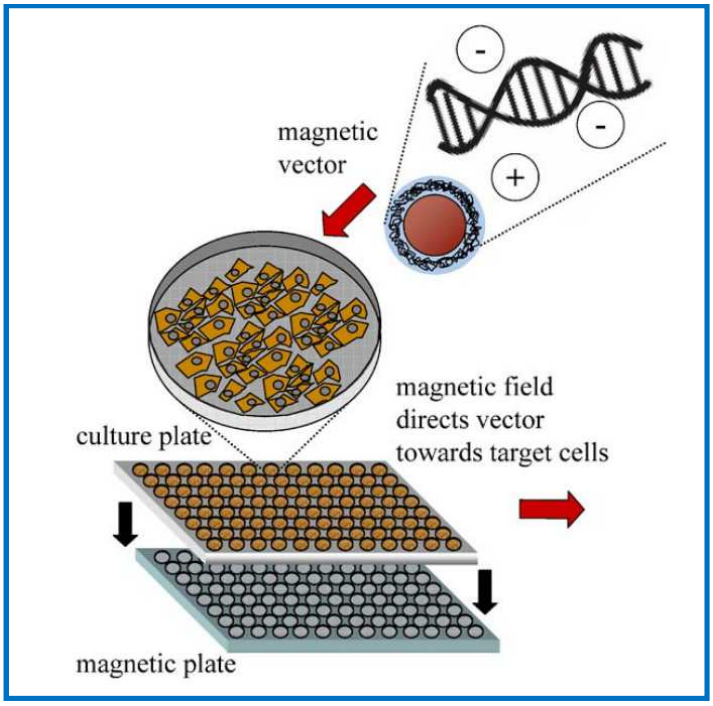

Figure 2.4: Scheme of the magnetofection for gene delivery *in vitro.* The vector is attached to magnetic nanoparticles, which are added to the cell culture under magnetic field (Schillinger et al. 2005).

Magnetic nanoparticles offer abundant opportunity in biomedical applications. First of all, their characteristic sizes range from a few nanometers to ten nanometers, placing them at dimensions analogous to the size of a virus (20–450 nm), a protein (5–50 nm), a cell (10–

100μm), or a gene (2 nm wide and 10–100 nm long) (Krishnan 2010). Secondly, nanoparticles are magnetic and obey Coulomb's law, which means they can be directed in any direction by using an external magnetic field gradient. For instance, for *in vivo* applications the nanoparticles can be localized in any internal target organs by using a strong external magnetic field. Thirdly, magnetic nanoparticles also have a large surface area, if it is properly engineered with an appropriate active surface groups, they can be easily shipped and targeted to any special biological target. Moreover, coating these particles with appropriate materials produces stable colloids that could be dispersed in any physiological solutions.

Through magnetofection, the expression of certain genes has been shown to increase up to hundred fold (Krotz, Sohn et al. 2003). Additionally, Scherer et al. (2002) demonstrated that the association of DNA vectors with superparamagnetic nanoparticles increased the transfection efficiency of a number of commercial transfection reagents *in vitro* and reduced the duration of gene delivery to minutes. The impact of various cell lines incubated with transMAG^{PEI} (polyethylenimine (PEI) associated superparamagnetic iron oxide) exposure to the magnetic field (Nd-Fe-B magnet) for 15 min was investigated by (Huth et al. 2004) using transmission electron microscope (TEM). The cells after five minutes exposure to the external magnetic field started to develop small finger-shaped extensions or narrow lamellae on the cell surface around the particles, especially in the parts where the particles attached. These cellular protrusions surround the particles during uptake and after 15 min there are particles found within the cell (Figure 2.5). The results of this study also showed that the cellular entry mechanism of the PEI coated SPIONs/DNA complexes were mediated via endocytosis which is a similar mechanism to PEI/DNA complex cellular entry. More specifically, Arsianti et al. demonstrated that the cellular entry mechanism of the SPIONs vector under magnetic field was mediated via clathrin endocytosis (Arsianti et al. 2010). This group also confirmed that despite different vector component assemblies (mixing order) comprising SPIONs, PEI, and DNA, all these vectors were taken up by the cells through the similar clathrin-mediated endocytosis mechanism. These results indicate that the magnetic field does not directly alter the cellular uptake mechanism of these particles, but just accelerates the sedimentation of magnetic particles on the cell surfaces (Huth, Lausier et al. 2004; Arsianti, Lim et al. 2010). A similar conclusion was drawn by

(Ang et al. 2011) who investigated the mechanism of magnetic gene transfection using modified forms of standard (normal) magnetic transfection protocol, namely reverse and retention magnetic transfection under magnetic field. The results of the normal, reverse and retention magnetic transfection experiments showed that the highest transfection efficiency was achieved in normal magnetic transfection mode due to clustering of the SPIONs/PEI particles on the cells whereas the particles were uniformly distributed in the reverse magnetic transfection (Figure 2.6). According to these results together with cell wounding assays, this group suggested that the mechanism of magnetic transfection is endocytosis rather than cell wounding (Ang, Nguyen et al. 2011).

By applying magnetofection, high gene expression can be achieved in a short duration time with remarkably low dose that can help to avoid cell toxicity of the gene complexes (Gersting et al. 2004). Magnetofection technique is a powerful tool in the gene delivery field especially for *in vivo* applications where very short duration time is required for high gene delivery before rapid filtration or systematic clearance takes place followed gene complex injection.

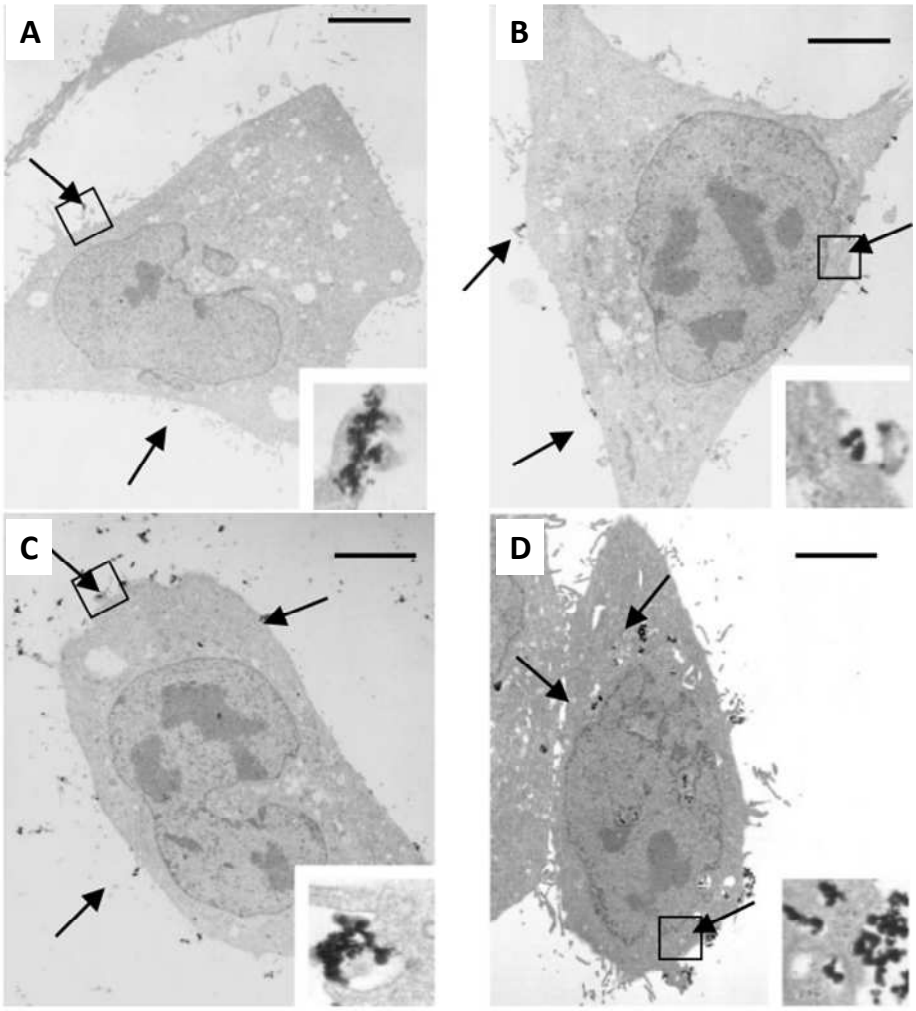

Figure 2.5: Electron microscopy for cellular trafficking of transMAG^{PEI} particles exposed to the magnetic field for (A) 1 min, (B) 5 min, (C) 15 min and (D) 15 min with further 24 h incubation. Arrows indicate transMAG^{PEI} particles (Huth, Lausier et al. 2004).

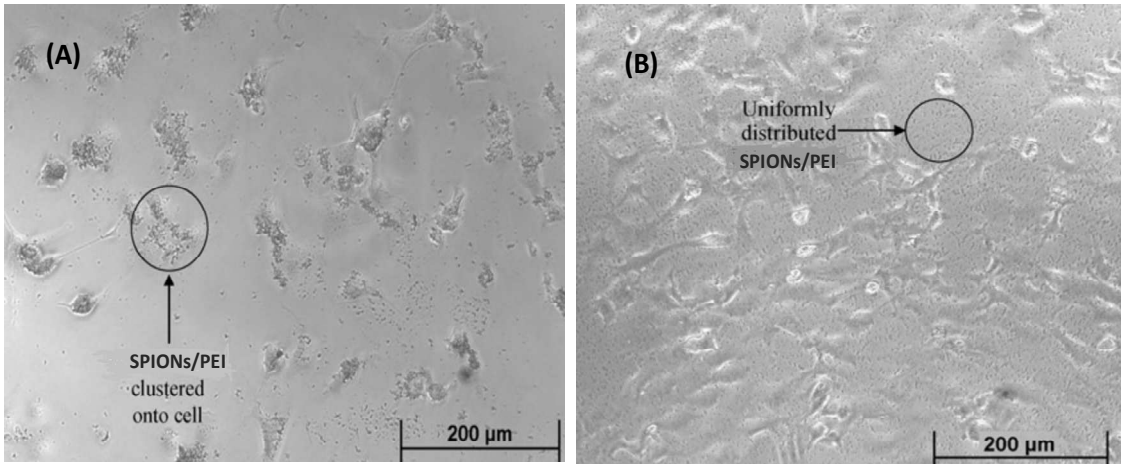

Figure 2.6: Typical optical micrographs showing the PEI/SPIONs distribution under (A) normal and (B) reverse magnetic transfection. PEI/SPIONs aggregates can be observed to cluster onto cells in (A), but are homogeneously distributed throughout the well in (B). The aggregate size of PEI/SPIONs in the normal magnetic transaction was larger than that for reverse magnetic transfection (Ang, Nguyen et al. 2011).

It has been shown that during the transfection process the type of applied magnetic fields could noticeably influence the transfection potency. A considerable increase in the transfection efficacy has been reported when the formulation of SPIONs with gene vectors were added to cells in the presence of static (permanent) magnetic field (Scherer, Anton et al. 2002; Gersting, Schillinger et al. 2004; Huth, Lausier et al. 2004). The magnetic gradient fields of the device are strong enough to sediment the magnetic vectors on the cell surfaces possibly within a few minutes. While for better transfection, a novel technique of combining a pulsating magnetic field to a static field has been achieved by (Kamau et al. 2006). This group coupled DNA fragments (PCR products) containing sequence encoding green fluorescent protein with polyethylenimine-coated SPIONs. The transfection has been done by applying a pulsating magnetic field for 5 min before or after placing the cells on the static magnetic plate for 20 min. The presence of these two types of magnetic field elevated the transfection dramatically up to 40 times when compared with the cells not exposed to magnetic fields. The efficiency of the high gene transfection was attributed to the fluctuation of nanoparticles in horizontal, perpendicular and oscillating movements within the pulsating magnetic field on the cell surface. The movements of particles seemed to enhance the particles transfer across the cell membrane. The further increase in

temperature on the surface of dynamic magnetic pulse generator could also contribute to enhance the gene transfection level (Pipes et al. 2005).

For biomedical applications, the size, charge and surface chemistry of magnetic particles are particularly important factors. The internalization and blood circulation time within the body greatly depend on the size of magnetic nanoparticles. *In vitro,* superior gene transfer rates were observed by Chorny et al. (2007) with large size magnetic nanoparticles in the range of 375 nm compared to 185 nm or 20 nm. Potentially, the large sized particles may have ability to escape lysosomal localization and release DNA in the pronuclear zone. In the same domain, higher gene transfection potency achieved by (Kamau, Hassa et al. 2006) with the size range of 200–250 nm using PEI coated magnetic particles after 5 min exposure to the magnetic field. The high gene transfection efficiency within the magnetic field was attributed to the particle size since larger sized particles required less time to sediment on the cells. These results corresponded with other studies which confirmed the efficiency of larger particle size on the gene transfection *in vitro* (Sung et al. 2003; Gersting, Schillinger et al. 2004; Huth, Lausier et al. 2004). However, for *in vivo* applications, injection of magnetic nanoparticles smaller than 1 mm in diameter not just enables them to pass through small capillaries which could be just a few microns wide but also transport them easily across the plasma membrane. The injected particles must be small enough to exclude any risk of the blockage of small arteries or capillaries. A broad range of *in vivo* gene delivery studies showed that the nanoparticles injected intravenously into mice result in gene expression that is stronger in a lung than liver, heart, spleen, or kidney, but this accompanied by high toxicity presumably because of the nanoparticles accumulation in the lung, which thereby resulted in death for most of the animals within the first 30 min (Wightman et al. 2001; Chollet, Favrot et al. 2002; Ito et al. 2008). The agglomeration of magnetic nanoparticles could be further induced by strong magnetic dipole–dipole attractions between the particles, leading to blockage of small capillaries within body tissues (Gupta and Gupta 2005). On the other hand, smaller particles in the size range (<15nm) are rapidly removed through extravasation and renal clearance (Stolnik et al. 1995; Gupta and Wells 2004). For that reason, the optimal particle size is from 10-100 nm, because it is small enough to avoid the reticuloendothelial system and able to penetrate

small capillaries within the body, thereby prolong the blood circulation time (Stolnik, Illum et al. 1995).

2.2.2 Synthesis of iron oxide particles for biomedical applications

In biotechnology the critical characteristics of magnetic nanoparticles are their nano-scale dimensions, magnetic properties, and ability to carry particular biomolecules for specific targets. Studies performed over the last decade with several types of iron oxides carried out in the field of nanosized magnetic particles, among them maghemite, γ-Fe_2O_3, or magnetite, Fe_3O_4, which consist of a single domain of about 5–20 nm in diameter have been widely used in the field of nanotechnology (Tartaj et al. 2003; Roca et al. 2009). Magnetite Fe_3O_4, is the most common magnetic iron oxide candidate because its biocompatibility in biological system has already been proved (Schwertmann and Cornell 1991). This form of iron oxide has stability in the water and physiological saline under natural pH conditions with large surface area that can be properly modified to attach biological agents (Ankamwar et al. 2010). These nanoparticles with suitable surface coating materials can disperse properly in suitable solvents to produce a homogenous suspension called ferrofluid that permits further biochemical functionalization.

Numerous synthesis methods have been used to produce magnetic nanoparticles for bio-applications including: Co-precipitation, microemulsions, polyols, sol-gel synthesis, sonochemical synthesis, hydrothermal, hydrolysis, thermolysis of organic precursors, flow injection, and electrospray (Tartaj, Morales et al. 2003; Laurent et al. 2010). These methods have been used to prepare magnetic particles with homogeneous composition and narrow size distribution. However, the most common method for synthesis magnetite particles in solution within the nanometer range is chemical co-precipitation technique of iron salts. The Co- technique is probably the simplest and most efficient wet chemical routes to obtain magnetic particles for biomedical applications (Gupta and Gupta 2005). This method involves synthesis via co-precipitation of ferrous and ferric salts in an alkaline solution by the addition of a base such as concentrated ammonium hydroxide (NH_4OH) or sodium hydroxide (NaOH) under non-oxidizing environment (N_2 gas atmosphere) (Massart 1981;

Massart et al. 1995). Control over size and shape of nanoparticles depends on the Fe^{2+} and Fe^{3+} ratio, the type of salts (e.g. Sulphate, nitrate, chloride, etc.) and the pH of reaction media (Gupta and Gupta 2005).

One important feature of using iron oxide nanoparticles in bioapplications is controlled over the narrow size distribution and magnetic properties of these particles. Recently, Gupta and Wells (2004) produced iron oxide in narrow size range with uniform chemical and physical properties inside a non-polar solvent aqueous cores of reverse micellar droplets at low temperature ($4 - 6^{\circ}C$) in the presence of N_2 gas. The size of the inner core of reverse micelles is in a nanometer range, so that the produced particles will be in a very small size (<15 nm) and narrow size distribution with high magnetization value. The main advantage of using a microemulsions system is to produce particles with controlled size by modulating the size of aqueous core (Munshi et al. 1997).

2.2.3 Surface modification of magnetic nanoparticles for biomedical applications

The stabilization of iron oxide nanoparticles is an important feature for obtaining stable ferrofluid colloid with no aggregation in both biological media and the magnetic field. Due to the hydrophobic surface of magnetic particles with a large surface area to volume ratio, these particles in the absence of coating materials tend to interact with each other to form large clusters, resulting in increase of aggregate size. Strong magnetic dipole-dipole interaction arises between these clusters when each of them comes in the magnetic field of the neighbor and gets more magnetized displaying ferromagnetic behavior. However, this makes magnetic particles unsuitable for biomedical applications (Hamley 2003; Gupta and Gupta 2005). For that reason, coating these nanoparticles is a key requirement of the stable magnetic colloid solution. Some stabilizer such as a surfactant or a polymer is usually added during the time of preparation to prevent aggregation of the particles, while another added after synthesis of particles.

These coating layers here not only provide stability to the nanoparticles in solution but also help for binding various biological ligands on the particle surface for various biomedical applications. Many types of linkages were used to couple magnetic nanoparticles to the

nucleic acids, and the simplest one is by employing a physical method based on an electrostatic interaction between positively charged magnetic particles due to the cationic polymer coating layer and negatively charged nucleic acid. Various materials have been used as protective coatings for the magnetic nanoparticles, as discussed in the following section.

2.2.3.1 Surface modification with non-polymeric organic stabilizers

Several authors have reported the adsorption of alkanesulfonic, octadecanephosphonic and alkanephosphonic acid surfactants on the surfaces of amorphous Fe_2O_3 nanoparticles as stabilizers for the particles (Yee et al. 1999; Portet et al. 2001). While another study done by Sahoo et al (2001) reported the use of oleic acid (OA), lauric acid, dodecylphosphonate, hexadecylphosphonate, and dihexadecylphosphate as surfactants to stabilize magnetite nanoparticles with a 6-8 nm average diameter and disperse them in organic solvents. This study suggested that carboxylate surfactants provide the particles with better isolation and dispersibility as compared to phosphonate surfactants.

2.2.3.2 Surface modification with inorganic materials

Iron oxide nanoparticles can be coated with inorganic materials such as gold (Lin et al. 2001; Chen et al. 2003) (Figure 2.7A), silica (Tartaj et al. 2002; Zhang et al. 2007) (Figure 2.7B) and gadolinium (III) (Morawski et al. 2004). These particles have an inner core of magnetic particles and the surface metallic shell of inorganic materials. The coating materials not only increase magnetic particle stability in solution but also help in binding other biological ligands on the nanoparticle surface for various biomedical applications.

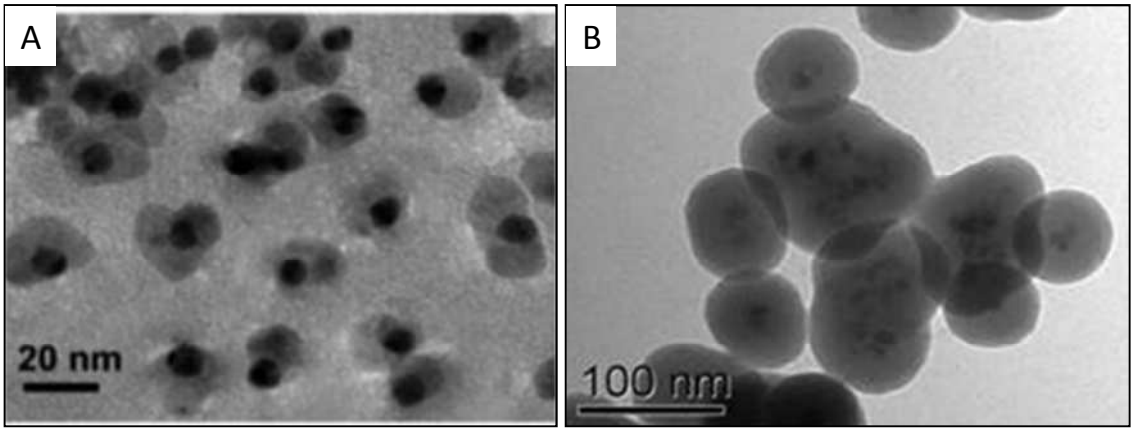

Figure 2.7: TEM images of (A) Gold-coated Fe_3O_4 nanoparticles (Xu et al. 2008); (B) Silica-coated γ-Fe_2O_3 nanoparticles (de Almeida et al. 2010).

2.2.3.3 Surface modification with polymers

Although different studies have focused on developing small magnetic particles with surfactants as a coating layer up to now, polymers coated magnetic particles have also received much attention. The major advantages of using polymer coating are increasing repulsive forces between particles that generally balance magnetic and van der Waals attractive (Wu, He et al. 2008). In addition, polymer coating on the magnetic nanoparticle surface offers a high potential in the application of several fields.

A variety of approaches have been developed to coat magnetic nanoparticles with polymers, including 'in situ' coating during particle synthesis and post synthesis absorption. For example**,** Mahmoudi et al. (2009) have developed a co-precipitation method for iron oxide nanoparticles in the presence of PVA polymer, producing magnetic particles with narrow size distribution and stable dispersion in the PVA polymeric substrate. Although *in situ* coating technique can produce stable dispersions of particles in aqueous media with small size, this approach may limit the crystallinity of the formed particles that may negatively affect their magnetic susceptibility (Veiseh et al. 2010). In addition, surface modification of the magnetic nanoparticles with a PVA polymer by precipitation of iron salts in different concentration of the PVA aqueous solution was also reported by (Lee et al. 1996), but they found that the crystallinity of magnetic nanoparticles decreases with the

increasing of PVA concentration. A novel method of preparing uniform polymer coated magnetic nanoparticles with polyethylene glycol (PEG) polymeric shell has been presented by Gupta and Wells (2004) using an inverse microemlusion polymerization process. Since the absorption of polymer on the particles takes place inside small droplets of the aqueous core of AOT/n-Hexane reverse micelles this method gives uniformly encapsulate cores and highly stable polymeric coating. More recently, *in situ* fabrication of magnetic gene vectors in the presence of PEI polymer was also evaluated by (Shi et al. 2010) to study the efficiency of magnetofection using PEI coated magnetic particles in COS-7 cells *in vitro*.

On the other hand, the post-synthesis modification consists of adsorbing or grafting the polymer on the surface of magnetic particles after they have been synthesized. The most common coating materials for these nanoparticles is polyethylene glycol (PEG) polymer. PEG is the most widely used synthetic polymers for *in vivo* application due to its hydrophilicity, biocompatibility, non-toxicity, non-antigenicity, and non-immunogenicity.
The attachment of PEG contains, e.g., hydroxyl or amino functional groups on the magnetic particle surface increases their ability to evade engulfment by the reticuloendothelial cells or circulating macrophages, and at the same time minimize or eliminate blood protein adsorption on the surface due to the natural charge of PEG, having better therapeutic efficacy (Zhang et al. 2002; Gupta and Wells 2004; Herve et al. 2008). In addition, it has been verified that PEG-surface modification facilitates the particle internalization into the cells resulting in a better cellular response (Gupta and Wells 2004). The possible mechanism for this uptake is that PEG can dissolve in both polar and non-polar solvents and has a high solubility in the cell membranes (Yamazaki and Ito 1990). Various polymers, e.g. polyvinyl alcohol (PVA), poly (lactic-co-glycolic acid), polyvinylpyrrolidone (PVP), poly (vinyl alcohol) (PVA), are typical examples of synthetic polymers used for magnetic nanoparticles coating (Miller et al. 1983; Ruiz and Benoit 1991; Kumar et al. 2006; Liu et al. 2007). While, natural polymer systems include the use of dextran, gelatin, chitosan, pullulan, etc. (Schwick and Heide 1969; Jeong et al. 1999; Massia et al. 2000; Berry and Curtis 2003).
The previous polymers have been often used as a coating materials mostly because of their non-toxicity, highly biocompatibility, biodegradability, and their capability to increase the stability and biocompatibility of the magnetic particles in the blood circulation (Lacava et

al. 2001). The major approach in the gene delivery system has drawn much interest on coating magnetic iron oxide nanoparticles with water-soluble cationic polymer such as poly-L-lysine, poly [2-(dimethylamino) ethyl methacrylate] (PDMAEMA) or, in particular, polyethylenimine (PEI) (Hu et al. 2009; Arsianti, Lim et al. 2010; Wu et al. 2011). Although several polymers display efficient DNA condensation properties, the high positive charge density of cationic polymers leads also to increase the cell toxicity (Mintzer and Simanek 2009).

2.2.3.4 Gene complexes modification with polyethylenimine (PEI)

The recent years have witnessed rapid development of non-viral gene vectors based on PEI and derivatives which possess properties addressing delivery problems associated with gene therapy. Polyethylenimine, is one of the prominent examples among cationic polymers for proficient gene transfection, often considered as a "gold standard" transfection agent, though it has appreciable toxicity (Godbey, Wu et al. 1999). High molecular weight PEI has high gene transfection efficiency, but unfortunately this process is accompanied by a high cytotoxic effect. The cytotoxicity of PEI is related to its high positive surface charge and low biodegradability especially at high concentration inside the cells, where it may interfere with the cellular activity and cause cell toxicity (Neu, Fischer et al. 2005). The first successful polyethylenimine-mediated gene transfer achieved by (Boussif, Lezoualch et al. 1995), followed by using PEI derivatives to improve the physicochemical and biological properties of gene complexes (Godbey, Wu et al. 1999; Neu, Fischer et al. 2005). PEI polymer is known to form cationic complexes that can interact nonspecifically with negatively charged DNA and enter the cell via endocytosis (Godbey, Wu et al. 1999). In contrast to other cationic polymer, PEI has high transfection efficiencies even in the absence of endosomolytic agents such as fusogenic peptides or chloroquine which facilitates the cellular uptake (Kichler et al. 2001).

Polyethylenimine polymer comes in two forms: branched and linear structure (Figure 2.8**)**. It has a 1:2:1 ratio of primary/secondary/tertiary amines in its structure, with every third nitrogen being protonable at various pHs. This structure results in a polymer with a very high positive charge density at various pHs (Eliyahu 2005). PEI is also a classic example of

a cationic polymer with endosome buffering capacity which is probably one of the most important hypothesis to elucidate the high gene transfection efficiency obtained with this polymer (Boussif, Lezoualch et al. 1995). The so called “proton sponge” hypothesis has found widespread acceptance in recent years, and some publications have challenged this hypothesis (Behr 1997; Varkouhi et al. 2011) (Figure 2.9**)**. In this case, PEI would be able to buffer the interior of endosomes through accumulation of protons brought in by the endosomal ATPase enzyme, which also results in an influx of chloride anions.

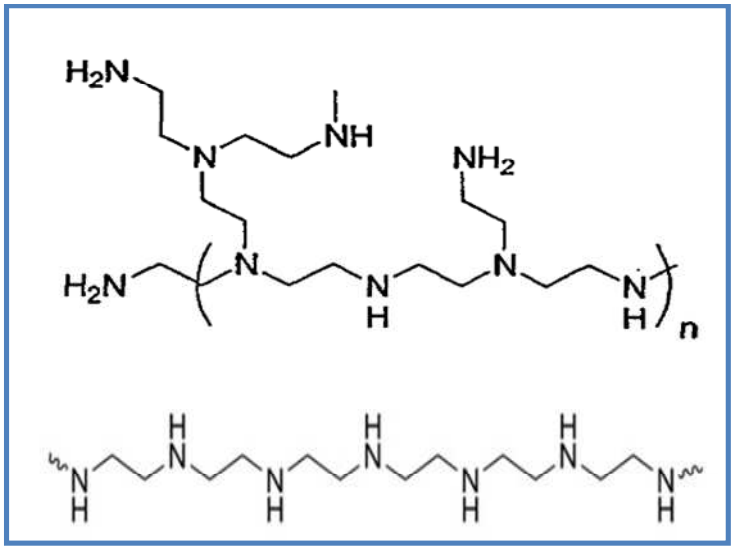

Figure 2.8: Chemical structure of branched (upper) and linear (lower) polyethylenimine (PEI)

As a consequence, water rushes in endosomes to decrease the gradient resulting in swelling and rupturing or leaking of the endosome membrane and this allows the entrapped DNA molecules to escape before lysosomal degradation can occur (Boussif, Lezoualch et al. 1995). Another mechanism for DNA endo-lysosomal escape was reported by Bieber et al. (2002), evidence of small local membrane damages has been observed using electron microscopy which was linked to the direct interaction between PEI (molecular weight 800 kDa) and endosomal membrane. The authors suggested that low molecular weight of PEI (25kDa) also causes minor membrane damage, but those holes may be quickly resealed. In

another study, the membrane damage was found to be depended on the PEI concentration used to prepare PEI/DNA complexes (Clamme et al. 2003).

The presence of multiple protonated amino groups in the PEI polymer structure increases the interaction of the positively charged polycations with the negatively charged phosphate groups in the DNA molecule's backbone producing small and compact transfection complex. The gene complexes are readily endocytosed by cells via electrostatic interactions of the polycation with the negatively charged cell membrane (Kabanov and Kabanov 1995). Once the complexes enter the endo-lysosomal compartment, PEI protects the DNA molecules from degradation and guides them efficiently to the nucleus.

An important part of polymer-DNA complexes transfection efficiency is the physico-chemical properties of the gene complexes such as size, structure and surface charge. Moreover, all these properties strongly depend on the nature of polycation such as structure, molecular weight, charge density and charge-to-mass ratio of the polymer and DNA molecules (Godbey, Wu et al. 1999).

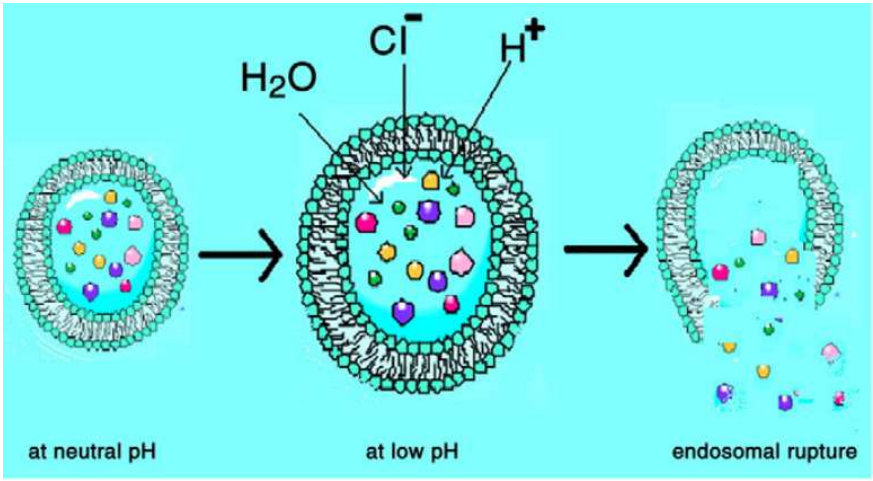

Figure 2.9: The proton-sponge hypothesis: H+ and Cl- entry into the endosomes leads to osmotic swelling and finally to endosome rupture (Varkouhi, Scholte et al. 2011)

Regarding the influence of the PEI different molecular weight on various biophysical characteristics of gene complexes, it seems that there is no significant difference in the surface charge determined by zeta-potential measurement for complexes prepared with

branched or linear PEI using the different molecular weight of PEI molecules, e.g. linear 22 kDa or branched 25 kDa, compared to branched 800 kDa at a similar N/P ratio of 6 (Kircheis et al. 2001). Whereas, the same study showed that PEI/DNA complexes formed with branched PEI (25 or 800 kDa) produced small (50 nm – 80 nm) or medium-sized (100 nm to few hundreds of nm) complexes depending on the DNA concentration. However, complexes formed by the linear PEI 22 produced a large complex, and the size increased with increasing incubation time (Kircheis, Wightman et al. 2001). The same behavior of the gene complexes was described by (Mady et al. 2011). The authors showed that when the molecular weight of PEI was increased, the DNA condensation ability and surface charge density increased while the size of the complexes decreased. Using scanning force microscopy, Dunlap and others described the complexation and DNA condensation behavior induced by linear and branched form of PEI (Dunlap et al. 1997; Wang et al. 2011). They reported that the branched form of PEI was more effective condensing agent for DNA molecules than linear one because of its high primary amine content that play a significant role in the DNA condensation capacity and the complexation efficiency (de Ilarduya et al. 2010). Primary amines are known to condense DNA better than another type of amine groups, due to their higher protonation under neutral pH condition (Wolfert et al. 1999). Further studies demonstrated that the binding capacity of PEI with DNA could be connected to the number of PEI's primary amines (Tang and Szoka 1997), and the stability of produced complex in physiological solutions could increase with increasing primary amine group content leading to higher gene transfection efficiency (Reschel et al. 2002).

The differences between complex sizes prepared with linear or branched PEI has a great influence on the transfection activity *in vitro* and *in vivo*. For example, in some cell lines such as colon carcinoma (MCA-38) and melanoma (B16F10), the transfection efficiency of linear PEI22 kDa/DNA was generally greater than that of branched PEI25kDa/DNA when the complexes generated in salt containing buffer (Wightman, Kircheis et al. 2001). In part, this effect was attributed to the linear PEI22 kDa/DNA complex's apparent ability to aggregate under salt conditions, since these large aggregates were seen either within the cytoplasm or associated with the nucleus using fluorescence microscopy. Because of the branched PEI25kDa/DNA complex stability under salt conditions, these small particles

remained as small distinct particles associated with the cell membrane or in the cytoplasm (Wightman, Kircheis et al. 2001).

In general, remarkably higher gene transfection efficiency for branched PEI/DNA complexes compared to linear PEI/DNA complexes at low N/P ratio (N/P= 3) was reported, while both linear and branched PEI showed similar high transfection efficiency at relatively high N/P ratios of 10 and 15, although the cellular uptake of the linear PEI complexes was significantly lower (Dai et al. 2011). Excess amount of PEI due to high N/P ratio can associate with the condensed particles, leading to a highly positive zeta potential that enhance the transfection efficiency for both types of PEI.

Regarding the impact of cell media on the biophysical properties of gene complexes, linear PEI22 kDa/DNA and even branched PEI25 kDa/DNA complexes generated under the salt-free 5% glucose buffer showed reduced gene delivery activity *in vitro* application, while they were highly active *in vivo* (Poulain et al. 2000; Wightman, Kircheis et al. 2001). Both PEI22 kDa/DNA and PEI25 kDa/DNA complexes generated in salt-free 5% glucose had small size particles making them particularly suitable for *in vivo* applications. Although salt-free PEI22 kDa/DNA consequently grew into aggregates when the salt was added, branched PEI25 kDa/DNA complexes were rather stable with small complexes in salty conditions and grew very slowly (Wightman, Kircheis et al. 2001). The tendency of complex sizes to increase with increasing saline concentration is thought to reflect low-affinity DNA-binding. Although some studies reported that the large particles might be favorable for *in vitro* use (Ogris et al. 1998) attributing that to the higher cellular uptake due to gravity and large particle sedimentation (Arsianti et al. 2010), the *in vivo* application of such large aggregates may not be feasible.

PEI molecular weight has also demonstrated the high influence on the gene transfection. Abdallah et al. worked quite well for testing three different molecular weights of PEI *in vivo*. The authors reported that the branched form of PEI with the molecular weight of 25kDa demonstrated the highest gene transfection efficiency than 50 and 800 kDa (Abdallah et al. 1996). Comparative studies also indicated the effectiveness of branched PEI25kDa transfection performance, although the branched form demonstrated high cytotoxicity compared to its linear counterparts (Remy et al. 1998; Jeong, Nah et al. 1999). It is generally believed that the most suitable molecular weight of PEI for gene transfer

ranges between 5 kDa and 25 kDa, while higher molecular weights lead to increase gene complex cytotoxicity (Fischer et al. 2003). The efficiency of gene transfection of lower molecular weights of PEI was examined by (Godbey et al. 1999) who verified that the transfection efficiency was significantly low but started to increase when PEI molecular weight was increased from 600 to 70,000 Da. However, at a very high or very low molecular weight of PEI, gene transfection significantly decreases. Generally, the low molecular weight produces unstable complexes with more tendency to dissociate in saline medium due to its lower condensation capability (Papisov and Litmanovich 1989). In contrast, increasing PEI molecular weight could also decrease its gene transfection efficiency because this will be accompanied by a high cytotoxic effect that might be attributed to the polymer high net positive charge (Bieber, Meissner et al. 2002).

For *in vivo* gene delivery applications, the use of PEI is limited by high toxicity and low gene expression due to its highly positive charges (Neu, Fischer et al. 2005). The positive charges cause disruption and permeabilization of cellular membranes (Godbey, Wu et al. 1999). In addition, decreased transfection efficiency of PEI *in vivo* is partly attributable to serum protein-induced aggregation and blood cell coagulation (Ito et al. 2006). Since PEI/DNA complexes are still highly positively charged, they are able to interact with negatively charged serum proteins such as opsonins in the blood, adversely affecting *in vivo* gene expression (Wightman, Kircheis et al. 2001; Chollet, Favrot et al. 2002). Therefore, different groups have attempted to avoid such unfavorable interactions of PEI/DNA complexes with target cells by shielding positive charges of PEI with other polymers to create a steric barrier against aggregation (Kichler 2004). For example, PEGylation of PEI via covalently attached polyethylene glycol (PEG) segments increase complexes circulation time *in vivo* due to reduce non-specific interactions with serum albumin and cellular components in the bloodstream, and also increase the water-solubility (Kichler 2004; Lee and Kim 2005). Recently, cationic PEI derivatives of hyaluronic acid HA (HA/PEI) have been used as a delivery system of siRNA and antisense oligonucleotides (Han et al. 2009). The modification of this polycation by polyanions such as hyaluronic acid has been reported to decrease the cytotoxicity and diminish the non-specific interaction with the serum protein, accordingly to reduce the aggregation in the physiological fluid (Yao et al. 2010). At the same time, gene transfer efficiency of this type of gene complexes to the desired tissue can be enhanced by

receptor-mediated endocytosis (Hornof et al. 2008; Jiang et al. 2009). Toll-like receptor including TLR 4 and TLR2, CD44, RHAMM, TNFIP6, HARE and LYVE-1 have been described as HA main cell surface receptors for different biological functions (Oh et al. 2010).

The mixing order of PEI , DNA, HA components in the complexes also showed a great influence on the complexes aggregate size and consequently their gene delivery function. Ito et al. (2008) verified that the pre-addition of HA to DNA prior to PEI effectively diminished the aggregation, and the complexes remained as small particles with a diameter below 80 nm. They hypothesized that the high dispersion stability of this complex under physiological condition could be attributed to the presence of the hydrated shell of HA polymer which promotes fine dispersivity of the complexes. HA interaction with water molecules result in fully ionized carboxylic groups of HA forming hydrogen bonds between the two molecules (Ito, Iida-Tanaka et al. 2006), which enhance the dispersion of these complexes in solution.

The influence of the N/P ratio might also be another factor which influences the transfection efficiency and cell toxicity of the complex (Zhao et al. 2009). The extent of condensation depends remarkably on the polymer: DNA ratio (Minagawa et al. 1991). The compact particles with smaller size are generally obtained at higher N/P ratios with high positive net charge leading to higher gene delivery than loosely complexed DNA (Godbey, Wu et al. 1999). This is because, firstly, all negatively charged DNA molecules are neutralized and no repulsion with negatively charged cell membrane takes place. Secondly, the small complexes size improves their cellular uptake by endocytosis and might also afford better protection against nuclease degradation (Ogris et al. 2001). Finally, the higher amount of PEI in the complexes creates local membrane damage leading to transfer PEI/DNA complexes over membrane barriers (Bieber, Meissner et al. 2002).

In recent years, magnetic nanoparticles have generated great interest in the scientific community because of their potential application in the biotechnology (O'Grady 2009; Pankhurst et al. 2009; Roca, Costo et al. 2009). In the field of gene delivery, the introduction of functional groups on the magnetic particle surface became very important to make these particles as potential non-viral gene vector with stable and smaller size in the physiological solution. Polyethylenimine, amongst other cationic polymers that have been

used as a coating material for superparamagnetic nanoparticles, since the SPIONs core would provide magnetic-targeting and PEI polymer can carry DNA through the electrostatic interaction (Abdallah, Hassan et al. 1996).

The most straightforward step for preparation the negatively charged SPIONs is using chemical co-precipitation method modified with sodium citrate or citric acid (Wang et al. 2009). Citric acid has carboxyl groups which facilitate its adsorption onto the iron oxide particles (Xu et al. 2006). Ionized carboxyl groups in the aqueous solution would increase the stability and at the same time provide the particles with a high negative charge, facilitating stronger attachments with cationic polyelectrolytes such as PEI (Figure 2.10). Becoming highly positive makes the SPIONs/PEI complex able to interact with the negatively charged DNA molecules. This approach appeared promising because PEI is known to be an excellent reagent to promote gene delivery. A previous work of (Plank et al. 2003) proved that PEI coated magnetic particles are the most effective vectors for gene delivery. The gene transfer efficiency of this type of reagent was also assessed by Krotz and others, who reported enhanced luciferase gene delivery in cultured human umbilical vein endothelial cells (HUVEC) which are virtually transfection resistant cells (Krotz, Sohn et al. 2003). The transfection efficiency of a luciferase reporter gene increased up to 360-fold compared to various conventional transfection systems, while there was only up to 1.6-fold increase in the cell toxicity caused by SPIONs/PEI complex in HUVEC culture (Krotz, Sohn et al. 2003). However, SPIONs/PEI complexes showed significant toxicity at concentrations around 500 ng PEI/ml compared to SPIONs/PEI/DNA complexes in HeLa cells after 24 h exposure in RPMI or DMEM media (Boussif, Lezoualch et al. 1995). The toxic effect of PEI/SPIONs complexes was notably decreased only when DNA added to the outer layer of PEI/SPIONs complex. This result may belong to the presence of external chains of DNA molecules around the complexes that minimize the high positive charge density of PEI to a level able to reduce cell toxicity while maintaining high gene delivery (Boussif, Lezoualch et al. 1995; Arsianti, Lim et al. 2010). The advantage of increased transfection efficiency of any gene delivery vector should be balanced with its cytotoxicity, especially when the vector is used for *in vivo* application.

It is important to keep the combination strength between the charged SPIONs and the PEI/DNA complexes at a reasonable level in order to achieve the optimal gene delivery. In

the magnetofection complexes, there should be enough amount of SPIONs to let controllable magnetic manipulation to pull the gene complex to the cells and balanced amount of PEI not only to stabilize the formed complex, but also to allow the release of DNA from the SPIONs/PEI/DNA complex. Electrophoretic mobility study of magnetofectins in a culture medium showed that the SPIONs/PEI/DNA complexes were stable at the N/P ratio over 10, while decreasing the N/P ratio to 5 the free DNA was visualized from magnetic complexes (Ma et al. 2011). Premature DNA release outside the cells and timely dissociation of DNA molecules from the complex to access cellular transcriptional machinery are very critical for optimal gene transfection. Therefore, high N/P ratios are important for stable and efficient gene expression to a point that the complexes are able to release DNA to access the transcription machinery. Very stable complexes due to strong electrostatic interactions between DNA and PEI seems to be unfavorable for gene delivery, since tightly bounded complexes will not dissociate and release DNA template to provide access for DNA polymerase (Prasad et al. 2003; Derouazi et al. 2004; Danielsen et al. 2005).

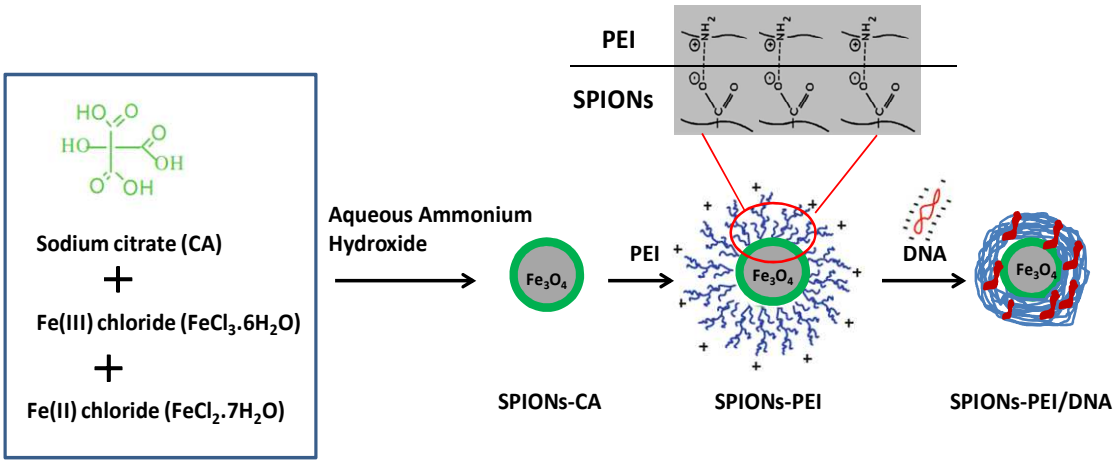

Figure 2.10: Schematic figure of synthesis of SPIONs/PEI/DNA complex

A novel non-viral gene delivery system based on PEI coated on the surface of bacterial magnetic nanoparticles (BMPs) obtained from *Magnetospirillum gryphiswaldense* MSR-1 (made of Fe_3O_4) has been introduced by (Xiang et al. 2007) (Figure 2.11A). The BMPs/PEI/DNA vector has delivered plasmid DNA effectively *in vitro* and *in vivo* with

low cell toxicity compared to PEI/DNA/DNA complex. Both BMPs/PEI and BMPs/PEI/DNA complexes were shown to have no negative effects on the different cell lines viability in contrast to the cytotoxic effect of PEI alone. The low toxic effect of PEI in these complexes might be due to neutralizing the positive charges of PEI by the negative charge of cytoplasmic membrane on BMPs (Xiang, Bin et al. 2007) (Figure 2.11B). Moreover, the same previous group also demonstrated a significantly high level of humoral and cellular immune responses against the target antigen *in vivo* when the BMPs/PEI/DNA complexes have been injected into the muscle and a Nd- Fe- B neodymium-iron-boron magnet was placed onto the site of injection for 20 min.

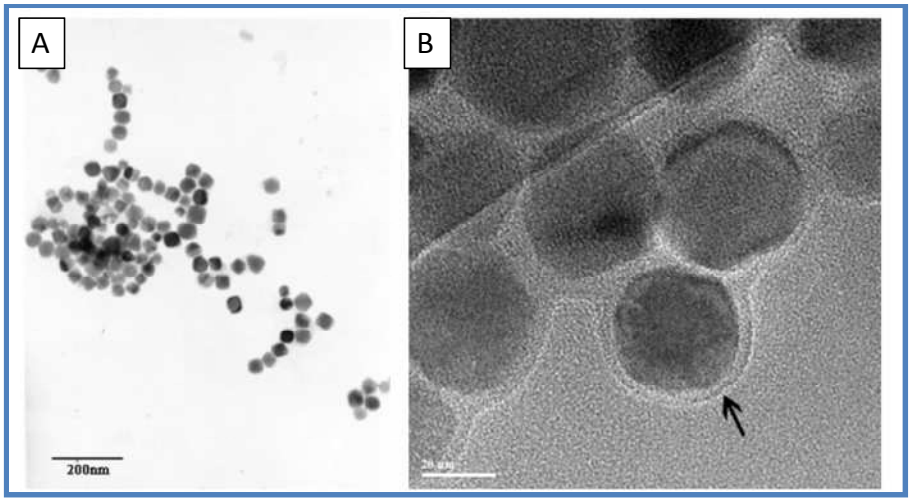

Figure 2.11: (A) Bacterial magnetic particles BMPs were obtained from *M.gryphiswaldense* MSR-1. (B) Cytoplasmic membrane coated BMPs (arrow) (Xiang, Bin et al. 2007).

The major problem related to the magnetic nanoparticles is the aggregation and dissolution in the biological fluids. For *in vivo* uses, this is quite crucial since agglomeration of particles within the vasculature has the possibility of problems such as embolization (Ramchand 2012). Consequently, numerous studies have been performed to overcome the problem of particle aggregation since the size of the aggregates may influence their biological function for *in vitro* and *in vivo* applications.

In terms of the SPIONs/PEI complexation pH values effects on the properties of the resulting particles, Wang et al. (2009) obtained particles de-aggregation when the SPIONs/PEI complexes were prepared under acidic condition. The process of particle de-

aggregation was irreversible and re-neutralization did not result in re-aggregation of the complexes (Figure 2.12). They suggested according to Vonzelewsky et al. (1993) theory (Figure 2.13) that under acidic conditions the mutual charge repulsion between the protonated amine groups leads the molecular chains of PEI polymer to become more stretched increasing the steric repulsion between the particles. Thus, after complexes acidifictaion both electrostatic and steric interactions participate in the de-aggregate formation. Particles de-aggregation under acidic condition has a big effect on their performance in magnetofection, with less aggregated particles being more effective in enhancing the gene transfection (Wang, Zhou et al. 2009).

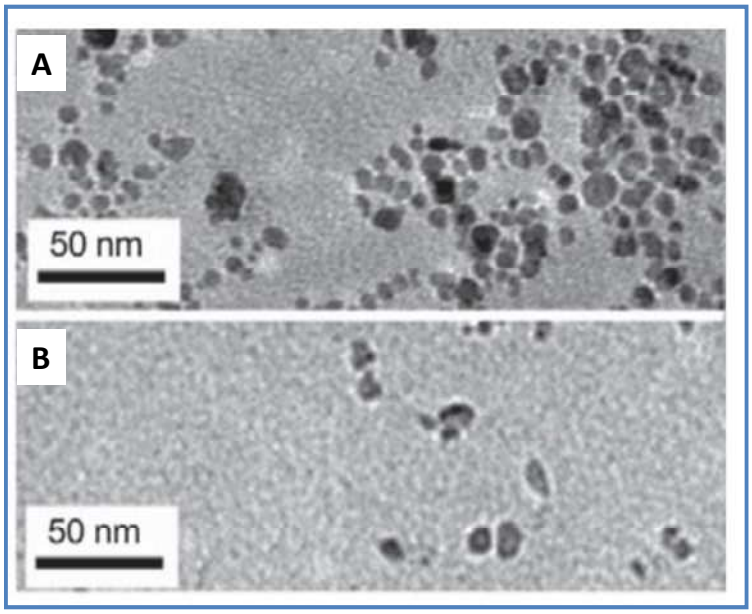

Figure 2.12: TEM images of the SPIONs-PEI under (A) neutral condition and (B) acidic condition (Wang, Zhou et al. 2009).

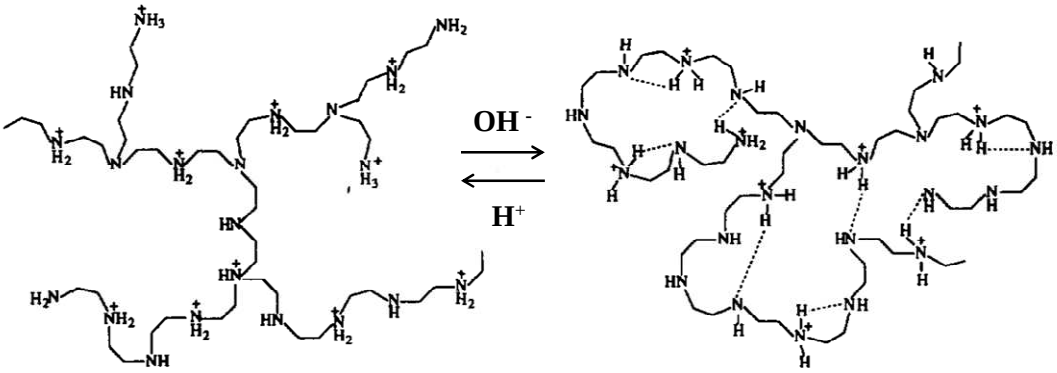

Figure 2.13: Schematic figure for PEI polymer under acidic and neutral conditions (Vonzelewsky, Barbosa et al. 1993)

These results verified that beside the small aggregate size of SPIONs/PEI complexes under acidic condition, they also have the capability to hold and entrap increased amount of DNA molecules. The open polymeric structure of branched PEI molecules at low pH due to mutual charge repulsion between positively charged amine groups is preferable for embedding large amount of the DNA molecules inside the polymer structure and protect them from enzymatic degradation (Vonzelewsky, Barbosa et al. 1993).

The assembly order of different magnetic gene vectors comprising SPIONs, PEI, and DNA also showed considerable effects on the vector size, surface charge, cellular uptake, and the level of gene expression. Recently Arsianti et al. reported that the complexation of DNA with PEI before coupling with the magnetic particle (SPIONs+PEI/DNA) resulted in an increase in the population of cells containing DNA than other vector assembly orders under external magnetic field (Arsianti, Lim et al. 2010). Here, the superiority of SPIONs+PEI/DNA vector for delivery DNA to cells *in vitro* was attributed to its larger size which improve cellular uptake of the particles due to enhanced gravitational and magnetic aided sedimentation onto the cells.

2.3 Summary and remarks

Magnetic nanoparticles have been used for different bio-medical applications such as drug and gene delivery. The efficiency of magnetic particles for *in vitro* and *in vivo* gene delivery is dependent on a number of parameters, including particle size, surface charge and surface chemistry of the particles etc., most of which can be determined or influenced by the coating materials which are used, including both inorganic and polymeric materials. These coating materials have to be not only non-toxic and biocompatible but also allow a targetable delivery of genes with particle localization in a specific area. The emphasis on PEI cationic polymer as a coating layer for magnetic nanoparticles is described in this chapter. This was due to its great ability to condense DNA into a compact structure which might afford protection from nuclease digestion and effective buffer capacity over a wide range of pHs. These properties presumably allowed a greater number of plasmids to reach the nucleus for transcription, as evidenced by increased transfection efficiency. High gene transfection efficiency of PEI coated superparamagnetic nanoparticles is due to their inherent magnetic properties that enhance *in vitro* gene transfection through magnetically sedimentation of particles on the cell surface and targeting the particles to any special internal target organs *in vivo* through the application of a strong external magnetic field.

It can be seen that although various studies have addressed different aspects of non-viral gene delivery systems and also various materials have been used for the transfer foreign genetic material to the cells, there are still many optimizations that need to be done in particular for a magnetic gene delivery system in order to achieve optimal delivery.

The literature showed that enhancing and targeting the gene vector delivery by a magnetic force *in vitro* and *in vivo* can reduce the DNA degradation and increase the efficiency of gene transfection. Different studies have been done in the malaria gene delivery with a number of reports which have indicated that immunization with naked malaria DNA vaccine induces only sub-optimal immune responses which fail to protect from an experimental parasite challenge. Therefore this study has focused on use of PEI coated magnetic gene vector to deliver the malaria DNA vaccine for *in vitro* and *in vivo* applications.

Many approaches have been made in magnetic gene complex's design for *in vitro* and *in vivo* gene delivery. The limitations of the presented studies can be summarized as follows.

No magnetic gene delivery studies are published for targeting malaria DNA vaccine delivery *in vitro* or *in vivo* specifically to investigate the effect of SPIONs/PEI complexation conditions on the malaria gene transfection efficiency.

The route of vaccine delivery is one of several factors that determine the type and efficiency of immune responses elicited by DNA vaccination. Different studies have been done in this field in order to detect the type of immune response induced by naked DNA after the different routes of administration. However, there is no specific study has compared the type and potency of immune response elicited by magnetic gene complexes after different injection routes.

The stability of magnetic particles in the biological media played a critical role in producing gene vectors with narrow size distributions and high stability. A few studies on magnetic nanoparticles stability for biomedical applications have been achieved, although most of them have investigated the stability of bare particles without any coating layers.

The present work is motivated by the above limitations of previous studies and also the increasing need for stable gene delivery systems using magnetic particles for *in vitro* and *in vivo* applications. Thus, including these sections could provide better and comprehensive strategies to develop a magnetic malaria gene delivery system. This system is not only stable in different cell media but also incorporates cell-binding ligands to combine intrinsic transfection activity with receptor-mediated cellular uptake mechanisms.

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CHAPTER 3

Superparamagnetic Nanoparticles for Effective Delivery of Malaria DNA Vaccine

3.1 Introduction

Superparamagnetic iron oxide nanoparticles (SPIONs) have attracted significant attention in gene delivery applications because of their relatively low toxicity, low cost of production, ability to immobilize biological materials on their surfaces, and the potential for direct targeting using external magnets. Magnetofection originated from the concept of magnetic drug delivery in the late 1970s, with the technique demonstrating applicability in gene delivery with viral and non-viral vectors (Scherer, Anton et al. 2002). Magnetic particles have proven their feasibility to elevate any gene delivery vector, while the duration of the transfection process can be significantly reduced down to 10 min, compared to 4 h incubation required with standard protocols (Scherer, Anton et al. 2002). Thus, magnetofection is an appropriate tool for rapid and specific gene transfection with low dose *in vitro* and site-specific *in vivo* applications (Plank, Scherer et al. 2003; Dobson 2006).

PEI polymer is known to form cationic complexes that interact non-specifically with negatively charged DNA and enter the cell via endocytosis (Boussif, Lezoualch et al. 1995; Behr 1997; Arsianti, Lim et al. 2010; Arsianti, Lim et al. 2010), with the ratio of PEI nitrogen to DNA phosphate (N/P) influencing the transfection efficiency and toxicity to transfected cells (Zhao, Chen et al. 2009). The use of PEI-coated SPIONs for gene delivery has been shown to increase the efficiency of gene delivery since the complexation and condensation of DNA with PEI offer good protection from degradation by nucleases (Arsianti, Lim et al. 2010; Arsianti, Lim et al. 2010), while the particles can also be magnetically directed to the specific target site *in vivo* (Kievit et al. 2010). A main

challenge in using nanoparticles is the formation of aggregates, since the transfection functions such as the endocytosis rate, cytotoxicity, and velocity of cytoplasmic movement are determined by the size of the gene vector (Prabha et al. 2002). Rejman et al. (2004) showed that 50–100 nm beads could be internalized into cells within 30 min. In the case of magnetic nanoparticles, the magnetic force that plays an important role in enhancing gene delivery is also affected by the particle size (Prabha, Zhou et al. 2002).

Recent studies have investigated the effects of size and surface charge of the magnetic vectors with PEI (Wang, Zhou et al. 2009; Wang et al. 2009), and the arrangement of SPIONs/PEI/DNA vectors on the efficiency of gene delivery (Arsianti, Lim et al. 2010; Arsianti, Lim et al. 2010). These studies have utilized genes encoding fluorescent proteins to elucidate the cellular entry mechanisms and the uptake of the vectors. Here, the magnetic targeting of a gene vector was investigated *in vitro* to identify approaches to increase the efficiency of delivery of the malaria DNA vaccine. Successful outcomes would suggest an approach to overcome the challenges associated with the poor immunogenicity of current malaria DNA vaccines. This study focuses on the effects of complexation pH (acidic and neutral conditions) on preparation of SPIONs/PEI vectors and the resulting effect on particle size, surface charge, and ability to condense DNA. Acidification during vector preparation was shown previously to help reduce the extent of particle aggregation (Wang, Zhou et al. 2009), an effect also noted in this study. In this work, an open polymeric structure at low pH condition is proposed to be preferable for binding and protecting DNA during transfection, resulting in significantly higher gene expression with comparable or less cytotoxicity than the leading non-viral reagent.

3.2 Materials and methods

3.2.1 Materials

Polyethylenimine with an average molecular weight (PEI, MW: 25 kDa, branched) and trisodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$) were purchased from Sigma Aldrich. RPMI 1640 medium (GIBCO), 0.05% trypsin-EDTA, L-glutamine, penicillin/ streptomycin, foetal calf serum, and Lipofectamine 2000 (Gibco-BRL, Gathersburg, MD) were supplied by Invtrogen (Carlsbad, CA). Fe(III) chloride ($FeCl_3$.6H20) and Fe(II) chloride

($FeCl_2$.7H2O) were purchased from Ajax Finechem and Ajax Chemicals, respectively. Mammalian expression vector VR1020 (Vical Inc., San Diego, CA), plasmid VR1020-PyMSP1$_{19}$, and COS-7 cell lines (African green monkey kidney cells) were kindly provided by Prof. Ross Coppel's group (Department of Microbiology, Monash University, Australia). The VR1020-PyMSP1$_{19}$ plasmid was amplified in *Escherichia coli* (strain DH5α) and purified using an endotoxin-free Mega-prep plasmid kit (Qiagen) according to the manufacturer's instruction. Arrays of permanent magnets of neodymium iron boron (Nd–Fe–B) in the format of a 6-well plate were used for *in vitro* transfection experiments. The magnets were circular disc Nd–Fe–B magnets (diameter 25 mm, height 5 mm) glued onto the bottom of a 6-well plate.

Methods

3.2.2 Synthesis and characterization of SPIONs

The synthesis of superparamagnetic iron oxide nanoparticles (SPIONs) was done by alkaline co-precipitation of Fe (III) chloride ($FeCl_3$ .6H_2O, Ajax Finechem) and Fe (II) chloride ($FeCl_2$.7H_2O, Ajax Chemicals) (1:2 molar ratios) (He et al. 2007) in aqueous solution in the presence of trisodium citrate ($C_6H_5Na_3O_7$.2H_2O, Sigma Aldrich) as an electrostatic stabilizer, with the chemical reaction given in:-

Eq: $Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O$

In brief, iron salts, ferric chloride (0.005 mol) and ferrous sulfate (0.0025 mol) were dissolved in 20 ml deionized (DI) water and the precipitation was performed by dropwise addition of iron salt solutions to the mixed sodium hydroxide solution (20 ml, 1.5 M, including 0.005 mol trisodium citrate) under vigorous stirring (1500 rpm) for 1 h at 80°C. Oxygen was removed from the solution by flowing N_2 gas through the reaction medium in a closed system during the synthesis reaction. The resulting black precipitates were collected and removed from solution by applying an external magnet and then washed four times, first with de-ionized (DI) water, twice with ethanol, and finally with DI water to remove excess ions and salts from the suspension. The washed precipitate was then dispersed in 20 ml DI water. Zetasizer Nano ZS (Malvern Instruments Ltd., UK) was used

to determine the hydrodynamic diameter and zeta potential of these particles in suspension, while transmission electron microscope (TEM CM20 Philips) was used to confirm the size and morphology of dry particles. X-ray powder diffraction (XRD) (Philips 1140 PW diffractometer with nickel-filtered Cu Kα radiation (λ = 1.5405 A°) was used to determine the crystallinity and phase of iron oxide particles. Magnetic saturation was measured using a vibrating sample magnetometer (VSM, RIKEN DENSHI) under a magnetic field of up to 15 kOe at the room temperature.

3.2.3 Preparation of plasmids DNA

The VR1020-PyMSP1$_{19}$ plasmid (5234 base pairs and relative molecular mass of 3.177×10^6 Daltons (g/mol)) (Appendix A (Figure A.1)), was amplified using *Escherichia coli* DH5α. A single colony of *E. coli* harbouring plasmid VR1020-PyMSP1$_{19}$ was picked out from a freshly streaked selective plate and inoculated in a 10 ml starter culture medium (LB broth containing 10 g NaCL, 10 g Bacto Tryptone, 5 g yeast, and 100 µg/ml of Kanamycin) for 8 h at 37 °C with vigorous shaking at 200 rpm. The starter culture was then diluted into 1000 ml of LB medium and incubated overnight at 37 °C with vigorous shaking of 200 rpm. The plasmids VR1020-PyMSP1$_{19}$ was purified from *E. coli* cells using an endotoxin-free QIAGEN Mega plasmid purification kit (QIAGEN) according to the manufacturer's protocol.

3.2.4 Preparation of SPIONs/PEI/DNA complexes

The iron oxide suspension (0.1 mg/ml) was mixed with 10% (w/v) PEI solution (25 kDa branched polyethyleneimine), with PEI/Fe mass ratios (R) of 0.6, 0.8, 1, 2, 3, 5, 10, 15, 20, 25, and 30, during which they were sonicated for 1 min. The SPIONs/PEI complexes were dialyzed using Spectra/Por® membranes (MWCO = 12,000 – 14,000) with deionized water for 3 days to remove any unbound / excess PEI. Following the method of (Wang, Zhou et al. 2009) to disperse the aggregates of SPIONs/PEI complexes, each mixture was acidified to pH 2.0 using 0.5 mol/l HCl and kept at this pH for 10 min to stabilize. After 10 min, each sample was divided into two aliquots: the pH of one part was increased to 4.0

(referred to as SPIONs/PEI-A) while the other part was neutralized to pH 7.0 (referred to as SPIONs/PEI-N) using 0.5 mol/l NaOH. The average hydrodynamic diameter and the zeta potential of the samples were determined using a Malvern Zetasizer ZS (Malvern Instruments Ltd., UK).

SPIONs/PEI complexes in the mass ratio (R) of 10 were mixed with plasmid DNA encoding VR1020-PyMSP1$_{19}$ gene at different N/P ratios (i.e. the molar ratio of PEI nitrogen to DNA phosphate) using a DNA concentration of 10 μg/ml in a phosphate buffer (PBS, pH 7.4). The average hydrodynamic diameter, zeta potential, and the stability of SPIONs/PEI/DNA complexes at PEI: SPIONs ratio of 10 and different N/P ratios were determined for SPIONs/PEI-A/DNA and SPIONs/PEI-N/DNA.

3.2.5 DNA binding assay

The DNA binding capabilities of SPIONs/PEI-A/DNA and SPIONs/PEI-N/DNA were determined using 0.8% agarose gel electrophoresis. SPIONs/PEI-A and SPIONs/PEI-N with VR1020-PyMSP1$_{19}$ plasmid were formed at N/P ratios of 0.5 to 30. In each case, the appropriate amount of SPIONs/PEI was mixed with 0.5 μg plasmid DNA in 20 μl PBS buffer. These solutions were incubated at 37 °C for 30 min and mixed with 1 μl of the loading dye (bromophenol blue/ xylene cyanol) solution before loading into agarose gel (0.8% agarose in Tris-borate EDTA buffer containing 5 μl ethidium bromide). Electrophoresis was carried out at 60 V for 90 min. The DNA bands were visualized with a UV illuminator.

3.2.6 Cell cultures and treatments

COS-7 monkey kidney cell lines were cultivated in the complete RPMI 1640 medium (Roswell Park Memorial Institute medium) supplemented with 10% fetal calf serum, 2 mM of L-glutamine, 100 μg/ml streptomycin and 100 μg/ml of penicillin at 37°C in a gassed incubator with 5% CO_2 before transfection. All incubations were performed under these conditions. COS-7 cells at a density of 2×10^5 per well in a 6-well plate were seeded a day before magnetofection. SPIONs/PEI-A/DNA and SPIONs/PEI-N/DNA complexes were

prepared with a fixed amount of plasmid DNA encoding VR1020-PyMSP1$_{19}$ gene (1 μg) for each well plate and incubated in PBS buffer for 30 min at room temperature. When the cells were 80% confluent (degree of coverage), the medium was removed. The SPIONs/PEI/DNA complexes were mixed into serum-free medium and then added into the 6-well plates with a neodymium-iron-boron magnet positioned under each well for 2 h. Lipofectamine 2000^{TM} was used as a positive control according to the manufacturer's instructions. After 5 h, the serum free-medium containing nanoparticles were removed from each well and replaced with 2 ml of fresh medium containing 10% serum was added and incubated for 48 h at 37 °C under 5% CO_2. Gene delivery mediated by SPIONs/PEI/DNA complexes in this work was examined with or without the application of the magnetic field during the transfection process. After 48 h, COS-7 cells were washed with PBS buffer, and trypsin was added to the collection of the cells to evaluate the transfection efficiency using the western blot technique. The harvested COS-7 cell pellets were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis under reduced conditions and then electrophoretically transferred to PolyScreen polyvinylidene difluoride transfer membrane. Subsequently the membrane was probed with antiserum and horseradish peroxidase-conjugated antibody, respectively, and then visualized by using Lumi-light western blotting substrate. The molecular size of the protein was estimated from the distance travelled by protein through the gel. The intensities of the fluorescence bands associated with the immunoblotted proteins were quantified as the total pixels within a defined boundary drawn on the image by Image J (Version 1.41, National Institutes of Health, USA). All experiments were performed at least in triplicate. The cells subjected to magnetofection and normal PEI transfections were also observed using fluorescence microscopy.

3.2.7 Evaluation of cell viability

Evaluation of the cytotoxicity was performed via MTT assay in COS-7 cells. The cytotoxicity of SPIONs/PEI/DNA complexes was evaluated in comparison with lipofectamine-DNA complex and naked SPIONs solutions of 0.1, 0.5 and 1 mg/ml. Cells were seeded at a density of 2×10^4 cells / well on a 96-well microtiter plate at 37 °C in 5%

CO_2 atmosphere overnight. SPIONs/PEI/DNA complexes, DNA/Lipofectamine 2000 complex, and naked SPIONs solutions were added for further 24 h incubation. The control well was a culture medium with no particles. 5 µl of MTT dye solution at a concentration 5mg/ml in phosphate buffer was added to each well plate. The plate was incubated for a further 4 h at 37 °C in 5% CO_2. After 4 h incubation the medium was removed from each well and the cells were rinsed with phosphate buffer. 100 µl of dimethylesulphoxide (DMSO) was added to each well with incubation for 1 h. The absorbance of the formazan product formed by viable cells was read at wavelengths of 570 nm and 690 nm simultaneously using a microplate reader (Magellan, Tecan, Austria). The relative cell viability (%) related to the control well containing the cell culture medium without nanoparticles was calculated as viability (%) = (Means absorbance of sample / Means absorbance of control) × 100%.

3.3 Results and discussion

3.3.1 Characterization of SPIONs/PEI/DNA complexes

The average diameter of the SPIONs was around 10 ± 3 nm, while the measured hydrodynamic diameter indicating particle size in suspension was predominantly around 85 ± 5 nm. The SPIONs were negatively charged with zeta potential of around –42 ± 2 mV. Both SPIONs and SPIONs/PEI particles showed magnetization of > 65 emu/g under 15 kOe applied magnetic field at room temperature with 0.01 emu/g remanance, indicating superparamagnetic behavior (Appendix A. **(**Figure A.2)), while X-Ray Diffraction pattern indicated the magnetite (Fe_3O_4) phase (Appendix A. **(**Figure A.3)).

When polymer was added to the nanoparticles, the adsorption of PEI polymer onto SPIONs occurred by electrostatic attraction between the negatively charged SPIONs (due to the presence of carboxylic groups) and the positively charged PEI. After PEI adsorption, the surface charge of SPIONs was converted from highly negative (–42 ± 2 mV) to positive (16 ± 2 mV), while the particle size increased to approximately 555 ± 20 nm due to polymer adsorption on the surface of magnetic particles and aggregation between particles. The

extent of aggregation may be attributed to the lack of repulsive forces between the slightly positive charged particles at around pH 6.8 – 7.0.

To reduce aggregation, acidification step for SPIONs/PEI complexes was done at pH 2.0, as the protonation of the amine groups on PEI under acidic condition would induce electrostatic repulsion between the various amine groups, leading to better dispersion (Vonzelewsky, Barbosa et al. 1993; Wang, Zhou et al. 2009). After reducing the pH to 2.0, the complexes were re-suspended at pH 4.0 and 7.0 to investigate the effects of pH on the properties of the gene vectors. At low pH, the mutual charge repulsion between the protonated amine groups could lead to more stretching of the polymer molecular chains, while at neutral pH the polymer tends to contract because of the hydrogen bonding between the amine groups (Vonzelewsky, Barbosa et al. 1993; Yankov et al. 2009). As proposed by (Vonzelewsky, Barbosa et al. 1993), polyethylenimine at pH 7.0 exists as a stiff stable structure with six-membered rings due to the hydrogen bonding between the neighboring free and charged amine groups.

Figure 3.1 showed the TEM images of as-synthesized SPIONs and SPIONs/PEI (R=10). As a result of the acidification step, the average hydrodynamic sizes of SPIONs/PEI complexes were shown to decrease significantly from 555 ± 20 nm to around 100 nm – 150 nm at different mass ratios of PEI to SPIONs (Figure 3.2A). There was no significant difference in the average hydrodynamic diameters of the complexes for SPIONs/PEI-A and SPIONs/PEI-N. This implied that the de-aggregation process was irreversible when the pH was increased from 2.0 to 4.0, or even to 7.0 in agreement with Wang et al. (2009). The zeta potentials of SPIONs/PEI complexes were measured under both pH conditions at a SPIONs concentration of 0.1 mg/ml. The SPIONs/PEI-A complexes showed slightly higher positive charges than SPIONs/PEI-N complexes (Figure 3.2B) because of the protonation of amine groups under an acidic condition.

The dual roles of PEI on SPIONs were to increase their stability in aqueous solution and at the same time to act as a condensing agent for pDNA. The extent of DNA condensation with PEI depends on a number of factors such as the ratio of PEI nitrogen to DNA phosphate (N/P), PEI molecular weight, and the structure of PEI. The average hydrodynamic sizes of SPIONs/PEI-A/DNA and SPIONs/PEI-N/DNA (Figure 3.3A) were relatively similar to the size of complexes prior to DNA addition, indicating the stability of

the vectors. At N/P ≤ 1, the measured hydrodynamic size appeared to be larger while the surface charge was slightly negative due to the presence of DNA in excess of PEI, which could induce aggregation between the particles. With a higher N/P ratio, the charge became increasingly positive, providing enough repulsion to prevent further aggregation. The naked VR1020-PyMSP1$_{19}$ molecules with the negative zeta potential of –59 mg were condensed onto the SPIONs/PEI to form complexes by increasing the N/P ratio to reach a maximum zeta potential value of around +30 mV at N/P ratio ≥ 15 (Figure 3.3B). Despite the fact that SPIONs/PEI complexes at pH 4.0 should have more capability to condense DNA than the complexes at pH 7.0 because of their more positive charge, there was no significant difference between the zeta potentials of the complexes especially at N/P ≥ 15 (Figure 3.3B). This implied that as the N/P ratio of ≥ 15, the complexes have reached the saturated charge ratios of the cationic polymer to DNA.

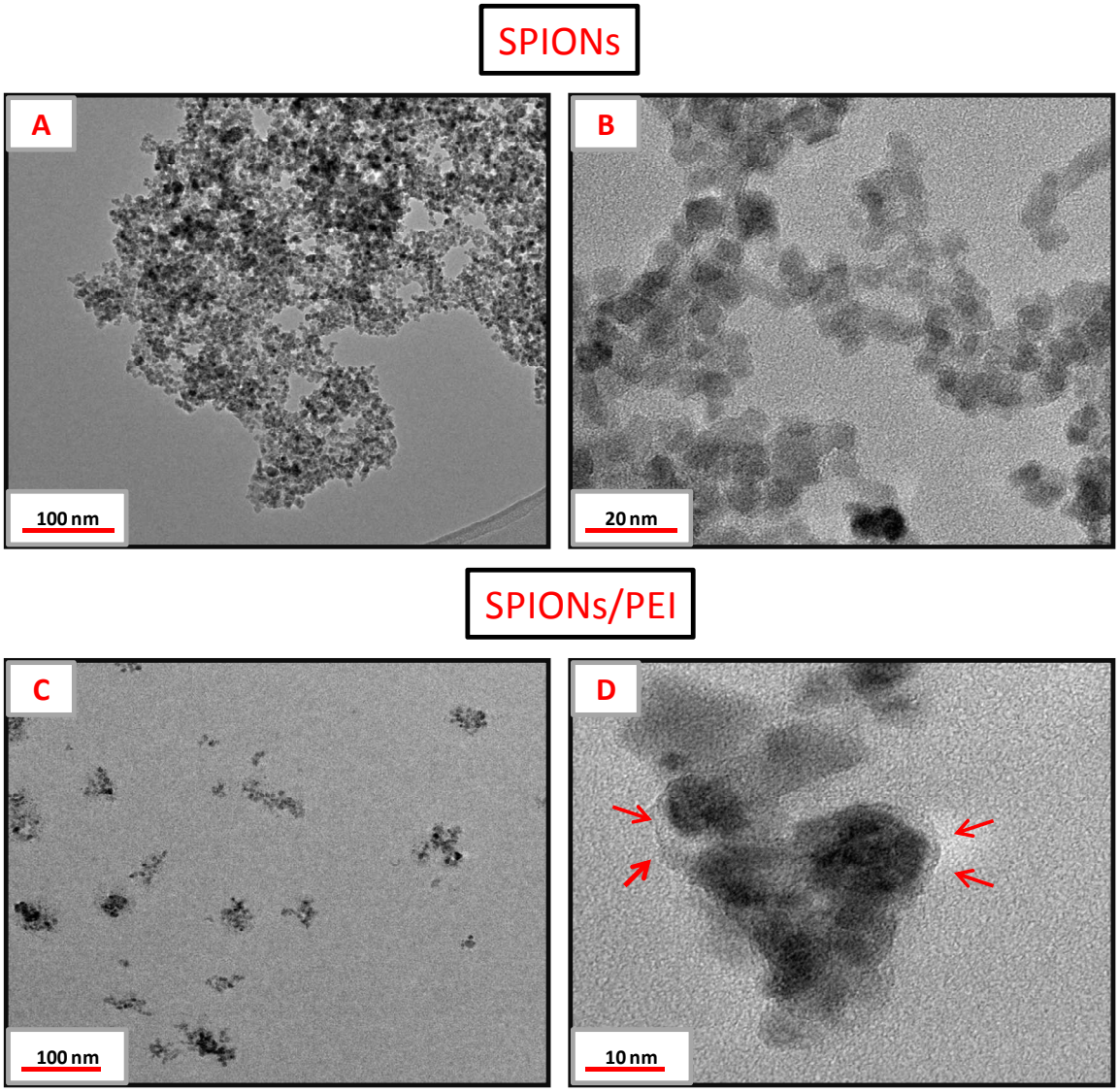


Figure 3.1: TEM images of (A & B) as-synthesized SPIONs and (C & D) SPIONs/PEI (ratio = 10) at pH 4 displaying better dispersion (arrows indicating layer of adsorbed PEI).

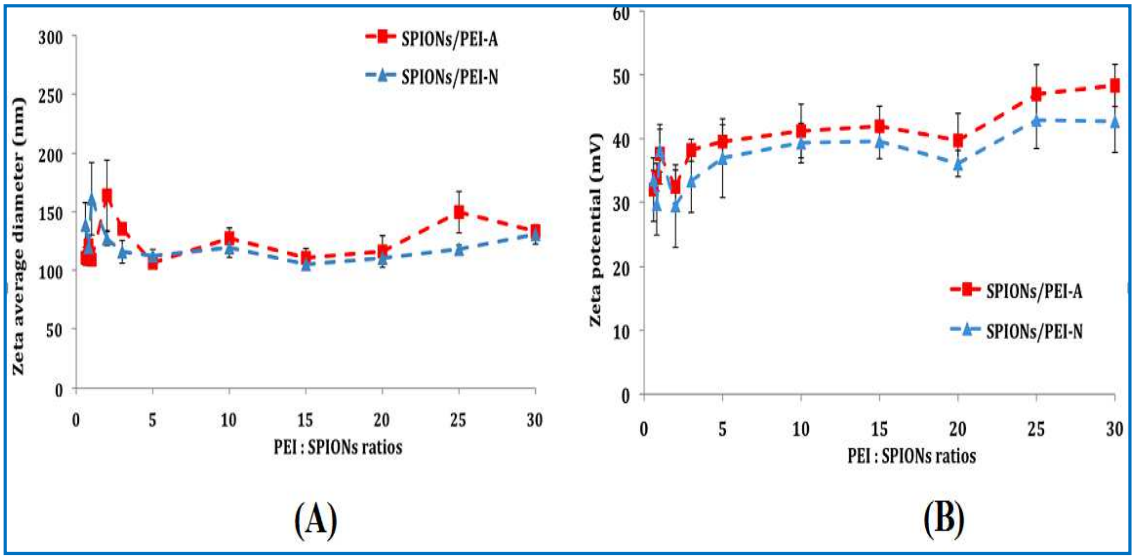


Figure 3.2: (A) Hydrodynamic diameter (nm) of SPIONs/PEI-A and SPIONs/PEI-N complexes; (B) zeta potential (mV) of SPIONs/PEI-A and SPIONs/PEI-N complexes with different PEI: SPIONs mass ratios. All data measured at least in triplicate.

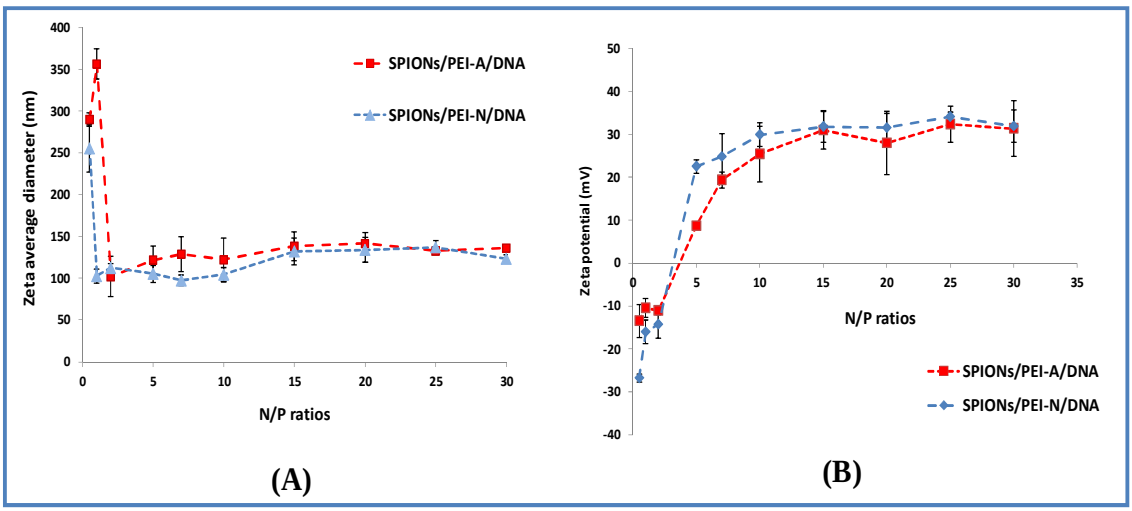


Figure 3.3: (A) Effects of N/P ratios on hydrodynamic diameter (nm) and (B) zeta potential (mV) of SPIONs/PEI/DNA complexes. All data measured at least in triplicate.

3.3.2 DNA binding assay

The complexation degree of DNA with cationic PEI was confirmed via agarose gel electrophoresis (Figure 3.4). SPIONs/PEI-A/DNA complexes with low molar ratios of PEI to DNA at N/P < 5 showed the intensity of ethidium bromide fluorescence in the application slots without migration of DNA (no band appeared toward anode) (Figure 3.4A). This indicated an incomplete condensation of DNA with PEI at low N/P because of an insufficient amount of PEI to condense DNA completely. Further increase of PEI at N/P ratio > 7 led to a loss of fluorescence intensity in the loading wells, which then completely disappeared. The data indicated that at N/P ratio > 7, ethidium bromide could not intercalate inside the DNA or even reduce their approach to the DNA structure, since all DNA molecules were wrapped within the PEI molecules. Due to the mutual charge repulsion between the amine groups, the six ringed structure of branched PEI polymer at low pH is proposed to be more stretched or open, as shown in Figure 3.5 (Vonzelewsky, Barbosa et al. 1993). The expansion of PEI molecular chain at acidic condition probably enabled the complexes to capture more DNA molecules. Thus, the behavior of branched PEI might increase the amount of genetic material that the particles could carry, not only by condensing the DNA molecules on the surface of the particles, but also by embedding the molecules inside the structures. This extended structure of PEI molecules should be able to absorb more protons and to form an electrostatic (and physical) barrier to DNases responsible for degradation of the transfected DNA (Godbey et al. 2000).

On the other hand, SPIONs/PEI-N/DNA showed the relatively higher intensity of fluorescence for N/P ratios < 10 (Figure 3.4B). Decreasing the fluorescence intensity was observed in the slots with increasing N/P ratios, although the intensity only completely disappeared at N/P ≥ 25. Hence, for this system, ethidium bromide existed in large excess from the gel into the DNA complexes at almost all N/P ratios except at the N/P ratios of 25 and 30 where no fluorescent bands ascribed to the un-complexed plasmid DNA could be observed. These results indicated the lower binding ability between DNA and SPIONs/PEI complexes at pH 7.0, possibly due to the stiff-membered rings

structure of the polymer under neutral condition (Vonzelewsky, Barbosa et al. 1993).

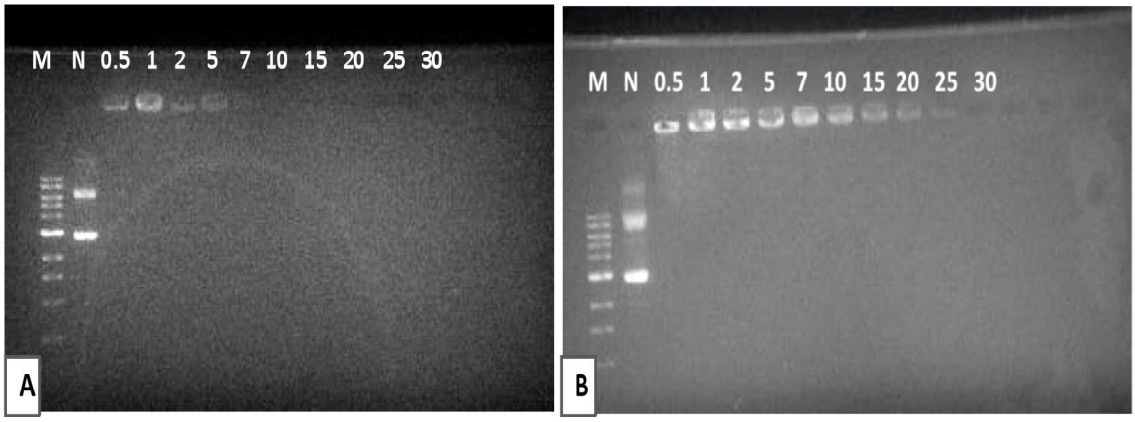


Figure 3.4: Effects of N/P ratios on the stability of (A) SPIONs/PEI-A/DNA; (B) SPIONs/PEI-N/DNA. Lane M: λ H/E molecular weight size marker. Lane N: plasmid VR1020-PyMSP1$_{19}$. Lanes 0.5-30 correspond to N/P ratios.

3.3.3 Transfection efficiency

Western blot analysis of the expression of VR1020-PyMSP1$_{19}$ in COS7 cell line is shown in Figure 3.6. Numerical analysis of western blot detection for VR1020-PyMSP1$_{19}$ (Figure 3.7) confirmed that the SPIONs/PEI-A/DNA complexes showed dramatically higher gene expression than SPIONs/PEI-N/DNA, especially with magnetofection. The SPIONs/PEI-A/DNA complexes at N/P ratios of 10 and 15 had the highest differences in transfection efficiency compared to SPIONs/PEI-N/DNA complexes at the same ratios. Gersting et al. found that the highest amounts of DNA led to maximum transfection efficacy, while decreasing DNA concentrations led to a rapid decrease of the complexes' ability for gene transfection (Gersting, Schillinger et al. 2004). In this case, the gene expressed at N/P ratios >10 for SPIONs/PEI-A/DNA complexes were of similar magnitude as at N/P = 10. Presumably stronger complexation of DNA with highly protonated amine groups of PEI at acidic pH could cause gene blocking which would not much improve the transfection efficiency at high N/P ratio.

Arsianti et al.(Arsianti, Lim et al. 2010; Arsianti, Lim et al. 2010) observed low gene expression when DNA was condensed on the surface of SPIONs/PEI under physiological

pH (neutral pH), as the unprotected DNA from the vector was degraded with nuclease before transport into cells. This finding also agreed with our observation for SPIONs/PEI-N/DNA complexes, since the PEI existed as a stiff stable structure with six-membered rings with the DNA possibly present at the surface. In contrast, SPIONs/PEI-A complexes could entrap the DNA molecules within the branched structure of the polymers, because of protonation under acidic condition that expanded the polymeric network (Figure 3.5). This type of structure also prevented DNA from early release from the complexes before entering the cells, while the extended structure of the PEI molecules should be able to absorb more protons leading to fast endosome swelling known as the “proton sponge” effects, which protected the DNA from degradation (Boussif, Lezoualch et al. 1995; Behr 1997). These results verified that SPIONs/PEI complexes prepared under acidic condition were more useful as gene vectors due to the structure of branched polymers that increased the amount of entrapped genetic material and subsequently, gene expression.

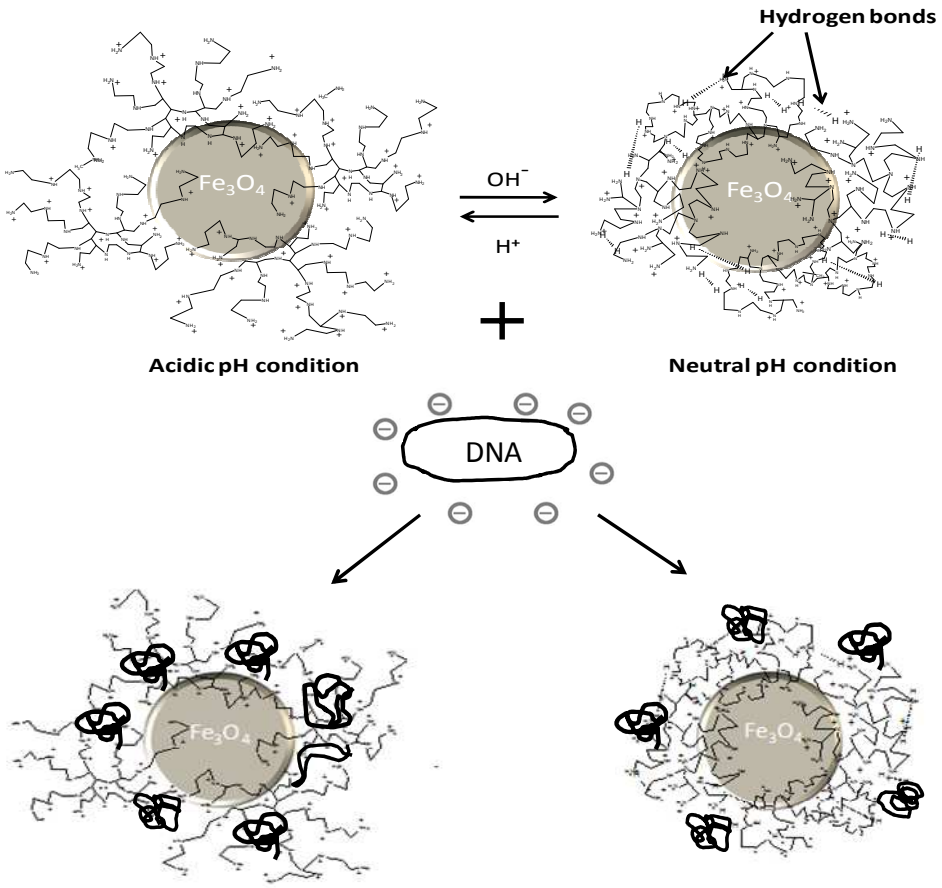


Figure 3.5: Shematic demonstrating PEI structure under acidic and neutral pH conditions, with a relatively branched structure due to mutual charge repulsion between the amine groups under acidic condition and stiff structure under neutral pH condition (Vonzelewsky, Barbosa et al. 1993), and possible entrapment of DNA in the responsive structure.

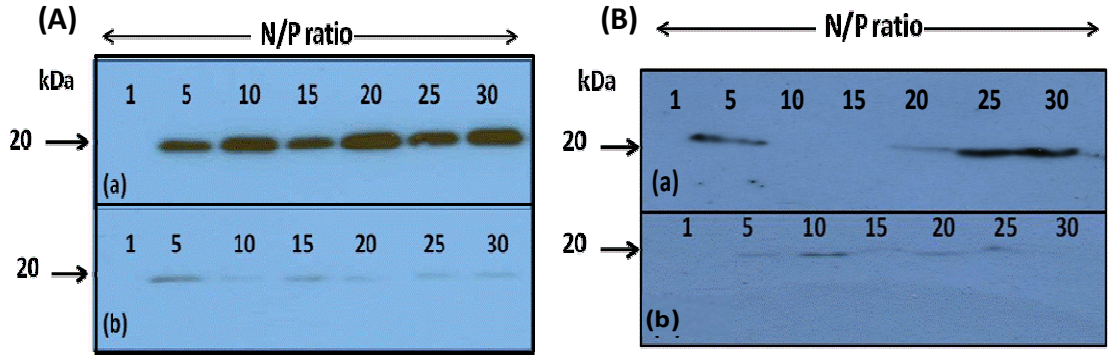


Figure 3.6: Western blot detection of (A) SPIONs/PEI-A/DNA; (B) SPIONs/PEI-N/DNA. Lane 1, 5, 10, 15, 20, 25 and 30 correspond to different N/P ratios of SPION/PEI/DNA complexes: (a) With magnet; (b) Without magnet.

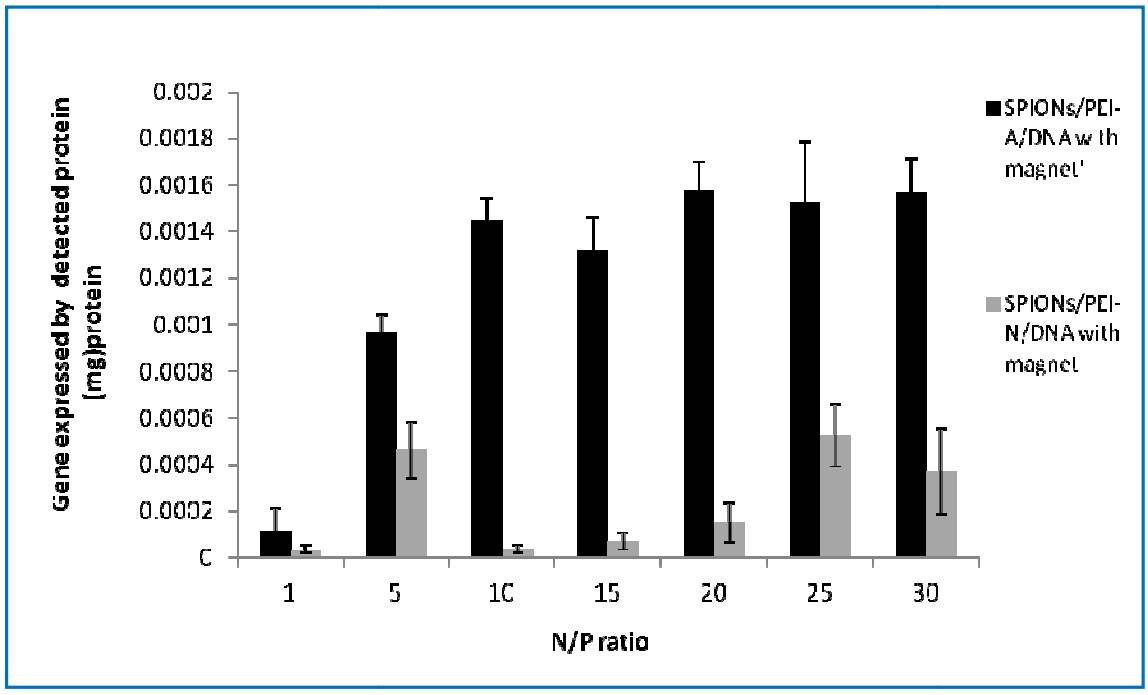


Figure 3.7: Densitometry results for PyMSP1$_{19}$ produced by SPIONs/PEI-A/DNA and SPIONs /PEI-N/DNA complexes with the application of the magnetic field during the gene transaction process. Experiments were performed at least in triplicate.

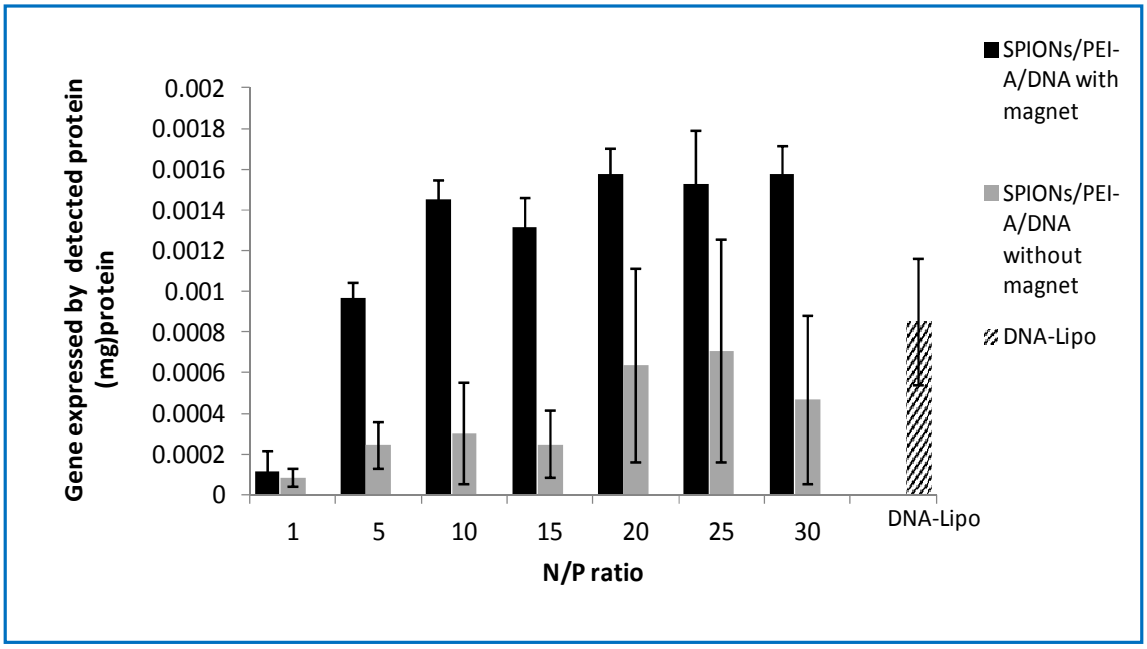


Figure 3.8: Densitometry results for PyMSP1$_{19}$ produced by SPIONs/PEI-A/DNA complexes with or without application of the magnetic field during the gene transaction process, and by Lipofectamine 2000 reagent. Experiments were performed at least in triplicate.

3.3.4 Effect of magnetofection on Malaria gene expression PyMSP1$_{19}$ in COS-7 cell line

The main concern for insufficient gene concentration on the target tissue for nonviral gene transfection is the insufficient accumulation of genes at the cell surface *in vitro*, and targeting gene at the specific site *in vivo*. Magnetofection can be used to accumulate the magnetic gene vector on the target tissue by applying an external magnetic field (Plank, Schillinger et al. 2003). To investigate the impact of magnetofection in the malaria gene delivery, gene transfections of both SPIONs/PEI-A/DNA and SPIONs/PEI-N/DNA complexes were compared with or without the use of neodymium-iron-boron magnets positioned under each well for about 2 h. Under the influence of magnetic field on COS-7 cells, SPIONs/PEI/DNA complexes showed noticeably higher transfection efficiency compared to the transfection without magnetic field (Figure 3.6A). Numerical analysis of western blot detection for expression of VR1020-PyMSP1$_{19}$ in the SPIONs/PEI-A/DNA complexes (Figure 3.8) confirmed the significant enhancement at almost all N/P ratios,

possibly because the magnetic field drew the complexes onto the surface of the cells leading to an increase in their cellular uptake. The gene expression of SPIONs/PEI-A/DNA complexes without magnetic field was lower than the transfection with Lipofectamine reagent, while the reverse was true when the magnetic field was applied. In addition, magnetofection also showed an efficient gene delivery with low vector dose for plasmid DNA at a shorter incubation time which greatly improved its dose–response profile (Scherer, Anton et al. 2002; Gersting, Schillinger et al. 2004). In this work, although a relatively low dose of DNA of about 1 μg was used, high gene transfection with magnetofection was achieved in comparison to transfection with Lipofectamine. Application of magnetic field drastically enhanced the efficiency of gene transfection, with the effect more pronounced with increasing N/P ratios although the sizes of the complexes were similar (~150 nm). When the N/P ratio was 1, low gene expressions with and without magnetofection were detected, possibly due to the lower DNA binding capability at this ratio. However with the increasing PEI amount, gene expression with magnetofection was found to be more effective than transfection without magnetic field. Although particle size is undoubtedly important for high gene expression, this study indicated that the presence of charged polymers protecting and binding the DNA with the application of external magnetic field could play a combined role to achieve elevated gene expression.

Evidence of the high protein expression enhancement by magnetofection was also observed by fluorescence microscopy using COS-7 cells transfected with YFP gene combined with two types of vectors: SPIONs/PEI and PEI polymer alone with N/P ratios of 5 and 10, respectively. The observation with fluorescent microscopy (Figure 3.9) indicated that COS-7 cells transfected with SPIONs/PEI/DNA complexes at N/P ratios of 5 and 10 exhibited significantly higher fluorescence compared with cells transfected with PEI/DNA alone. The higher level of gene expression at N/P of 10 may be attributed to more condensation of DNA into positively charged particles that increased the rate of their uptake by the cells.

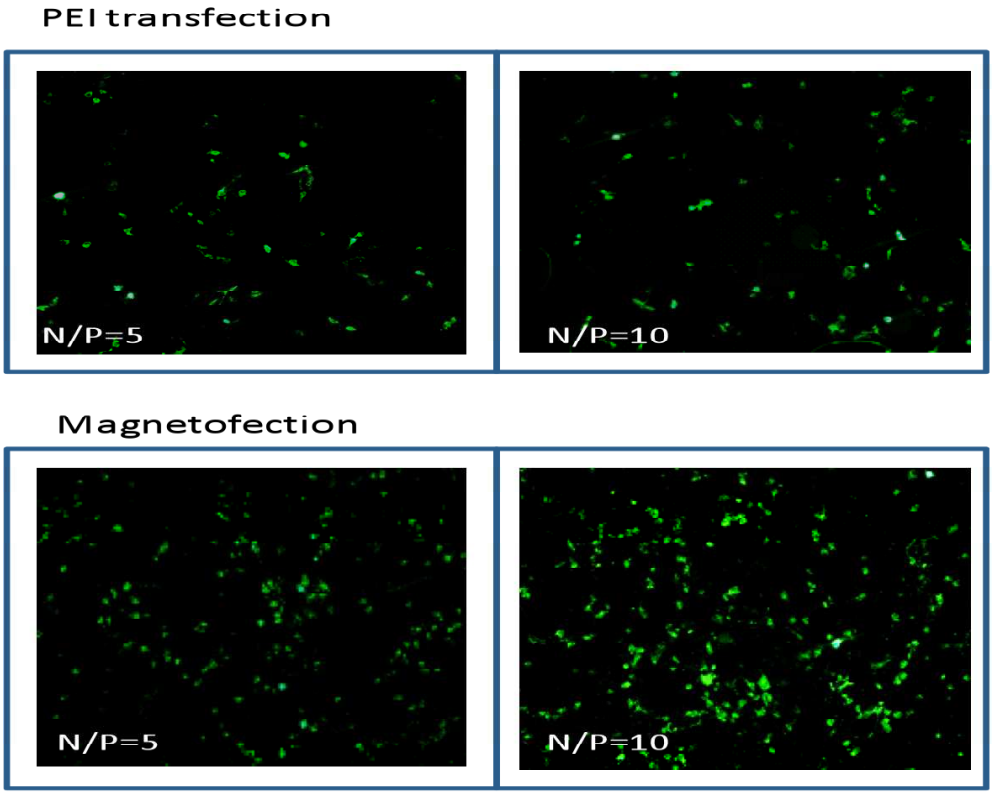


Figure 3.9: Expression of yellow fluorescent gene (YFP) in COS-7 cells. The upper row shows the effect of the PEI polymer transaction alone; the lower row shows the effect of magnetofection with SPIONs /PEI-A/DNA complexes.

3.3.5 *In vitro* cytotoxicity assay

In vitro cytotoxicity of SPIONs/PEI/DNA complexes and superparamagnetic iron oxide solution were assessed with different concentrations onto the COS-7 cell cultivated in RPMI medium. The influence of SPIONs/PEI/DNA on the COS-7 cell viability after 24 h exposure (Figure 3.10) showed that there was no significant difference between SPIONs/PEI-A/DNA and SPIONs/PEI-N/DNA complexes, with more than 60% of the cells remaining viable. This indicated that the pH of complexation of these gene complexes induced no statistically significant difference in terms of effects on cell viability. The toxic effects of the complexes on cell viability were mainly associated with a strong net positive charge of the complexes due to presence of PEI polymer, leading to strong interactions of PEI with the cell surface causing disruption to the cellular plasma membrane (Florea et al.

2002; Petri-Fink et al. 2008; Zintchenko et al. 2008). Figure 3.10 showed that cell viability was slightly less than 80% at N/P = 1 due to the low concentration of PEI that reduced toxicity however the cell viability decreased to around 60% with increasing N/P ratios, with no significant statistical difference in cell viability at N/P ratios above 10, in agreement with previous works (Wang, Zhou et al. 2009; Arsianti, Lim et al. 2010). The presence of SPIONs seemed to reduce the toxicity of PEI in comparison to *in vitro* cell viability studies using similar PEI/DNA systems (Fischer, Li et al. 2003; Zhao, Chen et al. 2009). Notably, more than 83% of cells remained alive when they were incubated with SPIONs solution, reaching 100% viability at 1 mg/ml concentration. The protection against cell toxicity could be due to the presence of citrate groups (Lacava et al. 1999).

Lipofectamine 2000 (cationic liposome) is the most effective non-viral reagent examined that yielded consistently high transfection rates accompanied with slightly higher toxicity (Djurovic et al. 2004). The cytotoxicity of Lipofectamine is due to the four protonatable amines on its headgroup at physiological pH (Dokka et al. 2000). Here no obvious difference was observed in the cell toxicity between Lipofectamine 2000 and SPIONs/PEI/DNA complexes even for N/P ratios > 15. In contrast, SPIONs/PEI/DNA complexes showed dramatically higher gene expression than Lipofectamine, indicating that they were more effective gene transfection agents than Lipofectamine, particularly with magnetofection.

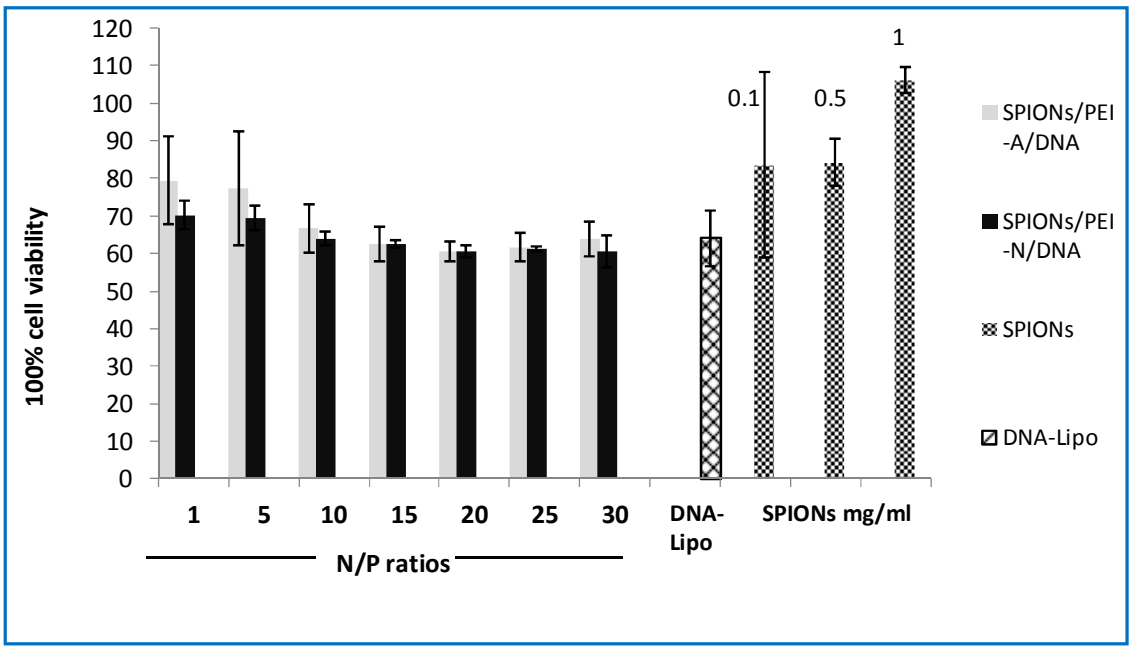


Figure 3.10: Cell viability from MTT assay results of treated COS-7 cells with different complexes. Experiments were performed at least in triplicate.

3.4 Conclusion

The use of magnetofection for the delivery of malaria DNA vaccine encoded merozoite surface protein $MSP1_{19}$ has been investigated *in vitro*. The vectors were composed of SPIONs and PEI complexed under different pH conditions of 4.0 and 7.0. The procedure resulted in stable particles with a narrow size range in aqueous media, rendering them suitable for the gene delivery system. SPIONs/PEI complexes produced under acidic condition showed the best DNA binding and gene transfection efficiency compared with those generated under neutral pH, possibly due to the protonated structure of the branched polymer that entrapped and protected the DNA. The cellular uptake of SPIONs/PEI/DNA also increased dramatically with the application of the external magnetic field during the gene transfection process. In summary, the high transfection potential of these nanoparticles as demonstrated by the expression of $MSP1_{19}$ protein *in vitro* indicated the

possibility of using these vectors with magnetofection technique as an efficient malaria gene $MSP1_{19}$ delivery carrier for *in vivo* application.

CHAPTER 4

On the Efficacy of Malaria DNA Vaccination with Magnetic Gene Vectors

4.1 Introduction

The development of a robust malaria vaccine could be a cost-effective intervention to reduce the transmission of infection and the burden of malaria. Vaccination using DNA has emerged as a potentially practical approach for the prevention of malaria because of its flexibility and ability to prime appropriate immunity (Liu 2011). Of particular interest, the membrane-associated 19-kD COOH-terminal fragment of merozoite surface protein MSP1 molecule ($MSP1_{19}$) is one of the leading malaria vaccine candidates. MSP1 is present in approximately all species of malaria parasites as a major component on the merozoite surface that is carried into red blood cells during merozoites invasion (Hodder, Crewther et al. 1996). Several *in vitro* and in *vivo* studies have shown that $MSP1_{19}$ is the target of protective immune responses against asexual blood stages of malaria parasites (Daly and Long 1995; Kumar, Yadava et al. 1995) and antibodies against $MSP1_{19}$ are thought to act through the direct inhibition of merozoite invasion into red blood cells (O'Donnell, de Koning-Ward et al. 2001).

Although DNA immunization is capable of inducing antigen-specific immune responses, it is now generally accepted that naked (uncomplexed) DNA immunization induces a relatively low level immunity due to different biological barriers that hinder DNA from reaching its destination in the cell nucleus for gene expression. The development of nano-carrier-based DNA vaccine formulation has become one of the most predominant areas of vaccine research (Vijayanathan, Thomas et al. 2002). Different studies have verified that non-viral carrier systems in the nanometer-size range can be successfully used as

transfection agents to deliver nucleic acids to antigen presenting cells for both *in vitro* and *in vivo* applications (Vijayanathan, Thomas et al. 2002). Magnetic particle assisted gene delivery, also known as magnetic transfection or magnetofection, has been proven to improve both the efficiency and the speed of gene delivery to different tissues (Krotz, Sohn et al. 2003).

Notably, the application of magnetofection could induce up to a 360-fold increase in luciferase gene transfer (Krotz, Sohn et al. 2003) compared to conventional transfection methods. The previous studies demonstrate the design of stable magnetic gene vectors for DNA vaccine delivery (Al-Deen et al. 2013), and also a promising proof-of-concept for magnetofection using polyethyleneimine (PEI)-coated superparamagnetic iron oxide nanoparticles (SPIONs) for malaria DNA vaccine delivery platforms *in vitro* (Al-Deen et al. 2011).

Different factors play a critical role in the immune response elicited by particulate gene delivery systems, including route of administration, the amount of antigen, the delivery system, the immunization interval, and adjuvant (Deck et al. 1997; Brice et al. 2007; Mohanan et al. 2010; Parween et al. 2011). The poor immunogenicity of malaria DNA vaccines has attracted considerable interest to develop various complexes and immunostimulatory molecules for malaria DNA vaccine delivery (Liu, Danquah et al. 2010; Al-Deen, Ho et al. 2011), and to induce better immune responses in animal models without the need for an adjuvant (Cherif, Shuaibu et al. 2011; Parween, Gupta et al. 2011). The focus on vaccine administration routes has also increased (Lodmell et al. 2000; Johansson et al. 2004), in order to determine optimal routes to induce a high antibody response that is accepted as of major importance to induce protection against asexual blood-stage malaria infection.

In this study, PEI-coated SPIONs formulation was used to deliver $PyMSP1_{19}$ via mice studies. The first stage of the study was done to determine the optimum PEI/DNA ratio for transfection, while the second stage was to compare efficacy and the type of immune responses generated by these magnetic vectors upon administration. The impacts of different administration routes on the immunogenicity from plasmid DNA vaccinations in homologous (DNA vaccination alone) as well as heterologous (DNA prime-protein boost)

regimens were studied. The understanding gained from this work should help in the development of optimal DNA vaccine delivery protocols for diverse diseases where antibodies play a protective role, including blood-stage malaria.

4.2 Materials and methods

4.2.1 Materials

Polyethylenimine with an average molecular weight (PEI, MW: 25 kDa, branched) and trisodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$) were purchased from Sigma Aldrich. Fe (III) chloride ($FeCl_3$.6H20) and Fe (II) chloride ($FeCl_2$.7H2O) were purchased from Ajax Finechem and Ajax Chemicals, respectively. Mammalian expression vector VR1020 (Vical Inc., San Diego, CA), plasmid VR1020-PyMSP1$_{19}$, were kindly provided by Prof. Ross Coppel's group (Department of Microbiology, Monash University, Australia). The VR1020-PyMSP1$_{19}$ plasmid was amplified in *Escherichia coli* (strain DH5α) and purified using an endotoxin-free Mega-prep plasmid kit (Qiagen) according to the manufacturer's instruction. Permanent magnets of neodymium iron boron Nd–Fe–B (a circular 3mm x 1mm disc magnet with a pull strength of 108 g in special bandage) were used for *in vivo* gene transfection experiments.

Methods

The preparation and characterization of SPIONs and VR1020-PyMSP1$_{19}$ plasmid were done as previously described in the sections (3.2.2 and 3.2.3).

4.2.2 Preparation of gene complexes

To prepare SPIONs/PEI complex, the iron oxide suspension (0.1 mg/ml) was mixed with 10% (w/v) PEI solution (25 kDa branched polyethyleneimine), with PEI/Fe mass ratios (R)=10 and during which they were sonicated for 5 min. The SPIONs/PEI complexes were

dialyzed using Spectra/Por® membranes (MWCO = 12,000 – 14,000) with deionized water for 3 days to remove any unbound / excess PEI.

Plasmid DNA encoding $PyMSP1_{19}$ gene is mixed separately with PEI at N/P (i.e., the molar ratio of PEI nitrogen to DNA phosphate) ratio of 15, SPIONs/PEI at different N/P ratios (10, 15, 20, and 30), suspended in 5% glucose buffer (water containing 5% glucose) (pH 7.4) for characterization and injection. The average hydrodynamic diameter (expressed as dynamic light scattering (DLS)) and the zeta potential of the complexes was determined using a Malvern Zetasizer ZS. Using this technique, the volume size distribution of the aggregates was also determined for SPIONs before and after coating with PEI polymer.

4.2.3 Recombinant $EcPyMSP1_{19}$ protein purification

Recombinant proteins $PyMSP1_{19}$ (Appendix A. **(**Figure A.1)) were expressed in *E. coli* and purified using TALON Metal Affinity Resin (Clontech). Transformed *E. coli* BL21 cells were cultivated in Super broth with Kanamycin (50µg/ml) at 37ºC, and protein expression was induced with 1mM Isopropyl β-D-1-thiogalactopyranoside for 3h. After cell lysis by French Press, the lysate was centrifuged and the supernatant was incubated with TALON for 1h at 4ºC. After two washes with IMAC-5 (immobilized metal ion affinity chromatography with 5mM imidazole), the bound proteins were eluted with IMAC-200 (200mM imidazole). Proteins were analyzed by SDS-PAGE and the concentrations were determined by the Bio-Rad Bradford assay.

4.2.4 Immunization of mice

Female BALB/c mice aged from 6 to 8 weeks were purchased from the Central Animal Services of Monash University and were kept in a specific pathogen free (SPF) environment. They were used at 6–8 weeks of age according to the protocol approved by the Monash University Animal Ethics Committee requirements and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Permit Number

MARP/2011/011). All efforts were made to minimize suffering, and animals were humanely sacrificed under anesthesia.

In the first stage of the study, each group containing 5 mice was intramuscularly immunized through their thigh skeletal muscle at two sites with different formulations of naked DNA, PEI/DNA at N/P (nitrogen in PEI / phosphate in DNA) ratio of 15 and SPIONs/PEI/DNA complexes at N/P ratios of 10, 15, 20, and 30, according to the immunization schedule (3 times at 2-week interval). Mice were prime-immunized at day 0 and two subsequent boosters of SPIONs/PEI/DNA complexes (25 µg of $PyMSP1_{19}$ plasmid/mouse for each immunization) were given at day 14 and 28 (Figure 4.2).

A total volume of 100-200 µl suspended in 5% glucose buffer with the $PyMSP1_{19}$ mass of 25 µg / mouse was used for each immunization. A small neodymium-iron-boron Nd-Fe-B permanent magnet was tightly attached for 1 h onto the surface of the injection site for the groups of mice injected with SPIONs/PEI/DNA complexes with different N/P ratios (Xiang, Bin et al. 2007). Two weeks after the last immunization (2^{nd} boost) (day 42), pooled sera were collected from all the mice for analysis by ELISA.

In the second stage, groups of 6-8 week-old female BALB/c mice (n = 5 per group) were immunized with either SPIONs/PEI/DNA complexes at N/P ratio of 15, or naked DNA in a 5% glucose buffer with $PyMSP1_{19}$ mass of 100 µg / mouse (for each immunization) according to the immunization schedule (3 times at 2-week interval) (Figure 4.3). The animals were prime-immunized by SPIONs/PEI/DNA complexes or naked DNA (either subcutaneously, intradermally, intramuscularly, intraperitoneally, and intravenously) at day 0 and two subsequent boosters of SPIONs/PEI/DNA complexes or naked DNA was given at day 14 and 28 (Figure 4.3). Nd-Fe-B permanent magnet was attached above the injection site with SPIONs/PEI/DNA complexes for 1 h depending on the injection site. Complexes were produced in different final volumes depending on the injection route but with equal dosing DNA (100 µg/dose). The total volumes were 100-200 µl for i.p., i.m., s.c. groups and 100 µl for i.d. group, respectively. Two weeks after the second immunization (1^{st} boost) on day 28 and the last immunization on day 42 (2^{nd} boost), pooled or individual sera were collected from all the mice for ELISA (Figure 4.3).

To test the efficiency of heterologous DNA prime-protein boost strategy in stimulating antibody immune responses against $PyMSP1_{19}$ antigen, two weeks after the final immunization (2^{nd} boost) with SPIONs/PEI/DNA complexes via i.p., i.m., i.d., and s.c. routes, all mice were immunized intraperitoneally with a single dose of 25 µg / mouse of recombinant $EcPyMSP1_{19}$ (*Escherichia coli* $PyMSP1_{19}$) protein emulsified in an incomplete Freund's adjuvant (Figure 4.6A). Purification of recombinant $EcPyMSP1_{19}$ protein is described in detail in section 4.2.3. Pooled sera were collected 2 weeks (day 56) after the final protein booster.

4.2.5 Antibody determination by enzyme-linked immunosorbent assay (ELISA)

To detect $PyMSP1_{19}$ specific antibodies, polyvinyl chloride microtiter plates pre-coated with recombinant $EcPyMSP1_{19}$ (5µg/ml) in 0.2 M sodium bicarbonate buffer (pH 9.6) by incubating at 37 ^{o}C for 2 h (or at 4^{o}C overnight) as a coating antigen for determination antibody titre and IgG profiles. The plates were blocked with blocking buffer (5% skim milk powder in phosphate buffered saline PBS) for 1 h at 37 °C, washed 5 times with PBS containing 0.05% (v/v) Tween-20 (Sigma Aldrich) and incubated for 2 h at room temperature with serial dilutions of pooled and individual mice sera. The plates were then washed and incubated with horseradish-peroxidase (HRP) -conjugated rabbit anti-mouse IgG (Invitrogen) for 1 h at room temperature RT. Antigen-specific IgG1, IgG2a, IgG2b and IgG3 subclasses were also assayed using a panel of HRP-conjugated rat anti-mouse immunoglobulin subclass IgG1, IgG2a, IgG2b and IgG3 (BD, Pharmingen) for 1 h at RT. After 1 h, the plates were washed again before the addition of a developing buffer containing substrate (TMB) (Invitrogen) for 30 min. The reaction was then stopped at 30 min by the addition of 1 M HCl, and absorbance was read at 450nm using Thermo Scientific Multiskan microplate reader. Data is presented as a mean standard deviation (SD) for each dilution of the sample, or as an antibody endpoint titre. The result of antibody endpoint titres was defined as the inverse of the highest dilution reaching an absorbance equal to or higher than the mean OD_{450} plus 3 × standard deviations (SD) of naïve mice.

4.2.6 Statistics

Statistical analysis of differences between the mean of the inverse of titer by ELISA for different immunized animal groups were compared using one-way ANOVA and the Tukey post-test. Comparisons were considered statistically significant at $p < 0.05$. All data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA).

4.3 Results and discussion

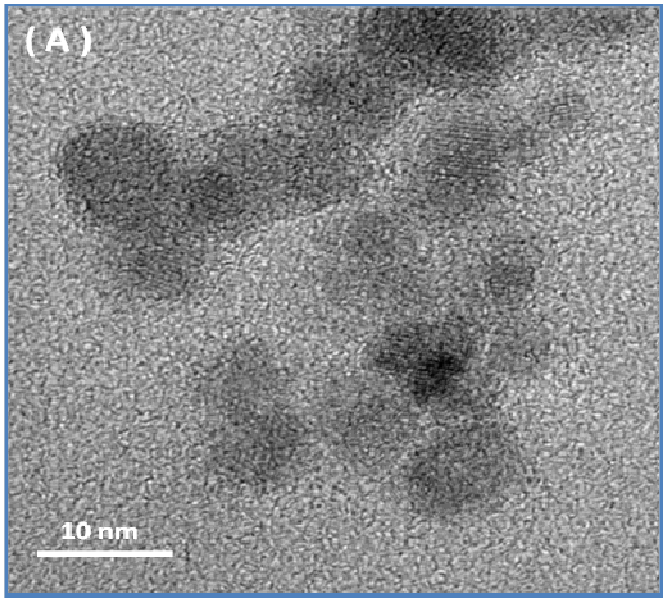
4.3.1 Size and zeta potential of magnetic gene vectors

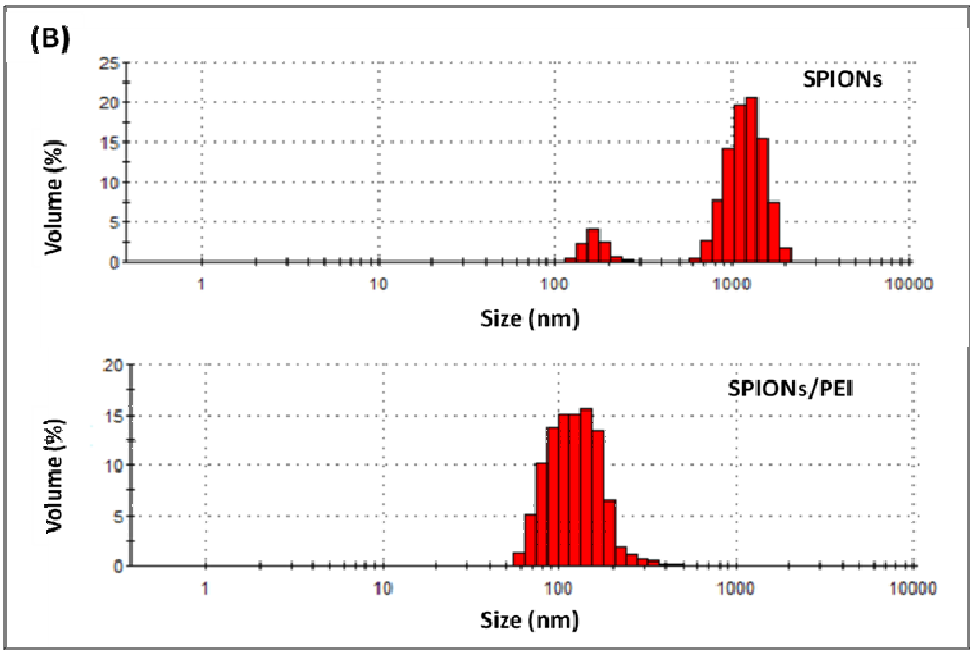
The co-precipitation of Fe (II) and Fe (III) in alkaline in the presence of trisodium citrate resulted in formation of SPIONs, with the average hydrodynamic diameter in suspension of around 160 ± 5 nm. SPIONs were negatively charged with zeta potential around -42 ± 2 mV due to the presence of carboxylic groups on the surface that would enhance the electrostatic interaction with positively charged PEI polymer. As reported previously (Al-Deen, Ho et al. 2011), as-synthesized SPIONs were predominantly superparamagnetic at room temperature with a specific saturation magnetism of >62 emu/g under 15 kOe applied magnetic field and 0.01 emu/g remanance (Appendix A. **(**Figure A.2)), while the X-Ray diffraction pattern of the sample was identical to the standard X-Ray diffraction pattern for pure magnetite JCPDS (Card No. 01-072-6170) (Appendix A. **(**Figure A.3)). Transmission electron microscopy (TEM) showed that freshly prepared magnetic nanoparticles are clusters of distinctive particles with an average core size of 10 ± 2 nm (Figure 4.1A). Upon adding PEI, the surface charge of SPIONs was converted from highly negative to highly positive 37.7± 1.9 mV, indicating PEI polymer adsorption on SPIONs (Figure 4.1C).

Figure 4.1B shows the volume-based size distribution of SPIONs aggregates in suspension as determined by Dynamic Light Scattering (DLS). In the absence of PEI, SPIONs aggregates have a wide size distribution ranging from 110 nm to several microns. On the other hand, when PEI was added to SPIONs with PEI/Fe mass ratios (R) of 10, the population of large aggregates (>1µm) disappeared almost entirely, with most of the particles demonstrating narrower size distributions with aggregate sizes predominantly in

the 50 nm – 500 nm range (volume-based). The presence of cationic polymer PEI on SPIONs has dual roles for increasing SPIONs stability in aqueous solution and at the same time acting as a condensing agent for plasmid DNA (Figure 4.1C). The ratio of PEI nitrogen atoms to DNA phosphate (N/P) is an important factor influencing the degree of DNA complexation on particles and consequently the transfection efficiency (Zhao, Chen et al. 2009). In this study SPIONs/PEI/DNA vectors were prepared by adding DNA to SPIONs/PEI at the different N/P ratio in 5% glucose buffer to investigate the optimal N/P ratio for our *in vivo* gene delivery (Table 4.1).

The naked plasmid VR1020-PyMSP1$_{19}$ molecules exhibited a mean hydrodynamic diameter of 162.5± 9.2 nm and negative zeta potential of -54.2± 2.9 mV. Adding highly negatively charged plasmid DNA molecules to PEI polymer at N/P of 15 resulted in small particles with a hydrodynamic diameter of around 64.3± 2.3 nm and positive zeta potential of 37.5± 1.4 mV, indicating the compact structure of the complex between PEI and plasmid DNA molecules. On the other hand, adding DNA to PEI-coated SPIONs at N/P ratio of 10 reduced the high positive charge of PEI/SPIONs particles to around 14.2 ± 2.4 mV, indicating DNA molecules condensation on the surface of magnetic complexes. At N/P of 10, the measured hydrodynamic size appeared to be larger around 174± 1.4 nm, while the surface charge was slightly positive due to the presence of DNA in excess of PEI, which could induce some aggregation between particles. The zeta potential increased to 30.4± 8.6 mV at a N/P ratio of 30, providing enough repulsion to avoid further aggregation (Table 4.1).


(A)
10 nm


(B)
SPIONs
Volume (%)
25
20
15
10
5
0
1
10
100
1000
10000
Size (nm)
SPIONs/PEI
Volume (%)
20
15
10
5
0
1
10
100
1000
10000
Size (nm)

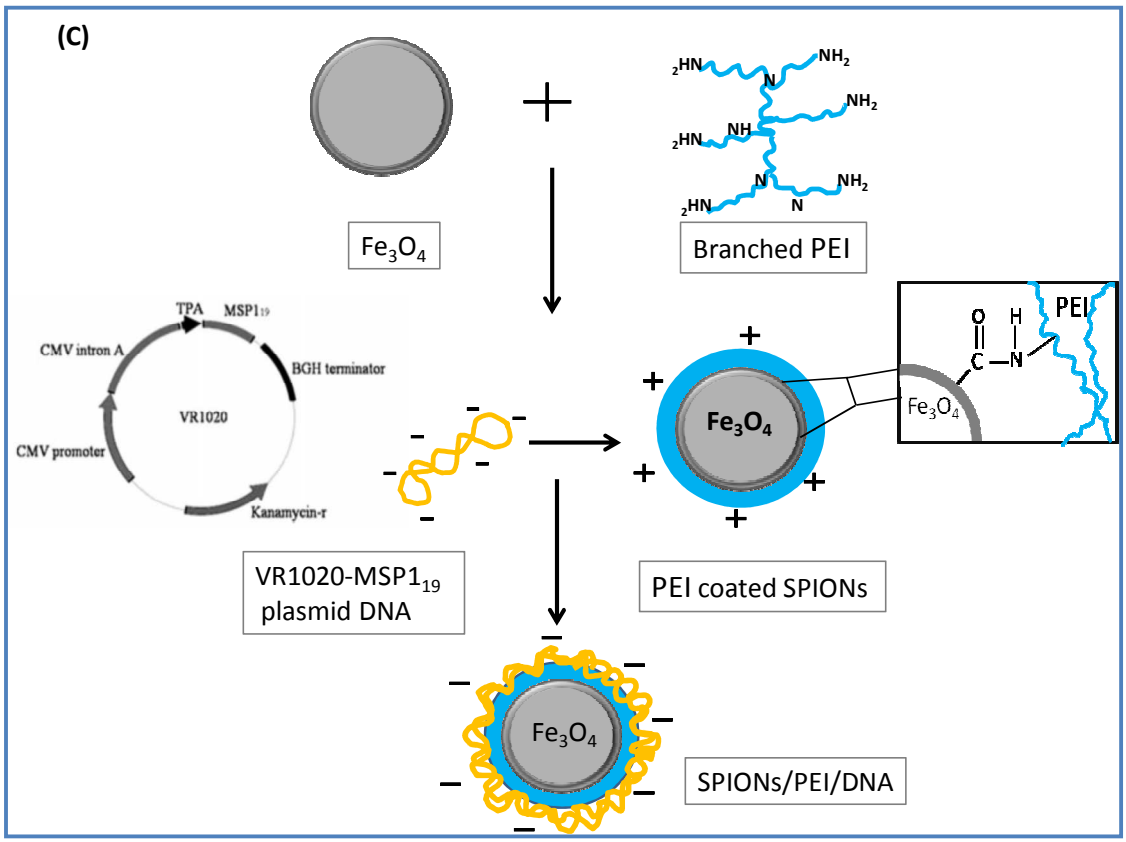


Figure 4.1: (A) TEM micrographs (JEOL 2011) of as-synthesized SPIONs; (B) Size distributions of magnetic nanoparticles before and after coating with PEI, suspended in water at pH 7.4 at 37 C; (C) A schematic representation of surface modification of SPIONs with PEI and DNA through electrostatic interactions.

Table 1
Characteristics of the tested complexes

Characteristics		Mean particles size (nm)	Zeta potential (mV)
	DNA	162.5 ± 9.2	-54.2 ± 2.9
	PEI/DNA	64.3 ± 2.3	37.5 ± 1.4
SPIONs/PEI/DNA N/P ratios	10	174.0± 1.4	14.2 ± 2.4
	15	147.0 ± 8.5	20.9 ± 1.7
	20	132.5 ± 7.8	23.1 ± 1.2
	30	104.1 ± 16.8	30.4 ± 8.6

Table 4.1: Characteristics of the tested complexes

4.3.2 Determination of the N/P ratio for SPIONs/PEI/DNA complexes based on antibody responses to PyMSP1$_{19}$

Early studies have shown that the muscle was a favored route for DNA vaccine delivery because skeletal muscle cells have an unusual capacity to take up and express foreign plasmid DNA *in vivo* without any special delivery mechanism (Danko and Wolff 1994). Here the muscle tissue was chosen for the administration of the SPIONs/PEI/DNA complexes to determine a suitable N/P ratio for malaria PyMSP1$_{19}$ gene expression *in vivo.*

To test elicited antibody levels, OD values of total IgG antibodies specific for PyMSP1$_{19}$ from each group of mice were measured by ELISA, with recombinant EcPyMSP1$_{19}$ as the coating antigen to determine the total serum IgG against PyMSP1$_{19}$. Figure 4.2 shows that the levels of IgG antibody response *in vivo* were significantly higher for vectors injected with magnetofection than without magnet. In addition, the level of antibody induced by

magnetofection was higher than those induced by PEI/DNA complexes at the N/P ratio of 15, whereas the level of antibody induced by naked DNA was very low compared to other groups.

The specific reason for magnetofection to improve immunization reactions *in vivo* remains unclear. Zhou et al. claimed that the external magnetic field could improve the distribution of magnetic gene complexes within the skeletal muscle tissue by drawing complexes across the tissue and accelerating the accumulation of gene complexes on the cell surface by magnetic force (Zhou et al. 2007). Although antigen expression in myoblasts may have a role in regulating immune responses following DNA vaccination, there is a clear evidence that only professional antigen presenting cell (APC) such as dendritic cells (DCs) are actually able to prime immune responses after DNA vaccination (Iwasaki et al. 1997). DCs could uptake a large number of magnetic nanoparticles in a wide range of size distribution from 10-200 nm (Goya et al. 2008). Considering that antigen-presenting cells (APCs) are less available in the muscle than in the skin, rapid gene transfection of magnetic gene complexes under an external magnetic field could be attributed to a higher transfection of APCs in the muscle tissue expressing relatively large amounts of antigen. In addition, activation of both B and T cells take place either by a direct uptake of plasmid DNA and the expression of protein in professional APCs, or by transfer of the expressed protein from myocytes to APCs in order to process and present them on MHC molecules (cross-priming) (Doe et al. 1996). For these reasons, the use of magnetofection in DNA vaccination strategy could enhance both APCs and myocytes transfection, resulting in higher immune responses.

The highest level of IgG antibody response against $PyMSP1_{19}$ was induced by magnetofection using SPIONs/PEI/DNA complexes at a N/P ratio of 15 ($p < 0.001$) compared with other groups. Magnetofection with SPIONs/PEI/DNA complexes at N/P ratio of 20 also induced comparatively high levels of IgG response, although they were still lower than those at the N/P ratio of 15 ($p < 0.05$) (Figure 4.2). The data were relatively consistent with a previous *in vitro* magnetofection study of $PyMSP1_{19}$ expression in COS-7 cell line, where the gene expressions of SPIONs/PEI/DNA complexes at N/P ratios of 10 and 15 were higher than those from other N/P ratios (Al-Deen, Ho et al. 2011).

In the *in vivo* delivery, a balanced interaction between PEI and plasmid DNA is important. The influence of PEI on IgG antibody response of PEI/DNA complex is moderate (Figure 4.2), suggesting that PEI by itself could not act alone as an efficient carrier for gene transfection *in vivo*, although it has a high buffering capacity in the endosome, known as the proton sponge effect (Godbey, Wu et al. 1999). The amount of PEI should be enough to condense plasmid DNA against enzymatic degradation, whilst at the same time weak enough to allow for a complete release of plasmid DNA inside the cell. SPIONs/PEI/DNA complexes at moderate N/P ratios were previously demonstrated to form loose-structured complexes, which still offered space to allow other materials such as polymerase to interact with DNA for gene expression (Prasad, Gopal et al. 2003). In contrast, a stronger complexation of DNA with large amounts of highly protonated amine groups in PEI molecules could cause gene blocking that may reduce SPIONs/PEI/DNA transfection efficiency / protein expression. Although magnetofection using SPIONs/PEI/DNA complexes at the N/P ratio of 15 and 20 together showed highest IgG antibodies against $PyMSP1_{19}$ (Figure 4.2), the highest endpoint titres were obtained when N/P ratio was 15 (Appendix B. (Figure B.1)). Therefore, the N/P ratio of 15 was used in the second stage to investigate the delivery of SPIONs/PEI/DNA complexes via different administration routes.

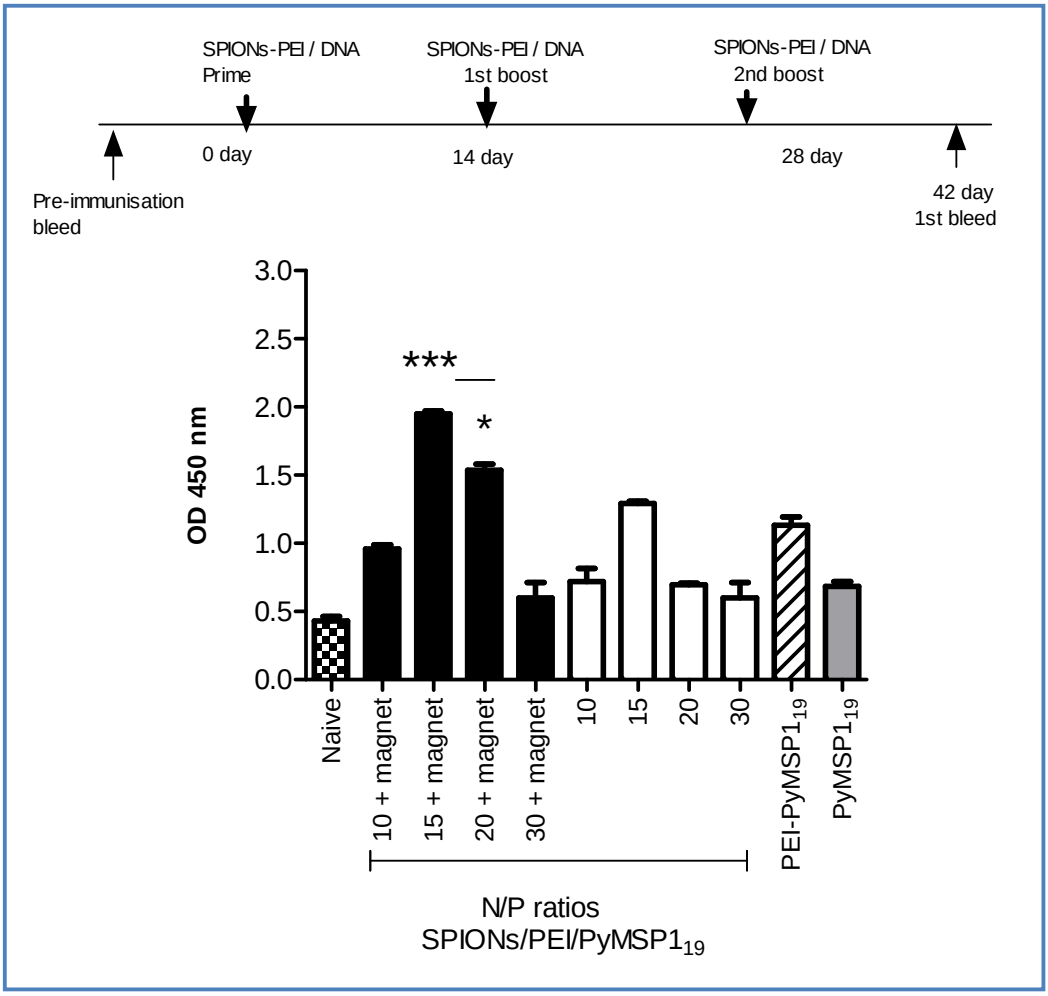


Figure 4.2: Total IgG obtained from BALB/c mice immunized intramuscularly with different combinations of PyMSP1$_{19}$ (naked DNA, DNA-PEI, at a N/P ratio of 15 and SPIONs/PEI/DNA at different N/P ratios with or without magnet). Two weeks after the last immunization (day 42), sera were collected from immunized mice in each group (n = 5), pooled sera were analyzed for IgG by ELISA as OD$_{450nm}$ at 1:150 dilution using recombinant protein as a capture antigen. Values are mean ± SD of duplicate measurement. One-way analysis of variance (ANOVA) and Tukey multiple comparison test were used to find the difference between different groups. Statistical significance is designated as *p < 0.05, *** p < 0.001.

Administration routes of SPIONs/PEI/PyMSP1$_{19}$ complexes

The type of administration route is of interest for particulate gene delivery systems in nanometers or small micrometre range, since they can be naturally phagocytised by various APCs toward an effective immune response against the target antigen. Gene complexes with small particle size are able to drain freely from the injection site to the lymph nodes, which is a key trigger of immunity in genetic immunization for stimulation and regulation of adaptive immune responses (Manolova et al. 2008). Most reported studies for DNA vaccine administration using PEI/DNA or SPIONs/PEI/DNA complexes were via intramuscular or intravenous injections using syringes (Chollet, Favrot et al. 2002; Xiang, Bin et al. 2007; Zhou, Liu et al. 2007). Here the effect of magnetofection on the level of IgG antibody responses against PyMSP119 antigen was investigated using SPIONs/PEI/DNA complexes via different administration routes (intraperitoneal (i.p.), intramuscular (i.m.), subcutaneous (s.c.), intradermal (i.d.), and intravenous (i.v.)).

4.3.3 Safety evaluation

The safety of SPIONs/PEI/DNA complexes was evaluated by monitoring the appearance of mice directly after injection, while the general health of mice was also checked twice a week after immunization. SPIONs/PEI/DNA complexes injected with different administration routes, except for i.v., did not adversely influence the survival of mice, with all animals remaining in good health until the end of the experiment. On the contrary, i.v. immunizations of SPIONs/PEI/DNA complexes caused transient shock with most animals dying in the first 30 min. These results suggested that Balb/c mice strain in this experiment was sensitive to SPIONs/PEI/DNA complexes toxicity through i.v. immunization, especially at a high concentration (100 µg/ mL DNA). The observation was in agreement with Collet et al., which showed that the toxicity of a high dose (more than 100 µg) of PEI/DNA complexes after i.v. injection was more pronounced in Balb/c strains than in Swiss nude mice where all treated animals died of shock in the first 30 min (Chollet, Favrot et al. 2002).

Since the same concentration of complexes delivered with other routes did not show any symptoms of toxicity, the prevailing hypothesis was that the aggregation of SPIONs/PEI/DNA complexes could cause a physical blockage of lung capillaries (Ogris et al. 1999). Different studies showed that PEI/DNA nanoparticles injected intravenously into mice resulted in gene expression that was stronger in the lung than liver, heart, spleen, or kidney, and was accompanied by high toxicity due to the accumulation of PEI/DNA nanoparticles in the lung resulting in death for most of the animals within the first 30 min after injection (Wightman, Kircheis et al. 2001; Chollet, Favrot et al. 2002). At the concentration used for *in vivo* experiments, micrometer-sized particles could be formed when DNA are mixed with positively charged PEI. Since PEI/DNA complexes are still highly positively charged, they are able to interact with negatively charged serum proteins such as opsonins in the serum, adversely affecting *in vivo* gene expression (Wightman, Kircheis et al. 2001; Chollet, Favrot et al. 2002). The agglomeration of magnetic PEI-coated nanoparticles in this study could be further induced by strong magnetic dipole–dipole attractions between particles, leading to a blockage of small capillaries within body tissues (Gupta and Gupta 2005).

4.3.4 Antibody responses induced by SPIONs/PEI/DNA complexes

The pre-immunization serum sample (depicted by week 0 in Figure 4.3) did not contain any detectable antibody response against recombinant protein EcPyMSP1$_{19}$ in ELISA. Generally, mice that were vaccinated by SPIONs/PEI/DNA complexes with the 3 DNA boosting protocol developed IgG antibody response. No significant difference was noted for IgG endpoint titers observed after the 1st boost for all administration routes. After the 2nd boost, the IgG antibody response via i.p. route was significantly higher than other groups (Figure 4.3), with IgG endpoint titers rising more than 21- fold higher ($p < 0001$) than the 1st booster injection. In comparison, IgG endpoint titres after 2nd booster injection were only moderately elevated (8- fold higher) compare with the 1st injection for i.m., i.d. and s.c. routes (Figure 4.3).

Mice immunized with SPIONs/PEI/DNA complexes intraperitoneally with the application of external magnetic field developed significantly higher antibody responses after the 2nd booster injection with endpoint titres of 11.047 $\times 10^3$ compared to 54.07 for control mice (naïve) ($p < 0.001$) (Figure 4.4). The antibody responses induced by i.m., i.d. and s.c. routes under the applied magnetic field were comparatively lower than those induced by i.p. (endpoint titres of 3.126×10^3, 1.096×10^3, 1.118×10^3 compared to 11.047 $\times 10^3$, $p < 0.001$) (Figure 4.4). The lower immunogenicity of magnetic gene complexes via i.m., i.d. and s.c. routes implies the inherent difficulty in anticipating gene expression after injection, even for the same gene complexes, probably because of the decreased immuno-accessibility of the antigen, since the main dose portion remained at the site of injection or was degraded before reaching secondary lymphoid organs.

Repeated intraperitoneal administration of SPIONs/PEI/DNA complexes were found to be highly reproducible for high gene expression, possibly due to its 'depot effect' for nanoparticles in the peritoneum, as proposed previously for PEI/DNA complexes (Intra and Salem 2008). Intraperitoneal administration of gene complexes has several advantages for transfection efficacy to antigen presenting cells APCs by SPIONs/PEI/DNA due to 1) high accessibility to APCs in the peritoneal cavity and lymph nodes; and 2) few bio-components that reduce transfection activity (Hattori et al. 2006). The application of an external magnetic field after injecting the gene complexes possibly captures and retains particles for a longer duration in the peritoneal cavity to remain in contact with peritoneal cells and lymphoid tissues. The stronger antibody responses induced in the intraperitoneal group could be due not only to the adjuvant effect of SPIONs/PEI/DNA complexes with the application of a magnetic field, but also because of the physiology of peritoneum as a major reservoir for macrophages (Weck et al. 1999) and dendritic cells (Rezzani et al. 1999), which are key triggers for activation of the adaptive immune system through the processing to present the antigen as peptide–MHC complexes to antigen-specific T cells. Taking into consideration the prolonged and strong interactions between these cells and i.p. administered SPIONs/PEI/DNA complexes in the peritoneal cavity under a magnetic field, the opportunity for cellular uptake of these complexes and generation of long-term gene expression would increase. The fact that an external magnetic field could enhance the cellular uptake capacity of macrophages and dendritic cells for magnetic particles has been

reported under *in vitro* (Chapman, Hassa et al. 2008; Goya, Marcos-Campos et al. 2008) and *in vivo* (Guedes et al. 2005) conditions. The intraperitoneal injection of SPIONs/PEI/DNA complexes possibly enhanced the concentration of these complexes in the peritoneal fluid and adjacent lymphoid tissue such as lymphoid organs and local mesenteric lymph nodes inside the peritoneal cavity by a rapid accumulation of the full vector dose within a close vicinity of target cells in a few minutes (Zhou, Liu et al. 2007). Since the intraperitoneal cavity is a good storage for self-replenishing cells that play a significant role in the mucosal immune response (Kroese et al. 1989), the gene delivery formulation (SPIONs/PEI/DNA) could work as well in the mucosal route of delivery system.

Different studies have reported the immediate distribution of magnetic nanoparticles in the liver and spleen after injection, with negligible traces reported in the brain, heart, and kidney (Kim et al. 2006; Zhao et al. 2012). The biological distribution and toxicity effects of 50 nm magnetic nanoparticles through intraperitoneal injection in mice has been studied histologically by (Kim, Yoon et al. 2006), reporting that magnetic particles were rapidly and widely distributed in different organs, with the highest concentrations in the liver and spleen without any apparent toxicity even after 4 weeks, while the spread into the lungs was minimal. The size of magnetic gene vectors in fluid used in this work (Table 4.1) implied that fast and free drainage of these particles into different lymphoid organs and local mesenteric lymph nodes were possible after intraperitoneal injection. The impact of particle size drainage to lymph nodes has been described by (Manolova, Flace et al. 2008), who reported that nanoparticles in the range of 20 – 200 nm were able to drain freely and directly to lymph nodes penetrating deeper into areas surrounding B cell follicles. In contrast, the magnetic susceptibility of our magnetic particles to an external magnetic field (Appendix A. **(**Figure A.1)) might prevent most of these particles from freely moving from the peritoneal cavity until the permanent magnet was removed from the injection site after 1 h. The response of magnetic gene complexes to an external magnetic field should enhance the efficiency of gene delivery to peritoneal cells, although their interactions depended on various parameters, including physiological fluid flow velocity inside the body, particle size, magnetic field properties (placement, size, shape, and strength), crucial for targeting magnetic particles (Nacev et al. 2010). The direct intraperitoneal injection of PEI/DNA was

also demonstrated by (Aoki et al. 2001) to be a promising delivery method to transduce a gene into disseminated cancer nodules in the peritoneal cavity. Intraperitoneal injections of PEI/DNA complexes have been shown to effectively deliver a luciferase gene (Intra and Salem 2008) and siRNA against human melanoma tumor growth (Aigner et al. 2002) in mice.

The results obtained here are in good agreement with those reported by (Cherif, Shuaibu et al. 2011), where intraperitoneal immunization of DNA/PEI/γ-PGA complexes as a delivery system for plasmid DNA encoding *P. yoelii* MSP1-C-terminus resulted in significantly higher levels of IgG and subclass antibodies. The same study also showed that i.v. injection of DNA/PEI/γ-PGA complexes stimulated antibody responses against MSP1, although at a lower level than those by i.p. injection. In their case, negatively charged DNA/PEI/γ-PGA gene vectors (–14.8 ± 0.7 mV) were used (Cherif, Shuaibu et al. 2011), avoiding the aggregation of complexes with blood components and ensuing blockage of small capillaries in the lungs, in contrast to our case with strongly positively charged SPIONs/PEI/DNA complexes (20.9 ± 1.7 mV) (Table 4.1).

The magnetic DNA gene transfer vaccine approach provides a unique and simple tool to increase the efficiency and the speed of gene delivery to different tissues, subsequently provides robust, consistent antigen expression and immune responses, therefore increase the vaccine efficacy. With good safety, tolerability and reproducibility data showed in this study as well as clinically acceptable administration, such an approach has a great translational potential to the clinic in the future.

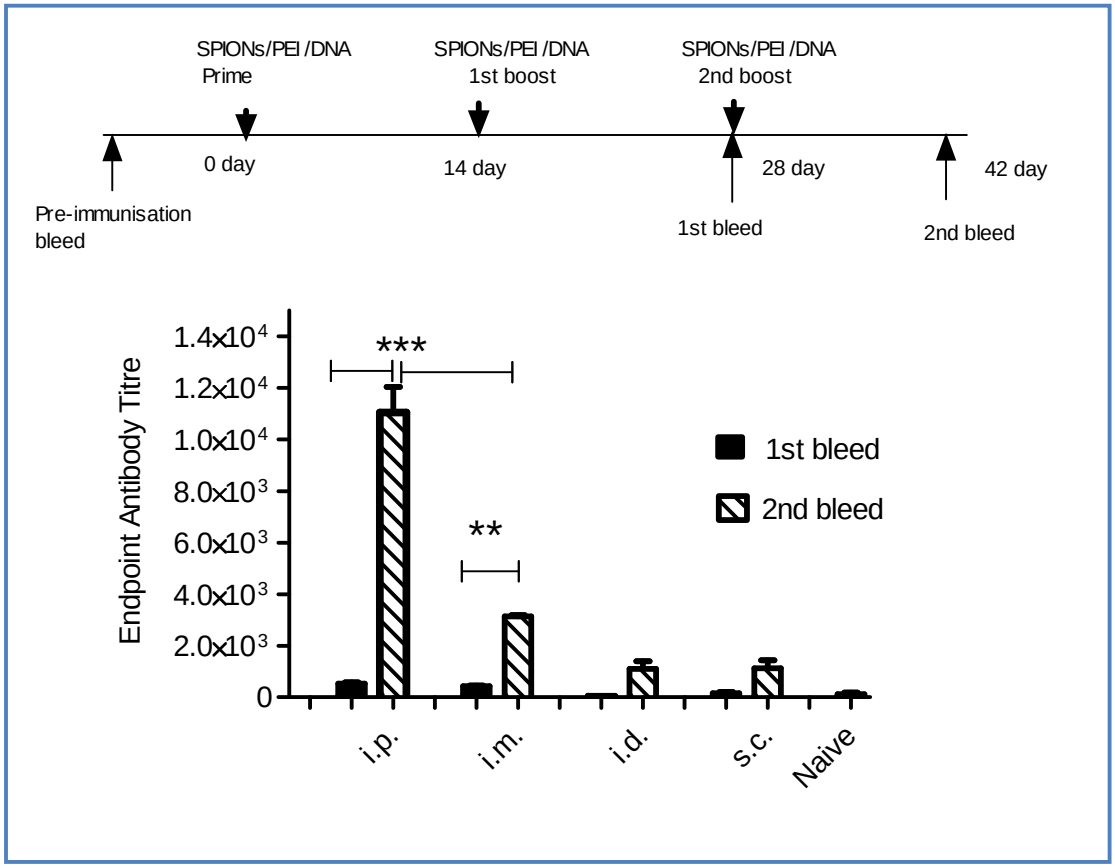


Figure 4.3: Effects of administration routes on total IgG produced after the second and third boost in BALB/c mice immunized with SPIONs/PEI/DNA through different routes of administration with magnet. Two weeks after the second immunization (day 28) and the third immunization (day 42), sera were collected from immunized mice in each group (n = 5), pooled sera were analyzed for the level of IgG antibodies against recombinant protein as capture antigen. The results shown are the last dilution of sera at which the OD_{450nm} is higher than mean+3SD of control mice. The results are expressed as means ± SD of duplicate. Statistical significance is designated as **p < 0.01, *** p < 0.001.

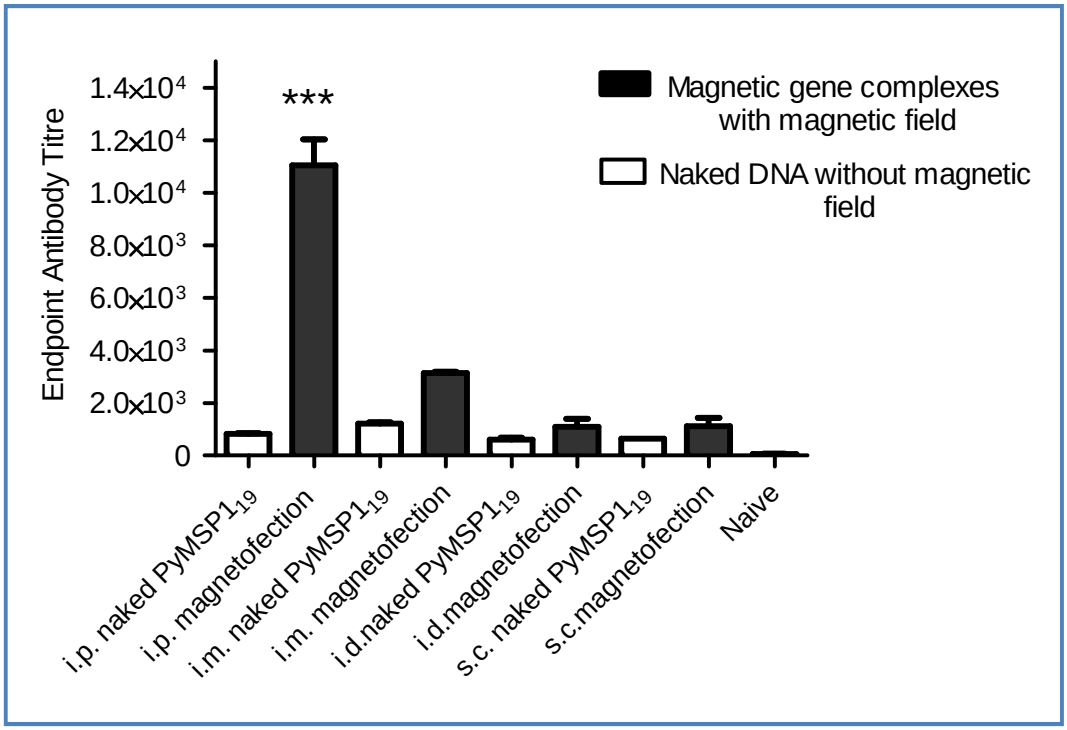


Figure 4.4: Effects of magnetofection on total IgG obtained in BALB/c mice immunized with naked DNA or SPIONs/PEI/DNA complexes at the N/P ratio of 15 through different administration routes after the third immunization. Two weeks after the third immunization (day 42), sera were collected from immunized mice in each group (n = 5), pooled sera were analyzed for the level of IgG antibodies against recombinant protein as a capture antigen. The results shown are the last dilution of sera at which the OD_{450nm} is higher than mean+3SD of control mice. One-way analysis of variance (ANOVA) and Tukey multiple comparison test were used to find the difference between magnetofection and naked groups. Results are expressed as means ± SD of duplicate. Statistical significance is designated as *** $p < 0.001$.

4.3.5 Isotype distributions of antibodies to $PyMSP1_{19}$

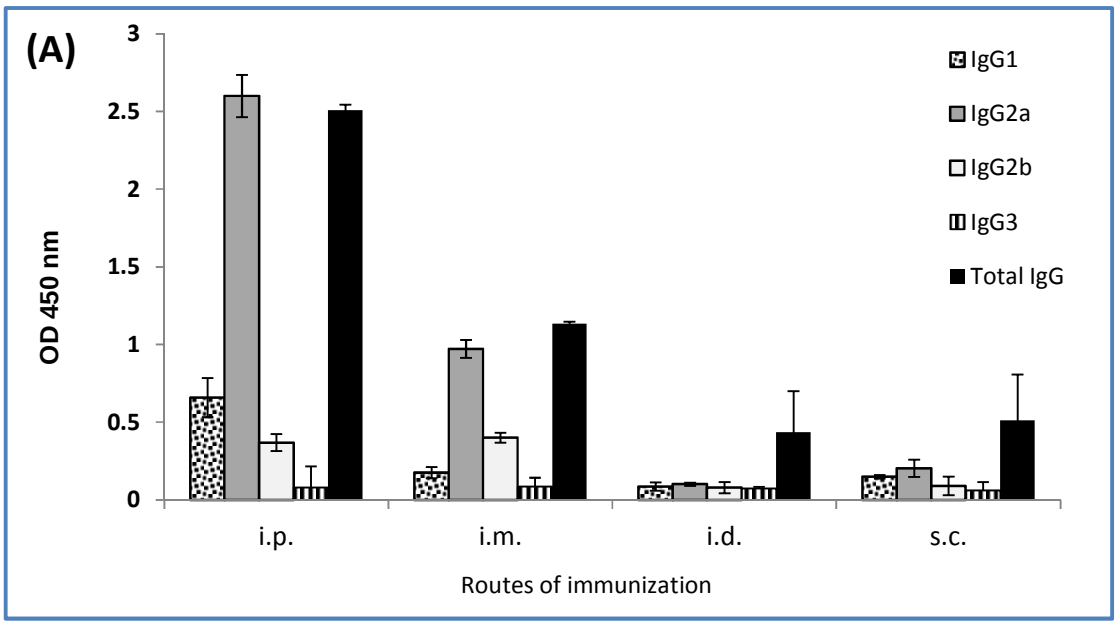
The immunoglobulin (IgG) subclass distribution of anti-$PyMSP1_{19}$ antibodies is an important parameter for protection against blood stage malaria infection. Pooled sera obtained on day 42 after the last booster via intraperitoneal and intramuscular routes demonstrated significantly higher IgG2a responses compared with IgG1 and IgG2b subclasses (Figure 4.5A). Both intradermal and subcutaneous routes failed to show any differences between IgG subclass profiles because of low IgG responses produced against

both complexed and naked PyMSP1$_{19}$ (Figure 4.5A). Since i.p. and i.m. groups showed differences between IgG subclass levels in pooled sera, variations between individual mouse IgG antibody subclasses were analyzed within these two groups for IgG1, IgG2a, IgG2b and IgG3 antibody levels (Figure 4.5B). There were some differences in the mean antibody titers between pooled and individual mice sera, attributed to assay variability. Significant differences were observed in levels of IgG2a, IgG1, and IgG2b responses between SPIONs/PEI/DNA complexes and naked DNA administered intraperitoneally, with little difference observed for the intramuscular route (Figure 4.5B).

Isotype profiles for pooled sera from mice injected intramuscularly revealed that the IgG2a was the predominant subclass for SPIONs/PEI/DNA complexes compared to IgG1, IgG2b, and IgG3 (Figure 4.5A). High IgG2a level after DNA vaccination may be attributed to unmethylated CpG dinucleotide motifs present in bacterial genomes, responsible for driving immune responses toward Th1-type responses as a sign of serum IgG2a antibody production (Chu et al. 1998). Previously others reported that IgG2a subclass were frequently found after i.m. injection of plasmid DNA (Pertmer et al. 1996; Feltquate et al. 1997). However, unlike the responses recorded for pooled sera, no significant differences for IgG2a (and even other subclasses) were observed here between complexed and naked PyMSP1$_{19}$ in the individual mice vaccinated via the i.m. route (Figure 4.5B), due to the variability in IgG responses between individual mice. On the other hand, the intraperitoneal injection of complexed PyMSP1$_{19}$ induced significantly higher IgG2a, IgG1, and IgG2b subclasses, respectively in individual mice, with predominant IgG2a responses compared to the naked PyMSP1$_{19}$. When comparing IgG1 and IgG2b, it was evident that IgG1 level was higher than IgG2b for i.p. route, whereas IgG1 level was lower than IgG2b for i.m. route.

Several studies indicated that the IgG2a response is predominantly responsible for protection observed against *P. yoelii* (White et al. 1991; Bouharountayoun and Druilhe 1992; Ling et al. 1997). Highest level of IgG2a response is the most favorable for therapeutic applications since it activates antibody-dependent cellular cytotoxicity, and confers protection against parasite invasion (Lim 2006). Furthermore, a high level of IgG2a has a main role in modulating *P. yoelii* parasitemias in passive transfer study (White, Evans et al. 1991). It is also believed that IgG2a subclass has a crucial role in antimalarial immunity especially against *P. yoelii* (Ling, Ogun et al. 1997). Bouharountayoun et al.

(1992) reported that antibody-dependent cell-mediated cytotoxicity (ADCC) against the blood-stage phase (merozoite) of the *P. yoelii* parasite is mediated by IgG2a, but not IgG1 in mice. It has been reported that induction of the IgG1 isotype in mice is driven by Th2-type cytokines, whereas the IgG2a isotype is directed by Th1-type cytokines (Coffman et al. 1988; Carcaboso et al. 2004). This analysis is an indicator of whether immune responses are biased toward Th1- or Th2-type (Sin et al. 2000). Although IgG2a response is important for antimalarial immunity, associations between IgG1 and IgG2b subclasses to $PyMSP1_{19}$ and low peak parasitemias were also demonstrated by (Hirunpetcharat et al. 1997), suggesting that both cellular and humoral immune responses have frequently been correlated with protection against *Plasmodium* infection. In addition, although antibody immune responses mediate blood-stage protection, the IFNγ production as a mark of Th1 activation is also associated in blood-stage protection that can eliminate intracellular parasites (Plebanski and Hill 2000). These findings suggested that SPIONs/PEI/DNA complexes administered via the i.p. route induced a mixed Th1/Th2 response with a predominantly Th1 biased response in this study. The existence of IgG2a isotype with IgG1 and IgG2b observed here suggested that the use of magnetic nanoparticles for malaria gene delivery provided an advantage over traditional adjuvants to provoke both Th1 and Th2 type cell-mediated immunity in systemic compartments, particularly when delivered via intraperitoneal or intramuscular routes.



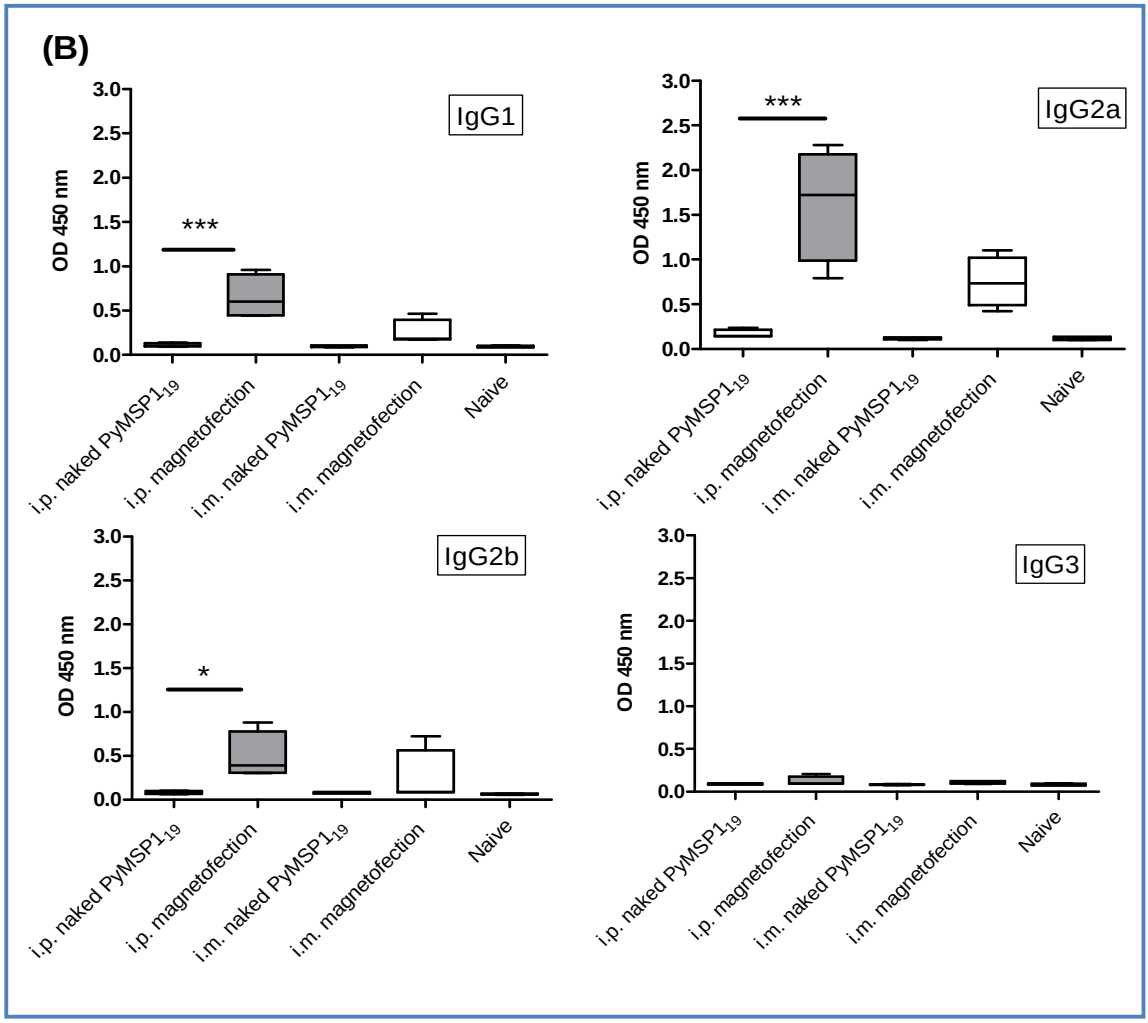


Figure 4.5: Effects of magnetofection on antibody IgG subclass profiles with SPIONs/PEI/DNA complexes: (A) IgG subclasses antibody titre in the pooled sera collected from immunized mice after the third immunization with different routes of administration; (B) Individual IgG subclass antibody titre in the groups of mice immunized with SPIONs/PEI/DNA complexes intraperitoneally and intramuscularly. One-way analysis of variance (ANOVA) and Tukey multiple comparison test were used to find difference between magnetofection and naked groups. Statistical significance is designated as * $p < 0.05$, *** $p < 0.001$.

4.3.6 Protein boost strategy

The immune responses generated by repeated DNA vaccination may be sub-optimal for protection because of the low amount of actual protein antigen synthesized in the host. DNA can be however an excellent priming agent to form memory cell that can then be recalled and boosted by other delivery modalities. Heterologous prime-boost strategies, for example by using consecutive DNA priming followed by a purified protein boosting have the potential to improve DNA-based vaccines dramatically (Ramshaw and Ramsay 2000). The most common is a heterologous booster using a recombinant protein boost formulated in an appropriate adjuvant after DNA vaccination (Hill et al. 2010). In this study, it has been hypothesized that this strategy may lead to improved antibody responses over groups immunized with DNA vaccine only. For consistency, recombinant EcPyMSP1$_{19}$ protein boost has been selected to test in combination with DNA vaccine for all groups of mice immunized with SPIONs/PEI/DNA complexes.

Figure 4.6A shows that the antibody response in vaccinated animals after 3 x DNA priming and 1 x protein boost regimen led to a significant boost in IgG responses for all groups (Appendix B (Figure B.2)). The last protein boosting with recombinant EcPyMSP1$_{19}$ significantly stimulated antibody responses in all (i.p., i.m., i.d., and s.c.) groups ($p< 0.001$) compared to only 3 x DNA immunization in terms of IgG endpoint titres, with the endpoint titre for i.p. group significantly higher than those of other groups ($p< 0.001$) (Figure 4.6A). Boosting with recombinant protein resulted in a significant increase in endpoint PyMSP1$_{19}$ specific antibody titres in i.p. group (IgG endpoint titers 3271922 ± 17) compared to only 3 x DNA immunization (IgG endpoint titers 11047.23 ± 1390) ($p< 0.0001$) (Figure 4.6A). Notably, a single inoculation of recombinant EcPyMSP1$_{19}$ alone in DNA unprimed mice failed to induce detectable anti-PyMSP1$_{19}$ antibodies ($p < 0.001$) compared to those of 3 x DNA + 1x protein group especially via i.p. route (Figure 4.6B). Mice receiving 3 x SPIONs/PEI/DNA complexes followed by a single recombinant EcPyMSP1$_{19}$ boost intraperitoneally generated significantly stronger levels of anti-PyMSP1$_{19}$ antibodies compared to the animals receiving 3 x naked DNA + 1 x protein boost via i.p. route group ($p < 0.001$) (Figure 4.6B). Together these results showed that naked DNA and single recombinant protein immunization induced only negligible antibody responses, but the

combination of magnetic vectors/DNA priming and recombinant protein single boosting induced much more rapid development of high-titer antibodies especially via the i.p. route of administration.

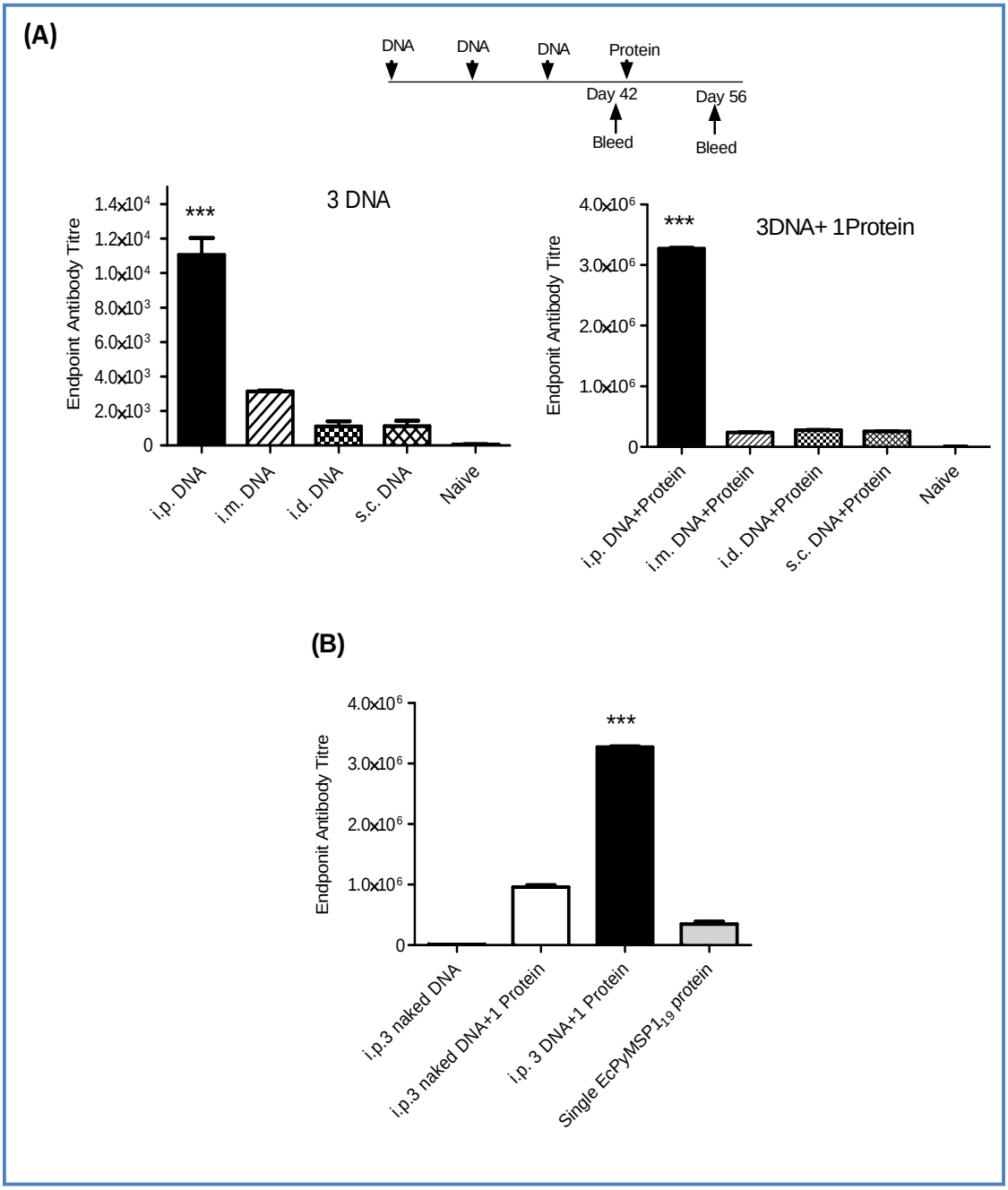
Regarding IgG subclass analysis of 3 x DNA + 1 x protein boost regime, the presence of a high level of IgG2a than IgG1 subclasses with IgG2a/IgG1 ratio of 1.9 was found for i.p. group, showing the same trend as 3 x DNA immunized mice with IgG2a/IgG1 ratio of 1.23 (Figure 4.6C, Appendix B (Figure B. 3B)). This was consistent with an earlier report by (Sin, Bagarazzi et al. 2000), describing the ability of DNA–DNA vaccination to significantly increase IgG2a levels, with higher IgG2a response level observed when the animals were primed with DNA and boosted with protein emulsified in an incomplete Freund's adjuvant intraperitoneally. The total IgG responses for vectors administered via i.p. route did not change in response to successive immunizations.

In contrast, 3 x DNA + 1x protein boost regime demonstrated a higher prevalence of IgG1 than IgG2a in i.m., i.d., and s.c groups of mice compared to 3 x DNA-immunization. Interestingly, the overall patterns of IgG isotype production were independent of the presence of the incomplete Freund's adjuvant (IFA). The protein boost induced a qualitative switch in relative levels of the antibody subclass profile toward IgG1 isotype in i.m., i.d., and s.c groups (Figure 4.6C, Appendix B (Figure B.3A)), with total IgG antibody response level higher than in 3 x DNA-vaccination.

The exact mechanism of protein boost switching IgG subclass from IgG2a to IgG1 in general is not understood. A possible explanation was that due to an initial weak antibody response and lack of significant differences between IgG subclass titres in the i.m., i.d., and s.c. routes after 3 x DNA-vaccination (Figure 4.6A), the last protein boost caused fluctuations in Th1 and Th2 cytokine production patterns affecting the original isotype profile. This enabled the protein boost to play a critical role in the subclass switching toward IgG1, as evident from the predominant IgG1 subclass in i.m., i.d., and s.c. Groups (Figure 4.6C, Appendix B (Figure B.3 A)). Furthermore, we could see a pattern of the isotype profile in which responses from the highest to the lowest were in the order of IgG1, IgG2a, IgG2b, and IgG3 in these three groups after the protein boost. This pattern was consistent with that found in a single unprimed protein group, confirming the superior role

of a protein boost (Appendix B (Figure B.3 A)). The observation that the IgG1, rather than IgG2a, was the predominant subclass elicited by recombinant EcPyMSP1$_{19}$ immunization was also reported by (Ma et al. 2012).

Diverging results after the protein boost in mice primed with the complexed DNA delivered via different routes are consistent with the idea that the type of antibody responses elicited following DNA-protein immunization regimen is dependent on the strength of the initial IgG response, the route of primed DNA administration, and the identity of the antigen. This study suggested that *in vivo* immune responses to DNA vaccine with magnetic vectors were characterized as mixed IgG2a/IgG1 subclasses, although IgG2a was the predominant subclass produced over other subclass. It has been reported that stimulation of the IgG1 subclass is driven by Th2-type cytokines, whereas the IgG2a subclass is directed by Th1-type cytokine (Coffman, Seymour et al. 1988; Sin, Bagarazzi et al. 2000). By introducing SPIONs/PEI/DNA complexes, or by boosting with recombinant PyMSP1$_{19}$ especially via i.p. route, both humoral and Th1 and Th2 type immune responses may be stimulated for protection against malaria infection in this case. Intraperitoneal injection of SPIONs/PEI/DNA complexes is attractive because it is simple, reproducible and might lead to a depot effect of gene complexes residing in the peritoneum for several hours (Aoki, Furuhata et al. 2001). The outcome is useful for developing protocols for a variety of diseases including cancer gene therapy. Ovarian, pancreatic, and gastric cancers are predominantly attractive targets for i.p. injections (Aoki, Furuhata et al. 2001; Kasuya et al. 2002; Jayne 2003; Louis et al. 2006), as the intraperitoneal injection of gene complexes allowed the straightforward distribution of plasmid in the whole peritoneal cavity (Louis, Dutoit et al. 2006).


(A)
DNA
DNA
DNA
Protein
Day 42
Day 56
Bleed
Bleed
3 DNA
3DNA+ 1Protein
Endpoint Antibody Titre
1.4x10⁴
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1.0x10⁴
8.0x10³
6.0x10³
4.0x10³
2.0x10³
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***
i.p. DNA
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i.d. DNA
s.c. DNA
Naive
Endponit Antibody Titre
4.0x10⁶
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s.c. DNA+Protein
Naive
(B)
Endponit Antibody Titre
4.0x10⁶
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i.p. 3 DNA+1 Protein
Single EcPyMSP1$_{19}$ protein

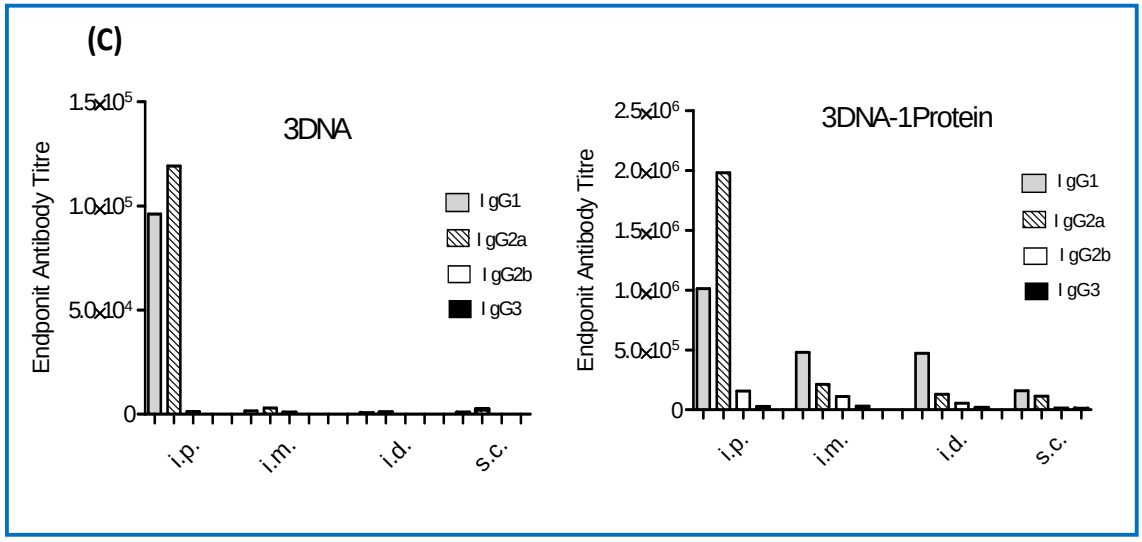


Figure 4.6: DNA prime-protein boost regime effect on IgG levels against PyMSP1$_{19}$ in mice immunized with SPIONs/PEI/DNA complexes. Groups of mice were immunized with three doses of SPIONs/PEI/DNA complexes via different routes of administration with magnet followed by a single boost with recombinant protein formulated in incomplete Freund's adjuvant injected intraperitoneally. Sera were obtained two weeks before and after protein boost and pooled for endpoint against recombinant protein: (A) Total IgG antibody titre obtained before and after protein boost for different groups; (B) Total IgG antibody titre obtained from pooled mice sera immunized intraperitoneally with three doses of either naked DNA or SPIONs/PEI/DNA followed by single dose of protein boost or primed with only single dose of protein; (C) IgG antibody subclass titre in the pooled sera collected from different groups before and after single protein boost. Results shown are the last dilution of sera at which the OD$_{450nm}$ is higher than mean+3SD of control mice. One-way analysis of variance (ANOVA) and Tukey multiple comparison test were used to find difference between groups. Results are expressed as means ± SD of duplicate. Statistical significance is designated as *** p < 0.001.

4.4 Conclusion

The efficacy and the type of *in vivo* immune responses elicited by magnetic gene vectors delivered via different routes were studied using PyMSP1$_{19}$ condensed on SPIONs/PEI particles. Intramuscular injection of SPIONs/PEI/DNA complexes at the N/P ratio of 15 with the application of the external magnetic field at the site of injection demonstrated the highest IgG antibody response compared to those generated with other N/P ratios. The stimulation of IgG2a and IgG1 subclass levels was also increased with delivery of SPIONs/PEI/DNA complexes with magnetofection, particularly for intraperitoneal and

intramuscular injections. Heterologous prime-boost strategies greatly enhanced IgG response titre and at the same time significantly enhanced IgG2a/ IgG1 ratio for i.p. route in a similar manner or even more than DNA immunization only. While the stimulation of IgG1 antibody immune responses after a single protein booster injection was observed for i.m., i.d., and s.c. routes. The stimulation of IgG2a immune responses was strongly dependent on the route of administration for the primed DNA and the total IgG level produced. Both IgG2a and IgG1 responses are involved in the protective immunity against malaria, thus these magnetic vectors could be an efficient immunological adjuvant. Antibody induction is a vital component for protection against toxin producing pathogens, as well as the key to prevent initial viral infection. In blood-stage malaria, antibodies are acknowledged as the primary stage of defense. It is therefore highly likely that the novel approaches described herein will support further studies towards the potential clinical applicability of magnetic DNA vaccine delivery for malaria and other diseases.

CHAPTER 5

On designing Stable Magnetic Vectors as Carriers for Malaria DNA Vaccine

5.1 Introduction

Several approaches have been targeted towards developing an effective malaria vaccine, with DNA vaccination emerging as the promising approach for the prevention and treatment of malaria and many other diseases (Doolan and Hoffman 2001; Moreno and Timon 2004). The main advantage of the DNA vaccine is its ability to induce both humoral and cellular immune responses against antigens encoded by recombinant DNA (Doolan and Hoffman 2001; Moreno and Timon 2004).

A variety of non-viral vectors have been used for gene delivery in order to increase transfection efficiencies. Magnetically guided gene transfection (magnetofection) has emerged as a promising strategy to improve the efficacy of gene delivery with low dose *in vitro* and site specific *in vivo* applications (Dobson 2006). Additionally, the duration of the transfection process can be significantly reduced by magnetofection compared to incubation time required with standard protocols. The applications of superparamagnetic iron oxide nanoparticles (SPIONs) for therapeutic purposes required them to be stable in aqueous conditions at physiological salinity and neutral pH 7.4. A major issue for magnetic nanoparticles is their tendency to aggregate especially under high-ionic strength biological media (Wiogo et al. 2011). Such colloidal stability affects particle size, which should be sufficiently small (< 200 nm) to increase cellular uptake and blood circulation time within the body. The surface properties also influence aggregation behavior, blood circulation time, and cytotoxicity of the magnetic particles in the body (Gupta and Gupta 2005).

A few studies on magnetic nanoparticles for biomedical applications have focused on the stability and magnetization of particles in biological media. Wiogo et al. (2011) examined the stabilization of magnetic iron oxide nanoparticles in a biological medium (RPMI-1640) via surface modification with fetal bovine serum (FBS), reporting that the aggregate size of the magnetic particles with surface carboxyl groups could be maintained at 190 nm ± 2 nm for up to 16 h in media containing ≥ 4 vol % FBS. Their stability in this medium was attributed to the presence of both hard and soft protein coronas of FBS on the particle surface, providing steric hindrance from magnetic and van der Waals forces. Other studies also found that the association of particles with serum protein in cell media affect their physicochemical interactions and provide better interactions with cellular receptors (Nel et al. 2009; Arsianti, Lim et al. 2010).

The stability of magnetic nanoparticles in a biological fluid can be improved by modifying their surface to increase repulsive forces between particles, thus balancing magnetic and van der Waals attractive forces. Polyethyleneimine (PEI) is a good candidate to modify the surface of SPIONs as the polymer belongs to one of the most efficient family of cationic compounds for delivery of plasmid DNA into mammalian cells due to their extensive buffering capacity through the "proton sponge" effect (Boussif, Lezoualch et al. 1995; Behr 1997). PEI has ability to capture protons that are pumped into endosomes, accompanied by an influx of chloride ions to maintain charge neutrality and subsequent osmotic swelling and disruption of the endosomes. This permits the delivery of plasmid from the degradative lysosomal trafficking pathway. On the other hand, the use of PEI *in vivo* is limited by the intrinsic high cytotoxicity and the relatively low gene expression of PEI is owing to the highly positive surface charge (Neu, Fischer et al. 2005). The toxic effect on cells is due to permeabilization of cellular membranes and disruption of phospholipids bilayers of the cell membrane (Godbey, Wu et al. 1999). The decreased transfection efficiency of PEI *in vivo* is partly attributed to serum protein-induced aggregation and coagulation of blood cells (Koyama et al. 2003). PEI-coated SPIONs usually aggregate immediately in commonly used cell media such as RPMI or DMEM (Dulbecco Modified Eagle's medium) in the presence of serum protein, due to the high amine contents of PEI that interact with negatively charged molecules such as opsonins in the serum (Neu, Fischer et al. 2005). Such non-specific interactions of PEI complexes with blood components and target cells

could be reduced by shielding positive charges of PEI with other polymers to create a steric barrier against aggregation (Kichler 2004). For example, PEGylation of PEI via covalently attached polyethylene glycol (PEG) segments to PEI molecules could increase their circulation time *in vivo* due to the reduction of non-specific interactions with serum albumin and cellular components in the bloodstream (Kichler 2004).

Another strategy is to develop a delivery system that not only resists aggregation but also incorporates cell-binding ligands to combine intrinsic transfection activity with receptor-mediated cellular uptake mechanisms. An example is the use of cationic PEI derivatives of hyaluronic acid (HA) (Han, Kang et al. 2009) that could decrease cytotoxicity, diminish non-specific interactions with serum protein in the physiological fluid (Yao, Fan et al. 2010), and enhance CD44 receptor-mediated endocytosis (Jiang, Park et al. 2009). Amphoteric HA derivative with PEI using high molecular weight of HA showed little aggregation in a phosphate buffer at the optimal charge ratio (Yao, Fan et al. 2010). The complexes also exhibited higher gene transfection efficiency with lower cellular toxicity in HepG2 cells than PEI/DNA alone, due to both better protection of DNA from nuclease and timely dissociation of DNA from complexes that resulted in increased DNA uptake by HA-specific receptor-mediated pathway. Ito et al. (2006) reported that amphoteric HA derivative with spermine side chains (Spn-HA) showed higher transcription-enhancing activity than HA alone, while PEI/DNA coated with Spn-HA exhibited 29-fold higher gene expression than PEI/DNA alone. The better performance of Spn-HA complexes could be due to their high mobility groups (protein-like transcriptional activation properties) that loosen the tightly compacted DNA/polycation complexes, thus enhancing plasmid transcription (Zappavigna et al. 1996). Ito et al. (2008) also found that the pre-addition of HA to DNA prior to PEI, successfully decreased the aggregation size of gene complexes, with some particles remaining without aggregation with a diameter of < 80 nm, even at a relatively higher concentration of DNA (200 mg / ml).

Shielding of cationic magnetic gene vectors such as PEI-coated SPIONs by HA through electrostatic interaction between amine (NH_2) groups in PEI and the carboxyl (COOH) groups in HA should influence their stability in different biological media and also the efficiency of gene transfection. To design effective magnetic malaria gene vectors, a simple *in vitro* assay was developed in this study to investigate the effects of order of assembly for

complexes comprising SPIONs, PEI, HA, and DNA in relation to their stability in different cell media. The complexes were formed as followed: *1st configuration vector*: layered assembly of PEI-coated SPIONs by DNA, followed by HA (SPIONs/PEI/DNA/HA); *2nd configuration vector*: coupling of PEI-coated SPIONs with DNA+HA mixture (SPIONs/PEI/DNA+HA); *3rd configuration vector*: coupling of bare SPIONs with PEI+DNA+HA mixture (SPIONs/PEI+DNA+HA). The stability of these gene vectors was measured in terms of size distribution, surface charge, and DNA binding capacities in water, in a 150 mM NaCl buffer solution, and in RPMI-1640 media with and without fetal bovine serum (FBS) at pH 7.4. Commercially available fetal bovine serum (FBS) was added to RPMI media as a model serum to test the stability of different magnetic vector configurations in a relatively identical environment to the human blood serum. Different gene configurations were also exposed to cleavage by DNase I to test the ability of these complexes in protecting DNA from digestion by DNase serum. The resistance of these vectors to polyanion-mediated dissociation by heparin was also investigated to understand the impact of the negatively charged compounds in a blood-like environment on the stability of the complexes.

5.2 Materials and methods

5.2.1 Materials

Polyethylenimine with an average molecular weight of 25 kDa (PEI branched), trisodium citrate dihydrate ($C_6H_5Na_3O.2H_2O$) and heparin sodium salt were purchased from Sigma Aldrich. RPMI 1640 medium (GIBCO), fetal bovine serum, Quant-iT PicoGreen dsDNA reagent and kits were supplied by Invitrogen (Carlsbad, CA). Sodium hyaluronate (HA) with an average molecular weight of <10 kDa was purchased from Life Core Biomedical LLC. Fe (III) chloride ($FeCl_3.6H_2O$) and Fe (II) chloride ($FeCl_2.7H_2O$) were purchased from Ajax Finechem and Ajax Chemicals, respectively. Mammalian expression vector VR1020 (Vical Inc., San Diego, CA), the VR1020-PyMSP1$_{19}$ plasmid was kindly provided by Prof. Ross Coppel's group (Department of Microbiology, Monash University). The plasmid VR1020-PyMSP1$_{19}$ (Appendix A (Figure A.1)) was amplified in *Escherichia coli*

(strain DH5α) and purified using an endotoxin-free Mega-prep plasmid kit (Qiagen) according to the manufacturer's protocol.

Methods

5.2.2 Preparation of magnetic gene vectors

Superparamagnetic iron oxide nanoparticles (SPIONs) were synthesized by alkaline co-precipitation of Fe (III) chloride ($FeCl_3.6H_2O$) and Fe (II) chloride ($FeCl_2.7H_2O$) (1:2 molar ratios) in an aqueous solution in the presence of trisodium citrate ($C_6H_5Na_3O_7.2H_2O$) as an electrostatic stabilizer, as described previously (Al-Deen, Ho et al. 2011). Details of SPIONs synthesis and purification of plasmids DNA can be found in the sections (3.2.2 and 3.2.3).

The iron oxide suspension (0.1mg/ml) was mixed with 10% (w/v) PEI solution (25 kDa branched polyethyleneimine), with PEI/Fe mass ratios (R) =10 and during which they were sonicated for 5 min. The SPIONs/PEI complexes were dialyzed using Spectra/Por membranes (MWCO = 12000 -14000) with deionized water for 3 days to remove any unbound/excess PEI.

Different magnetic vector configurations were prepared by changing the mixing order of vector components. The proposed vector configuration is illustrated in Figure 5.2. Ternary complexes (SPIONs/PEI/DNA) (Figure 5.3.B.1) were prepared by mixing SPIONs/PEI complexes with plasmid DNA encoding VR1020-PyMSP1_{19} gene (8 µg/ml) at N/P ratio of 10 in Milli-Q water for 30 min, a period of time in which DNA should have been entirely complexed (Al-Deen, Ho et al. 2011). The vectors were prepared as followed:

1st configuration: SPIONs/PEI/DNA/HA (Figure 5.3.B.2) was prepared by mixing ternary complexes with various amounts of HA solution, to adjust HA: PEI % charge ratio from 0.5% to 100%, incubated for 30 min at room temperature.

2nd configuration: SPIONs/PEI/DNA+HA (Figure 5.3.B.3) was prepared by first adding HA solution with various amounts (as mentioned above) to plasmid DNA (8 µg/ml) for 30 min at room temperature. The HA+DNA mixture was then added to SPIONs/PEI complexes, at N/P of 10 and incubated for 30 min at room temperature.

3rd configuration: SPIONs/PEI+DNA+HA (Figure 5.3.B.4) was prepared by firstly mixing PEI, DNA, and HA with various HA: PEI % charge ratios and N/P of 10. After 30 min incubation in Milli-Q water, the zeta potential of the complexes (PEI+DNA+HA) was measured using a Malvern Zetasizer ZS (Malvern Instruments Ltd., U.K.). Table 5.1 shows that PEI+DNA+HA mixtures were positively charged from +33 mV to +12.2 mV when HA : PEI % charge ratio varied from 0 to 25%, while the charge became negative from -8.89 mV to -20.23 mV with higher % HA : PEI between 50% and 100% due to the presence high amounts of polyanions. For these vectors, the positively charged PEI+DNA+HA complexes were mixed directly with negatively charged bare SPIONs (-40 mV ± 2 mV), producing vectors referred to as **SPIONs/PEI+DNA+HA (A-3rd configuration vectors 0-25%)**. On the other hand, the negatively charged PEI+DNA+HA complexes mixed with positively charged SPIONs/PEI complexes were referred to as **SPIONs/PEI/PEI+DNA+HA (B-3rd configuration vectors 50-100%)**.

PEI+DNA+HA complexes	Zeta potential (mV)
% HA: PEI charge ratio	
0	33 ± 3
5%	28 ± 2
10%	25.9 ± 3
25%	12.2 ± 7
50%	-8.89 ± 2
75%	-19.86 ± 2
100%	-20.23 ± 4

Table 5.1: Zeta potential PEI+DNA+HA complexes with different % HA : PEI charge ratios incubated in water for 30 min (n = 3, ± SD).

5.2.3 Characterization of magnetic gene vectors

Typically the vectors were suspended in Milli-Q water, in 150 mM NaCl, in RPMI, and in RPMI containing 10% FBS at pH 7.4. The pH of all media was adjusted to 7.4 with either the addition of 1.0 N NaOH or 1.0 N HCl. The samples were then incubated for 1 h at 37 ^{o}C prior to measurements. The average hydrodynamic diameters (expressed as dynamic light scattering (DLS)) of different configurations and their zeta potential in aqueous solution were measured using Zetasizer® Nano ZS (Malvern Instruments Ltd, Malvern, UK) at 37°C using a DTS 1060C clear disposable zeta cell (Malvern Instruments). Using this technique, the volume size distribution of the aggregates was also determined. Measurements were conducted on different samples (on average from 3 samples) of complexes with SPIONs concentration of 0.05 mg/ml suspended in four different media. Transmission electron microscopy (TEM) was performed using a JEOL 2011 microscope operated at 200 k. A drop from each suspension was deposited onto a carbon-coated copper grid and dried at room temperature for TEM observation.

5.2.4 DNA retardation assay

The effects of HA addition on the electrophoretic mobility of DNA incubated in different cell media were assessed using agarose gel electrophoresis. The vectors were incubated for 30 min in different cell media at room temperature prior loading into agarose gel (1% agarose in Tris-borate EDTA buffer containing 5 μL ethidium bromide, EtBr at 5 μg/ml). Electrophoresis was carried out at 60 V for 90 min, and DNA bands were visualized with a UV illuminator.

5.2.5 DNA binding assay

DNA binding to complexes was determined using a DNA-specific fluorescence reagent PicoGreen dye to quantify the amount of unbound DNA in solution (Ferrari et al. 2001; Arsianti, Lim et al. 2010). PicoGreen assay reagent was prepared according to the manufacturer's protocol: the PicoGreen dsDNA reagent (Invitrogen) was diluted (1: 200) in

10 mM TE buffer (10 mM Tris-HCL, 1mM EDTA, pH 7.4). Diluted PicoGreen reagent 500 µl was added to 500 µl of supernatant after centrifugation at 12,800 ×g for 2 min. Samples containing DNA alone and complexes without DNA were used as references. Samples were prepared in the duplicates and incubated at room temperature for 10 min in the dark. The intercalation of the PicoGreen reagent with free (unbound) DNA was detected using a fluorescence microplate reader SpectraMax M2 (Molecular Devices). The fluorescence was measured at an excitation wavelength of 480 nm and emission wavelength of 520 nm.

5.2.6 Stability of magnetic gene vectors

The effects of heparin with different concentrations on vector's stability in the presence of other glycosaminoglycans such as HA polymer were observed from the change in fluorescence intensity using a fluorescent probe (ethidium bromide, EtBr) with agarose gel electrophoresis at pH 8. The vectors were incubated in RPMI media for 30 min followed by the addition of different concentrations of heparin (0.1, 0.3, 0.5 mg/ml) to the solution. After a further incubation for 30 min at room temperature to study the possible destabilization of complexes, DNA in the supernatant was recovered via centrifugation for 2 min at 12,800 × g, and analyzed by agarose gel electrophoresis at 60 V for 90 min. DNA bands were visualized with a UV illuminator.

5.2.7 DNase sensitivity assay

To assess DNA protection for different magnetic gene vector configurations against degradation by DNase I, vectors with 50 µg/ml of DNA were first diluted with a volume of 2× DNase reaction buffer (100 mM Tris HCl (pH 7.5), 500 mM $MgCl_2$, 13 mM $CaCl_2$) containing RNase-free DNase I (Thermo scientific) at a final activity of 250 U/ml for 30 min at 37°C (both naked DNA and DNA complexes were incubated with DNase I) (Bertschinger et al. 2006). After incubation, the sample tubes were rapidly placed on ice and the enzymatic reaction was terminated by adding 500 mM EDTA.

To estimate the approximate amount of liberated DNA molecules from complexes which were possibly degraded by DNase, heparin solution at a concentration of 0.5 mg/ml was then added and the sample was incubated for 30 min at room temperature to release DNA molecules, followed by addition of DNase I for 30 min at 37°C before the enzymatic reaction was inactivated in the presence of 500 mM EDTA. The integrity of DNA molecules in the complexes after different treatments with DNase and heparin was determined by treating different complexes with DNase I for 30 min at 37°C (as described previously). Then DNase was inactivated in the presence of 500 mM EDTA, followed by addition of heparin (0.5 mg/ml) for 30 min at room temperature. For all preparations, 20 µl of the samples were analyzed by 1% agarose gel electrophoresis.

5.3 Results and discussion

5.3.1 Magnetic gene vectors

The hydrodynamic diameter of SPIONs indicated the size in suspension of 80 nm ± 5 nm. The particles were negatively charged with zeta potential of -40 mV ± 2 mV due to carboxylic groups on the surface. As-synthesized SPIONs were predominantly superparamagnetic at room temperature with a specific saturation magnetism of >62 emu/g under 15 kOe applied magnetic field and 0.01 emu g^{-1} remanance (Appendix A. **(**Figure A.2)), while X-ray diffraction pattern indicated a magnetite (Fe_3O_4) phase (Appendix A. **(**Figure A.3)). Addition of PEI to SPIONs reversed the surface charge to 35 mV ± 3 mV (Figure 5.2).

Upon adding HA to SPIONs/PEI complexes, electrostatic interactions between carboxylic groups in HA and amine groups in PEI (Figure 5.2) would lead to a decrease in the positive charge of the complexes. Bare SPIONs displayed clusters of distinctive particles with an average core size of 8 nm – 10 nm (Figure 5.1A). After assembling SPIONs, PEI, DNA, and HA with different mixing orders ((Figure 5.3), small clusters of several particles were observed. However due to the low atomic number of polymers (Sawyer et al. New York : Springer (2008)), there was an inherent lack of contrast to be able to clearly distinguish the morphology of different gene vectors (Figure 5.1 B-D).

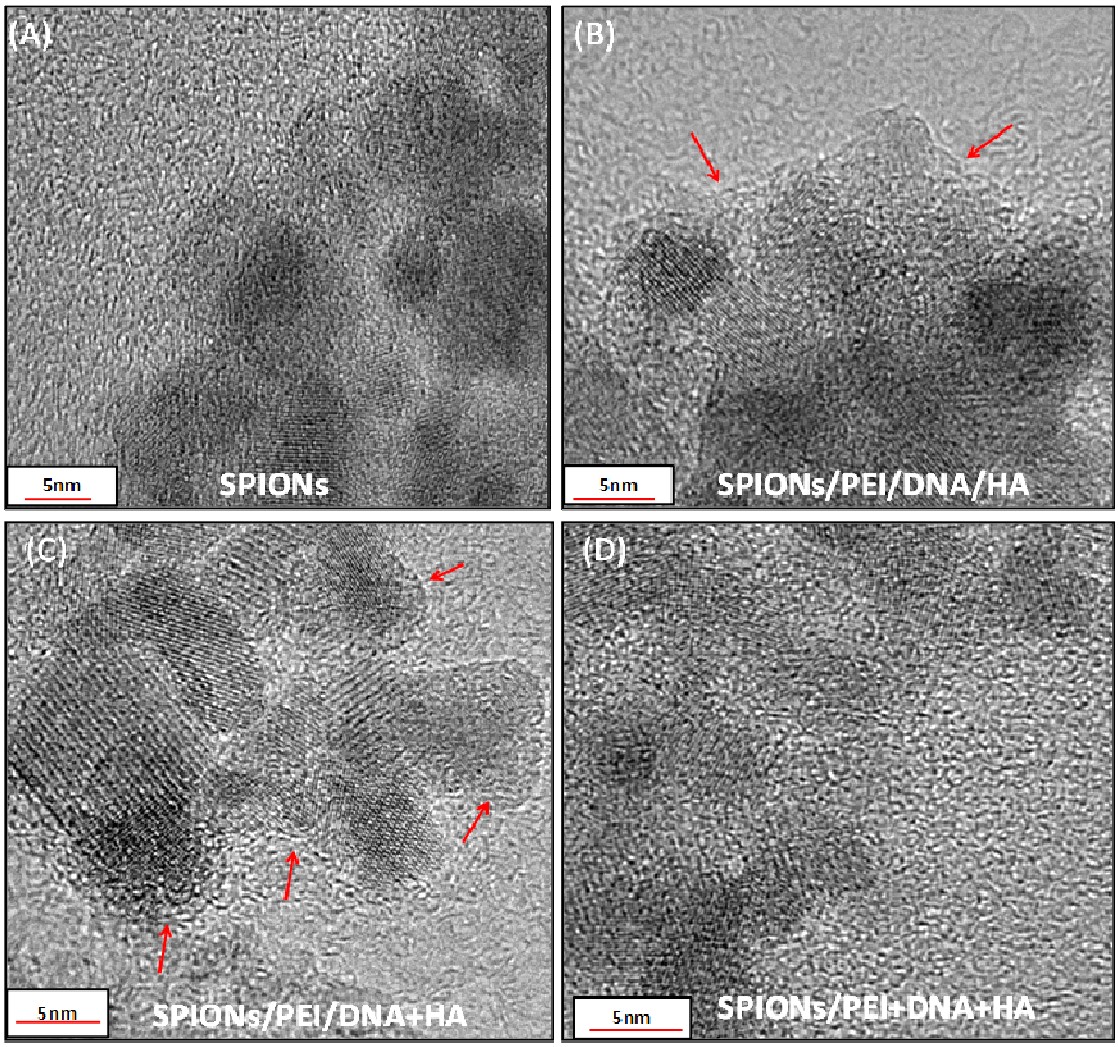


Figure 5.1: TEM micrographs (JEOL 2011) of (A) as-synthesized SPIONs; (B – D) magnetic vectors with different configurations in water at pH 7. (Arrows indicating layer of adsorbed PEI, DNA, and HA on SPIONs).

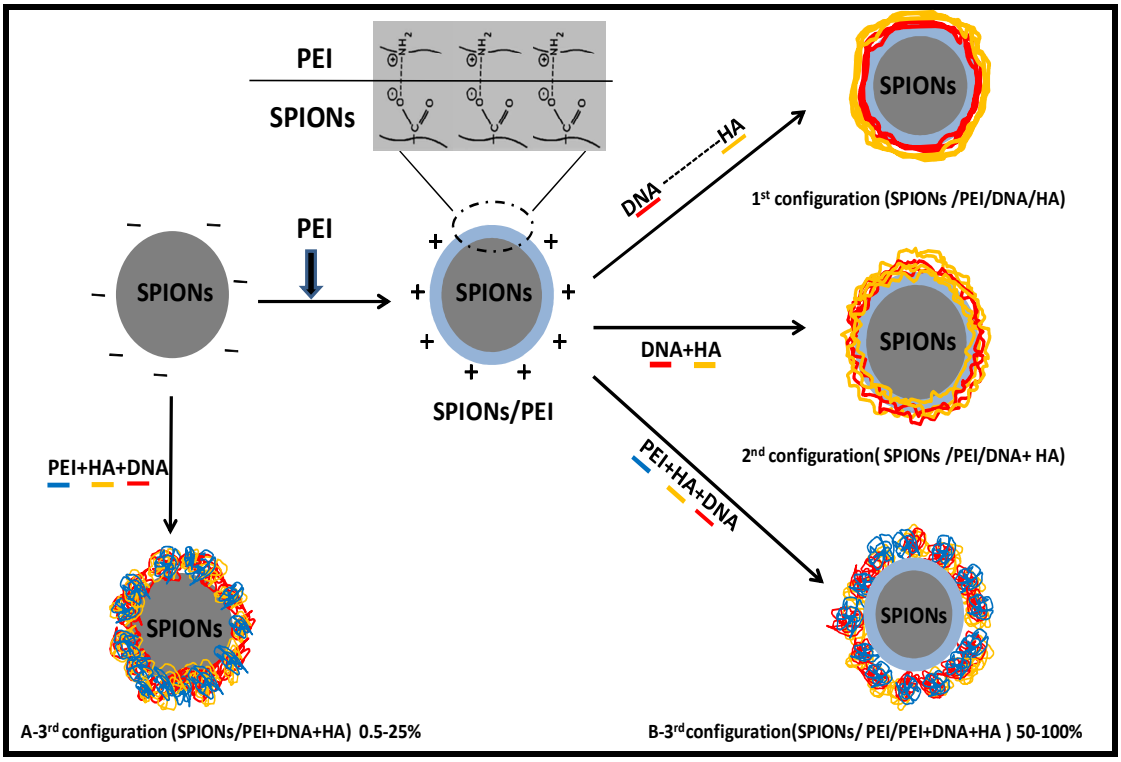


Figure 5.2: Schematic figure of surface modification of SPIONs with PEI and HA through electrostatic interactions.

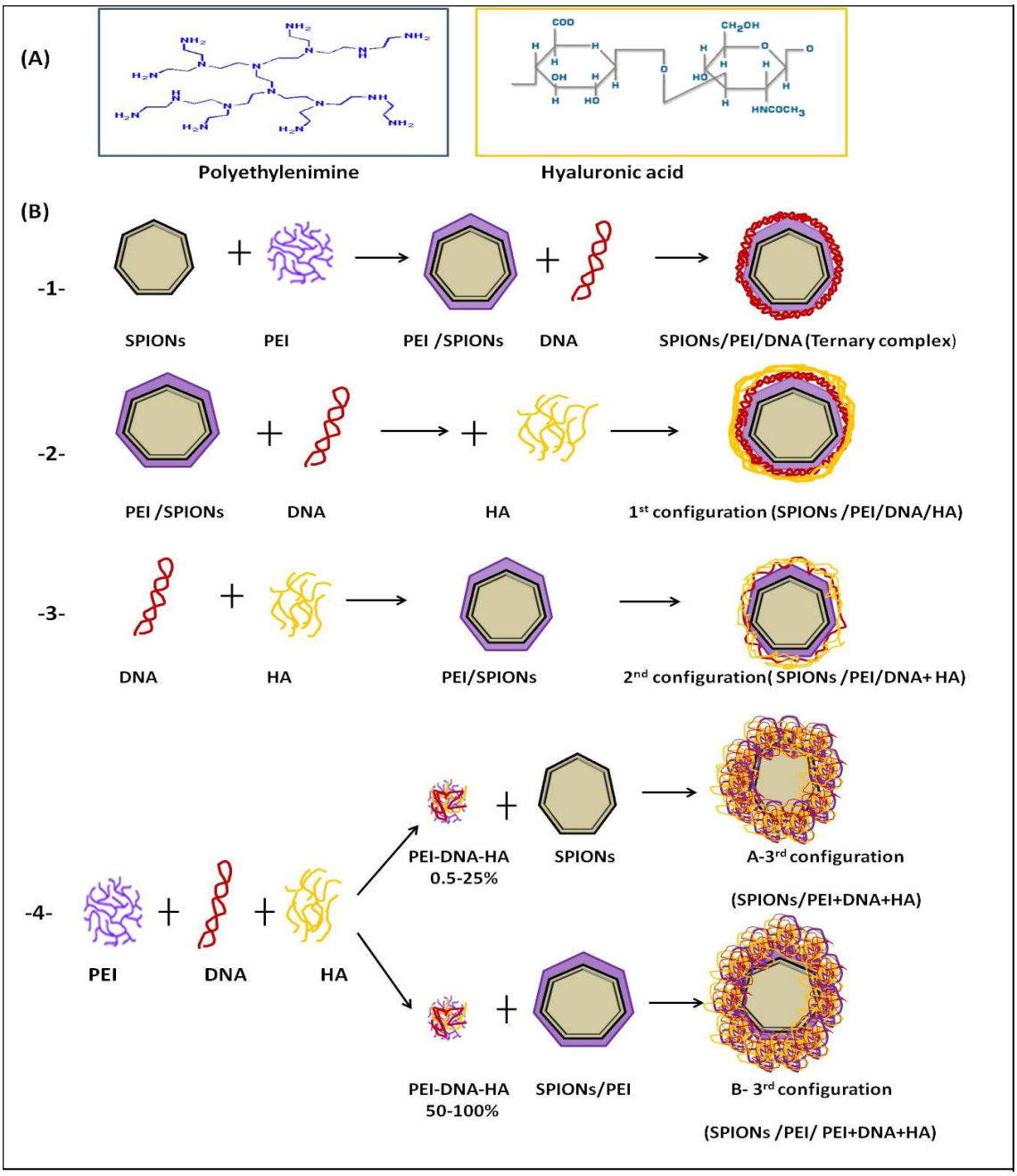


Figure 5.3: (A) Chemical structure of PEI and HA; (B) Magnetic vectors prepared with different mixing orders of SPIONs, PEI, DNA and HA:
(1) *Ternary complex* (SPIONs/PEI/DNA); (B) Magnetic vectors prepared with different mixing orders of SPIONs, PEI, DNA and HA:
(2) *1st configuration vector* (SPIONs/PEI/DNA/HA);
(3) *2nd configuration vector* (SPIONs/PEI/DNA+HA);
(4) *3rd configuration vector* A-(SPIONs/PEI+DNA+HA) with 0.5-25% HA : PEI;
B-(SPIONs/PEI/PEI+DNA+HA) with 50-100% HA : PEI.

5.3.2 Effects of different cell media on stability of magnetic gene vectors

In water: Figure 5.4 shows that magnetic gene vectors suspended in salt-free condition (Milli-Q water) underwent no significant agglomeration after 1 h incubation. Hydrodynamic diameters of 1st configuration vectors (SPIONs/PEI/DNA/HA) and 2nd configuration vectors (SPIONs/PEI/DNA+HA) increased slightly to ~300 nm at HA: PEI charge ratio of 25% (Figures 5.4A), however the size decreased with higher charge ratio, eventually to < 100 nm for 100% charge ratio.

Without HA, the vectors exhibited highly positive charge at ~32 mV in water (Figure 5.5A) due to protonation of the amine groups in repeating units of PEI (Sun et al. 2011). Increasing amounts of HA in the 1st and 2nd configuration vectors reduced their positive charge as the repulsive forces were reduced, until the vectors became negatively charged with further HA addition (Figures 5.5A). Both 1st and 2nd configuration vectors would have HA on the outer layer of the particles. In water, the backbone of HA would be stiffened due to hydrogen bonding between hydroxyl groups along the polymeric chain (Liao et al. 2005). The interactions with water molecules resulted in fully ionized carboxylic groups within D-glucuronic acid of HA forming hydrogen bonds between the two molecules (Liao, Jones et al. 2005; Necas et al. 2008), which enhanced the dispersion of these vectors in the solution (Ito, Iida-Tanaka et al. 2008). Thus the stability of these complexes especially at high HA: PEI ratio could be attributed to the presence of a hydrated shell of HA polymer.

On the other hand, the 3rd configuration vectors (A-3rd SPIONs/PEI+DNA+HA 0.5-25%, and B-3rd SPIONs/PEI/PEI+DNA+HA 50-100%) in water displayed a different behavior. All vectors exhibited relatively small hydrodynamic diameters of < 200 nm in water (Figure 5.4A), with the zeta potential measurement revealing positive surface charges of about 29 mV ± 4 mV (Figure 5.5A), possibly due to high protonated branched PEI that not only condensed the negatively charged DNA and HA, but also embedded them inside the polymeric structure. This finding was in agreement with the previous study on the ability of PEI/SPIONs complexes to entrap DNA molecules within the branched structure of PEI polymer as a result of protonation under physiological condition (Al-Deen, Ho et al. 2011). The A-3rd configuration vectors displayed a larger size of ~400 nm when they were without

HA or with a very small amount of HA (0.5-1% charge ratios). A similar finding has been reported elsewhere, with Arsianti et al. (2010) showing a significantly larger size of 1 – 2 μm when PEI+DNA mixture was added directly onto bare SPIONs. In this study, the coupling of SPIONs with PEI+DNA complexed with HA resulted in smaller sizes of less than 200 nm or even 100 nm, since HA could assist in dispersing small complexes due to their interaction with water molecules (Liao, Jones et al. 2005; Ito, Iida-Tanaka et al. 2008; Necas, Bartosikova et al. 2008).

In 150mM NaCl: All vectors showed relatively large aggregate sizes when they were suspended in NaCl compared to other media. The complexes were unstable and quickly agglomerated in NaCl, reaching several microns for the B-3rd configuration vector at 100 % HA: PEI charge ratio (Figure 5.4B). The larger hydrodynamic diameter of different vectors incubated in 150 mM NaCl buffer was attributed to the aggregation of polycation/DNA complexes through interparticle ionic bridging (Delacruz et al. 1995). Depending on the amount of added HA, their surface charges gradually decreased when the complexes were suspended in 150mM NaCl, to as low as ~ -8 mV at 100% HA : PEI charge ratio (Figure 5.5B). The aggregation of complexes in a high ionic strength buffer such as NaCl was due to the accumulation of ions that suppressed the electrical double layer around the particles (Kitchener 1972). Since an important property of HA in solution is its ability to bind to water and become hydrated to such an extent that a gel-like system, high ionic media may dehydrate HA coating layers. This causes the contraction of polymer chains by disruption of intermolecular interactions that reduces the entanglement between HA molecules, thus inducing aggregation (Eberbeck et al. 2010).

In RPMI media: The stability of magnetic complexes was also tested in a commonly used cell medium, RPMI 1640, to mimic the interaction of particles with a real biological fluid. Generally, an agglomeration of complexes in RPMI media were markedly less than in 150 mM NaCl buffer after 1 hour incubation, except in several of 3rd configuration vectors where they showed slightly larger sizes in RPMI media than in 150 mM NaCl buffer (Figure 5.4C). The RPMI 1640 medium (GIBCO) is supplemented with different components such as vitamins, amino acids, salts, and other components specifically for different cell lines according to the manufacturer's formulation, with NaCl (103.45 mM)

and glucose (11.11 mM) present at much higher concentrations than the rest of the trace elements. Therefore, NaCl and glucose should play more considerable roles in the agglomeration of magnetic vectors suspended in a RPMI medium. The presence of sugars such as glucose in RPMI could retard the disentanglements of HA molecular chains induced by another component such NaCl in the same medium (Mo et al. 1999; Liao, Jones et al. 2005), and thus strengthening the transient network structure to promote hydrogen bonding via polymer–polymer or polymer– glucose groups (Mo, Takaya et al. 1999), consequently improving their stability.

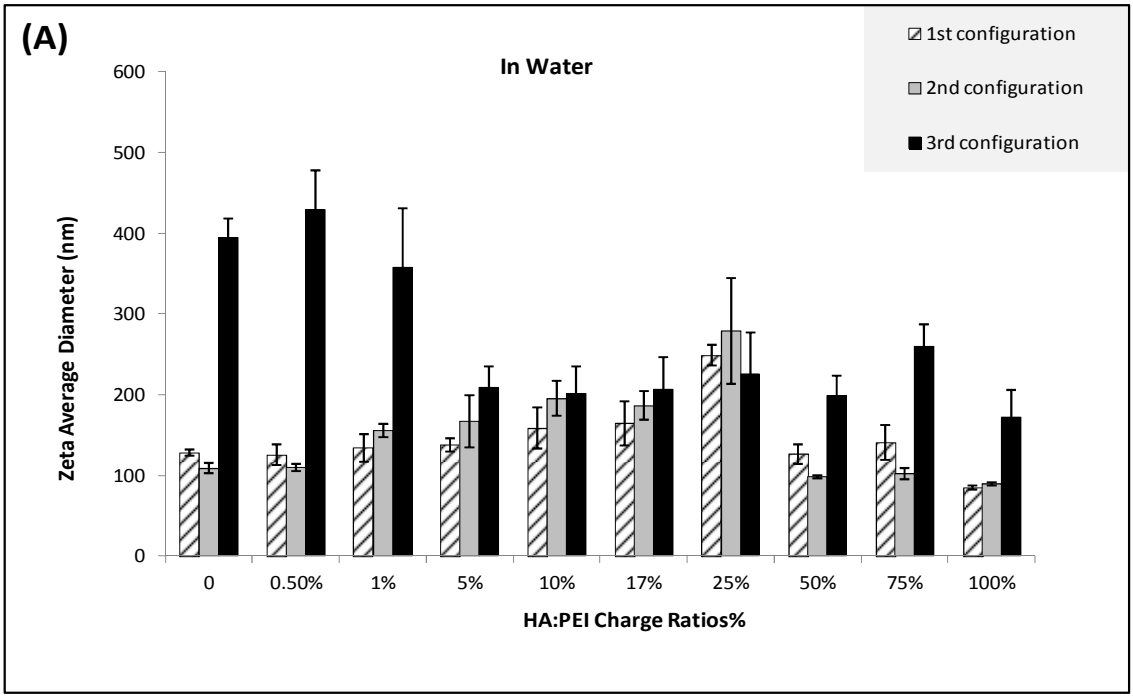
The increasing amounts of HA led to more stable complexes in the 1st and 2nd configuration vectors (Figure 5.4C), suggesting that HA adsorption on the complexes was sufficient to provide steric stabilization under a physiological condition. Smaller aggregates were obtained for 2nd configuration vectors by pre-addition of HA to DNA prior to SPION/PEI (Figure 5.4C), with size reaching ~100nm at HA: PEI charge ratios >50 % in RPMI media. Although RPMI solution contained salts that could induce particle aggregation (Schweiger et al. 2011), adding HA significantly reduced the aggregate size by maintaining the negative zeta potential of complexes from around -10 mV to -20 mV (Figure 5.5C), inhibiting salt-induced aggregation particularly for 2nd configuration vectors (Sun et al. 2009).

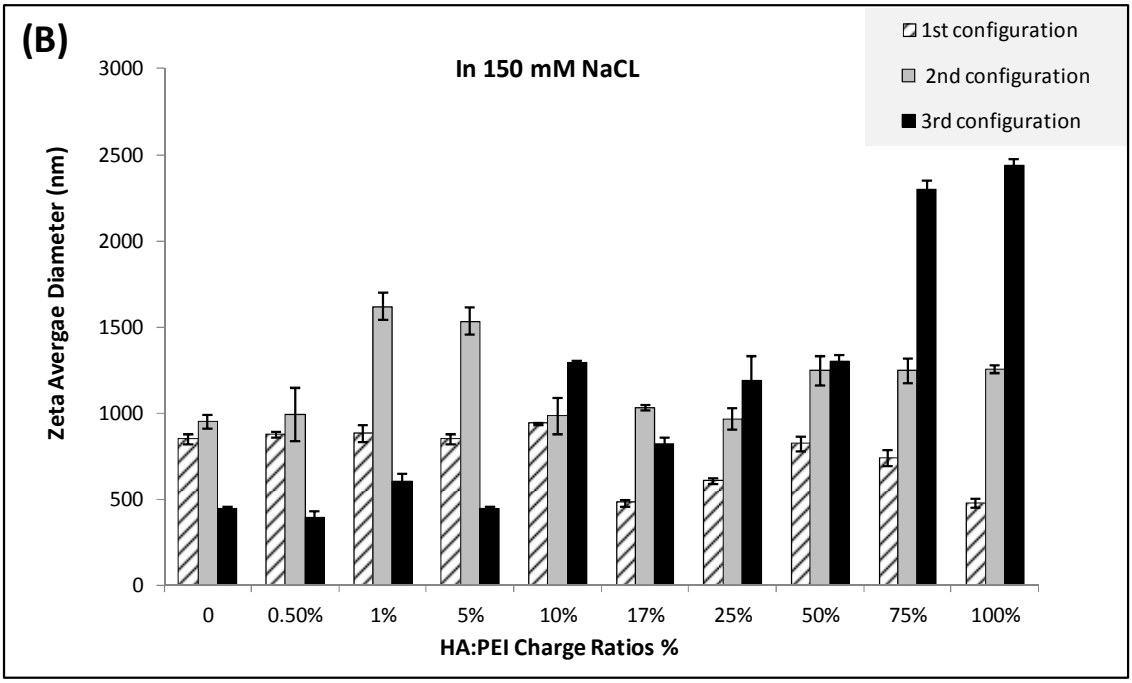
In 10% FBS supplemented RPMI media: Incubation of complexes in RPMI medium supplemented with 10% FBS was done to demonstrate the stability of magnetic gene vectors in conditions normally encountered *in vivo*. All complexes were generally more stable in this medium, compared to other media, with complexes without HA displaying the largest aggregate size at 150 nm ± 20 nm (Figure 5.4D) and lowest zeta potential at ~ -18 mV (Figure 5.5D). The complexes with HA showed surface charges of around ~ -10 mV regardless of % HA: PEI charge ratios or mixing orders of different components (Figure 5.5D). Upon incubation, sizes of around ~ 40 nm were observed for most complexes (Figure 5.4D). The HA coating had been shown to prevent protein fouling and provided steric hindrance against aggregation (Ito, Iida-Tanaka et al. 2006; Ito, Iida-Tanaka et al. 2008; Sun, Ma et al. 2009). The high stability of these complexes could be caused by the effective reduction in absolute protein adsorption levels by depletion stabilization through

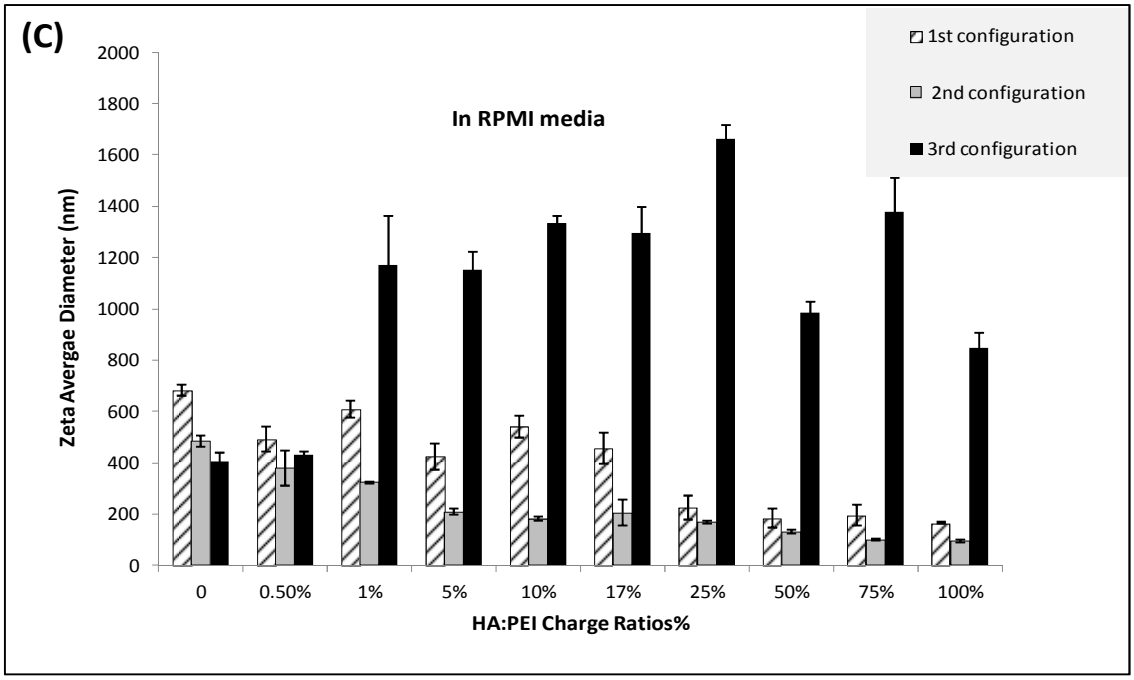
unanchored (free) proteins component of serum, creating repulsive forces that prevented particles from aggregating (Petri-Fink, Steitz et al. 2008).

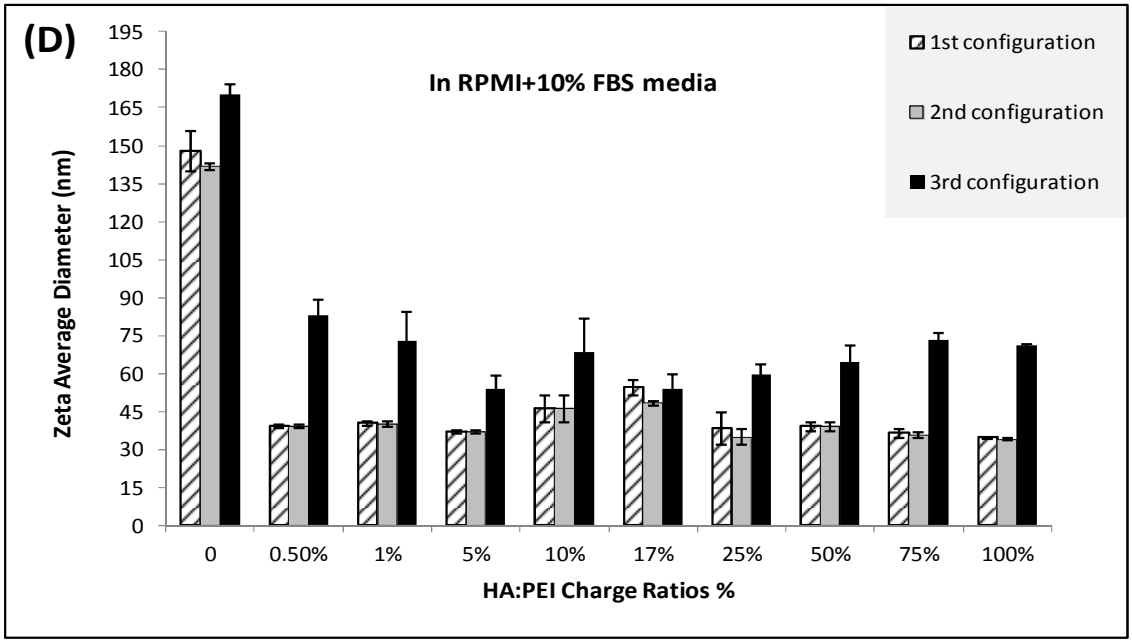
The 3rd configuration vectors showed slightly larger sizes (~80nm) than others (Figure 5.4D). Since HA was possibly embedded to some extent inside branched PEI molecular chains due to protonation of PEI under physiological conditions (Sun, Tang et al. 2011), some unmasked PEI amine groups could remain to interact with serum proteins, inducing agglomeration (Petri-Fink, Steitz et al. 2008). These data confirmed that even small amounts of HA could provide relatively high colloidal stability of complexes in serum-containing media. Since the composition of RPMI with 10% FBS medium closely simulated ionic contents of the biological fluid and was identical to the human blood serum assembly, these results indicated promising *in vivo* applications for HA-modified complexes.

***Size distribution of 2nd configuration vector (SPIONs/PEI/DNA+HA) in RPMI media*:** as 2nd configuration vectors showed more stability in RPMI media than the other vectors, size distributions of these vectors in RPMI media were also present (Figure 5.4E). The average sizes of SPIONs/PEI/DNA complexes were relatively large in the absence of HA with a wide size distribution from 200 nm to several microns (in volume). On the other hand, pre-addition of HA to DNA resulted in the reduction of the population of large aggregates, to give an average of < 90 nm (in volume) at HA : PEI charge ratio of 100%. Since the colloidal stability of gene complexes in different cell media or in body fluids is an important parameter for efficient gene delivery, the 2nd configuration formulation displayed the desired degree of stability in terms of particle aggregate size in RPMI media, rendering it a promising vector for gene delivery *in vivo*.


(A)
In Water
1st configuration
2nd configuration
3rd configuration
Zeta Average Diameter (nm)
600
500
400
300
200
100
0
0
0.50%
1%
5%
10%
17%
25%
50%
75%
100%
HA:PEI Charge Ratios%


(B)
In 150 mM NaCL
1st configuration
2nd configuration
3rd configuration
Zeta Avergae Diameter (nm)
3000
2500
2000
1500
1000
500
0
0
0.50%
1%
5%
10%
17%
25%
50%
75%
100%
HA:PEI Charge Ratios %


(C)
In RPMI media
1st configuration
2nd configuration
3rd configuration
Zeta Avergae Diameter (nm)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
0.50%
1%
5%
10%
17%
25%
50%
75%
100%
HA:PEI Charge Ratios%


(D)
In RPMI+10% FBS media
1st configuration
2nd configuration
3rd configuration
Zeta Average Diameter (nm)
195
180
165
150
135
120
105
90
75
60
45
30
15
0
0
0.50%
1%
5%
10%
17%
25%
50%
75%
100%
HA:PEI Charge Ratios %

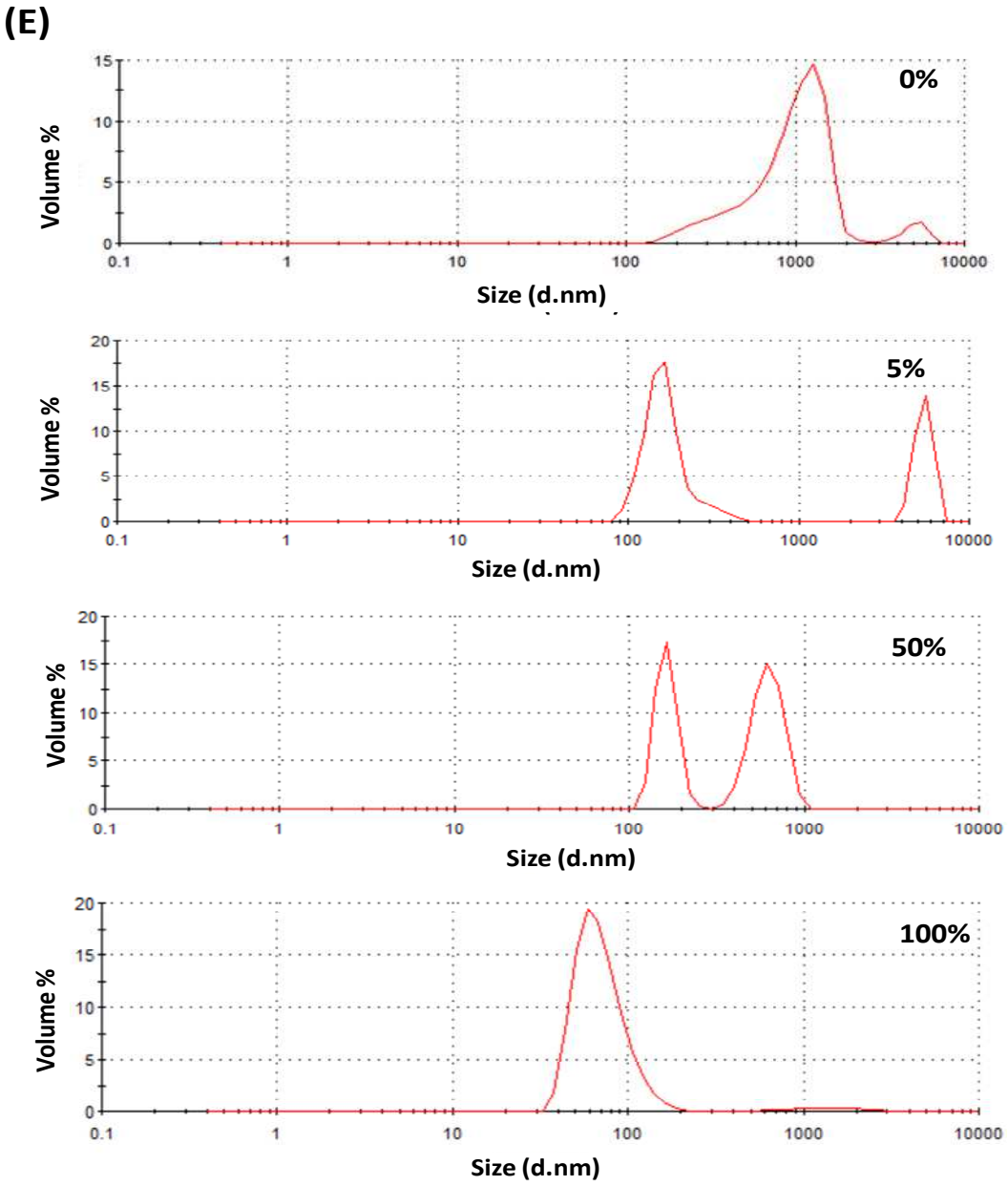
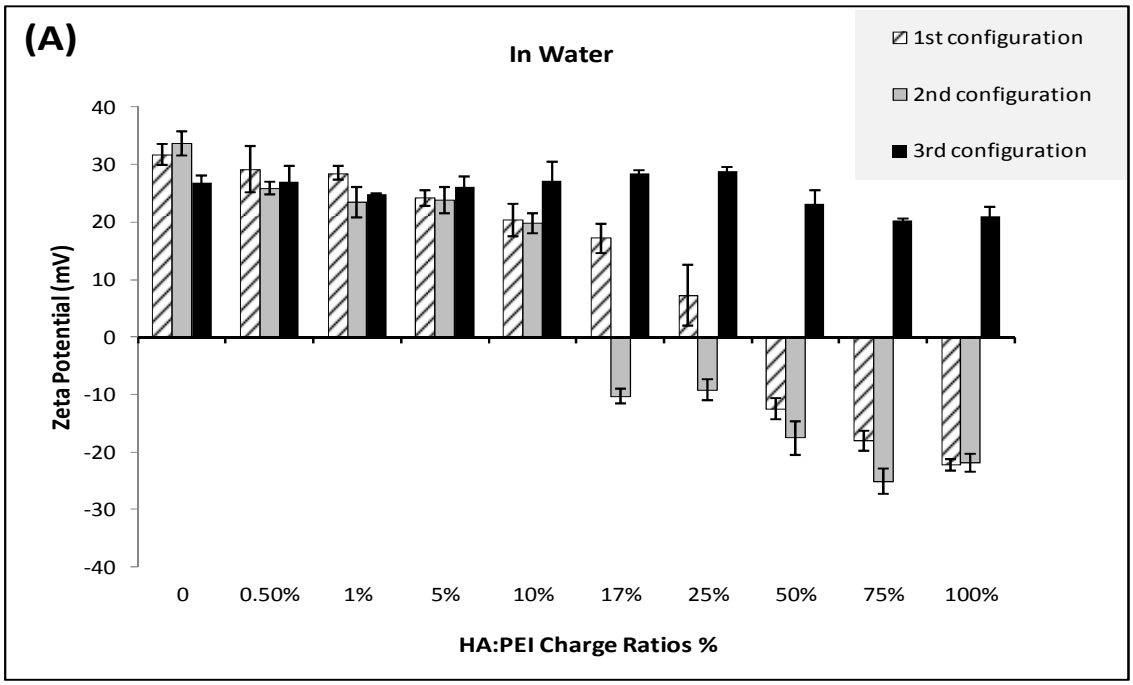
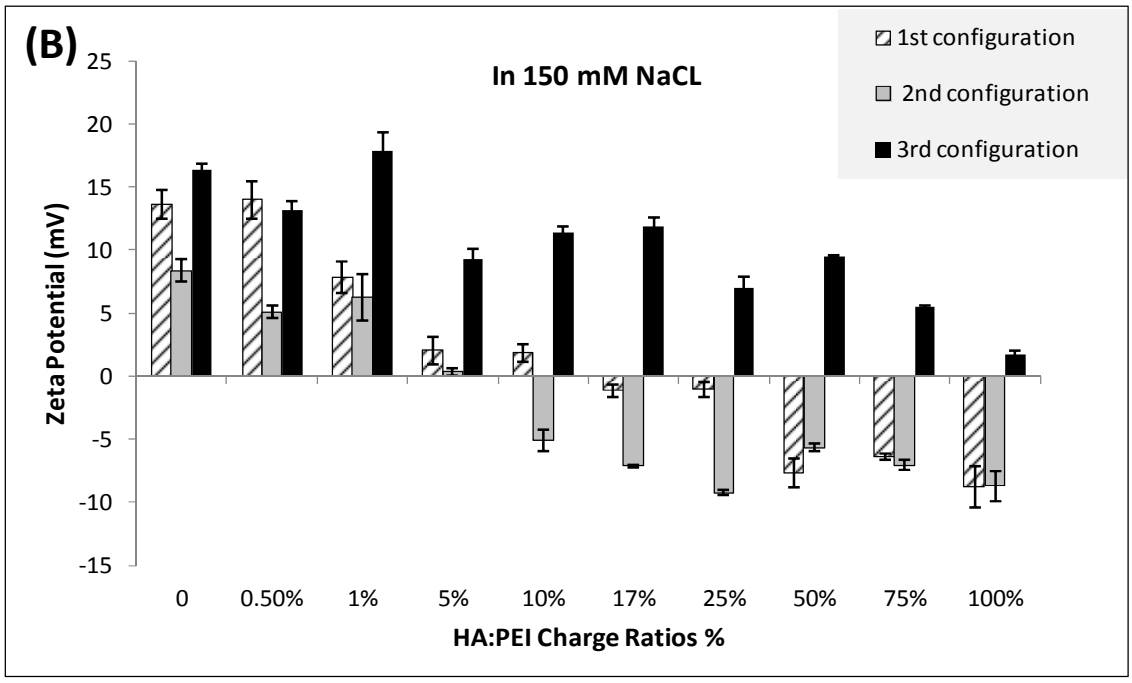


Figure 5.4: Hydrodynamic diameters of different configuration vectors after 1 h incubation in different media with pH maintained at 7.4: (A) In water (B) In 150mM NaCL; (C) In RPMI ; (D) *In* 10% FBS supplemented RPMI; Hydrodynamic diameters of different vectors after 1 h incubation. Error bars represent means ± SD for n = 3. (E) Volume-based size distributions of 2nd configuration vectors in RPMI.


(A)
In Water
1st configuration
2nd configuration
3rd configuration
Zeta Potential (mV)
40
30
20
10
0
-10
-20
-30
-40
0
0.50%
1%
5%
10%
17%
25%
50%
75%
100%
HA:PEI Charge Ratios %


(B)
In 150 mM NaCL
1st configuration
2nd configuration
3rd configuration
Zeta Potential (mV)
25
20
15
10
5
0
-5
-10
-15
0
0.50%
1%
5%
10%
17%
25%
50%
75%
100%
HA:PEI Charge Ratios %

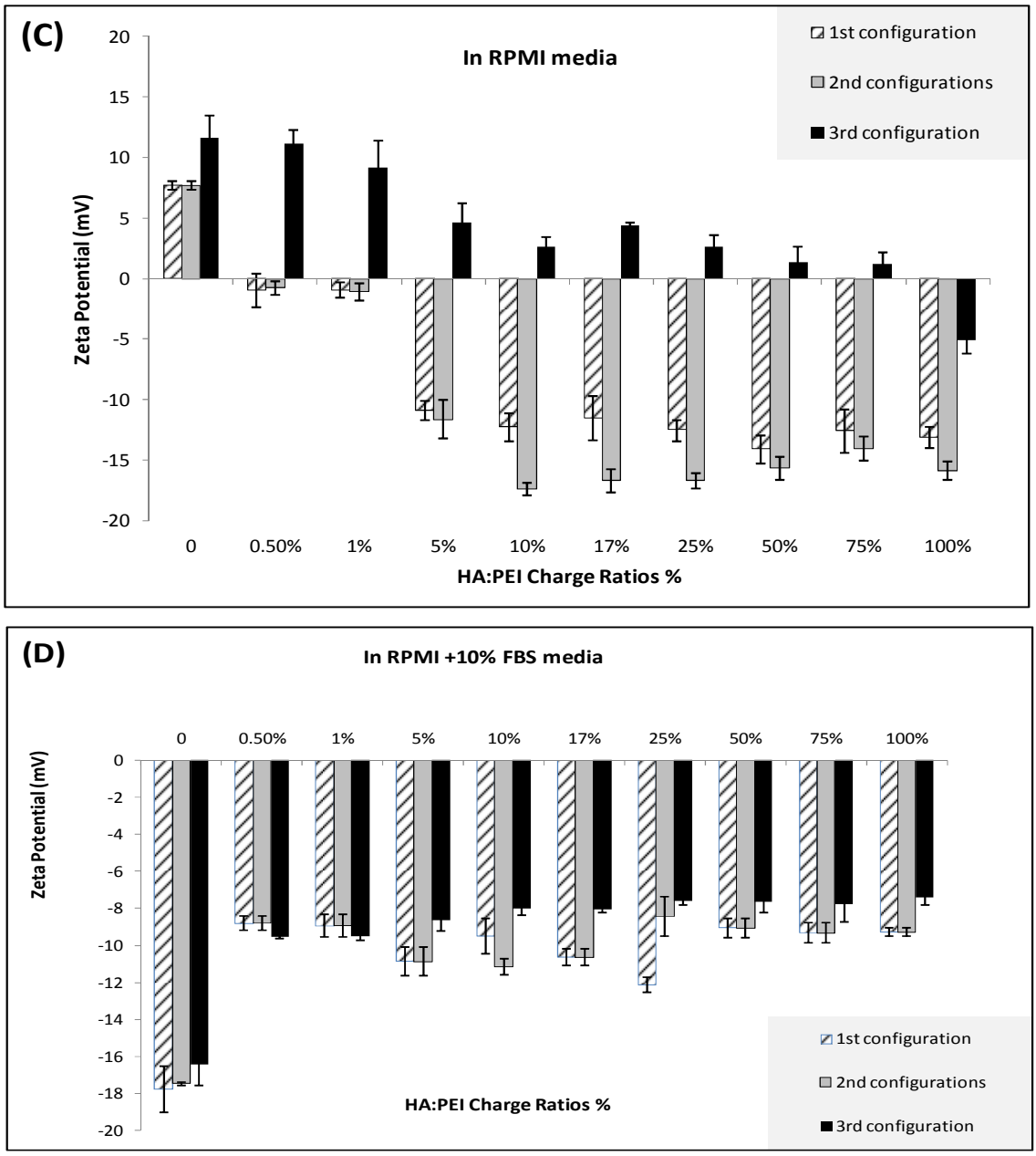


Figure 5.5: Zeta potential of different configuration vectors after 1 h incubation in different media, with pH maintained at 7.4: (A) In water (B) In 150mM NaCL ; (C) In RPMI ; (D) In 10% FBS supplemented RPMI. Error bars represent means ± SD for n = 3.

5.3.3 DNA retardation assays

In water: The release of plasmid DNA was detected for 1st configuration vectors when the charge ratio increased above 25% (Figure 5.6A). The released DNA had the same electrophoretic mobility as super-coiled DNA, indicating that they were no longer associated with PEI. In contrast, at ≤ 17% charge ratio, no release of DNA was observed. Partial disassembly of DNA was also observed for 2nd configuration vectors without any band of free DNA, indicating that a complete disassembly of complexes did not occur (Figure 5.6A). This was probably due to the fact that low charge density of HA was not strong enough to release DNA molecules from the complexes (Ruponen et al. 1999). No release of DNA or even partial disassembly of complexes was observed in the 3rd configuration vectors (Figure 5.6A).

The accessibility of DNA molecules to ethidium bromide in water after 1 h incubation indicated either de-condensation of DNA in the complexes or charge reversal. With the high amount of HA on the surface, the 1st and 2nd configuration vectors possessed highly negative charges (Figure 5.5A) that would promote electrophoretic mobility through the agarose gel. The presence of HA as a coating layer seemed to loosen DNA binding (Ito, Iida-Tanaka et al. 2008). These results suggested that interactions with deionized water could be an important mechanism to release plasmid DNA due to osmotic swelling effects following water penetration to the matrix (Surini et al. 2003; Liao, Jones et al. 2005). Increasing DNA release from 1st configuration vectors with increasing HA indicated that swelling of HA participated in the disassociation of DNA. However, no DNA release from the 3rd configuration further confirmed that DNA molecules were possibly embedded inside the PEI+DNA+HA matrix in a compact structure. DNA molecules could also interact with components from complexes that prevented their electrophoretic mobility, when their negative charges were neutralized **(**Figure 5.6A).

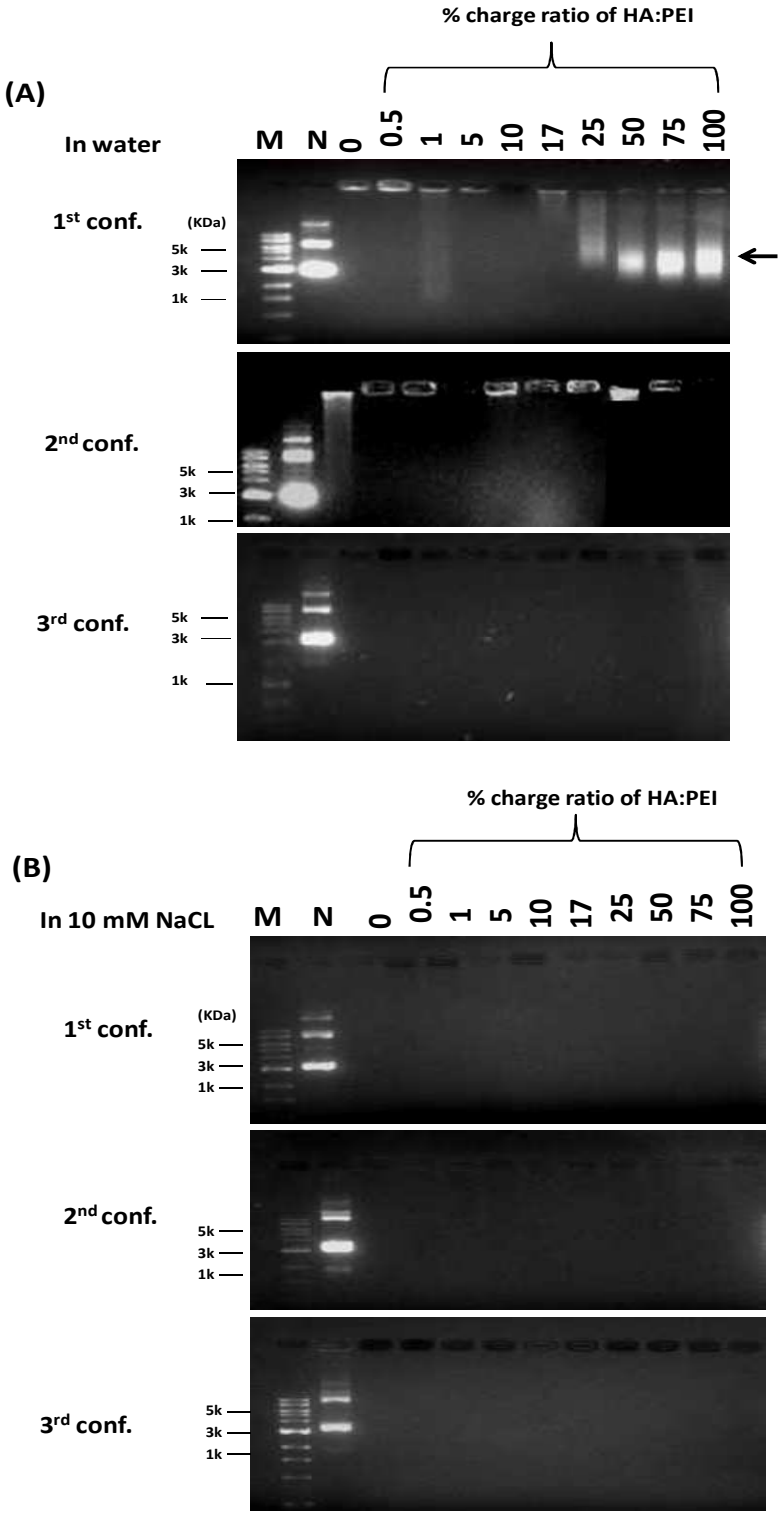
In 150 mM NaCl solution**:** No releases of DNA were observed from any of the complexes when incubated in NaCl buffer solution for 1h (Figure 5.6B). These results demonstrated that the aggregation of complexes in NaCl could affect the release of plasmid DNA. Particles that were incubated in NaCl buffer did not result in any liberation of plasmid

DNA, even at a high amount of HA (Derouazi, Girard et al. 2004). It was noted by Danielsen et al. (2005) that complexes appeared to be more easily dissociated in water than the buffer solution, due to the tight binding of DNA to the vectors because of the contraction of HA chains in NaCl-containing solution that might entrap DNA molecules inside more compact structures (Mo, Takaya et al. 1999). For DNA delivery applications, this would reduce their transfection efficiency due to inaccessibility of the DNA template to the polymerase (Prasad, Gopal et al. 2003; Derouazi, Girard et al. 2004).

***In RPMI*:** Incubation for 1 h could loosen plasmid DNA binding depending on the mixing order of complexes. Figure 5.6C shows that the relative fluorescence intensity was detected in 1st and 2nd configuration vectors. The 2nd configuration vectors clearly displayed fluorescence intensity in the wells with higher HA dosage (charge ratio > 17%). With increasing HA amounts, DNA condensation became less compact, thus increasing its accessibility to ethidium bromide. The loose DNA complexes in RPMI indicated that this medium could be suitable for transfection of HA-coated complexes. The fact that 1st and 2nd configuration vectors demonstrated a relatively good stability in RPMI media in terms of small aggregate sizes (Figure 5.4C), combined with loose binding of DNA in the presence of HA, should favor the gene transfection efficiency of these complexes in this media.

***In 10% FBS supplemented RPMI*:** Figure 5.6D indicates that DNA complexation in 1st and 2nd configurations was more susceptible toward partial disassembly than in the 3rd configuration (Bertschinger, Backliwal et al. 2006). The fluorescence signal increased slightly with HA dosage, indicating interactions between serum components and HA molecules. For almost all lanes of the 1st configuration vectors, a spot was observed (Figure 5.6D), which could be due to some of the released fractions of external DNA when the complexes were incubated in RPMI with 10% FBS. On the other hand, for almost all 3rd configuration vectors, no obvious fluorescence signal was observed. These data again confirmed that adding HA as a mixture with PEI and DNA before adding to SPIONs caused DNA molecules to form more compact structures that prevented their release. Too stable complexes are not very favorable in gene delivery as they would not disassociate and

release DNA intracellularlly to gain access to the cellular transcriptional machinery (Prasad, Gopal et al. 2003).



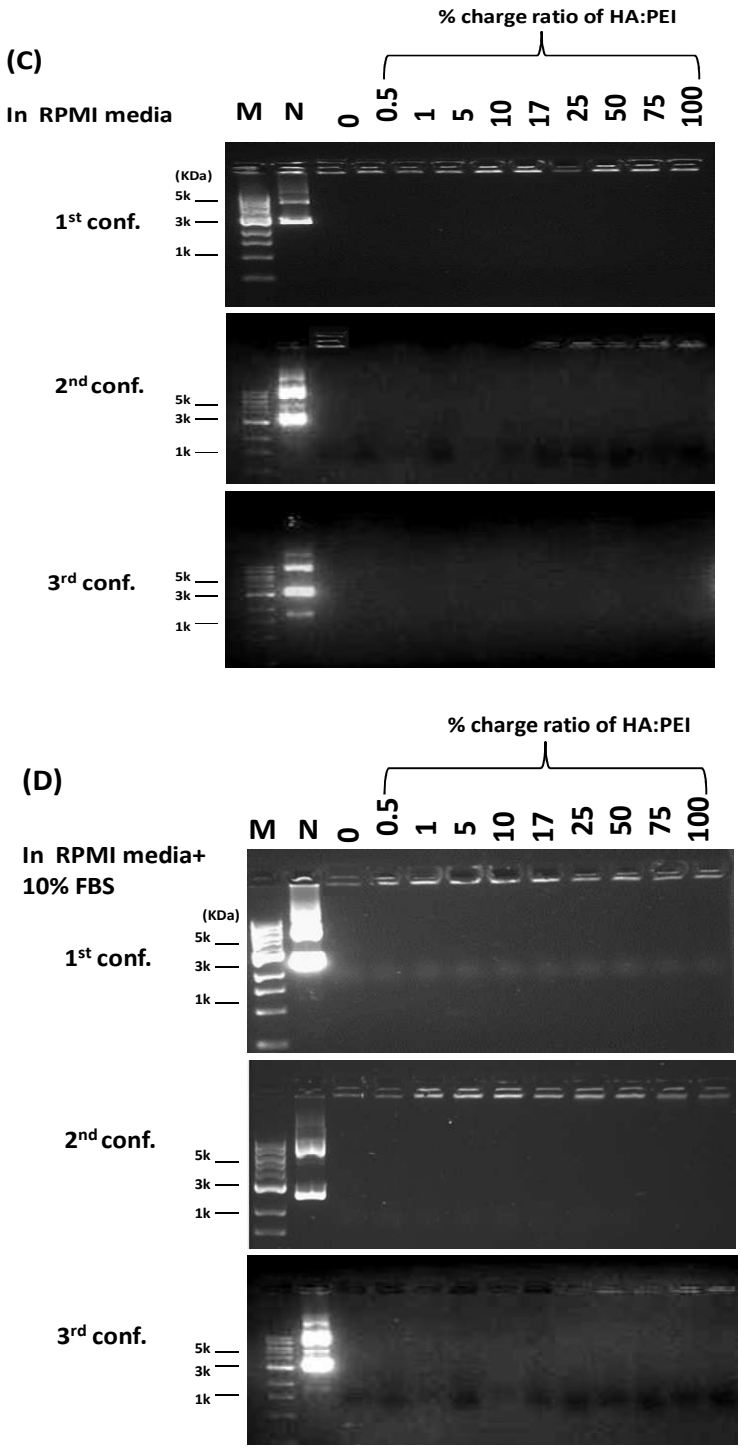


Figure 5.6: Agarose gel electrophoresis data showing the effects of 1 h incubation in different cell media on DNA stability in complexes. Lane M: λ H/E molecular weight size marker; Lane N: plasmid VR1020-PyMSP1$_{19}$; Lane 0: ternary complexes without HA; Lane 0.5 – 100: complexes with different % charge ratios of HA : PEI.

5.3.4 PicoGreen assay

This assay was performed using 5% and 100% charge ratio of HA: PEI to test the effects of different HA quantities on the unbound DNA in suspension. The percentages of unbound DNA for different configurations after 1h incubation in RPMI medium are presented in Table 5.2. In general, all SPIONs vectors retained DNA after 1 h incubation in RPMI with negligible amount of unbound DNA remaining in the supernatant after centrifugation at 12,800 × g for 2 min. The lowest percentage of unbound DNA was found in complexes without HA and in the supernatant for 3rd configuration vectors with very little amounts of DNA (~1%) released into the solution. These results revealed that the these complexes completely reduced DNA release at a N/P ratio of 10 (Al-Deen, Ho et al. 2011), indicating strong interactions between DNA and protonated PEI that entrapped DNA molecules within the branched structure of polymer without any negative effects from HA on DNA condensation on the complexes (Kircheis et al. 2001; Al-Deen, Ho et al. 2011). On the other hand, incubation of 1st and 2nd configuration vectors in RPMI showed slightly increased amounts of unbound DNA (Table 5.2). Pre-addition or even post-addition of HA to DNA as a coating layer on SPIONs/PEI complexes may loosen the strong complexation between PEI and DNA.

Sample		HA:PEI charge ratio	% Bounded DNA
SPIONs/PEI/DNA	Ternary complexes	0	99 ± 1 %
SPIONs/PEI/DNA/HA	1st configuration	5%	96 ± 3 %
		100%	95 ± 2 %
SPIONS/PEI/DNA+HA	2nd configuration	5%	94 ±1 %
		100%	93 ±3 %
SPIONs/PEI+HA+DNA	3rd configuration	5%	99 ±0.5 %
SPIONs/PEI/PEI+DNA+HA		100%	99 ±1 %

Table 5.2: Percentage of bounded DNA in different configurations (incubated in a RPMI medium for 1 h) as detected in the supernatant after centrifugation for 2 min at 12,800 × g using a PicoGreen assay (n = 3, ± SD).

5.3.5 Effect of heparin on the stability of complexes

Interactions between glycosaminoglycans (GAGs) and gene delivery system could impede gene transfer towards the target cell and reduce cellular uptake. Negatively charged GAGs bind to positively charged gene complexes, inducing DNA dissociation, or by changing the size and surface charge to cause dissociation of the complexes (Danielsen, Strand et al. 2005). Heparin and HA are glycosaminoglycans (GAGs) that are present in the extracellular matrix and on the surface of different cell types with different concentrations (Kolset et al. 2004; Kuberan 2011). Although incorporating HA polyanions does not disassociate DNA from complexes, it is important to achieve sufficient stabilization against the extracellular environment with the ability to dissociate inside the cell. Therefore, the ability of competitive anionic moiety heparin with DNA to bind to complexes in the presence of other polyanions (HA) would be an indication of their stability in an extracellular environment.

It was reported that the release of DNA by heparin is concentration-dependent (Moret et al. 2001). Therefore, the exposure of these vectors to increasing amounts of heparin (0.1, 0.3, 0.5 mg/ml) was tested here. After incubation in RPMI and adding heparin solution, the amount of heparin at which DNA was released was used to evaluate the stability of the complexes. Figure 5.7 shows that DNA was released from the ternary complexes when the heparin concentration was 0.1 mg/ml and from 1st and 2nd configuration vectors at 0.3 mg/ml, but no release of DNA was detectable in 3rd configuration vectors until heparin concentration reached 0.5 mg/ml. DNA was released from B-3rd configuration vectors when HA : PEI charge ratio was 100% (SPIONs/PEI/PEI+DNA+HA), while 5% HA : PEI (SPIONs/PEI/ DNA+HA) (A-3rd configuration vector) did not show any release. The high amount of HA in the B-3rd configuration at 100% charge ratio could render it more susceptible to heparin disruption than 5% complexes by loosening the tight binding of DNA, yielding a partial destabilization of complexes that facilitated the approach of heparin to release DNA after 1 h exposure. The significant difference in the capabilities of heparin and HA to dissociate the complexes as observed here was attributed to the much higher anionic charge density of heparin than that of HA, which could disturb the integrity

of cationic gene complexes, in agreement with previous studies (Ruponen et al. 2001; Ruponen et al. 2003).

The amount of heparin needed to release DNA from the complexes without HA (0.1 mg/ml) was less than that for complexes with HA (0.3-0.5 mg/ml) (Figure 5.7). As evident for 3rd configuration vectors, HA could hinder DNA release by heparin, possibly by entrapping DNA inside strong complexes of HA fragments with PEI molecular chains that provided some protection, while only fractions of free DNA were released. Polyanionic glycosaminoglycans such as heparin and heparan sulfate have been reported to preferably bind with PEI and thus release DNA binding from complexes (Moret, Peris et al. 2001), but this interaction could be less important when the surface charge was reduced due to coating with HA (Hornof, de la Fuente et al. 2008). Thus complexes with HA were more resistant to disruption by heparin than those formed in the system without HA.

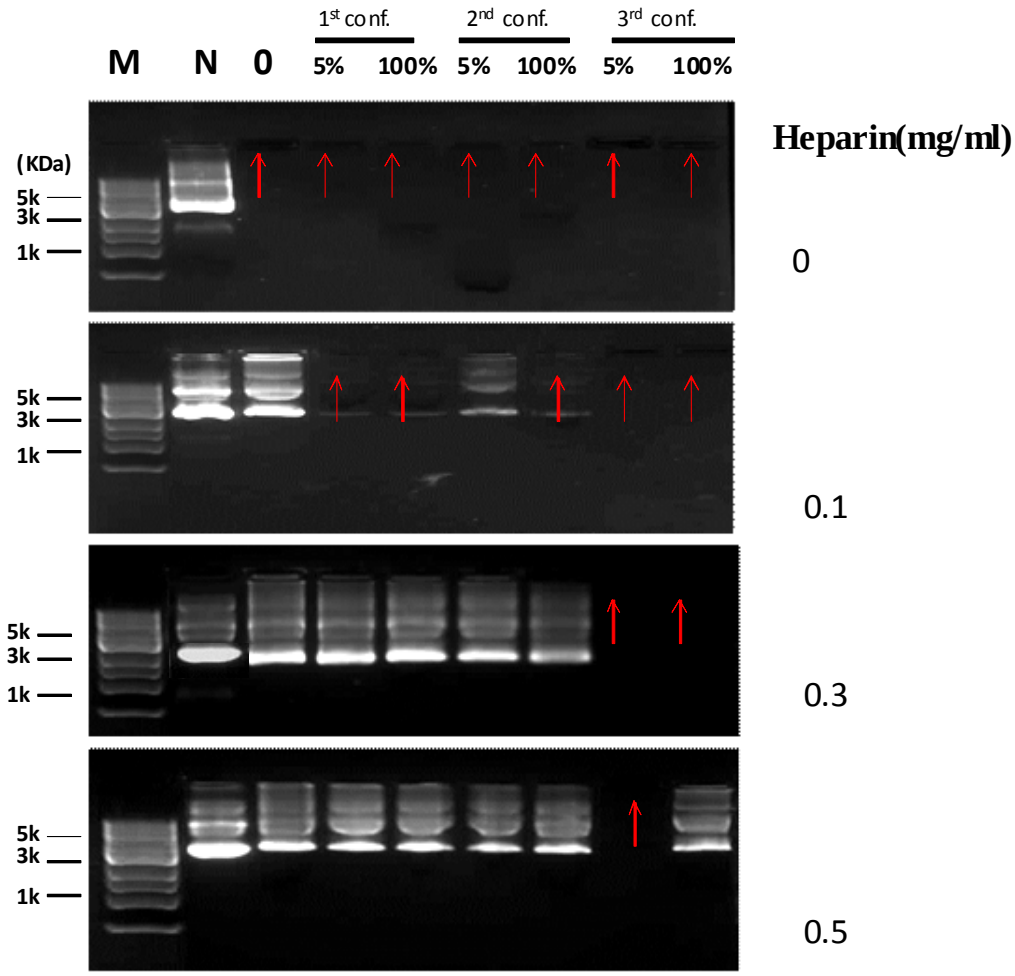


Figure 5.7: Agarose gel electrophoresis showing the effects of heparin on the stability of the complexes. Samples were diluted with RPMI and various amounts of heparin solution (as indicated) were added to the suspension for 1 h at room temperature. After centrifugation, DNA in the supernatant was analyzed by agarose gel electrophoresis. The arrow in each panel indicates the position of complexed DNA without the release by heparin. Lane M: λ H/E molecular weight size marker; Lane N: plasmid VR1020-PyMSP1$_{19}$; Lane 0: ternary complexes without HA; Lane 5%-100%: complexes with different % charge ratios of HA: PEI in different configurations.

5.3.6 Stability against nuclease

A good gene delivery system should be able to offer the protection of DNA against degradation by nuclease in the cellular environment. Figure 5.8A shows the agarose gel electrophoresis from DNase I digestion for different vectors to monitor plasmid DNA degradation. Complexation of DNA with PEI and HA in different configurations appeared to protect DNA from DNase degradation, while naked DNA was completely destructed to nucleic acid debris when treated with DNase I for 30 min.

The amount of free DNA molecules liberated from different complexes by heparin (0.5 mg/ml) and the efficiency of DNase I enzyme to digest them were also observed. Figure 5.8B shows that almost all of free DNA molecules released from complexes were fragmented to nucleic acid debris after 30 min of enzyme treatment. However, the intensities of fragmented DNA band in lane 0 and above were significantly less compared to the bright band from naked DNA in lane N/D (Figure 5.8B). These results indicated that only small portions of DNA molecules were released from each complex to be degraded by DNase I, in the presence of high amount of heparin. No bands of fragmented DNA molecules were observed in the 3rd configuration vectors, indicating that regardless of the amount of HA, these complexes were able to condense DNA in more compact structure against heparin release and DNase I degradation.

Since preserving the structural integrity of plasmid DNA is crucial for transfection efficiency, the most stable component of plasmid DNA is the supercoiled configuration (Weintraub et al. 1986; del Barrio, Novo et al. 2003). It is thus very important to control the stability of plasmid DNA during different treatments. Thus, the possibility of compromising DNA integrity with DNase I on the complexes was investigated, following the enzymatic treatment. Figure 5.8C shows that condensed DNA molecules were released by heparin, while more intact DNA survived the DNase degradation. These results indicated that PEI should provide a sufficient protection against enzymatic degradation without any negative interference by HA. However, no migration of free DNA was observed again in lanes 5% and 100% (3rd configuration) vectors (Figure 5.8C), indicating that the condensed DNA was not released from 3rd configuration vectors by heparin, again confirming the compact condensation of DNA inside these particular complexes.

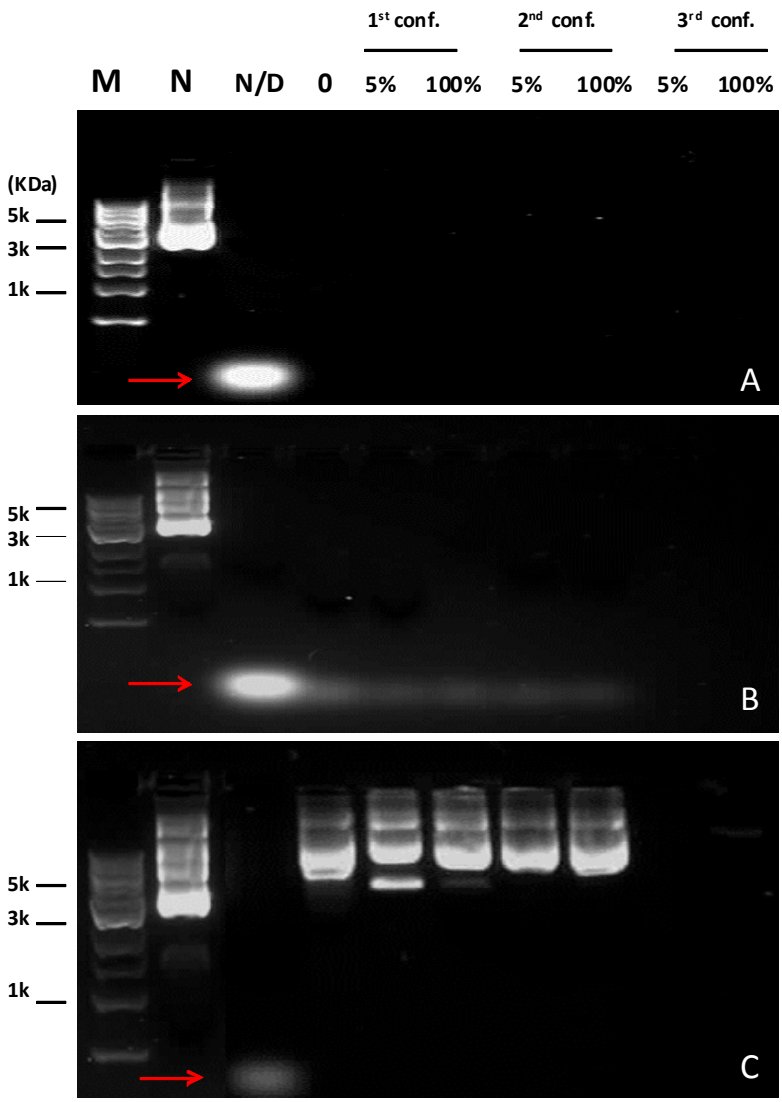


Figure 5.8: Stability of complexes against DNase I, with arrows indicating plasmid VR1020-PyMSP1$_{19}$ degradation: (A) Samples were diluted with 100 µl of 1× DNase buffer containing DNase I 250 U/ml at 37 °C for 30 mins; (B) Samples were diluted with 100 µl of RPMI containing 5 mg/ml heparin for 15 mins and 100 µl of 1× DNase buffer containing 250 U/ml of DNase I was added for 30 mins; (C) Samples were diluted with 100 µl of 1× DNase buffer containing 250 U/ml of DNase I for 30 mins and 100µl of 5 mg/ml heparin was added for 30 mins.

5.4 Conclusion

This study demonstrated that size, surface charge, colloidal stability in different cell media, and polyanions-mediated dissociation by heparin for superparamagnetic DNA vectors were highly dependent on the assembly order of different components comprising complexes for gene delivery. Depositing HA as a coating layer improved their stability and enhanced the loosening of tightly compacted DNA/polycations inside the complexes. The pre-addition of HA to DNA in the 2^{nd} configuration vectors, in particular, played a critical role in producing vectors with narrow size distributions and high stability in RPMI media via electrostatic and steric effects. All complexes with HA regardless of the assembly order showed a reduced ability to interact with serum protein in RPMI supplemented with 10% FBS, indicating the role of HA in minimizing non-specific interactions with serum proteins through depletion stabilization. It is also critical to prevent premature DNA release outside the cells, and at the same time to control the dissociation and release of DNA to gain access to cellular transcriptional machinery. Adding HA to gene complexes as a coating layer could decrease their strong cationic charge, which should allow for timely dissociation of DNA–polymer complexes prior to nuclear entry. At the same time, incorporation of HA protected complexes against polyanions-mediated dissociation by heparin and preserved plasmid DNA in the supercoiled configuration against DNase degradation. This study provided a template to design non-viral vectors with polymers incorporating cell-binding ligands for optimal plasmid DNA vaccine encoding the 19-kDa C-terminal fragment of malaria merozoite surface protein 1 ($MSP1_{19}$) transfection. It is recommended that further work be conducted on the use of the 2^{nd}configuration magnetic gene vectors to efficiently target dendritic cells (DCs) that require high efficiency of binding and up-take of the malaria vaccine by cells, followed by DC activation and maturation.

CHAPTER 6

Design of Magnetic Polyplexes Taken Up Efficiently by Dendritic Cell for Enhanced DNA Vaccine Delivery

6.1 Introduction

A valuable characteristic of DNA vaccination is the capacity to induce both cellular and antibody immunity (Liu 2011). This is particularly useful when attempting to protect against diseases where an effective cellular immune response is crucial in controlling intracellular pathogens, including malaria, AIDS, and tuberculosis (Seder and Hill 2000). Various non-viral vectors have been utilized to deliver genes *in vitro* and *in vivo* (Park, Jeong et al. 2006), including lipid-based and polymer-coated gene vectors. High levels of gene expression *in vitro* were also generated with biodegradable poly (lactic-co-glycolic acid) (PLGA) microparticles of polyethylenimine (PEI)- condensed plasmid DNA encoding the blood-stage malaria protein PyMSP4/5 (Liu, Danquah et al. 2009), attributed to the narrow particle size distribution (0.8–1.9 μm) and high DNA encapsulation efficiencies (82%–96%). The same group subsequently reported high levels of gene expression and moderate cytotoxicity in COS-7 cells using a similar carrier with plasmid DNA encoding PyMSP1$_{19}$ formulated via ultrasonic atomization (Liu, Danquah et al. 2010).

Previously, it has been shown that magnetofection significantly improved the transfection efficiency of DNA encoding *P. yoelii* MSP1$_{19}$ (VR1020-PyMSP1$_{19}$) in COS-7 cells (Al-Deen, Ho et al. 2011). The vector compositions of superparamagnetic iron oxide nanoparticles (SPIONs) and PEI complexed under acidic condition showed better DNA transfection than at neutral condition, possibly due to the protonated structures of PEI

polymer that entrapped and condensed higher amounts of DNA. More importantly, the application of an external magnetic field during the gene transfection process dramatically increased the COS-7 cell transfection efficiency. It is to be noted however that the transfection of COS cells can only be indicative of the potential of any DNA particle-based vaccine to transfect specialized antigen presenting cells (APCs) involved in inducing immunity, and specifically the critical primers of immunity, the dendritic cells (DCs), which contain unique particle uptake pathways, many of which are not present in COS cells (Xiang et al. 2008).

Insights into optimizing the transfection of DC, the most powerful APC, are needed if we are to develop effective vaccines that target DC *in vivo*, as well as optimize DC transfection for use in ex-vivo therapies. Although the uptake of DNA or DNA-loaded vaccine carriers by DCs is a vital step for the transfection of these cells, it is by itself not enough to ensure a successful expression of the antigen in DC, or ensure that the DC are activated and able to interact positively with T cells, usually monitored by surface expression of CD86 and MHC class II molecules. In general, the transfection efficiency of non-viral gene delivery systems in non-dividing primary cells like DCs decreases considerably when compared to transformed cell lines (Hamm et al. 2002). A study recently done by Chapman et al. (2008) found that the application of permanent and pulsating magnetic fields could significantly enhance the efficiency of gene delivery in primary cells such as macrophages, synoviocytes, chondrocytes, and others isolated from different organs if polyethylenimine-coated magnetic nanoparticles were used.

To achieve optimal gene transfection specific for DCs through improved cell entry, various barriers including DCs cellular targeting, particle uptake, and DNA endosomal escape have to be overcome. DCs further needs to be in a mature/activated state to express high levels of major histocompatibility molecules MHCI and MHCII, as well as T cell costimulatory molecules such as CD86, to effectively stimulate CD8 and CD4 T cell immunity. With respect to transfection of DCs, certain plasmid DNA formulations or delivery systems may be able to target DCs for improved DNA uptake — for example, polyplexes with longer diblock copolymer chain showed the highest cellular uptake by DCs compared to a short chain followed by effective gene transfection (Tang et al. 2010).

Hyaluronic acid (HA) is a high molecular weight mucopolysaccharide naturally present in all living organisms, as the most important space-filling element concentrated in the extracellular matrix and mammalian joint liquids (Necas, Bartosikova et al. 2008). It consists of a linear, unbranched, polyanions formed by D-glucuronic acid and N-acetyl-D-glucosamine repetitive units, proven to be low toxicity, non-antigenic, non-immunogenic, biodegradable, and biocompatible (Necas, Bartosikova et al. 2008). Toll-like receptors (TLR) including TLR4 and TLR2, CD44, RHAMM, TNFIP6, HARE and LYVE-1 have been described as the main receptors for HA for different biological functions (Oh, Park et al. 2010). A gene delivery system consisting of SPIONs/PEI/DNA-HA polyplexes can therefore be used to target DCs. The uptake of these polyplexes would be facilitated by HA receptor-mediated endocytosis on the surface of DCs, while at the same time maintaining the endosomal escape capacity of the PEI polymer (Godbey, Wu et al. 1999). Such a system could also provide an additional layer of targeting by combining magnetic nanoparticles, which initiate the surface of DCs under an external magnetic field, with targeting of specific DC receptors by HA ligands on the surface of the magnetic vectors.

Here for the first time the results of utilizing a novel system of SPIONs/PEI/DNA-HA malaria gene complexes were reported to target and transfect DC. Given the magnetic core, and the potential opportunity offers for directional transfection, the transfection efficacy has been further analyzed with or without the added variable of an external magnetic field. Additionally, the maturation/activation state of the DCs *in vitro* was assessed after interaction with these nanoparticles and magnetic fields. It has been focused on a quaternary system (SPIONs/PEI/DNA-HA) for blood-stage malaria DNA vaccine that encodes *P. yoelii* merozoite surface protein $MSP1_{19}$ (VR1020-Py$MSP1_{19}$). The effects of combining different molecular weights of HA with different percentage charge ratios of HA: PEI on the stability, cytotoxicity, and DC transfection/ maturation efficiency of the polyplexes were all investigated *in vitro*. This illustrates how the interactions of such well-designed quaternary complexes under magnetic field may markedly influence the delivery of genetic cargos to DCs.

6.2 Materials and methods

6.2.1 Materials

Sodium hyaluronate (HA) with average molecular weight of <10 kDa and 900 kDa was purchased from Life Core Biomedical LLC. RPMI 1640 medium, 0.05% trypsin-EDTA, penicillin/ streptomycin, L-glutamine, Lipofectamine and fetal calf serum 2000 were from Gibco-BRL (Gathersburg, MD). Hamster-anti-mouse APC-CD11c antibody, rat-anti-mouse biotinylated-CD86 (B7.2), rat anti-mouse APC-CD44 and rat-anti-mouse PE-MHC Class I were purchased from BD Pharmingen, while granulocyte and macrophage colony stimulating factor (GM-CSF) were ordered from PeproTech. PE-Cy7-streptavidin was from BD Bioscience while rat-anti-Mouse APCCy7-MHC Class II was from eBioscience. Live /Dead fixable dead cell stains kit (Aqua LIVE/DEAD; Invitrogen) and Lipofectamine 2000 (Gibco-BRL, Gathersburg, MD) were supplied by Invitrogen (Carlsbad, CA). Mammalian expression vector VR1020 (Vical Inc., San Diego, CA), VR1020-PyMSP1_{19} and VR1020-YFP plasmids were obtained from collaborators at the Coppel's lab (Department of Microbiology, Monash University). C57BL/6 mice (aged 6–8 weeks) were supplied by the animal facilities in the Alfred Medical Research and Education Precinct (AMREP). Arrays of permanent magnets of neodymium iron boron (Nd-Fe-B) in the format of a 6-well plate were used for *in vitro* transfection experiments. The magnets were circular disk Nd-Fe-B magnets (diameter 25 mm, height 5 mm) glued onto the bottom of a 6-well plate.

Methods

The SPIONs were prepared and characterized as previously described in the sections 3.2.2, while SPIONs were coated with PEI polymer as described previously in the section 5.2.2.
VR1020-PyMSP1_{19} and VR1020-YFP plasmids were amplified using *Escherichia coli* DH5α and prepared as reported before in the section 3.2.3.

6.2.2 Preparation of SPIONs/PEI/DNA-HA quaternary polyplexes

To prepare the quaternary polyplexes, $MSP1_{19}$/YFP plasmids mixtures, prepared by mixing $MSP1_{19}$ with YFP plasmid in the ratio 1:1 (w: w) with a total mass of 2 µg of DNA, were added to each well in the 6 wells plate. The $MSP1_{19}$/YFP plasmids mixture was then mixed with equal volumes (e.g. 100 µl) of HA solutions containing HA <10 kDa (low molecular weight LMW) or HA 900 kDa (high molecular weight HMW) to form DNA-HA mixture, and then incubated for 10 min at room temperature (RT). At the molar ratio of PEI nitrogen to DNA phosphate (N/P) of 10, DNA-HA matrices were added to 100 µl of SPIONs/PEI solutions. The amount of HA was adjusted according to the different % charge ratio of -COOH (carboxylic group of Hyaluronan): N (from amine group of PEI). In this study, 5% and 100% charge ratios of HA : PEI were used. The final mixtures were then mixed and incubated at room temperature RT for 30 min to construct the SPIONs/PEI/DNA-HA quaternary polyplexes. The resulting suspension was adjusted to 1 ml at the pH value of 7.4 by the addition of RPMI medium before use. For comparison, SPIONs/PEI/DNA ternary complexes were also prepared by adding100 µl of $MSP1_{19}$/YFP plasmids mixtures solutions (2 µg plasmid/ml) to 100 µl of SPIONs/PEI solutions, before incubating at RT for 30 min. Lipofectamine 2000 reagent (Invitrogen) was used as a positive control according to the manufacturer's instructions.

6.2.3 Characterization of SPIONs/PEI/DNA-HA quaternary polyplexes

Size distribution and zeta potential of the polyplexes in aqueous solution were measured by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments, UK). Nanoparticle suspension was diluted to 1 ml with RPMI 1640 medium at 2 µg DNA/ml and ultrasonicated for 30 min before the samples were measured. The results were presented as average values of three replicates. The morphology of nanoparticles was also observed by transmission electron microscopy (TEM) using a JEOL 2011 TEM microscope.

6.2.4 DNA retardation assays

DNA binding capabilities of the quaternary polyplexes (SPIONs/PEI/DNA-HA) were determined by gel electrophoresis (1% agarose gel). SPIONs/PEI/DNA-HA was formed at a N/P ratio of 10 and HA: PEI charge ratios of 5% and 100%. In each case, the appropriate amount of 0.5 μg plasmid $MSP1_{19}$/ HA mixture was mixed with SPIONs/PEI complex in 20 μll PBS buffer. These solutions were incubated at 37 °C for 30 min and mixed with 1 μl of the loading dye (bromophenol blue/ xylene cyanol) solution before loading into agarose gel (1.0% agarose in 1 × TBE Tris-borate EDTA buffer containing 0.5 μg/ml ethidium bromide). Electrophoresis was carried out at 60 V for 90 min. DNA bands were visualized and photographed using a UV transilluminator and Molecular Imager Gel Doc XR System imaging system (BIO-RAD).

6.2.5 Nuclease resistance

The DNase I sensitivity assay was carried out to evaluate the protection of DNA molecules in the complexes against DNase degradation. Free DNA, ternary complex SPIONs/PEI/DNA, and quaternary polyplexes with 5% and 100% charge ratios with two different molecular weights of HA (total mass of pDNA ($MSP1_{19}$) of 50 μg per ml) were formed at N/P ratio of 10. The complexes were then allowed to mature in RPMI medium for 30 min at 37^{o}C. Free DNA and the DNA complex solution were separately incubated with the DNase I reaction buffer (pH 7.5, 100 mM Tris HCl, 500 mM $MgCl_2$, 13 mM $CaCl_2$) included RNase-free DNase I (250 U/ml) for 30 min at 37^{o}C. After incubation, the sample tubes were rapidly placed on ice and the enzymatic reaction was terminated by adding 100 μl of stop solution of (500 mM EDTA) at pH 8 per milliliter of extract and mixed well. Then the samples were centrifuged at 12,800 ×g for 2 min, and twenty microliters of the supernatant were analyzed by 1% by agarose gel electrophoresis. To further ensure DNA super-coil integrity in the complexes following DNase digestion, the complex solutions were diluted by the addition of heparin solution to 5 mg/ml for 30min at 37^{o}C after the termination of the DNase I enzymatic reaction. Then the solutions were

centrifuged at 12,800 ×g for 2 min and twenty microliters of the supernatant were analyzed by 1% agarose gel electrophoresis.

6.2.6 Cytotoxicity assay

The toxicity of SPIONs/PEI/DNA-HA quaternary polyplexes were studied by co-culturing the polyplexes with COS-7 cells, to determine the cell variability by the MTT colorimetric assay (Jaracz et al. 2005). For comparison, SPIONs/PEI/DNA ternary complexes and naked DNA solutions were also tested. The cells were cultivated in the complete RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM of L-glutamine, 100 μg/m1 streptomycin, and 100 μg/ml of penicillin at 37 °C incubator with 5% CO2. COS-7 cells were seeded onto 96-well plates at a density of 2×10^4 cells/well and incubated for 24 h. The cells were then treated with SPIONs/PEI/DNA-HA quaternary polyplexes, SPIONs/ PEI/DNA ternary complexes, and naked DNA respectively. After another 24 h culture, 5 μl of MTT solution at a concentration of 5 mg/ml in phosphate buffer was added to each well, with a further incubation for 2 h. Then the supernatant was aspirated, and the formazan crystals were suspended in 100 μl of dimethyl sulfoxide (DMSO) with an incubation period of 1 h. The intensity of color was measured spectrophotometrically at wavelengths of 570 and 690 nm simultaneously using an ELISA plate reader (Magellan, Tecan, Austria). Untreated cells were taken as control with 100% viability. The relative cell viability (%) compared to control cells was calculated according to: (*means absorbance of sample*/*means absorbance of control*) ×100%. All experiments were repeated at least three times (Al-Deen, Ho et al. 2011).

6.2.7 Generation of murine bone marrow–derived dendritic cells DCs

Bone marrow-derived dendritic cells (BM-DC) were generated as previously described with minor alterations (Inaba et al. 1993). Briefly, to harvest the bone marrow cells, four to six-week-old C57BL/6 mice were sacrificed and their femur and tibia bones were removed, cleaned, and soaked in 70% ethanol for 1 min. The bones were then washed twice and transferred into a complete RPMI 1640 medium containing 2% HEPES buffer, 0.1 mM 2-ME, 100 U/ml penicillin, 100 µg /ml streptomycin, 2 mM glutamine and 10% Fetal Calf Serum (FCS) (all from GIBCO, Gathersburg, MD). The bones were cut at both ends and the marrow was flushed out using complete RPMI 1640 medium. The marrows were then filtered through cell strainer (100 µm nylon mesh, BD Falcon, Franklin Lakes, NJ, USA), centrifuged at 1500 rpm at RT for 5 min, and treated with lysing buffer (0.15M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2 EDTA, pH 7. 2) to lyse the erythrocytes. The harvested cells were then washed with excess volume of complete RPMI 1640 medium and centrifuged to remove the supernatant. The cells were subsequently cultured in complete RPMI 1640 medium supplemented with 10 ng/ ml GM-CSF (PeproTech, Rocky Hill, NJ. USA) at a cell density of 5 x 10^5 cells/ml in 6-well plate (3ml/well), and maintained at 37 °C in a humidified incubator containing 5% CO_2 for 5 days.

6.2.8 Magnetofection-based DCs transfection and maturation *in vitro*

To examine the effects of quaternary polyplexes on DCs transfection and maturation state, on day 5 of the BM-DC culture, the culture plates were centrifuged and the medium was removed as above. The cells in each well were washed with serum free RPMI 1640 medium. Freshly prepared ternary and quaternary polyplexes of SPIONs/PEI/DNA-HA with two different molecular weights of HA and two different ratios of HA: PEI (charge) at N/P of 10 was diluted in serum free RPMI 1640 medium for 15 min at RT. The polyplexes were then added to the cells with a fixed amount of $MSP1_{19}$ /YFP plasmid mixtures of 2 µg

per well. The cells were cultured with a neodymium-iron-boron magnet positioned under each well for two hours. After 5 hours, the culture media containing polyplexes were removed from each well and replaced with 2 ml of the fresh complete medium containing 10 ng/ml (GM-CSF) and cultured for a further 24 h in 37 °C incubator with 5% CO_2. DCs transfection efficiency by quaternary polyplexes was examined with or without the application of the magnetic field during the process. Transfection of DCs with plasmid DNA alone without complexation in the polyplexes was also examined for comparison. To examine the transfection efficiency of quaternary polyplexes in DCs and maturation of CD11c+ DCs, the non-adherent cells were harvested and washed with PBS after 24 h. The cells were stained using the following antibodies: APC-CD11c, biotinylated-CD86, APC-CD44, PE-MHC Class I, APC-Cy7-MHC Class II, while dead cells were excluded using live/dead fixable dead cell stain kit (Aqua LIVE/DEAD, Invitrogen)**.** Biotin-labeled mAbs was developed with PE-Cy7-streptavidin and analyzed by flow cytometry (FACSCalibur). Untreated DCs were also included as negative controls.

6.2.9 Statistical analysis

Statistical analyses were conducted with GraphPad Prism (version 5, Graph Pad Software) for the preparation of graphs and statistical analysis. Comparison of two relevant groups was conducted using an unpaired t-test. The significance for all statistical analyses performed was set at *$p < 0.05$, ***$p < 0.001$ and ****$p < 0.0001$.

6.3 Results and discussion

The magnetic particles showed a magnetization of > 63 emu/g under 15 kOe of applied magnetic field at room temperature, with 0.01 emu/g remanance indicating superparamagnetic behavior (Appendix A. **(**Figure A.2)), while Appendix A. **(**Figure A.3) shows that the X-Ray diffraction pattern of the sample was identical to the standard X-Ray diffraction pattern for pure magnetite JCPDS (Card No. 01-072-6170).

6.3.1 Stability of SPIONs/PEI/DNA-HA quaternary polyplexes

The quaternary polyplexes were synthesized using a layer-by-layer approach via electrostatic interactions between the components. In the first step, the negatively charged SPIONs were coated by the cationic polymer PEI, and then the negatively charged mixture of DNA and HA were added onto the PEI-coated SPIONs. Figure 6.1A showed the TEM image of the synthesized SPIONs, with average diameters of 10 nm ± 2 nm, whereas the hydrodynamic diameter (as measured by dynamic light scattering) was predominantly 85 nm ± 3 nm, representing the particle size in suspension. The particles were negatively charged with an average zeta potential of -40 mV ± 3 mV, thereby enhancing the interaction with PEI to form a stable coating on the surface of the magnetic particles (Al-Deen, Ho et al. 2011). When PEI was added to the nanoparticles with PEI/Fe mass ratio (R) of 10, the adsorption of PEI polymer onto SPIONs decreased the surface charge from highly negative to positive (15 mV ± 3 mV). In the second step, when negatively charged DNA solution was added to PEI-coated SPIONs with N/P ratio of 10, the surface charge of the polyplexes decreased slightly to 10 mV ± 2 mV, signifying DNA adsorption (Figure 6.1B). To examine what occurs in the culture medium, ternary complexes of SPIONs/PEI/DNA were incubated in RPMI 1640 medium for 1 hour. Upon incubation, the hydrodynamic particle size increased to approximately 450 nm ± 30 nm (Figure 6.1B), while the zeta potential was 10 mV ± 2 mV, indicating a rather weak net positive charge. Wiogo et al. (2011) proposed that the aggregation of magnetic particles incubated in a high ionic strength medium, such RPMI-1640 or PBS buffer, was due to the suppression of the double layer around the particles which decreased the electrostatic repulsion. From the TEM image (Figure 6.1A), it appeared that by adding the DNA-HA mixture, a thick layer could be seen covering the surface of the magnetic particles. Similar hydrodynamic size distributions were displayed for quaternary polyplexes with 5% and 100% charge ratio (HA : PEI) using different molecular weights of HA in RPMI 1640 medium, giving a mean size of 150 nm. Addition of HA caused the reduction of the positive zeta potential of the complexes from 10 mV ± 2 mV to ⁻15 mV ± 3mV (Figure 6.1B), thus minimizing the interaction with the components of RPMI medium that could induce aggregation (Sun, Ma et al. 2009).

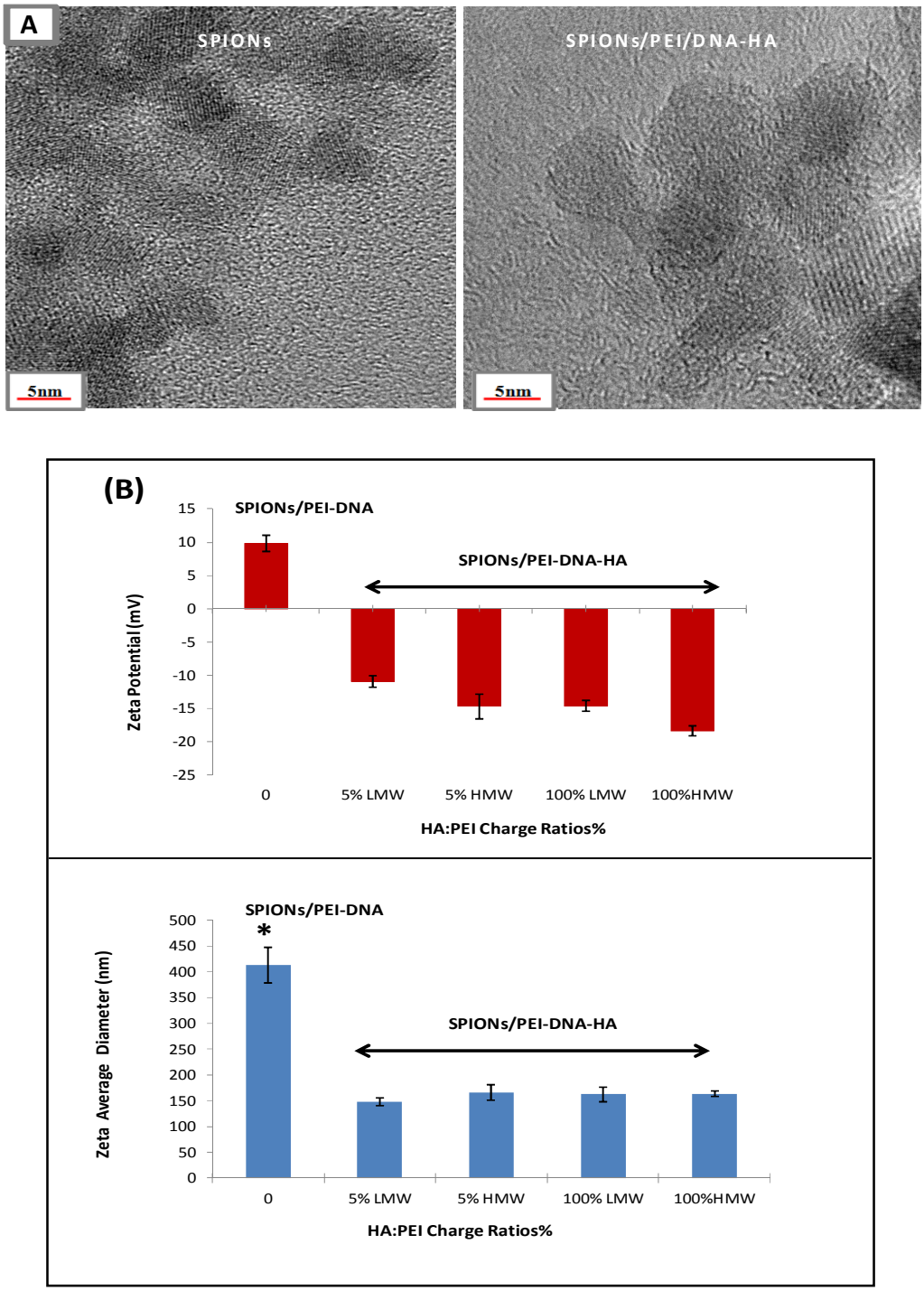


Figure 6.1:Characterization of magnetic gene complexes (A) TEM images of as-synthesized SPIONs, SPIONs/PEI/DNA-HA complexes; (B) Zeta potential (mV) of SPIONs/PEI/DNA-HA polyplexes and hydrodynamic diameter (nm) of SPIONs/PEI/DNA-HA polyplexes, measured in RPMI media at pH 7.3 at 37 ^{o}C. The data were analyzed statistically using one-way analysis of variance (ANOVA), with comparison of means conducted using Tukey multiple comparison test. Results are expressed as means ±S.D. (n =3), * P<0.05. All measurements were measured at least in triplicate.

6.3.2 DNA condensation in the polyplexes

The image from the gel retardation assay is shown in Figure 6.2A, with a slight intensity of ethidium bromide fluorescence in the application slots, indicating tightly associated DNA in the ternary complex SPIONs/PEI/DNA at N/P ratio of 10. No free DNA was detected in the polyplexes with a low molecular weight of HA (LMW), confirming that the disassembly of quaternary polyplexes did not occur (Figure 6.2A) (Sun, Ma et al. 2009). On the other hand, large amounts of DNA stayed in the wells with small fluorescent trails formed (as indicated by arrows in Figure 6.2A) for the polyplexes with high molecular weight HA (HMW). This could be due to the increasing molecular length and negative charge of high molecular weight HA, which could compete with DNA molecules to interact with PEI without releasing DNA completely from the polyplexes (Ito, Iida-Tanaka et al. 2006). The short fluorescence trails of free DNA implied that the disassociated DNA from polyplexes was not in free-form, but might be complexed with high molecular weight HA molecules, whereas the large size of these complexes prevented them from migrating through the gel pores (Kim et al. 2003). Notably, DNA–HA complexes prevented DNA release as no clear band was noted in the same proximity as the naked plasmid DNA.

6.3.3 Nuclease resistance

The stability of plasmid DNA in the complexes from DNase degradation was examined using DNase I. Figure 6.2B shows the agarose gel electrophoresis from DNase I digestion of DNA with and without complexes. It was expected that the plasmid DNA in either ternary or quaternary complexes would be more resistant to DNase I degradation than free DNA. Free DNA (uncomplexed) was rapidly degraded by DNase I and almost all DNA molecules were fragmented with enzyme treatments as shown in Figure 6.2B, while DNA complexes displayed no fluorescent bands either for ternary or quaternary complexes. The complexes maturation in RPMI medium and incubation with the DNase I reaction buffer (pH 7.5, 100 mM Tris HCl, 500 mM $MgCl_2$, 13 mM $CaCl_2$) for 30 min may also participate to entrap DNA molecules inside the polymers structure. It was noted by (Al-Deen, Selomulya et al. 2013) that no complete or even partial release of DNA molecules from

SPIONs/PEI/DNA or SPIONs/PEI/DNA-HA complexes has observed when these complexes were incubated in the salt containing media due to the contraction of the polymers chains in a salt-containing solution that might entrap DNA molecules inside more compact structures (Mo, Takaya et al. 1999). These results indicated that the complexes could protect DNA from nuclease degradation, while the presence of HA in quaternary complexes did not adversely affect PEI efficiency in protecting DNA from degradation.

A further study of the effects of the DNase I digestion on the integrity of plasmid DNA molecules in the complexes was also evaluated by the addition of heparin. As shown in Figure 6.2C, the entire supercoiled structure of plasmid DNA remained un-degraded in all complexes after DNase I treatment. Low electrophoretic mobility of the released DNA from some complexes, especially from ternary and 5% LMW (HA with low MW) polyplexes compared to the free DNA, suggested that the plasmid remained bound to PEI or HA, but in conformations that allowed interaction with ethidium bromide. The released DNA from 100% LMW (HA with low MW), 5% HMW and 100% HMW (HA with high MW) polyplexes displayed different forms from the natural conformation after DNase I treatment, as indicated by smearing on the gel image (Figure 6.2C). These smears may indicate that the released DNA was not in free-form but possibly complexed with PEI or HA molecules, thus preventing them from migrating through the gel pores. In general, these results demonstrated that the complexation of DNA with ternary or quaternary complexes effectively protected supercoiled DNA molecule structures from DNase I.

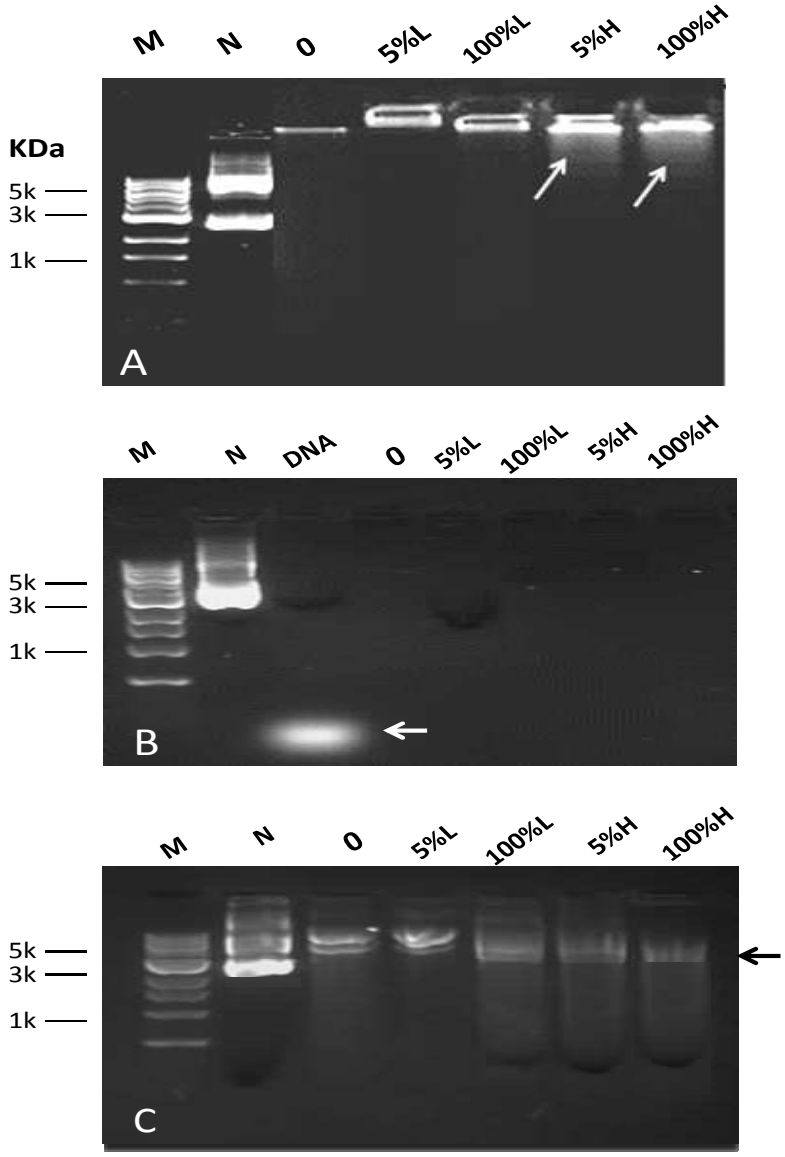


Figure 6.2: Gel retardation assay of DNA complexes. Lane M: λ H/E molecular weight size marker; Lane N: plasmid VR1020-PyMSP1$_{19}$; Lane 0: SPIONs/PEI/DNA; Quaternary polyplexes: Lane: 5% LMW; 100% LMW; 5% HMW; 100% HMW. (A) DNA condensation in the polyplexes, arrows indicating fluorescent trails formed by disassociated DNA that might be complexed with HMW HA, preventing the migration through the gel pores; (B) DNase I treated DNA complexes; samples were diluted with DNase reaction buffer including DNase I for 30 min, and then centrifuged. DNA in the supernatant was analyzed by agarose gel electrophoresis, while an equivalent amount of free DNA as used in particle formation was loaded on the gel as a control (Lane DNA). The arrow indicates degraded DNA molecules in uncomplexed free DNA after DNase treatment; (C) After samples were treated with DNase I for 30 min, heparin solution was added for 30 min. The arrow indicates the position of supercoiled DNA MSP1$_{19}$ molecules. (5%, 100% indicate HA : PEI charge ratio; HMW, LMW indicate high and low HA molecular weights, respectively).

6.3.4 Cytotoxicity assay

Cell cytotoxicity increased significantly in COS-7 cells when co-cultured with SPIONs/PEI/DNA at N/P ratio of 10 with cell viability reduced to ~60% (Figure 6.3), in agreement with previous work (Arsianti, Lim et al. 2010; Al-Deen, Ho et al. 2011). The cytotoxicity was significantly reduced when HA polymer was added at a physiological concentration similar to that of the naturally occurring HA *in vivo*. Figure 6.3 shows that the cells treated with polyplexes containing HA polymer gave approximately similar or even better viability than the unmodified DNA-treated cells or control cells (untreated cells). Adding HA to the SPIONs/PEI/DNA complexes reduced the toxicity of complexes by partly shielding the highly positive charges of complexes, as shown by the zeta potential measurement (Figure 6.1). Furthermore, there was no significant difference in cell viability when comparing different charge ratios of HA : PEI or even different molecular weights of HA in complexes.

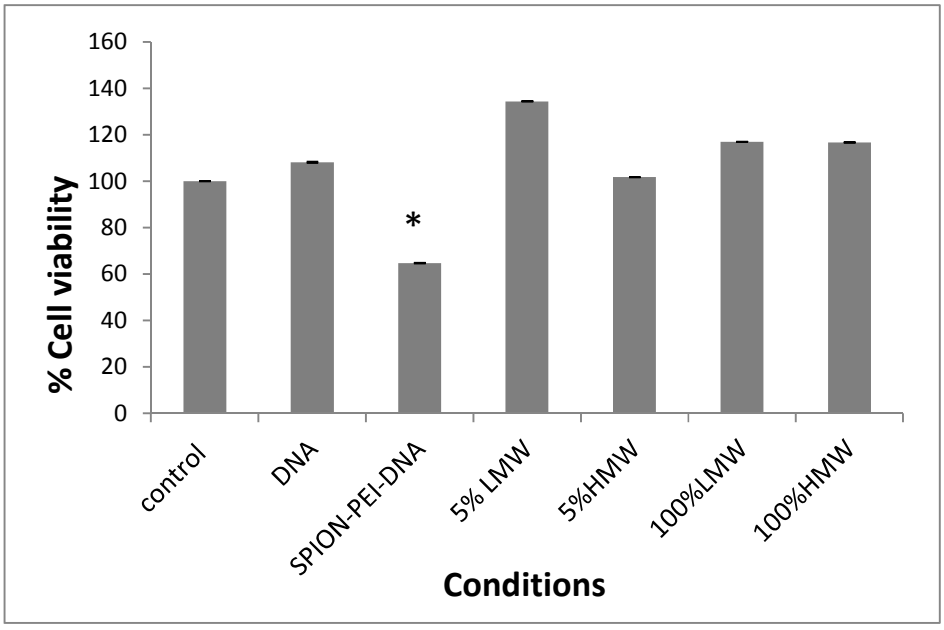


Figure 6.3: Cell viability as measured by MTT assays in COS-7 cells treated with different complexes. Experiments were performed at least three times. The data were analyzed statistically using one-way analysis of variance (ANOVA), with comparison of means conducted using a Tukey multiple comparison test. Results are expressed as means ±S.D (n =3), * P<0.05. All measurements are measured at least in triplicate. Quaternary polyplexes: 5% LMW; 100% LMW; 5% HMW; 100% HMW.

6.3.5 Magnetofection-based DCs transfection and maturation *in vitro*

The rationale for selecting this system of gene delivery comprising SPIONs, PEI, hyaluronic acid (HA), and DNA with special assembly order of SPIONs/PEI/DNA+HA has been discussed in the recent publication (Al-Deen, Selomulya et al. 2013).

The effects of magnetofection using quaternary polyplexes on mouse bone marrow-derived DC transfection and subsequent maturation (assessed by expression of MHCI, MHCII and CD86) after five hours incubation with two hours application of an external magnetic field have been examined. As shown in (Figure 6.4), the typical purity of CD11c+DCs which is the transmembrane protein found at high levels on the most dendritic cells in the cultures was approximately 70-80%. Treatment with different DNA complexes, especially quaternary polyplexes, showed no significant negative effects on the expression of CD11c on DCs compared to the normal control. Internalization of exogenous antigens is a prerequisite for APCs transfection with the antigen of interest and would improve their antigen presentation to T cells.

An important concern resulting in insufficient gene delivery of non-viral gene vectors *in vitro* is the low accumulation of gene complexes at the cell surface. Here an external magnetic field was applied on the quaternary magnetic gene vector to accumulate the gene vector on DCs in the culture media (Scherer, Anton et al. 2002; Plank, Schillinger et al. 2003). DNA encoding a fluorescent protein (YFP) mixed at 1:1 ratio with the $MSP1_{19}$ expressing vector was used to allow reading out the efficiency of DNA uptake. Western blot analysis using anti-$PyMSP1_{19}$ antibodies was further performed on transfected DCs pellets to identify the identity of the recombinant proteins produced by $PyMSP1_{19}$ plasmid in CD11c+ DCs. The result showed that expressed protein was equal to the $PyMSP1_{19}$ protein expressed in *Escherichia coli* (Appendix C. (Figure C.1)).

Flow cytometry analysis results of YFP expression in CD11c+DCs showed that almost all SPIONs/PEI/DNA-HA polyplexes increased significantly the transfection efficiency under the magnetic field, compared to transfection with other DNA complexes and even quaternary complexes without an applied magnetic field, regardless of the HA : PEI charge

ratio or HA molecular weight (Figure 6.5 A, C). This is possibly due to the gene delivery system playing dual roles; increasing the sedimentation of gene vectors on DCs and also facilitating the receptor-mediated endocytosis into DCs of magnetic vector via HA recognition (Scherer, Anton et al. 2002; Plank, Schillinger et al. 2003; Al-Deen, Ho et al. 2011). A trend was further observed between DC cellular uptake via magnetofection and increasing the molecular weight of HA in the quaternary polyplexes.

The ability of DCs to drive antigen-specific immunity is dependent on their degree of maturation. Flow cytometric analyses provided evidence that generally, the treatment of DCs cells with plasmid DNA or even plasmid DNA loaded onto nanoparticles enhanced the expression of CD86 in total CD11c+DCs. In particular, CD11c+DCs treatment with naked plasmid DNA showed a significantly higher CD86 maturation signal compared to untreated DC (Figure 6.5B, $p < 0.0001$). This is because bacterial plasmid DNA possesses inherent immune adjuvant activity based on its unmethylated CpG motifs in the plasmid DNA backbone, previously shown to act as a potent DNA vaccine adjuvant in rodents (Hartmann et al. 1999). On the other hand, culturing DCs with quaternary polyplexes, although it did not induce substantial up-regulation of CD86 in the overall culture, it specifically increased both the efficiency of YFP gene transfection in CD11c+DCs and concomitant maturation through up-regulated expression of costimulatory molecule CD86 and MHC molecules (Figure 6.5C, D).

In contrast, DCs treatment with different DNA complexes excluding quaternary polyplexes did not enhance YFP gene expression and also the level of stimulatory markers (CD86, MHC molecules) on the CD11c+DCs expressing YFP, suggesting that internalization was the main contributor in quaternary polyplexes-induced concomitant DC gene transfection and maturation (Figure 6.5C). The YFP gene expression and phenotypical maturation signals observed during DCs exposure to quaternary polyplexes were much higher compared to those of ternary polyplexes. These results supported the notion that factors such as endocytosis via HA receptors may play a role in DNA complexes-induced DC maturation, since HA was present in the quaternary system. In addition, unmethylated CpG motifs of plasmid DNA are known to be recognized by the Toll-like receptor 9 (TLR9) in DCs which play an important role in long-term vaccination protocols (Spies et al. 2003). It is likely that upon endocytosis, plasmid DNA becomes at least partially degraded by DNase

II in the endosomal-lysosomal compartment to release CpG motifs. As TLR9 is not present on the surface of the cells, targeted delivery of plasmid DNA by means of nanoparticles to the endosomal compartment might be important for potential association of DNA with the TLR9 receptor in the endosome and trigger DC maturation followed by the immune response.

The effects of polyplexes gene transfection on DCs transfection and maturation in the *in vitro* culture correlated with the extent of *in vivo* DC maturation through the consequent adjuvant effect on the immune response towards the antigen of interest. The data obtained highlight (for the first time) the direct interactions of DC with magnetic gene complexes coated with PEI and HA polymers in promoting DCs maturation. Therefore, suitable surface modifications by HA should allow specific endocytosis of gene complexes to affect the pathways of intracellular processing and trafficking. It also suggests that the prime mechanism by which the gene complexes enhance an adaptive immune response is the maturation of the antigen presenting cells, specifically DC, such that they become efficient at antigen presentation and T-cell stimulation to generate an immune response to the antigen of interest.

As these results suggested that quaternary polyplexes were stimuli of DC maturation through internalization, probably by specific HA receptors, further investigation of the effects of HA molecular weight and magnetic field applications on the uptake of quaternary polyplexes by murine DCs was made, to determine to what extent the particle endocytosis played a role in DC maturation. As shown in Figure 6.5A, C, SPIONs/PEI/DNA-HA polyplexes at high molecular weight HA and 100% charge ratio of HA : PEI (100% HMW polyplexes) displayed the highest YFP gene expression, accompanied also by maturation through up-regulated expression of CD86, compared to transfection with other complexes. The efficiency was much higher than DNA alone ($p< 0.0001$). Although SPIONs/PEI/DNA ternary complexes-mediated transfection was also done under a magnetic field, they showed a significantly lower transaction efficiency than SPIONs/PEI/DNA-HA quaternary polyplexes and DNA-Lipofectamine 2000 (a standard gene delivery agent) ($p< 0. 05$) (Figure 6.5 C).

Interestingly, 100% HMW polyplexes showed approximately the same levels of YFP and CD86 expression under or in the absence of magnetic field. The physicochemical properties

of HA are related to its molecular weight (Liao, Jones et al. 2005). Higher molecular weight is associated with longer molecular chains that in turn increase the mucoadhesive properties of HA in solution. Lim et al (2002) also found that the incorporation of HA with the molecular weight of 8.5×10^5 Da into macro-particles increased mucoadhesive properties more than chitosan-based systems. High molecular weight HA in quaternary polyplexes is likely to increase the mucoadhesive and viscoelastic properties of the complexes, accordingly enhances large molecular chains of HA ligands accessibility to the DC cell receptors and promotes the multivalent binding to receptors (Laurent et al. 1986), regardless of the presence or absence of the magnetic field. The efficiency of HA polymer with high molecular weight in accessing DC cells was associated with their ability to bind to DCs receptors, with a previous study showing that high molecular weight HA binding was irreversible to CD44 cell receptors, while the binding became weaker and reversible with decreased HA molecular weight (Wolny et al. 2010).

On the other hand, applying an external magnetic field could enhance the accumulation and attachment of low molecular weight (LMW) quaternary polyplexes on the surface of DCs, resulting in significant increases of the cellular uptake of the complex ($p < 0.001$), accompanied by the improved phenotypic maturation of DCs (Yao, Fan et al. 2010). Indeed, the effects of magnetic field on LMW (low molecular weight HA) quaternary polyplexes were more pronounced in the 5% LMW polyplexes (Figure 6.5C). This new observation is in good agreement with a previous study by Qhattal et al (2011) showing that the DC cellular targeting efficiency of HA-liposomes nanoparticles depended strongly upon HA molecular weight and grafting density, with high molecular weight HA resulting in better cellular adhesion and internalization of HA-liposomes to DCs.

In this study, the concentration of hyaluronic acid was in the range of 0.012 - 0.14 µg/ml, approximately similar to the range used by Do et al (2004) who found that the addition of 0.01 - 1 µg/ml of HA in DCs culture were very effective in growing cells, while high HA concentration of 0.5 µg/ml inhibited the growth of DCs. In agreement with our data, the polyplexes with these concentrations could interact properly with DCs and promote their maturation. In addition, the goal of this study was to determine whether HA-coated quaternary polyplexes could increase the functional uptake of DNA by dendritic cells for targeted gene expression and antigen presentation. After DC transfection, the produced

antigen was processed and presented by major histocompatibility complex MHC class I and MHC class II to stimulate cytotoxic T-cells (CTLs) and helper T-cells, respectively (Banchereau and Steinman 1998). These intracellular produced-antigens by DCs cells are hard to detect and present as MHC class I and II, since the molecular endocytosis of antigen is not necessarily sufficient to ensure efficient antigen presentation (Watts 1997). However, here the quaternary polyplexes showed considerably enhanced antigen uptake and presentation by DCs, probably via HA-mediated endocytosis that has been enhanced by the application of an external magnetic field. The use of quaternary polyplexes under a magnetic field significantly upregulated MHC class I and MHC class II expressions on DCs expressing YFP, supporting further their advanced maturation profile (Figure 6.5D).

HA molecules may also act as a condensation agent for DNA in a similar manner to PEI, especially as DNA molecules were mixed with HA polymer before addition to SPION/PEI complexes. Kim et al. showed by gel electrophoresis that DNA could interact to some degree with HA fragments, with only small portions of the DNA molecules able to be released from the DNA-HA matrix (Kim, Checkla et al. 2003). It is also known that the positively charged PEI condenses DNA into a compact colloid while affording a good protection from DNase digestion (Godbey, Wu et al. 1999). Thus, the interaction of DNA with HA and then condensation together with PEI-coated SPIONs could well increase the stability of DNA in the complexes, and at the same time protect DNA from DNase degradation. However, strong interactions between PEI and DNA may delay DNA release from complexes or even prevent all DNA molecules reaching the nucleus, leading to a decrease in transfection efficiency and gene expression. HA might be useful in this regard as well behaving here like a transcriptional activator by lessening the electrostatic complexation between DNA and PEI polymer (refer to Figure 6.2A, gel retardation assay), thus facilitating the interaction of transcription factors with plasmid DNA.

Granulocyte macrophage colony stimulating factor (GM-CSF) was added at a final concentration of 10 ng /ml to the cell culture in order to stimulate DC differentiation. Adding this cytokine to DC culture has been described previously to activate DCs proliferation and cellular uptake (Hamilton and Anderson 2004). Adding this cytokine to the cell culture with the quaternary polyplexes might promote cellular uptake of the gene complexes, followed by gene expression in the DCs. The result of this study seemed to

confirm the finding by Ali et al. (2008) in suggesting that placing DC in the media containing low levels of GM-CSF (50 ng/ml) during PEI/DNA gene transfection could greatly enhance gene transfection, comparatively to high amounts of GM-CSF (500 ng/ml) that could promote the degradation of intracellular DNA and its protein products.

Modifying the magnetic gene vector with HA polymer also led to the higher stability of complexes in the DCs media. This could lead to more cellular uptake of the complexes with well-timed dissociation of the DNA–polymer complex. Other possible mechanisms of high gene expression which relate to the presence of HA here, was the notably decreased cytotoxicity (Figure 6.3) and the size of quaternary polyplexes in the cell media to around 150 nm at the optimal charge ratio of 100% (Figure 6.1B), leading to better distribution with high cellular uptake (Manolova, Flace et al. 2008).

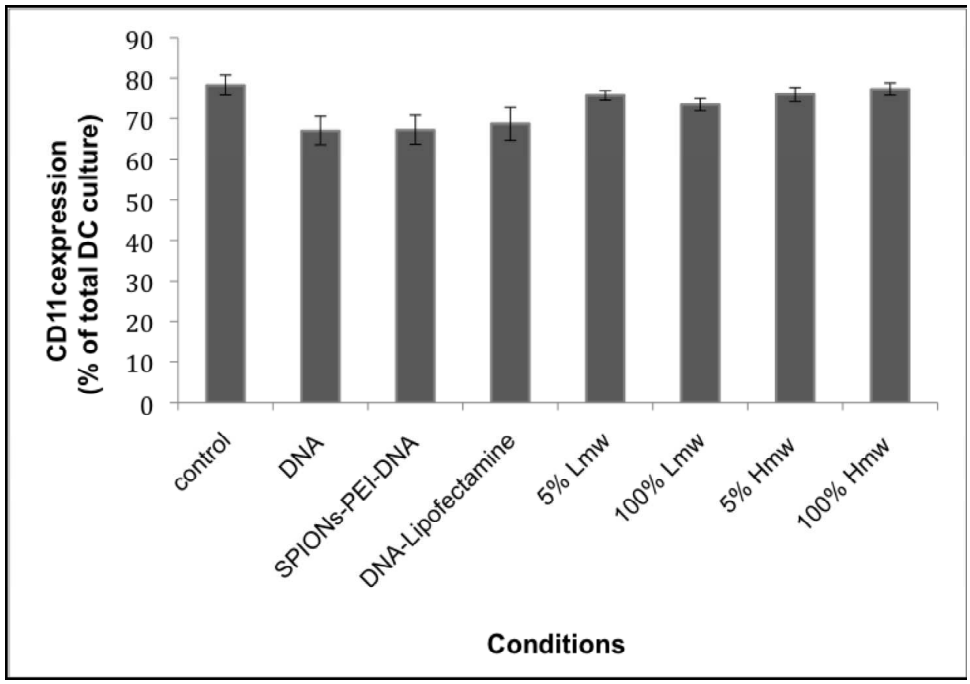
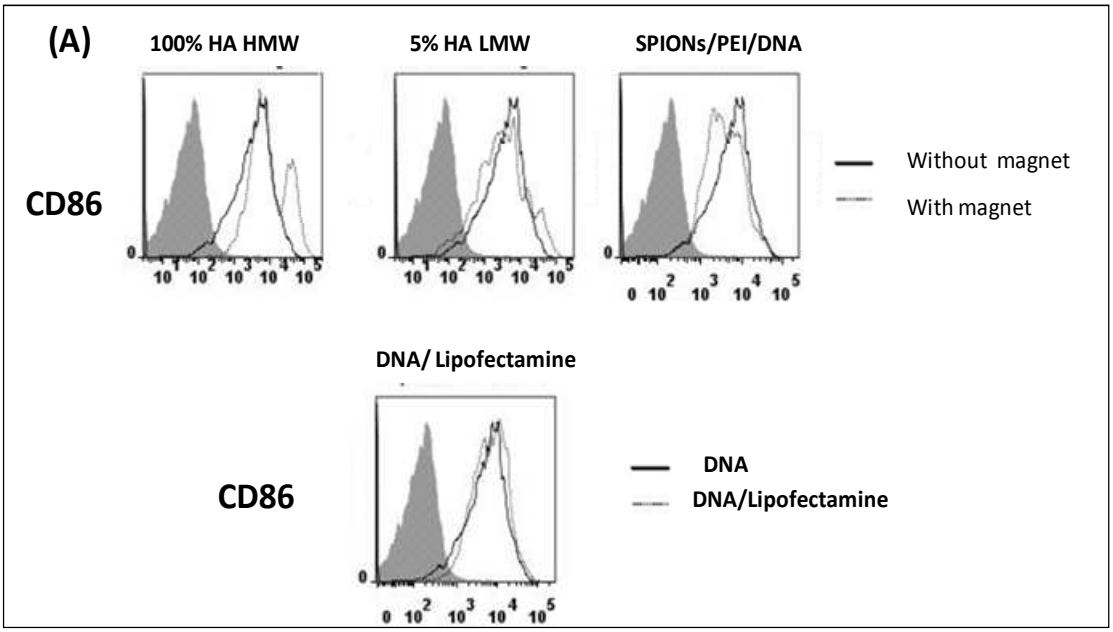
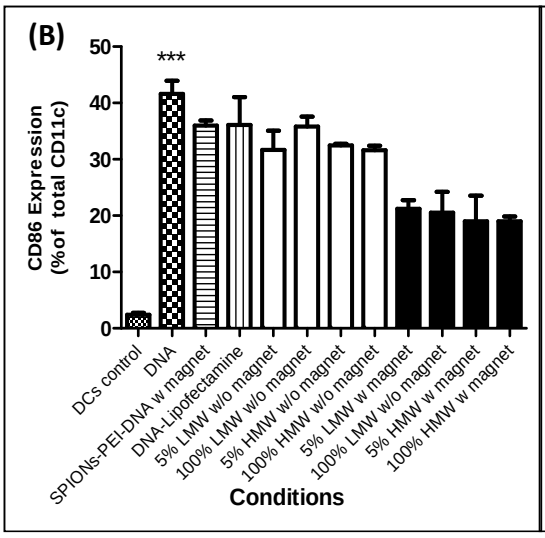
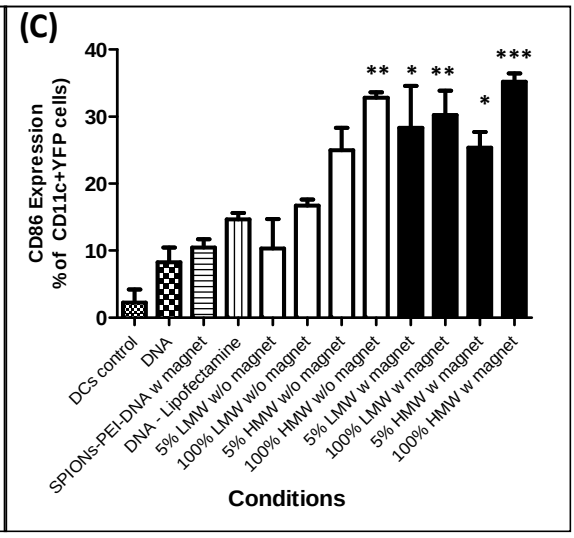


Figure 6.4: Percentage of CD11c marker expression on DCs derived from mouse bone marrow precursors under different culture conditions.


(A)
100% HA HMW
5% HA LMW
SPIONs/PEI/DNA
CD86
Without magnet
With magnet
DNA/ Lipofectamine
CD86
DNA
DNA/Lipofectamine


(B)
CD86 Expression (%of total CD11c)
***
DCs control
DNA
SPIONs-PEI-DNA w magnet
DNA-Lipofectamine
5% LMW w/o magnet
100% LMW w/o magnet
5% HMW w/o magnet
100% HMW w/o magnet
5% LMW w magnet
100% LMW w magnet
5% HMW w magnet
100% HMW w magnet
Conditions


(C)
CD86 Expression % of CD11c+YFP cells)
**
*
**
*
***
DCs control
DNA
SPIONs-PEI-DNA w magnet
DNA - Lipofectamine
5% LMW w/o magnet
100% LMW w/o magnet
5% HMW w/o magnet
100% HMW w/o magnet
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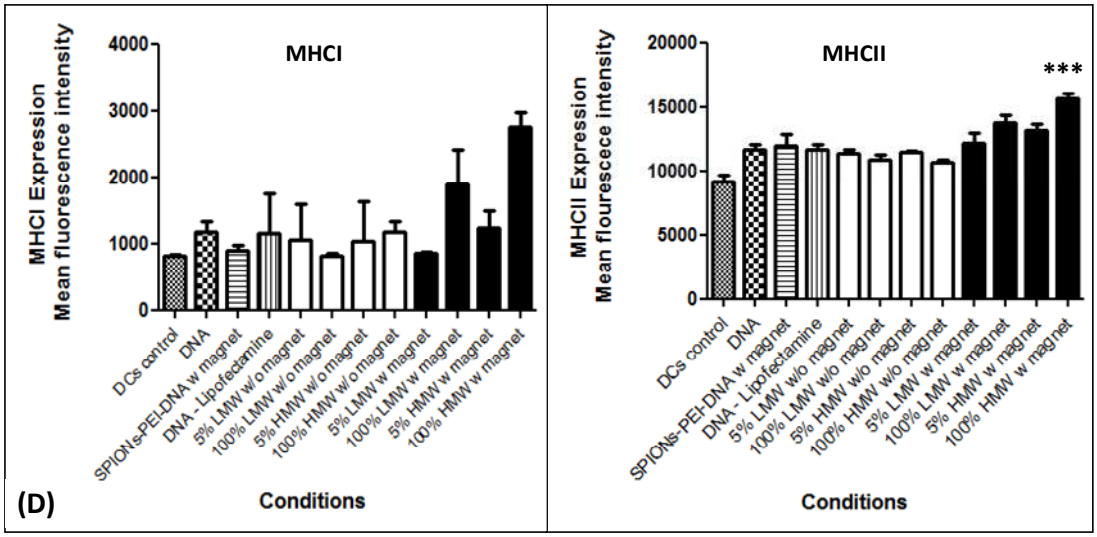

Figure 6.5: YFP expression and maturation of CD11c+DCs induced by functionalized DNA complexes added to cells at a mass of 2 µg per well. DCs were isolated from the old C57BL/6 mice and incubated with the indicated different DNA complexes. After 24 h, the cells were harvested and stained for the expressions of CD86, MHC class I and MHC class II and analyzed by flow cytometry: (A) Representative set of flow cytometry histograms of CD86 expression on CD11c+DCs expressing YFP. Gray shaded area: untreated cells; CD86 maturation markers on (B) total CD11c+DCs and (C) CD11c+DCs expressing YFP; (D) YFP expression level and MHCI, MHCII costimulatory molecule expression on CD11c+DCs expressing YFP. The data were analyzed statistically using one-way analysis of variance (ANOVA), and the comparison of means conducted using Tukey multiple comparison test. Results are expressed as means ±S.D. (n =3). * P<0.05, **p< 0.001, *** p<0.0001 (w magnet indicates application of an external magnetic field; w/o magnet indicates without application of an external magnetic field).

6.3.6 Effect of HA blocking on cellular uptake of the quaternary polyplexes with HA receptors

Quaternary polyplexes coated with HA can be easily dispersed in a cell medium, favorably responding to receptor-ligand interactions with HA receptors positive-cells such as DCs, while they could also loosen strong electrostatic interactions between DNA and PEI (Figure 6.2A, gel retardation assay). A way to prove the existence of these interactions was by deliberately blocking HA-receptors on the cells by pre-treatment with a saturable amount of free HA prior incubation with different plasmid complexes. Before adding different polyplexes to the cells, 200 µl of HA solutions (3 mg/ ml in PBS) (HMW or LMW) were added to DC cells in addition to 1 ml of RPMI medium. After incubation for 60 min at 37

°C, the cells were washed with RPMI media to remove unbound HA and then different complexes were added to the wells by application of an external magnetic field for 2 h, with similar treatment as previously referred to in the transfection assay. Flow cytometric analysis results of YFP expression in CD11c+DCs showed that the DC pre-treated with excess HA ligand had nearly 50% reduction in YFP gene expression compared to the transfection with cells pre-treated with PBS alone (Figure 6.6A). The YFP gene expression in CD11c+ DCs treated with HMW HA-polyplexes without pre-treated HA showed significantly the highest gene expression ($p < 0.0001$), while LMW HA-polyplexes with and without pre-treated HA did not show any significant difference in gene expression. This suggested that the YFP expression of HMW polyplexes could be mediated by HMW HA-specific receptors on the surface of DC cells, more than the LMW HA that could be competitively inhibited by excess free HA. Interestingly, Figure 6.6B showed that all wells treated with quaternary polyplexes pre-treated with PBS alone (without HA) upregulated CD86 maturation marker on CD11c+ DCs expressing YFP regardless of the HA molecular weight, in contrast to pre-treated HA wells. This was in agreement with Ito et al. (2006), who showed that the CHO cells (Chinese hamster ovary cell line) pre-treated with HA had diminished gene expression compared to the highly enhanced luciferase gene expression of HA-coated ternary complexes (DNA/PE/HA) pre-treated with only PBS. Taken together, these results suggested that the interactions between HA special receptors on DC specifically with HMW HA-coated polyplexes could encourage uptake via these receptors (Ito, Iida-Tanaka et al. 2006).

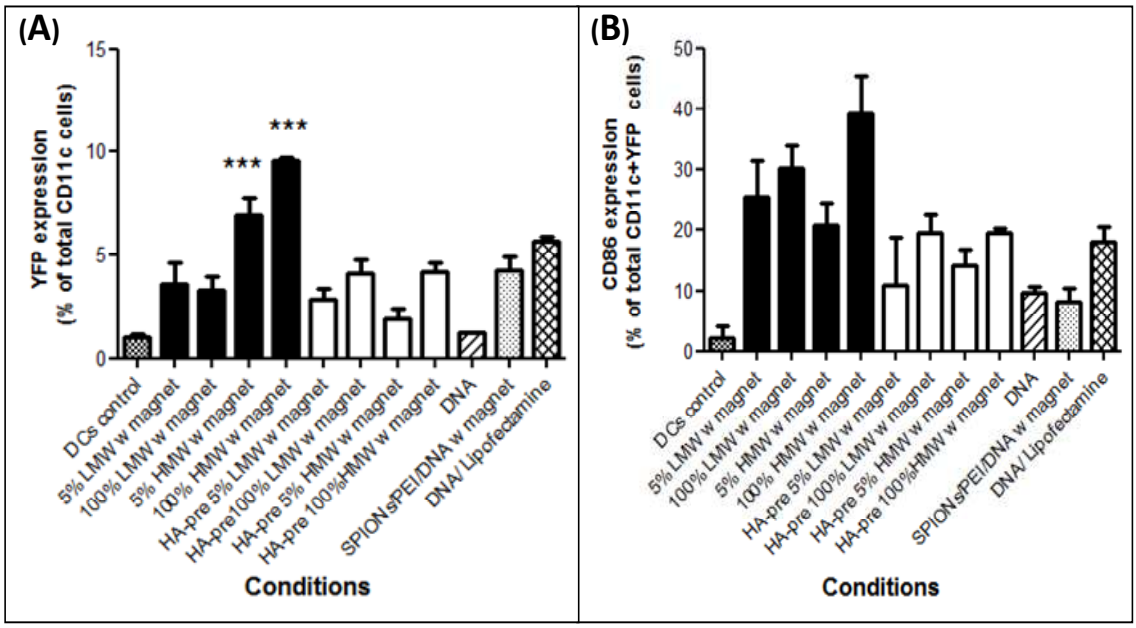

Figure 6.6: Effects of HA-pre-addition on the YFP expression and co-stimulatory surface marker (CD86) on CD11c+DCs mediated by quaternary polyplexes: (A) The percentage of total CD11c +DC cells expressing YFP; (B) The percentage of CD11c+ DCs expressing YFP with up-regulation of CD86 maturation signal (n = 3).
*****p< 0.0001 (Black columns indicate DCs pre-treatment with PBS alone; White columns indicate HA pre-treatment (high or low molecular weight HMW or LMW)).**

6.3.7 HA-coated polyplexes induced activation of DC independently of CD44

CD44 is a principal cellular HA receptor (Culty et al. 1992; Oh, Park et al. 2010), raising the question on whether CD44 could also mediate the HA-coated quaternary polyplexes uptake. We assessed the CD44 expression level on total CD11c+DCs and CD11c+ DCs expressing YFP after treatment with different gene complexes compared with CD44 expression level in untreated CD11c+DCs. Our results showed strong expression of surface CD44 receptor around 90% in all CD11c+DCs indicating the presence of this type of receptor in our DC cultures (Figure 6.7).

In general, untreated or treated CD11c+DCs with different DNA polyplexes showed approximately the same percentage of CD44 expression. The ternary polyplexes, DNA/Lipofectamine complexes, and even free DNA slightly, but not significantly, upregulated CD44 expression level on DCs especially with cells expressing YFP compared to control DCs. In contrast, DC pre-treated with high concentrations of HA before adding the quaternary polyplexes showed significant trends toward decreasing CD44 in total DCs or DCs expressing YFP compared to control DCs (Figure 6.7A, B). Adding HA at high concentration may potentially inhibit CD44 expression, as the previous study of Kaya et al. (2000) suggested that the abnormal accumulation of hyaluronate in superficial dermis due to some medical reasons was accompanied by the decline in CD44 expression in the keratinocytes, and it could be argued that a similar mechanism could promote low CD44 expression on DCs when pre-treated with high amounts of HA (Figure 6.7).

A previous report showed that under normal conditions, CD44 receptors are rapidly recycled back from the intracellular compartment to the plasma membrane following internalization and the release of cargos (G.Garg and Hales 2004). Therefore, the possibility of a lower expression of CD44 due to uptake of the HA coated quaternary polyplexes as a receptor for HA could be excluded, as the FACS analysis was performed 24 h after adding the polyplexes in the cell culture, allowing enough time for CD44 to recycle back to the cell surface.

The transfection study (Figure 6.5) showed that DCs treatment with the quaternary polyplexes especially under a magnetic field displayed significantly higher levels of YFP

gene expression accompanied by significant up-regulation in the CD86 expression, thus implying that quaternary polyplexes were actively uptake by DCs via different types of HA receptors than CD44. Most interestingly, there was no significant difference in CD44 expression levels between total CD11c+DCs and CD11c+DCs transfected with a YFP gene for cells treated with different HA coated quaternary polyplexes compared to control DCs. Taken together, these data potentially demonstrate that CD44 receptors were not actively involved in HA-quaternary polyplex uptake by DCs, thereby suggesting a possible role of other receptors of HA, such as toll like receptors -2 and -4 , CD38, LYVE-1, RHAMM or even unknown novel receptors (Oh, Park et al. 2010). Others have suggested that the mammalian endocytic HA receptor is HARE instead of CD44 (Weigel et al. 2002)**.** Furthermore, Do et al. previously reported that HA could activate DC through CD44 independent pathway, while CD44 on antigen Ag-specific T-cells played a critical role during interaction with DC to present antigen Ag and subsequently T-cells expansion (Do, Nagarkatti et al. 2004). Moreover, Termeer and colleagues (2000) found that CD44KO-derived DCs (knock-out mice) showed similar up-regulation of costimulatory molecules like wild type-derived DCs after HA treatment. It should be noted that these studies were different from the current study in that they primarily investigated the ability of the HA-treated DCs to activate alloreactive T-cells. While, the aim of the present study was to transfect DCs using malaria DNA vaccine condensed on HA-coated magnetic quaternary polyplexes, and at the same time to increase DCs maturation through upregulation of costimulatory molecules. HA on the surface of these quaternary complexes could act as ligands to enhance receptor-mediated endocytosis through specific HA receptors on DCs. However, it cannot be entirely eliminated the possibility that CD44 could make a minor contribution to the cellular uptake of quaternary polyplexes involved in the polyplexes transfection and maturation of wild type-derived DCs. Since the expressions by other receptors in mature or immature DC by RT-PCR were not detected, the effects of other HA receptors could be excluded. Further study will be necessary to determine the specific type of DCs receptor contributing to the uptake of quaternary polyplexes.

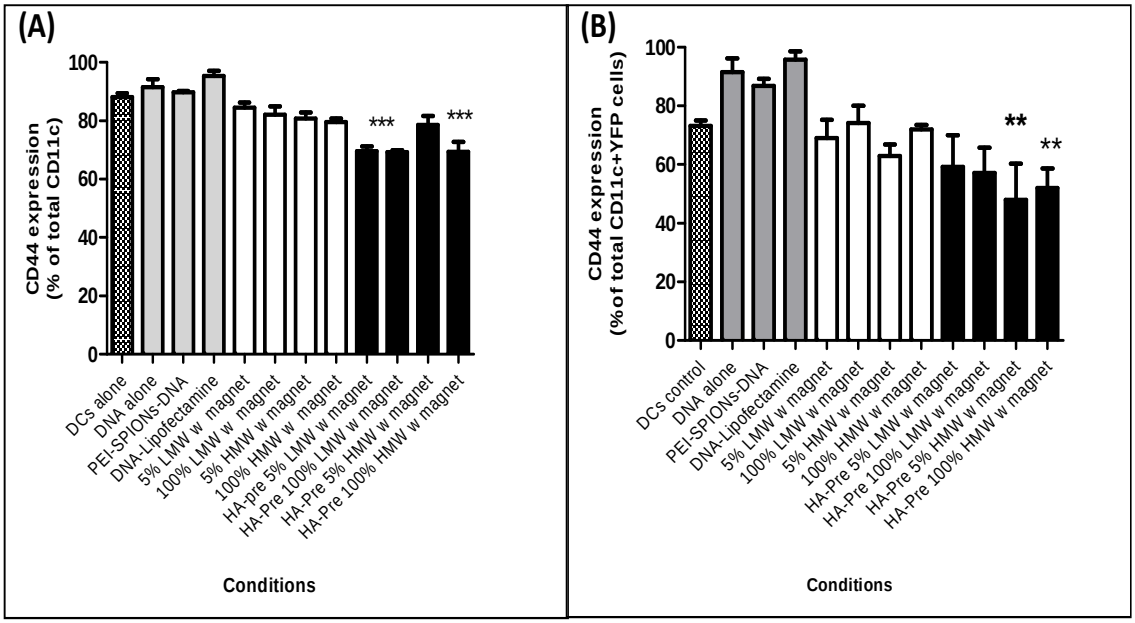

Figure 6.7: Effects of different DNA complexes on the expression of CD44 receptors on CD11c+ DCs population: (A) The percentage of total CD11c +DC cells; (B) The percentage of CD11c+ DCs expressing YFP. The data were analyzed statistically using one-way analysis of variance (ANOVA), and the comparison of means was conducted using Tukey multiple comparison test. Results are expressed as means ±S.D (n =3).
*****p< 0.001 (Black columns indicate DCs pre-treatment with PBS alone; White columns indicate HA pre-treatment (high or low molecular weight HMW or LMW).**

6.4 Conclusion

As it is reported in the literature that DC is problematic to transfect, however here it has been demonstrated that the quaternary magnetic polyplexes containing hyaluronic acid (HA) could be used to transfect DCs *in vitro* high effectively with a model DNA based malaria vaccine candidate. The transfection could be enhanced by increasing the HA molecular weight and the application of an external magnetic field. The size of the HA ligand on the polyplexes was an important determinant of its cellular uptake, with higher molecular weight of HA resulting in better cellular adhesion and internalization. Moreover, the presence of HA loosened the strong complexation between DNA and PEI polymer, but did not result in DNA disassembly, with higher cell viability compared to those treated without HA in the polyplexes. These quaternary polyplexes also had adjuvant activity that

encouraged DC maturation (specifically for the cells that had internalized the complexes), while the application of an external magnetic field improved gene delivery to DCs at low DNA doses, while also resulting in up-regulation of DC maturation markers. This new information will be crucial for the design of the DNA gene delivery tool to somatic cells such as DCs for potent immune activation, with further studies recommended to investigate the detailed uptake mechanisms and also the specific type of DCs receptor of this gene delivery system both *in vitro* and *in vivo.*

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CHAPTER 7
Conclusions and Recommendations

7.1 Conclusions

The initial work of this thesis shows that the magnetofection technique is promising for the delivery of malaria DNA vaccine. SPIONs/PEI complexes produced under an acidic condition showed less aggregation, better DNA binding, and high gene transfection efficiency than those generated under neutral pH, due to the protonated structure of the branched polymer that entrapped and protected DNA molecules from enzymatic digestion. The expression of VR1020-PyMSP1$_{19}$ in COS7 cell line showed that complexes formed under an acidic condition were able to deliver malaria gene better especially with magnetofection. The enhancement of gene delivery under an external magnetic field was possibly because the magnetic force drew the complexes onto the cell surface, leading to an increase in their cellular uptake. In addition, the SPIONs/PEI/DNA complexes containing moderate amounts of PEI at N/P ratio of 10 and 15 showed the highest transfection efficiency compared with others. This is attributed to sufficient amounts of PEI for condensation and timely release of DNA molecules from the complexes for nuclear localization.

SPIONs/PEI-DNA complexes containing VR1020-PyMSP1$_{19}$ were used for malaria DNA vaccine delivery *in vivo*. In terms of an immune response, the magnetofection technique was able to considerably improve the response in the animal model compared to the naked DNA. The applicability of SPIONs/PEI/DNA complexes for *in vivo* study was first studied by assessing the best N/P ratio to stimulate an immune response via intramuscular injection. Moderate amounts of PEI at N/P ratio of 15 and 20 stimulated the highest IgG antibody level against PyMSP1$_{19}$ under a magnetic field. The effects of magnetofection on the level of IgG antibody responses using SPIONs/PEI/DNA complexes via different routes of administration showed that the magnetic field significantly induces high IgG antibody

responses via intraperitoneal routes compared to intramuscular, subcutaneous and intradermal routes. These results were attributed to the physiology of peritoneum as a major storage of macrophages and dendritic cells, allowing better gene transfection opportunity in the peritoneal cavity under an external magnetic field. On the contrary, intravenous injection of SPIONs/PEI/DNA complexes caused a transient shock with most animals dying a short time after the injection. The high positive charge of complexes rendered them able to interact with negatively charged blood serum proteins such as opsonins in the serum, leading to aggregation that could cause a physical blockage of lung capillaries with an intravenous injection.

Heterologous DNA prime followed by a single recombinant EcPyMSP1$_{19}$ protein boost vaccination regime also significantly stimulated antibody responses in all immunization groups in terms of IgG endpoint titres, with the endpoint titre for the intraperitoneal group significantly higher than those of other groups, indicating the induction of appropriate memory immunity that can be elicited by protein on recall. In addition, heterologous prime-boost strategies greatly enhanced the IgG2a / IgG1 ratio for intraperitoneal route in a similar manner or even more than DNA immunization only. The stimulation of IgG2a immune responses was strongly dependent on the route of administration for priming DNA and the total IgG level.

A surface functionalization strategy of magnetic particles was also developed here to add anionic polymer such as hyaluronic acid in order to reduce the strong net positive charge of the PEI polymer. *In vitro* study showed that although SPIONs/PEI/DNA complexes exhibit high gene transfection in the COS-7 cell, these complexes also showed toxic effects on the cell viability which are mainly associated with the strongest net positive charge of the PEI polymer that lead to strong interactions and disruption of the plasma membrane. Therefore, a new system is developed here to decrease the PEI polymer cytotoxic effect and also to incorporate receptor-mediated cellular uptake mechanisms. Notably, hyaluronic acid was used in this study to functionalize PEI coated magnetic nanoparticles via a direct electrostatic binding with the surface amino groups of PEI. By adding hyaluronic acid to the SPIONs/PEI/DNA complexes, the cytotoxicity could be decreased by diminishing non-specific interactions with serum protein in the physiological fluid, and enhance receptor-mediated endocytosis via HA receptors on the cells.

The assembly orders of different magnetic vector configurations comprising SPIONs, PEI, and hyaluronic acid (HA), acting as carriers for the malaria DNA vaccine and their stability in different cell media were investigated. Generally, all complexes showed relatively small sizes in the water, whereas higher degree of aggregation was detected instantly after transferring to high-ionic strength media such as an NaCl buffer and RPMI media. However, pre-addition of HA to DNA prior to SPIONs/PEI configuration vectors effectively reduced the extent of aggregation in RPMI media particularly at the highest % HA : PEI charge ratio. Partial disassembly of some complexes was observed in all media except in NaCl buffer where all complexes preserved DNA molecules without showing any detectable release. This indicates that the incubation of complexes in the NaCl buffer prior to transfection may limit the intracellular release of plasmid DNA for gene transfection. DNase sensitivity assay showed that the plasmid DNA in the all configurations preserved their structural integrity without damage, even after DNase I treatment. The benefit of this characterization study is significant, particularly in handling these particles in malaria DNA vaccine delivery system for *in vivo* applications.

Magnetic gene vectors comprising SPIONs, PEI, DNA, and HA to target antigen-presenting cell APCs were applied to deliver malaria DNA vaccine to dendritic cell (DC) as a model of potent APCs. The cells were treated with these particles to facilitate receptor-mediated endocytosis via the hyaluronic acid surface receptor on DCs. The ability of these complexes to transfect dendritic cells has been tested using different molecular weights of HA and with different % charge ratios of HA: PEI under a magnetic field. Vectors formed by pre-addition of HA to DNA prior to SPION/PEI configuration were used for DCs transfection as they were more stable with smaller sizes under physiological conditions. Flow cytometry analysis was used to assess DC transfection and maturation by monitoring the up-regulation of the surface expression of CD86 plus major histocompatibility complexes MHC class I and MHC class II expression. Quaternary complexes with high molecular weight of HA and high charge ratio of HA: PEI yielded better transfection efficiency and maturation of DCs than others. The presence of magnetic fields also significantly enhanced DC transfection and maturation in DC cell cultures, particularly with low molecular weight hyaluronic containing complexes. The result was attributed to the stability and transfection efficiency of these complexes, due to long molecular chains

and higher mucoadhesive properties of high molecular weight HA, thus enhancing the HA ligands accessibility and promoting the multivalent binding to DCs receptors. As DCs are pivotal in the stimulation of a primary immunogenic response to plasmid encoded antigens, and they are far superior in the presenting antigen compared with other APC following DNA vaccination. The result of this study represents a big step forward to the transfection and simultaneous maturation of DCs, which offers a potential approach for treating different diseases via gene therapy.

7.2 Recommendations for future work

The promising results from this study warrant further investigation of magnetic nanoparticles for malaria gene delivery applications: Future work could address the following topics:

(1) Although the difference in the gene expression value mediated by SPIONs/PEI complexes formed under acidic and natural conditions have been tested *in vitro* (Chapter 3), no study has been done *in vivo* to determine if a different pH complexation also affects the level of stimulating IgG antibody production. The gene complexes under acidic condition displayed more stability in terms of small particle size and more DNA condensation capability making them ideally suitable for *in vivo* gene delivery condition, since compact complex structure can offer a good protection from degradation through nuclease in the blood circulation. Therefore, evaluating the antibody response value elicited by the complexes under different pH condition is very important to determine the best complexation condition for *in vivo* application, since the mechanisms of gene delivery *in vivo* were different than those *in vitro*.

(2) Since intraperitoneal administration of the complexes was found to stimulate higher immune responses under the external magnetic field (Chapter 4), *in vivo* assessment of the optimal N/P ratio via intraperitoneal injection should be done to enhance the response via this route of vaccination. Intraperitoneal vaccination is predominantly used in veterinary medicine due to the simplicity of administration compared to other routes and stimulation

of protective immune response in several types of animals. However, effective translation of this approach to a human clinical setting could be tasking and possibly controversial. Therefore, investigating other more practical administration routes such as oral or intradermal tattoo vaccination with a high immune response may be more appropriate under clinical conditions.

(3) The study of the properties and behavior of quaternary polyplexes comprising SPIONs, polyethylenimine, and hyaluronic acid as carriers for malaria DNA vaccine (Chapter 5) can be extended to include other competitors or conditions that gene complexes normally encounter in the bloodstream. Additions of these competitors such as RNA and DNA, or by changing the pH or osmolality of the cell culture media would provide a better prediction of the actual behavior under physiological conditions, particularly for high molecular weight HA-containing polyplexes that have shown better gene transfection and maturation in DCs.

(4) An optimized protocol for the efficient transfection of dendritic cells would be highly desirable, because transaction of this type of primary cells with plasmid DNA by using non-viral reagents is very difficult (Awasthi and Cox 2003). Effective gene tranfection to dendritic cells using quaternary polyplexes SPIONs/PEI/DNA+HA with high molecular weight of HA was demonstrated in Chapter 6. The most interesting aspect is that a considerably lower dose of condensed DNA in the polyplexes elicited significantly high DCs transfection and maturation under a magnetic field. Thus, the same polyplexes can be used for targeting DCs *in vivo*, since the success of DNA vaccination system hinges on the ability of targeting DNA vaccines to DCs. Furthermore, additional detection tests both *in vitro* and *in vivo* should be also conducted to determine the detailed uptake mechanisms and also the specific type of DCs receptor for this gene delivery system.

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References

Abdallah, B., Hassan, A., Benoist, C., Goula, D., Behr, J. P. and Demeneix, B. A. (1996). "A powerful nonviral vector for in vivo gene transfer into the adult mammalian brain: Polyethylenimine." Human Gene Therapy **7**(16): 1947-1954.

Aigner, A., Fischer, D., Merdan, T., Brus, C., Kissel, T. and Czubayko, F. (2002). "Delivery of unmodified bioactive ribozymes by an RNA-stabilizing polyethylenimine (LMW-PEI) efficiently down-regulates gene expression." Gene Therapy **9**(24): 1700-1707.

Al-Deen, F. N., Ho, J., Selomulya, C., Ma, C. and Coppel, R. (2011). "Superparamagnetic Nanoparticles for Effective Delivery of Malaria DNA Vaccine." Langmuir **27**(7): 3703-3712.

Al-Deen, F. N., Selomulya, C. and Williams, T. (2013). "On designing stable magnetic vectors as carriers for malaria DNA vaccine." Colloids and Surfaces B: Biointerfaces **102**(0): 492-503.

Ali, O. A. and Mooney, D. J. (2008). "Sustained GM-CSF and PEI condensed pDNA presentation increases the level and duration of gene expression in dendritic cells." Journal of Controlled Release **132**(3): 273-278.

Ang, D., Nguyen, Q. V., Kayal, S., Preiser, P. R., Rawat, R. S. and Ramanujan, R. V. (2011). "Insights into the mechanism of magnetic particle assisted gene delivery." Acta Biomaterialia **7**(3): 1319-1326.

Ankamwar, B., Lai, T. C., Huang, J. H., Liu, R. S., Hsiao, M., Chen, C. H. and Hwu, Y. K. (2010). "Biocompatibility of Fe3O4 nanoparticles evaluated by in vitro cytotoxicity assays using normal, glia and breast cancer cells." Nanotechnology **21**(7).

Aoki, K., Furuhata, S., Hatanaka, K., Maeda, M., Remy, J. S., Behr, J. P., Terada, M. and Yoshida, T. (2001). "Polyethylenimine-mediated gene transfer into pancreatic tumor dissemination in the murine peritoneal cavity." Gene Therapy **8**(7): 508-514.

Arcasoy, S. M., Latoche, J., Gondor, M., Watkins, S. C., Henderson, R. A., Hughey, R., Finn, O. J. and Pilewski, J. M. (1997). "MUC1 and other sialoglycoconjugates inhibit adenovirus-mediated gene transfer to epithelial cells." American Journal of Respiratory Cell and Molecular Biology **17**(4): 422-435.

Arsianti, M., Lim, M., Marquis, C. P. and Amal, R. (2010). "Assembly of Polyethylenimine-Based Magnetic Iron Oxide Vectors: Insights into Gene Delivery." Langmuir **26**(10): 7314-7326.

Arsianti, M., Lim, M., Marquis, C. P. and Amal, R. (2010). "Polyethylenimine Based Magnetic Iron-Oxide Vector: The Effect of Vector Component Assembly on Cellular Entry Mechanism, Intracellular Localization, and Cellular Viability." Biomacromolecules **11**(9): 2521-2531.

Ballou, W. R. (2009). "The development of the RTS,S malaria vaccine candidate: challenges and lessons." Parasite Immunology **31**(9): 492-500.

Banchereau, J. and Steinman, R. M. (1998). "Dendritic cells and the control of immunity." Nature **392**(6673): 245-252.

Barcena, C., Sra, A. K. and Gao, J. M. (2009). Applications of Magnetic Nanoparticles in Biomedicine.

References

Barry, M. E., Pinto-Gonzalez, D., Orson, F. M., McKenzie, G. J., Petry, G. R. and Barry, M. A. (1999). "Role of endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after naked DNA injection." Human Gene Therapy **10**(15): 2461-2480.

Behr, J. P. (1997). "The proton sponge: A trick to enter cells the viruses did not exploit." Chimia **51**(1-2): 34-36.

Berry, C. C. and Curtis, A. S. G. (2003). "Functionalisation of magnetic nanoparticles for applications in biomedicine." Journal of Physics D-Applied Physics **36**(13): R198-R206.

Bertschinger, M., Backliwal, G., Schertenleib, A., Jordan, M., Hacker, D. L. and Wurm, F. M. (2006). "Disassembly of polyethylenimine-DNA particles in vitro: Implications for polyethylenimine-mediated DNA delivery." Journal of Controlled Release **116**(1): 96-104.

Berzofsky, J. A., Ahlers, J. D. and Belyakov, I. M. (2001). "Strategies for designing and optimizing new generation vaccines." Nature Reviews Immunology **1**(3): 209-219.

Bieber, T., Meissner, W., Kostin, S., Niemann, A. and Elsasser, H. P. (2002). "Intracellular route and transcriptional competence of polyethylenimine-DNA complexes." Journal of Controlled Release **82**(2-3): 441-454.

Blackman, M. J., Heidrich, H. G., Donachie, S., McBride, J. S. and Holder, A. A. (1990). "A single fragment of a malaria merozoite surface protein remains on the parasite during red-cell invasion and is the target of invasion-inhibiting antibodies " Journal of Experimental Medicine **172**(1): 379-382.

Bouharountayoun, H. and Druilhe, P. (1992). "Plasmodium-falciparum malaria - evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity." Infection and Immunity **60**(4): 1473-1481.

Boussif, O., Lezoualch, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B. and Behr, J. P. (1995). "A versatile vector for gene and oligonucleotide transfer into cells and in-vivo-polyrthyleimine." Proceedings of the National Academy of Sciences of the United States of America **92**(16): 7297-7301.

Boyoglu, S., Vig, K., Pillai, S., Rangari, V., Dennis, V., Khazi, F. and Singh, S. R. (2009). "Enhanced delivery and expression of a nanoencapsulated DNA vaccine vector for respiratory syncytial virus." Nanomedicine-Nanotechnology Biology and Medicine **5**(4): 463-472.

Brice, G. T., Dobano, C., Sedegah, M., Stefaniak, M., Graber, N. L., Campo, J. J., Carucci, D. J. and Doolan, D. L. (2007). "Extended immunization intervals enhance the immunogenicity and protective efficacy of plasmid DNA vaccines." Microbes and Infection **9**(12-13): 1439-1446.

Bruder, J. T., Angov, E., Limbach, K. J. and Richie, T. L. (2010). "Molecular vaccines for malaria." Human Vaccines **6**(1): 54-77.

Bruder, J. T., Stefaniak, M. E., Patterson, N. B., Chen, P., Konovalova, S., Limbach, K., et al. (2010). "Adenovectors induce functional antibodies capable of potent inhibition of blood stage malaria parasite growth." Vaccine **28**(18): 3201-3210.

Bulte, J. W. M., Douglas, T., Witwer, B., Zhang, S. C., Strable, E., Lewis, B. K., et al. (2001). "Magnetodendrimers allow endosomal magnetic labeling and in vivo tracking of stem cells." Nature Biotechnology **19**(12): 1141-1147.

Carcaboso, A. M., Hernandez, R. M., Igartua, M., Rosas, J. E., Patarroyo, M. E. and Pedraz, J. L. (2004). "Potent, long lasting systemic antibody levels and mixed

Th1/Th2 immune response after nasal immunization with malaria antigen loaded PLGA microparticles." Vaccine **22**(11-12): 1423-1432.

Chapman, S. W. K., Hassa, P. O., Koch-Schneidemann, S., von Rechenberg, B., Hofmann-Amtenbrink, M., Steitz, B., Petri-Fink, A., Hofmann, H. and Hottiger, M. O. (2008). "Application of pulsed-magnetic field enhances non-viral gene delivery in primary cells from different origins." Journal of Magnetism and Magnetic Materials **320**(8): 1517-1527.

Chen, M., Yamamuro, S., Farrell, D. and Majetich, S. A. (2003). "Gold-coated iron nanoparticles for biomedical applications." Journal of Applied Physics **93**(10): 7551-7553.

Cherif, M. S., Shuaibu, M. N., Kurosaki, T., Helegbe, G. K., Kikuchi, M., Yanagi, T., Tsuboi, T., Sasaki, H. and Hirayama, K. (2011). "Immunogenicity of novel nanoparticle-coated MSP-1 C-terminus malaria DNA vaccine using different routes of administration." Vaccine **29**(48): 9038-9050.

Chollet, P., Favrot, M. C., Hurbin, A. and Coll, J. L. (2002). "Side-effects of a systemic injection of linear polyethylenimine-DNA complexes." Journal of Gene Medicine **4**(1): 84-91.

Chorny, M., Polyak, B., Alferiev, I. S., Walsh, K., Friedman, G. and Levy, R. J. (2007). "Magnetically driven plasmid DNA delivery with biodegradable polymeric nanoparticles." Faseb Journal **21**(10): 2510-2519.

Chu, R. S., Targoni, O. S., Krieg, A. M., Lehmann, P. V. and Harding, C. V. (1998). "CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity." Faseb Journal **12**(4): A612-A612.

Clamme, J. P., Krishnamoorthy, G. and Mely, Y. (2003). "Intracellular dynamics of the gene delivery vehicle polyethylenimine during transfection: investigation by two-photon fluorescence correlation spectroscopy." Biochimica Et Biophysica Acta-Biomembranes **1617**(1-2): 52-61.

Coffman, R. L., Seymour, B. W. P., Lebman, D. A., Hiraki, D. D., Christiansen, J. A., Shrader, B., et al. (1988). "The role of helper T-cell products in mouse B-cell differentiation and isotype regulation." Immunological Reviews **102**: 5-28.

Colombo, M., Carregal-Romero, S., Casula, M. F., Gutierrez, L., Morales, M. P., Bohm, I. B., Heverhagen, J. T., Prosperi, D. and Parak, W. J. (2012). "Biological applications of magnetic nanoparticles." Chemical Society Reviews **41**(11): 4306-4334.

Culty, M., Nguyen, H. A. and Underhill, C. B. (1992). " The hyaluronan receptor (CD44) participates in the uptake and degradation of hyaluronan." Journal of Cell Biology **116**(4): 1055-1062.

Dai, Z. J., Gjetting, T., Mattebjerg, M. A., Wu, C. and Andresen, T. L. (2011). "Elucidating the interplay between DNA-condensing and free polycations in gene transfection through a mechanistic study of linear and branched PEI." Biomaterials **32**(33): 8626-8634.

Daly, T. M. and Long, C. A. (1995). "Humoral response to a carboxyl-terminal region of the merozoite surface protein-1 plays a predominant role in controlling blood-stage infection in rodent malaria." Journal of Immunology **155**(1): 236-243.

Danielsen, S., Strand, S., Davies, C. D. and Stokke, B. T. (2005). "Glycosaminoglycan destabilization of DNA-chitosan polyplexes for gene delivery depends on chitosan

chain length and GAG properties." Biochimica Et Biophysica Acta-General Subjects **1721**(1-3): 44-54.

Danko, I. and Wolff, J. A. (1994). "Direct gene transfer into muscle." Vaccine **12**(16): 1499-1502.

de Almeida, M. P. S., Caiado, K. L., Sartoratto, P. P. C., Silva, D., Pereira, A. R. and Morais, P. C. (2010). "Preparation and size-modulation of silica-coated maghemite nanoparticles." Journal of Alloys and Compounds **500**(2): 149-152.

de Ilarduya, C. T., Sun, Y. and Duezguenes, N. (2010). "Gene delivery by lipoplexes and polyplexes." European Journal of Pharmaceutical Sciences **40**(3): 159-170.

Dean, D. A., Strong, D. D. and Zimmer, W. E. (2005). "Nuclear entry of nonviral vectors." Gene Therapy **12**(11): 881-890.

Deck, R. R., DeWitt, C. M., Donnelly, J. J., Liu, M. A. and Ulmer, J. B. (1997). "Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine." Vaccine **15**(1): 71-78.

del Barrio, G. G., Novo, F. J. and Irache, J. M. (2003). "Loading of plasmid DNA into PLGA microparticles using TROMS (Total Recirculation One-Machine System): evaluation of its integrity and controlled release properties." Journal of Controlled Release **86**(1): 123-130.

Delacruz, M. O., Belloni, L., Delsanti, M., Dalbiez, J. P., Spalla, O. and Drifford, M. (1995). "Precipittion of higly-charged polyelectrolyte solutions in presence of multivalent salts." Journal of Chemical Physics **103**(13): 5781-5791.

Derouazi, M., Girard, P., Van Tilborgh, F., Iglesias, K., Muller, N., Bertschinger, M. and Wurm, F. M. (2004). "Serum-free large-scale transient transfection of CHO cells." Biotechnology and Bioengineering **87**(4): 537-545.

Diggs, C. L., Ballou, W. R. and Miller, L. H. (1993). "The major merozoite surface protein as a malaria vaccine target " Parasitology Today **9**(8): 300-302.

Djurovic, S., Iversen, N., Jeansson, S., Hoover, F. and Christensen, G. (2004). "Comparison of nonviral transfection and adeno-associated viral transduction on cardiomyocytes." Molecular Biotechnology **28**(1): 21-31.

Do, Y., Nagarkatti, P. S. and Nagarkatti, M. (2004). "Role of CD44 and hyaluronic acid (HA) in activation of alloreactive and antigen-specific T cells by bone marrow-derived dendritic cells." Journal of Immunotherapy **27**(1): 1-12.

Dobson, J. (2006). "Gene therapy progress and prospects: magnetic nanoparticle-based gene delivery." Gene therapy **13**(4): 283-287.

Doe, B., Selby, M., Barnett, S., Baenziger, J. and Walker, C. M. (1996). "Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells." Proceedings of the National Academy of Sciences of the United States of America **93**(16): 8578-8583.

Dokka, S., Toledo, D., Shi, X. G., Castranova, V. and Rojanasakul, Y. (2000). "Oxygen radical-mediated pulmonary toxicity induced by some cationic liposomes." Pharmaceutical Research **17**(5): 521-525.

Doolan, D. L. and Hoffman, S. L. (2001). "DNA-based vaccines against malaria: status and promise of the multi-stage malaria DNA vaccine operation." International Journal for Parasitology **31**(8): 753-762.

Dunlap, D. D., Maggi, A., Soria, M. R. and Monaco, L. (1997). "Nanoscopic structure of DNA condensed for gene delivery." Nucleic Acids Research **25**(15): 3095-3101.

References

Eberbeck, D., Kettering, M., Bergemann, C., Zirpel, P., Hilger, I. and Trahms, L. (2010). "Quantification of the aggregation of magnetic nanoparticles with different polymeric coatings in cell culture medium." Journal of Physics D-Applied Physics **43**(40).

Eliyahu, H. (2005). "Polymers for DNA delivery." Molecules **10**(1): 34-64.

Feltquate, D. M., Heaney, S., Webster, R. G. and Robinson, H. L. (1997). "Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization." Journal of Immunology **158**(5): 2278-2284.

Ferrari, M. E., Rusalov, D., Enas, J. and Wheeler, C. J. (2001). "Trends in lipoplex physical properties dependent on cationic lipid structure, vehicle and complexation procedure do not correlate with biological activity." Nucleic Acids Research **29**(7): 1539-1548.

Fischer, D., Li, Y. X., Ahlemeyer, B., Krieglstein, J. and Kissel, T. (2003). "In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis." Biomaterials **24**(7): 1121-1131.

Florea, B. I., Meaney, C., Junginger, H. E. and Borchard, G. (2002). "Transfection efficiency and toxicity of polyethylenimine in differentiated Calu-3 and nondifferentiated COS-1 cell cultures." Aaps Pharmsci **4**(3).

Forde, G. M. (2005). "Rapid-response vaccines - does DNA offer a solution?" Nature Biotechnology **23**(9): 1059-1062.

Frenkel, J. and Dorfman, J. (1930). "Spontaneous and induced magnetisation in ferromagnetic bodies." Nature **126**: 274-275.

G.Garg, H. and Hales, C. A. (2004). "Chemistry And Biology Of Hyaluronan." 1st Edition **ELSEVIER Ltd.**(USA): 624 Pages.

Gade, T. P. F., Koutcher, J. A., Spees, W. M., Beattie, B. J., Ponomarev, V., Doubrovin, M., et al. (2008). "Imaging transgene activity in vivo." Cancer Research **68**(8): 2878-2884.

Garcon, N., Heppner, D. G. and Cohen, J. (2003). "Development of RTS,S/AS02: a purified subunit-based malaria vaccine candidate formulated with a novel adjuvant." Expert review of vaccines **2**(2): 231-238.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., et al. (2002). "Genome sequence of the human malaria parasite Plasmodium falciparum." Nature **419**(6906): 498-511.

Gaur, U., Sahoo, S. K., De, T. K., Ghosh, P. C., Maitra, A. and Ghosh, P. K. (2000). "Biodistribution of fluoresceinated dextran using novel nanoparticles evading reticuloendothelial system." International Journal of Pharmaceutics **202**(1-2): 1-10.

Gersting, S. W., Schillinger, U., Lausier, J., Nicklaus, P., Rudolph, C., Plank, C., Reinhardt, D. and Rosenecker, J. (2004). "Gene delivery to respiratory epithelial cells by magnetofection." Journal of Gene Medicine **6**(8): 913-922.

Gilson, P. R., Nebl, T., Vukcevic, D., Moritz, R. L., Sargeant, T., Speed, T. P., Schofield, L. and Crabb, B. S. (2006). "Identification and stoichiometry of glycosylphosphatidylinositol-anchored membrane proteins of the human malaria parasite Plasmodium falciparum." Molecular & Cellular Proteomics **5**(7): 1286-1299.

Godbey, W. T., Barry, M. A., Saggau, P., Wu, K. K. and Mikos, A. G. (2000). "Poly(ethylenimine)-mediated transfection: A new paradigm for gene delivery." Journal of Biomedical Materials Research **51**(3): 321-328.

References

Godbey, W. T., Wu, K. K. and Mikos, A. G. (1999). "Poly(ethylenimine) and its role in gene delivery." Journal of Controlled Release **60**(2-3): 149-160.

Godbey, W. T., Wu, K. K. and Mikos, A. G. (1999). "Size matters: Molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle." Journal of Biomedical Materials Research **45**(3): 268-275.

Good, M. F., Kaslow, D. C. and Miller, L. H. (1998). "Pathways and strategies for developing a malaria blood-stage vaccine." Annual Review of Immunology **16**: 57-87.

Goya, G. F., Marcos-Campos, I., Fernandez-Pacheco, R., Saez, B., Godino, J., Asin, L., et al. (2008). "Dendritic cell uptake of iron-based magnetic nanoparticles." Cell Biology International **32**(8): 1001-1005.

Guedes, M. H. A., Sadeghiani, N., Peixoto, D. L. G., Coelho, J. P., Barbosa, L. S., Azevedo, R. B., et al. (2005). "Effects of AC magnetic field and carboxymethyldextran-coated magnetite nanoparticles on mice peritoneal cells." Journal of Magnetism and Magnetic Materials **293**(1): 283-286.

Gupta, A. K. and Gupta, M. (2005). "Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications." Biomaterials **26**(18): 3995-4021.

Gupta, A. K. and Wells, S. (2004). "Surface-modified superparamagnetic nanoparticles for drug delivery: Preparation, characterization, and cytotoxicity studies." Ieee Transactions on Nanobioscience **3**(1): 66-73.

Gurunathan, S., Klinman, D. M. and Seder, R. A. (2000). "DNA vaccines: Immunology, application, and optimization." Annual Review of Immunology **18**: 927-974.

Gurunathan, S., Wu, C. Y., Freidag, B. L. and Seder, R. A. (2000). "DNA vaccines: a key for inducing long-term cellular immunity." Current Opinion in Immunology **12**(4): 442-447.

Hamilton, J. A. and Anderson, G. P. (2004). "GM-CSF biology." Growth Factors **22**(4): 225-231.

Hamley, I. W. (2003). "Nanotechnology with soft materials." Angewandte Chemie-International Edition **42**(15): 1692-1712.

Hamm, A., Krott, N., Breibach, I., Blindt, R. and Bosserhoff, A. K. (2002). "Efficient transfection method for primary cells." Tissue Engineering **8**(2): 235-245.

Han, S. E., Kang, H., Shim, G. Y., Kim, S. J., Choi, H. G., Kim, J., Hahn, S. K. and Oh, Y. K. (2009). "Cationic derivatives of biocompatible hyaluronic acids for delivery of siRNA and antisense oligonucleotides." Journal of Drug Targeting **17**(2): 123-132.

Hartmann, G., Weiner, G. J. and Krieg, A. M. (1999). "CpG DNA: A potent signal for growth, activation, and maturation of human dendritic cells." Proceedings of the National Academy of Sciences of the United States of America **96**(16): 9305-9310.

Hattori, Y., Kawakami, S., Nakamura, K., Yamashita, F. and Hashida, M. (2006). "Efficient gene transfer into macrophages and dendritic cells by in vivo gene delivery with mannosylated lipoplex via the intraperitoneal route." Journal of Pharmacology and Experimental Therapeutics **318**(2): 828-834.

Hay, S. I., Okiro, E. A., Gething, P. W., Patil, A. P., Tatem, A. J., Guerra, C. A. and Snow, R. W. (2010). "Estimating the global clinical burden of Plasmodium falciparum malaria in 2007." PLoS medicine **7**(6): e1000290.

He, C. X., Tabata, Y. and Gao, J. Q. (2010). "Non-viral gene delivery carrier and its three-dimensional transfection system." International Journal of Pharmaceutics **386**(1-2): 232-242.

He, R., You, X. G., Shao, J., Gao, F., Pan, B. F. and Cui, D. X. (2007). "Core/shell fluorescent magnetic silica-coated composite nanoparticles for bioconjugation." Nanotechnology **18**(31): 7.

Hedstrom, R. C., Doolan, D. L., Wang, R. B., Kumar, A., Sacci, J. B., Gardner, M. J., et al. (1998). "In vitro expression and in vivo immunogenicity of Plasmodium falciparum pre-erythrocytic stage DNA vaccines." International Journal of Molecular Medicine **2**(1): 29-38.

Helson, R., Olszewska, W., Singh, M., Megede, J. Z., Melero, J. A., Hagan, D. O. and Openshaw, P. J. M. (2008). "Polylactide-co-glycolide (PLG) microparticles modify the immune response to DNA vaccination." Vaccine **26**(6): 753-761.

Heppner, D. G., Kester, K. E., Ockenhouse, C. F., Tornieporth, N., Ofori, O., Lyon, J. A., et al. (2005). "Towards an RTS,S-based, multi-stage, multi-antigen falciparum malaria: progress at the Walter Reed Army Institute of Research." Vaccine **23**(17-18): 2243-2250.

Herve, K., Douziech-Eyrolles, L., Munnier, E., Cohen-Jonathan, S., Souce, M., Marchais, H., et al. (2008). "The development of stable aqueous suspensions of PEGylated SPIONs for biomedical applications." Nanotechnology **19**(46).

Hill, A. V. S., Reyes-Sandoval, A., O'Hara, G., Ewer, K., Lawrie, A., Goodman, A., et al. (2010). "Prime-boost vectored malaria vaccines Progress and prospects." Human Vaccines **6**(1): 78-83.

Hilleman, M. R. (2000). "Vaccines in historic evolution and perspective: a narrative of vaccine discoveries." Vaccine **18**(15): 1436-1447.

Hirunpetcharat, C., Tian, J. H., Kaslow, D. C., vanRooijen, N., Kumar, S., Berzofsky, J. A., Miller, L. H. and Good, M. F. (1997). "Complete protective immunity induced in mice by immunization with the 19-kilodalton carboxyl-terminal fragment of the merozoite surface protein-1 (MSP1(19)) of Plasmodium yoelii expressed in Saccharomyces cerevisiae - Correlation of protection with antigen-specific antibody titer, but not with effector CD4(+) T cells." Journal of Immunology **159**(7): 3400-3411.

Hodder, A. N., Crewther, P. E., Matthew, M., Reid, G. E., Moritz, R. L., Simpson, R. J. and Anders, R. F. (1996). "The disulfide bond structure of Plasmodium apical membrane antigen-1." Journal of Biological Chemistry **271**(46): 29446-29452.

Hoffman, S., Franke, E., Hollingdale, M. and Druilhe, P. (1996). Attacking the infected hepatocyte Malaria vaccine development: A multi-immune response approach.

Hoffman, S. and Miller, L. (1996). Perspectives on malaria vaccine development malaria vaccine development: A multi-immune response approach.

Holder, A. A. and Freeman, R. R. (1984). "Protective antigens of rodent and human bloodstage malaria." Philosophical Transactions of the Royal Society of London Series B-Biological Sciences **307**(1131): 171-177.

Horn, N. A., Meek, J. A., Budahazi, G. and Marquet, M. (1995). "Cancer gene-therapy using plasmid DNA - purification of DNA for human clinical-trials." Human Gene Therapy **6**(5): 565-573.

Hornof, M., de la Fuente, M., Hallikainen, M., Tammi, R. H. and Urtti, A. (2008). "Low molecular weight hyaluronan shielding of DNA/PEI polyplexes facilitates

CD44 receptor mediated uptake in human corneal epithelial cells." Journal of Gene Medicine **10**(1): 70-80.

Hu, F. X., Neoh, K. G. and Kang, E. T. (2009). "Synthesis of Folic Acid Functionalized PLLA-b-PPEGMA Nanoparticles for Cancer Cell Targeting." Macromolecular Rapid Communications **30**(8): 609-614.

Huth, S., Lausier, J., Gersting, S. W., Rudolph, C., Plank, C., Welsch, U. and Rosenecker, J. (2004). "Insights into the mechanism of magnetofection using PEI-based magnetofectins for gene transfer." Journal of Gene Medicine **6**(8): 923-936.

Inaba, K., Inaba, M., Deguchi, M., Hagi, K., Yasumizu, R., Ikehara, S., Muramatsu, S. and Steinman, R. M. (1993). "Granulocytes, macrophages, and dendritic cells arise from a common major histocompatibility complex class-ii-negative progenitor in mouse bone-marrow." Proceedings of the National Academy of Sciences of the United States of America **90**(7): 3038-3042.

Intra, J. and Salem, A. K. (2008). "Characterization of the transgene expression generated by branched and linear polyethylenimine-plasmid DNA nanoparticles in vitro and after intraperitoneal injection in vivo." Journal of Controlled Release **130**(2): 129-138.

Ito, J., Ghosh, A., Moreira, L. A., Wimmer, E. A. and Jacobs-Lorena, M. (2002). "Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite." Nature **417**(6887): 452-455.

Ito, T., Iida-Tanaka, N. and Koyama, Y. (2008). "Efficient in vivo gene transfection by stable DNA/PEI complexes coated by hyaluronic acid." Journal of Drug Targeting **16**(4): 276-281.

Ito, T., Iida-Tanaka, N., Niidome, T., Kawano, T., Kubo, K., Yoshikawa, K., Sato, T., Yang, Z. H. and Koyama, Y. (2006). "Hyaluronic acid and its derivative as a multi-functional gene expression enhancer: Protection from non-specific interactions, adhesion to targeted cells, and transcriptional activation." Journal of Controlled Release **112**(3): 382-388.

Iwasaki, A., Torres, C. A. T., Ohashi, P. S., Robinson, H. L. and Barber, B. H. (1997). "The dominant role of bone marrow-derived cells in CTL induction following plasmid DNA immunization at different sites." Journal of Immunology **159**(1): 11-14.

Jaracz, S., Chen, J., Kuznetsova, L. V. and Ojima, L. (2005). "Recent advances in tumor-targeting anticancer drug conjugates." Bioorganic & Medicinal Chemistry **13**(17): 5043-5054.

Jayne, D. G. (2003). "The molecular biology of peritoneal carcinomatosis from gastrointestinal cancer." Annals Academy of Medicine Singapore **32**(2): 219-225.

Jenner, E. (1798). "An inquiry into the causes and effects of variolae vaccinae, a disease discovered in some of the western counties of England." Sampson Low.

Jeong, Y. I., Nah, J. W., Na, H. K., Na, K., Kim, I. S., Cho, C. S. and Kim, S. H. (1999). "Self-assembling nanospheres of hydrophobized pullulans in water." Drug Development and Industrial Pharmacy **25**(8): 917-927.

Jiang, G., Park, K., Kim, J., Kim, K. S. and Hahn, S. K. (2009). "Target Specific Intracellular Delivery of siRNA/PEI-HA Complex by Receptor Mediated Endocytosis." Molecular Pharmaceutics **6**(3): 727-737.

Johansson, E. L., Bergquist, C., Edebo, A., Johansson, C. and Svennerholm, A. M. (2004). "Comparison of different routes of vaccination for eliciting antibody responses in the human stomach." Vaccine **22**(8): 984-990.

Kabanov, A. V. and Kabanov, V. A. (1995). "DNA complexes with polycations for the delivery of genetic material into cells." Bioconjugate Chemistry **6**(1): 7-20.

Kamau, S. W., Hassa, P. O., Steitz, B., Petri-Fink, A., Hofmann, H., Hofmann-Amtenbrink, M., von Rechenberg, B. and Hottiger, M. O. (2006). "Enhancement of the efficiency of non-viral gene delivery by application of pulsed magnetic field." Nucleic Acids Research **34**(5).

Kasuya, H., Nomoto, S. and Nakao, A. (2002). "The potential of gene therapy in the treatment of pancreatic cancer." Drugs of Today **38**(7): 457-464.

Kay, M. A., Glorioso, J. C. and Naldini, L. (2001). "Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics." Nature Medicine **7**(1): 33-40.

Kaya, G., Augsburger, E., Stamenkovic, I. and Saurat, J. H. (2000). "Decrease in epidermal CD44 expression as a potential mechanism for abnormal hyaluronate accumulation in superficial dermis in lichen sclerosus et atrophicus." Journal of Investigative Dermatology **115**(6): 1054-1058.

Khalafalla, S. E. and Reimers, G. W. (1980). "Preparation of dilution-stable aqueous magnetic fluids " Ieee Transactions on Magnetics **16**(2): 178-183.

Kichler, A. (2004). "Gene transfer with modified polyethylenimines." Journal of Gene Medicine **6**: S3-S10.

Kichler, A., Leborgne, C., Coeytaux, E. and Danos, O. (2001). "Polyethylenimine-mediated gene delivery: a mechanistic study." Journal of Gene Medicine **3**(2): 135-144.

Kievit, F. M., Veiseh, O., Fang, C., Bhattarai, N., Lee, D., Ellenbogen, R. G. and Zhang, M. Q. (2010). "Chlorotoxin Labeled Magnetic Nanovectors for Targeted Gene Delivery to Glioma." Acs Nano **4**(8): 4587-4594.

Kim, A., Checkla, D. M., Dehazya, P. and Chen, W. L. (2003). "Characterization of DNA-hyaluronan matrix for sustained gene transfer." Journal of Controlled Release **90**(1): 81-95.

Kim, J. S., Yoon, T. J., Kim, B. G., Park, S. J., Kim, H. W., Lee, K. H., Park, S. B., Lee, J. K. and Cho, M. H. (2006). "Toxicity and tissue distribution of magnetic nanoparticles in mice." Toxicological Sciences **89**(1): 338-347.

Kircheis, R., Wightman, L., Schreiber, A., Robitza, B., Rossler, V., Kursa, M. and Wagner, E. (2001). "Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application." Gene Therapy **8**(1): 28-40.

Kircheis, R., Wightman, L. and Wagner, E. (2001). "Design and gene delivery activity of modified polyethylenimines." Advanced Drug Delivery Reviews **53**(3): 341-358.

Kitchener, J. A. (1972). "Principles of action of polymeric flocculants." British Polymer Journal **4**(3): 217-229.

Kolset, S. O., Prydz, K. and Pejler, G. (2004). "Intracellular proteoglycans." Biochemical Journal **379**: 217-227.

Koyama, Y., Ito, T., Matsumoto, H., Tanioka, A., Okuda, T., Yamaura, N., Aoyagi, H. and Niidome, T. (2003). "Novel poly(ethylene glycol) derivatives with carboxylic acid pendant groups: synthesis and their protection and enhancing effect on non-

viral gene transfection systems." Journal of Biomaterials Science-Polymer Edition **14**(6): 515-531.

Krishnan, K. M. (2010). "Biomedical Nanomagnetics: A Spin Through Possibilities in Imaging, Diagnostics, and Therapy." Ieee Transactions on Magnetics **46**(7): 2523-2558.

Kroese, F. G., Butcher, E. C., Stall, A. M., Lalor, P. A., Adams, S. and Herzenberg, L. A. (1989). "Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the peritoneal cavity." Int Immunol **1**(1): 75–84.

Krotz, F., Sohn, H. Y., Gloe, T., Plank, C. and Pohl, U. (2003). "Magnetofection potentiates gene delivery to cultured endothelial cells." Journal of Vascular Research **40**(5): 425-434.

Kuberan, B. (2011). "Structures and functions of proteoglycans." Abstracts of Papers of the American Chemical Society **241**.

Kularatne, B. Y., Lorigan, P., Browne, S., Suvarna, S. K., Smith, N. O. and Lawry, J. (2002). "Monitoring tumour cells in the peripheral blood of small cell lung cancer patients." Cytometry **50**(3): 160-167.

Kumar, P. S., Ramakrishna, S., Saini, T. R. and Diwan, P. V. (2006). "Influence of microencapsulation method and peptide loading on formulation of poly(lactide-co-glycolide) insulin nanoparticles." Pharmazie **61**(7): 613-617.

Kumar, S., Epstein, J. E., Richie, T. L., Nkrumah, F. K., Soisson, L., Carucci, D. J. and Hoffman, S. L. (2002). "A multilateral effort to develop DNA vaccines against falciparum malaria." Trends in Parasitology **18**(3): 129-135.

Kumar, S., Yadava, A., Keister, D. B., Tian, J. H., Ohl, M., Perduegreenfield, K. A., Miller, L. H. and Kaslow, D. C. (1995). "Immunogenicity and in-vivo efficacy of recombinant Plasmodium-falciparum merozoite surface protein-1 in Aotus monkeys." Molecular Medicine **1**(3): 325-332.

Lacava, L. M., Lacava, Z. G. M., Da Silva, M. F., Silva, O., Chaves, S. B., Azevedo, R. B., et al. (2001). "Magnetic resonance of a dextran-coated magnetic fluid intravenously administered in mice." Biophysical Journal **80**(5): 2483-2486.

Lacava, Z. G. M., Azevedo, R. B., Martins, E. V., Lacava, L. M., Freitas, M. L. L., Garcia, V. A. P., et al. (1999). "Biological effects of magnetic fluids: toxicity studies." Journal of Magnetism and Magnetic Materials **201**: 431-434.

Lalani, J. and Misra, A. (2011). "Gene Delivery Using Chemical Methods: In : Ambikanandan Misra, editor. MethodsChallenges in Delivery of Therapeutic Genomics and Proteomics, Elsevier, 1st ed." Chapter 4.

Laurent, S., Forge, D., Port, M., Roch, A., Robic, C., Elst, L. V. and Muller, R. N. (2010). "Magnetic Iron Oxide Nanoparticles: Synthesis, Stabilization, Vectorization, Physicochemical Characterizations, and Biological Applications (vol 108, pg 2064, 2008)." Chemical Reviews **110**(4): 2574-2574.

Laurent, T. C., Fraser, J. R. E., Pertoft, H. and Smedsrod, B. (1986). "Binding of hyaluronate and chondroitin sulfate to liver endothelial-cells." Biochemical Journal **234**(3): 653-658.

Lee, J., Isobe, T. and Senna, M. (1996). "Preparation of ultrafine Fe3O4 particles by precipitation in the presence of PVA at high pH." Journal of Colloid and Interface Science **177**(2): 490-494.

Lee, M. and Kim, S. W. (2005). "Polyethylene glycol-conjugated copolymers for plasmid DNA delivery." Pharmaceutical Research **22**(1): 1-10.

Li, S., Locke, E., Bruder, J., Clarke, D., Doolan, D. L., Havenga, M. J. E., et al. (2007). "Viral vectors for malaria vaccine development." Vaccine **25**(14): 2567-2574.

Liao, Y. H., Jones, S. A., Forbes, B., Martin, G. P. and Brown, M. B. (2005). "Hyaluronan: Pharmaceutical characterization and drug delivery." Drug Delivery **12**(6): 327-342.

Lim, S.-Y. (2006). "Phytol-based novel adjuvants in vaccine formulation: 2. assessment of efficacy in the induction of protective immune responses to lethal bacterial infections in mice." Journal of Immune Based Therapies and Vaccines **4**(1): 5.

Lim, S. T., Forbes, B., Berry, D. J., Martin, G. P. and Brown, M. B. (2002). "In vivo evaluation of novel hyaluronan/chitosan microparticulate delivery systems for the nasal delivery of gentamicin in rabbits." International Journal of Pharmaceutics **231**(1): 73-82.

Lin, J., Zhou, W. L., Kumbhar, A., Wiemann, J., Fang, J. Y., Carpenter, E. E. and O'Connor, C. J. (2001). "Gold-coated iron (Fe@Au) nanoparticles: Synthesis, characterization, and magnetic field-induced self-assembly." Journal of Solid State Chemistry **159**(1): 26-31.

Ling, I. T., Ogun, S. A., Momin, P., Richards, R. L., Garcon, N., Cohen, J., Ballou, W. R. and Holder, A. A. (1997). "Immunization against the murine malaria parasite Plasmodium yoelii using a recombinant protein with adjuvants developed for clinical use." Vaccine **15**(14): 1562-1567.

Listner, K., Bentley, L., Okonkowski, J., Kistler, C., Wnek, R., Caparoni, A., et al. (2006). "Development of a highly productive and scalable plasmid DNA production platform." Biotechnology Progress **22**(5): 1335-1345.

Liu, F., Heston, S., Shollenberger, L. M., Sun, B., Mickle, M., Lovell, M. and Huang, L. (2006). "Mechanism of in vivo DNA transport into cells by electroporation: electrophoresis across the plasma membrane may not be involved (vol 8, pg 353, 2006)." Journal of Gene Medicine **8**(9): 1192-1192.

Liu, M. A. (2011). "DNA vaccines: an historical perspective and view to the future." Immunological Reviews **239**: 62-84.

Liu, S., Danquah, M. K., Forde, G. M., Ma, C., Wang, L. and Coppel, R. (2010). "Microparticle-Mediated Gene Delivery for the Enhanced Expression of a 19-KDa Fragment of Merozoite Surface Protein 1 of Plasmodium falciparum." Biotechnology Progress **26**(1): 257-262.

Liu, S., Danquah, M. K., Ho, J., Ma, C., Wang, L., Coppel, R. and Forde, G. M. (2009). "Preparation and characterization of poly(lactic-co-glycolic acid) microparticles containing DNA molecules encoding a malaria vaccine candidate." Journal of Chemical Technology and Biotechnology **84**(5): 782-788.

Liu, X. Q., Kaminski, M. D., Chen, H. T., Torno, M., Taylor, L. and Rosengart, A. J. (2007). "Synthesis and characterization of highly-magnetic biodegradable poly(D,L-lactide-co-glycolide) nanospheres." Journal of Controlled Release **119**(1): 52-58.

Lodmell, D. L., Ray, N. B., Ulrich, J. T. and Ewalt, L. C. (2000). "DNA vaccination of mice against rabies virus: effects of the route of vaccination and the adjuvant monophosphoryl lipid A (MPL (R))." Vaccine **18**(11-12): 1059-1066.

Louis, M. H., Dutoit, S., Denoux, Y., Erbacher, P., Deslandes, E., Behr, J. P., Gauduchon, P. and Poulain, L. (2006). "Intraperitoneal linear polyethylenimine (L-PEI)-mediated gene delivery to ovarian carcinoma nodes in mice." Cancer Gene Therapy **13**(4): 367-374.

References

Lubbe, A. S., Alexiou, C. and Bergemann, C. (2001). "Clinical applications of magnetic drug targeting." Journal of Surgical Research **95**(2): 200-206.

Lubbe, A. S., Bergemann, C., Riess, H., Schriever, F., Reichardt, P., Possinger, K., et al. (1996). "Clinical experiences with magnetic drag targeting: A phase I study with 4'-epidoxorubicin in 14 patients with advanced solid tumors." Cancer Research **56**(20): 4686-4693.

Lungwitz, U., Breunig, M., Blunk, T. and Gopferich, A. (2005). "Polyethylenimine-based non-viral gene delivery systems." European Journal of Pharmaceutics and Biopharmaceutics **60**(2): 247-266.

Luo, D. and Saltzman, W. M. (2000). "Enhancement of transfection by physical concentration of DNA at the cell surface." Nature Biotechnology **18**(8): 893-895.

Luo, D. and Saltzman, W. M. (2000). "Synthetic DNA delivery systems." Nature Biotechnology **18**(1): 33-37.

Lyon, J. A., Thomas, A. W., Hall, T. and Chulay, J. D. (1989). "Specificities of antibodies that inhibit merozoite dispersal from malaria-infected erythrocytes." Molecular and Biochemical Parasitology **36**(1): 77-85.

Ma, C., Wang, L. N., Webster, D. E., Campbell, A. E. and Coppel, R. L. (2012). "Production, characterisation and immunogenicity of a plant-made Plasmodium antigen-the 19 kDa C-terminal fragment of Plasmodium yoelii merozoite surface protein 1." Applied Microbiology and Biotechnology **94**(1): 151-161.

Ma, Y. J., Zhang, Z. J., Wang, X. L., Xia, W. L. and Gu, H. C. (2011). "Insights into the mechanism of magnetofection using MNPs-PEI/pDNA/free PEI magnetofectins." International Journal of Pharmaceutics **419**(1-2): 247-254.

Mady, M., Mohammed, W., EL-gundy, N. M. and ELsayed, A. (2011). "Effect of polymer molecular weight on the DNA/PEI polyplexes properties." Rom. J. Biophys **21**(2): 151-165.

Mahmoudi, M., Shokrgozar, M. A., Simchi, A., Imani, M., Milani, A. S., Stroeve, P., Vali, H., Hafeli, U. O. and Bonakdar, S. (2009). "Multiphysics Flow Modeling and in Vitro Toxicity of Iron Oxide Nanoparticles Coated with Poly(vinyl alcohol) (vol 113C, pg 2323, 2009)." Journal of Physical Chemistry C **113**(39): 17274-17274.

Manolova, V., Flace, A., Bauer, M., Schwarz, K., Saudan, P. and Bachmann, M. F. (2008). "Nanoparticles target distinct dendritic cell populations according to their size." European Journal of Immunology **38**(5): 1404-1413.

Massart, R. (1981). "Preparation of aqueous magnetic liquids in alkaline and acidic media." Ieee Transactions on Magnetics **17**(2): 1247-1248.

Massart, R., Dubois, E., Cabuil, V. and Hasmonay, E. (1995). "Preparation and properties of monodisperse magnetic fluids." Journal of Magnetism and Magnetic Materials **149**(1-2): 1-5.

Massia, S. P., Stark, J. and Letbetter, D. S. (2000). "Surface-immobilized dextran limits cell adhesion and spreading." Biomaterials **21**(22): 2253-2261.

Matuschewski, K. (2006). "Vaccine development against malaria." Current Opinion in Immunology **18**(4): 449-457.

McAllister, D. V., Allen, M. G. and Prausnitz, M. R. (2000). "Microfabricated microneedles for gene and drug delivery." Annual Review of Biomedical Engineering **2**: 289-313.

Mehier-Humbert, S. and Guy, R. H. (2005). "Physical methods for gene transfer: Improving the kinetics of gene delivery into cells." Advanced Drug Delivery Reviews **57**(5): 733-753.

Mendenhall, G. D., Geng, Y. P. and Hwang, J. (1996). "Optimization of long-term stability of magnetic fluids from magnetite and synthetic polyelectrolytes." Journal of Colloid and Interface Science **184**(2): 519-526.

Mettens, P., Dubois, P. M., Dernoitie, M. A., Bayat, B., Donner, M. N., Bourguignon, P., et al. (2008). "Improved T cell responses to Plasmodium falciparum circumsporozoite protein in mice and monkeys induced by a novel formulation of RTS,S vaccine antigen." Vaccine **26**(8): 1072-1082.

Miller, E. S., Peppas, N. A. and Winslow, D. N. (1983). "Morphological-changes of ethylene vinyl acetate-based controlled delivery systems during release of water-soluble solutes." Journal of Membrane Science **14**(1): 79-92.

Minagawa, K., Matsuzawa, Y., Yoshikawa, K., Matsumoto, M. and Doi, M. (1991). "Direct observation of the biphasic conformational change of DNA induced by cationic polymers." Febs Letters **295**(1-3): 67-69.

Mintzer, M. A. and Simanek, E. E. (2009). "Nonviral Vectors for Gene Delivery." Chemical Reviews **109**(2): 259-302.

Mo, Y., Takaya, T., Nishinari, K., Kubota, K. and Okamoto, A. (1999). "Effects of sodium chloride, guanidine hydrochloride, and sucrose on the viscoelastic properties of sodium hyaluronate solutions." Biopolymers **50**(1): 23-34.

Mohanan, D., Slutter, B., Henriksen-Lacey, M., Jiskoot, W., Bouwstra, J. A., Perrie, Y., Kundig, T. M., Gander, B. and Johansen, P. (2010). "Administration routes affect the quality of immune responses: A cross-sectional evaluation of particulate antigen-delivery systems." Journal of Controlled Release **147**(3): 342-349.

Morawski, A. M., Winter, P. M., Crowder, K. C., Caruthers, S. D., Fuhrhop, R. W., Scott, M. J., et al. (2004). "Targeted nanoparticles for quantitative imaging of sparse molecular epitopes with MRI." Magnetic Resonance in Medicine **51**(3): 480-486.

Moreno, S. and Timon, M. (2004). "DNA vaccination: an immunological perspective." Immunologia **123**: 41-55.

Moret, I., Peris, J. E., Guillem, V. M., Benet, M., Revert, F., Dasi, F., Crespo, A. and Alino, S. F. (2001). "Stability of PEI-DNA and DOTAP-DNA complexes: effect of alkaline pH, heparin and serum." Journal of Controlled Release **76**(1-2): 169-181.

Morgan, W. D., Birdsall, B., Frenkiel, T. A., Gradwell, M. G., Burghaus, P. A., Syed, S. E. H., Uthaipibull, C., Holder, A. A. and Feeney, J. (1999). "Solution structure of an EGF module pair from the Plasmodium falciparum merozoite surface protein 1." Journal of Molecular Biology **289**(1): 113-122.

Munshi, N., De, T. K. and Maitra, A. (1997). "Size modulation of polymeric nanoparticles under controlled dynamics of microemulsion droplets." Journal of Colloid and Interface Science **190**(2): 387-391.

Mykhaylyk, O., Antequera, Y. S., Vlaskou, D. and Plank, C. (2007). "Generation of magnetic nonviral gene transfer agents and magnetofection in vitro." Nature Protocols **2**(10): 2391-2411.

Nacev, A., Beni, C., Bruno, O. and Shapiro, B. (2010). "Magnetic nanoparticle transport within flowing blood and into surrounding tissue." Nanomedicine **5**(9): 1459-1466.

References

Necas, J., Bartosikova, L., Brauner, P. and Kolar, J. (2008). "Hyaluronic acid (hyaluronan): a review." Veterinarni Medicina **53**(8): 397-411.

Nel, A. E., Madler, L., Velegol, D., Xia, T., Hoek, E. M. V., Somasundaran, P., Klaessig, F., Castranova, V. and Thompson, M. (2009). "Understanding biophysicochemical interactions at the nano-bio interface." Nature Materials **8**(7): 543-557.

Neu, M., Fischer, D. and Kissel, T. (2005). "Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives." Journal of Gene Medicine **7**(8): 992-1009.

Nguyen, D. N., Green, J. J., Chan, J. M., Longer, R. and Anderson, D. G. (2009). "Polymeric Materials for Gene Delivery and DNA Vaccination." Advanced Materials **21**(8): 847-867.

Nielsen, O. S., Horsman, M. and Overgaard, J. (2001). "A future for hyperthermia in cancer treatment?" European Journal of Cancer **37**(13): 1587-1589.

Nussenzw.Rs, Vanderbe.J, Most, H. and Orton, C. (1967). "Protective immunity produced by injection of x-irradiated sporozoites of Plasmodium berghei." Nature **216**(5111): 160-&.

Nussenzweig, V. and Nussenzweig, R. S. (1989). "Rationale for the development of an engineered sporozoite malaria vaccine." Advances in Immunology **45**: 283-334.

Nuwaha, F. (2001). "The challenge of chloroquine-resistant malaria in sub-Saharan Africa." Health Policy and Planning **16**(1): 1-12.

O'Donnell, R. A., de Koning-Ward, T. F., Burt, R. A., Bockarie, M., Reeder, J. C., Cowman, A. F. and Crabb, B. S. (2001). "Antibodies against merozoite surface protein (MSP)-1(19) are a major component of the invasion-inhibitory response in individuals immune to malaria." Journal of Experimental Medicine **193**(12): 1403-1412.

O'Grady, K. (2009). "Progress in applications of magnetic nanoparticles in biomedicine." Journal of Physics D-Applied Physics **42**(22).

Ogris, M., Brunner, S., Schuller, S., Kircheis, R. and Wagner, E. (1999). "PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery." Gene Therapy **6**(4): 595-605.

Ogris, M., Steinlein, P., Carotta, S., Brunner, S. and Wagner, E. (2001). "DNA/polyethylenimine transfection particles: Influence of ligands, polymer size, and PEGylation on international and gene expression." Aaps Pharmsci **3**(3): art. no.-21.

Ogris, M., Steinlein, P., Kursa, M., Mechtler, K., Kircheis, R. and Wagner, E. (1998). "The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells." Gene Therapy **5**(10): 1425-1433.

Oh, E. J., Park, K., Kim, K. S., Kim, J., Yang, J. A., Kong, J. H., Lee, M. Y., Hoffman, A. S. and Hahn, S. K. (2010). "Target specific and long-acting delivery of protein, peptide, and nucleotide therapeutics using hyaluronic acid derivatives." Journal of Controlled Release **141**(1): 2-12.

Pankhurst, Q. A., Thanh, N. T. K., Jones, S. K. and Dobson, J. (2009). "Progress in applications of magnetic nanoparticles in biomedicine." Journal of Physics D-Applied Physics **42**(22).

References

Papisov, I. M. and Litmanovich, A. A. (1989). "Molecular recognition in interpolymer interactions and matrix polymerization." Advances in Polymer Science **90**: 139-179.

Park, T. G., Jeong, J. H. and Kim, S. W. (2006). "Current status of polymeric gene delivery systems." Advanced Drug Delivery Reviews **58**(4): 467-486.

Parween, S., Gupta, P. K. and Chauhan, V. S. (2011). "Induction of humoral immune response against PfMSP-119 and PvMSP-119 using gold nanoparticles along with alum." Vaccine **29**(13): 2451-2460.

Pathak, A., Vyasz, S. P. and Gupta, K. C. (2008). "Nano-vectors for efficient lover specific gene transfer." International Journal of Nanomedicine **3**(1): 31-49.

Patil, S. D., Rhodes, D. G. and Burgess, D. J. (2005). "DNA-based therapeutics and DNA delivery systems: A comprehensive review." Aaps Journal **7**(1): E61-E77.

Pertmer, T. M., Roberts, T. R. and Haynes, J. R. (1996). "Influenza virus nucleoprotein-specific immunoglobulin G subclass and cytokine responses elicited by DNA vaccination are dependent on the route of vector DNA delivery." Journal of Virology **70**(9): 6119-6125.

Petri-Fink, A., Steitz, B., Finka, A., Salaklang, J. and Hofmann, H. (2008). "Effect of cell media on polymer coated superparamagnetic iron oxide nanoparticles (SPIONs): Colloidal stability, cytotoxicity, and cellular uptake studies." European Journal of Pharmaceutics and Biopharmaceutics **68**(1): 129-137.

Pimentel, D., Cooperstein, S., Randell, H., Filiberto, D., Sorrentino, S., Kaye, B., et al. (2007). "Ecology of increasing diseases: Population growth and environmental degradation." Human Ecology **35**(6): 653-668.

Pipes, B. L., Vasanwala, F. H., Tsang, T. C., Zhang, T., Luo, P. and Harris, D. T. (2005). "Brief heat shock increases stable integration of lipid-mediated DNA transfections." Biotechniques **38**(1): 48-+.

Plank, C., Anton, M., Rudolph, C., Rosenecker, J. and Krotz, F. (2003). "Enhancing and targeting nucleic acid delivery by magnetic force." Expert Opinion on Biological Therapy **3**(5): 745-758.

Plank, C., Scherer, F., Schillinger, U., Bergemann, C. and Anton, M. (2003). "Magnetofection: Enhancing and targeting gene delivery with superparamagnetic nanoparticles and magnetic fields." Journal of Liposome Research **13**(1): 29-32.

Plank, C., Schillinger, U., Scherer, F., Bergemann, C., Remy, J. S., Krotz, F., Anton, M., Lausier, J. and Rosenecker, J. (2003). "The magnetofection method: Using magnetic force to enhance gene delivery." Biological Chemistry **384**(5): 737-747.

Plebanski, M. and Hill, A. V. S. (2000). "The immunology of malaria infection." Current Opinion in Immunology **12**(4): 437-441.

Portet, D., Denizot, B., Rump, E., Hindre, F., Le Jeune, J. J. and Jallet, P. (2001). "Comparative biodistribution of thin-coated iron oxide nanoparticles TCION: Effect of different bisphosphonate coatings." Drug Development Research **54**(4): 173-181.

Poulain, L., Ziller, C., Muller, C. D., Erbacher, P., Bettinger, T., Rodier, J. F. and Behr, J. P. (2000). "Ovarian carcinoma cells are effectively transfected by polyethylenimine (PEI) derivatives." Cancer Gene Therapy **7**(4): 644-652.

Pouton, C. W. and Seymour, L. W. (2001). "Key issues in non-viral gene delivery." Advanced Drug Delivery Reviews **46**(1-3): 187-203.

Prabha, S., Zhou, W. Z., Panyam, J. and Labhasetwar, V. (2002). "Size-dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles." International Journal of Pharmaceutics **244**(1-2): 105-115.

Prasad, T. K., Gopal, V. and Rao, N. M. (2003). "Structural changes in DNA mediated by cationic lipids alter in vitro transcriptional activity at low charge ratios." Biochimica Et Biophysica Acta-General Subjects **1619**(1): 59-69.

Prestvik, W. S., Berge, A., Mork, P. C., Stenstad, P. M. and Ugelstad, J. (1997). Preparation and application of monosized magnetic particles in selective cell separation.

Prud'homme, G. J., Glinka, Y., Khan, A. S. and Draghia-Akli, R. (2006). "Electroporation-enhanced nonviral gene transfer for the prevention or treatment of immunological, endocrine and neoplastic diseases." Current Gene Therapy **6**(2): 243-273.

Pusic, K., Xu, H. Y., Stridiron, A., Aguilar, Z., Wang, A. and Hui, G. (2011). "Blood stage merozoite surface protein conjugated to nanoparticles induce potent parasite inhibitory antibodies." Vaccine **29**(48): 8898-8908.

Qhattal, H. S. S. and Liu, X. (2011). "Characterization of CD44-Mediated Cancer Cell Uptake and Intracellular Distribution of Hyaluronan-Grafted Liposomes." Molecular Pharmaceutics **8**(4): 1233-1246.

Ramaswamy, S., Greco, J. B., Uluer, M. C., Zhang, Z. J., Zhang, Z. L., Fishbein, K. W. and Spencer, R. G. (2009). "Magnetic Resonance Imaging of Chondrocytes Labeled with Superparamagnetic Iron Oxide Nanoparticles in Tissue-Engineered Cartilage." Tissue Engineering Part A **15**(12): 3899-3910.

Ramchand, C. N. (2012). "Molecular Medicine-Prospects and Challenges." American journal of infectious diseases **8**(1): 19.

Ramshaw, I. A. and Ramsay, A. J. (2000). "The prime-boost strategy: exciting prospects for improved vaccination." Immunology Today **21**(4): 163-165.

Regules, J. A., Cummings, J. F. and Ockenhouse, C. F. (2011). "The RTS,S vaccine candidate for malaria." Expert Review of Vaccines **10**(5): 589-599.

Rejman, J., Oberle, V., Zuhorn, I. S. and Hoekstra, D. (2004). "Size-dependent internalization of particles via the pathways of clathrin-and caveolae-mediated endocytosis." Biochemical Journal **377**: 159-169.

Remy, J. S., Abdallah, B., Zanta, M. A., Boussif, O., Behr, J. P. and Demeneix, B. (1998). "Gene transfer with lipospermines and polyethylenimines." Advanced Drug Delivery Reviews **30**(1-3): 85-95.

Reschel, T., Konak, C., Oupicky, D., Seymour, L. W. and Ulbrich, K. (2002). "Physical properties and in vitro transfection efficiency of gene delivery vectors based on complexes of DNA with synthetic polycations." Journal of Controlled Release **81**(1-2): 201-217.

Rezzani, R., Rodella, L., Zauli, G., Caimi, L. and Vitale, M. (1999). "Mouse peritoneal cells as a reservoir of late dendritic cell progenitors." British Journal of Haematology **104**(1): 111-118.

Richie, T. L. and Saul, A. (2002). "Progress and challenges for malaria vaccines." Nature **415**(6872): 694-701.

Robinson, H. L. (2007). "HIV/AIDS vaccines: 2007." Clinical Pharmacology & Therapeutics **82**(6): 686-693.

Roca, A. G., Costo, R., Rebolledo, A. F., Veintemillas-Verdaguer, S., Tartaj, P., Gonzalez-Carreno, T., Morales, M. P. and Serna, C. J. (2009). "Progress in the preparation of magnetic nanoparticles for applications in biomedicine." Journal of Physics D-Applied Physics **42**(22).

References

Ruiz, J. M. and Benoit, J. P. (1991). "In vivo peptide release from poly(DI-lactic acid-co-glycolic acid) copolymer 50/50 microspheres." Journal of Controlled Release **16**(1-2): 177-185.

Ruponen, M., Honkakoski, P., Ronkko, S., Pelkonen, J., Tammi, M. and Urtti, A. (2003). "Extracellular and intracellular barriers in non-viral gene delivery." Journal of Controlled Release **93**(2): 213-217.

Ruponen, M., Ronkko, S., Honkakoski, P., Pelkonen, J., Tammi, M. and Urtti, A. (2001). "Extracellular glycosaminoglycans modify cellular trafficking of lipoplexes and polyplexes." Journal of Biological Chemistry **276**(36): 33875-33880.

Ruponen, M., Yla-Herttuala, S. and Urtti, A. (1999). "Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies." Biochimica Et Biophysica Acta-Biomembranes **1415**(2): 331-341.

Sachs, J. and Malaney, P. (2002). "The economic and social burden of malaria." Nature **415**(6872): 680-685.

Sahoo, Y., Pizem, H., Fried, T., Golodnitsky, D., Burstein, L., Sukenik, C. N. and Markovich, G. (2001). "Alkyl phosphonate/phosphate coating on magnetite nanoparticles: A comparison with fatty acids." Langmuir **17**(25): 7907-7911.

Sakai, T., Hisaeda, H., Nakano, Y., Zhang, M. X., Takashima, M., Ishii, K., et al. (2003). "Gene gun-based co-immunization of merozoite surface protein-1 cDNA with IL-12 expression plasmid confers protection against lethal Plasmodium yoelii in A/J mice." Vaccine **21**(13-14): 1432-1444.

Sawyer, L. C., Grubb, D. T. and Meyers, G. F. (New York : Springer (2008)). "Polymer Microscopy (Third Edition),Chapter 1.".

Scherer, F., Anton, M., Schillinger, U., Henkel, J., Bergemann, C., Kruger, A., Gansbacher, B. and Plank, C. (2002). "Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo." Gene Therapy **9**(2): 102-109.

Schillinger, U., Brill, T., Rudolph, C., Huth, S., Gersting, S., Krötz, F., Hirschberger, J., Bergemann, C. and Plank, C. (2005). "Advances in magnetofection—magnetically guided nucleic acid delivery." Journal of Magnetism and Magnetic Materials **293**(1): 501-508.

Schmidt, H. (2001). "Nanoparticles by chemical synthesis, processing to materials and innovative applications." Applied Organometallic Chemistry **15**(5): 331-343.

Schweiger, C., Pietzonka, C., Heverhagen, J. and Kissel, T. (2011). "Novel magnetic iron oxide nanoparticles coated with poly(ethylene imine)-g-poly(ethylene glycol) for potential biomedical application: Synthesis, stability, cytotoxicity and MR imaging." International Journal of Pharmaceutics **408**(1-2): 130-137.

Schwertmann, U. and Cornell, R. M. (1991). Iron oxides in the laboratory : preparation and characterization, Weinheim, New York:VCH.

Schwick, H. G. and Heide, K. (1969). "Immunochemistry and immunology of collagen and gelatin." Bibliotheca Haematologica(33): 111-&.

Sedegah, M., Hedstrom, R., Hobart, P. and Hoffman, S. L. (1994). "Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein." Proceedings of the National Academy of Sciences of the United States of America **91**(21): 9866-9870.

Sedegah, M., Rogers, W. O., Belmonte, A., Belmonte, M., Banania, G., Patterson, N., et al. (2006). "Vaxfectin™ enhances immunogenicity and protective efficacy of P. yoelii circumsporozoite DNA vaccines." Vaccine **24**(11): 1921-1927.

Sedegah, M., Rogers, W. O., Belmonte, M., Belmonte, A., Banania, G., Patterson, N. B., et al. (2010). "Vaxfectin® enhances both antibody and in vitro T cell responses to each component of a 5-gene Plasmodium falciparum plasmid DNA vaccine mixture administered at low doses." Vaccine **28**(17): 3055-3065.

Seder, R. A. and Hill, A. V. S. (2000). "Vaccines against intracellular infections requiring cellular immunity." Nature **406**(6797): 793-798.

Senyei, A., Widder, K. and Czerlinski, G. (1978). "Magnetic guidance of drug-carrying microspheres." Journal of Applied Physics **49**(6): 3578-3583.

Shi, Y. F., Zhou, L. Z., Wang, R. B., Pang, Y., Xiao, W. C., Li, H. Q., et al. (2010). "In situ preparation of magnetic nonviral gene vectors and magnetofection in vitro." Nanotechnology **21**(11).

Shuaibu, M. N., Cherif, M. S., Kurosaki, T., Helegbe, G. K., Kikuchi, M., Yanagi, T., Sasaki, H. and Hirayama, K. (2011). "Effect of nanoparticle coating on the immunogenicity of plasmid DNA vaccine encoding P. yoelii MSP-1 C-terminal." Vaccine **29**(17): 3239-3247.

Sin, J. I., Bagarazzi, M., Pachuk, C. and Weiner, D. B. (2000). "DNA priming-protein boosting enhances both antigen-specific antibody and Th1-type cellular immune responses in a murine herpes simplex virus-2 gD vaccine model (vol 18, pg 777, 1999)." DNA and Cell Biology **19**(1): 69-69.

Snow, R. W., Guerra, C. A., Noor, A. M., Myint, H. Y. and Hay, S. I. (2005). "The global distribution of clinical episodes of Plasmodium falciparum malaria." Nature **434**(7030): 214-217.

Spies, B., Hochrein, H., Vabulas, M., Huster, K., Busch, D. H., Schmitz, F., Heit, A. and Wagner, H. (2003). "Vaccination with plasmid DNA activates dendritic cells via toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice." Journal of Immunology **171**(11): 5908-5912.

Stechschulte, S. U., Joussen, A. M., von Recum, H. A., Poulaki, V., Moromizato, Y., Yuan, J., D'Amato, R. J., Kuo, C. and Adamis, A. P. (2001). "Rapid ocular angiogenic control via naked DNA delivery to cornea." Investigative Ophthalmology & Visual Science **42**(9): 1975-1979.

Stolnik, S., Illum, L. and Davis, S. S. (1995). "Long circulation micrparticulate drug carriers." Advanced Drug Delivery Reviews **16**(2-3): 195-214.

Sun, C., Tang, T., Uludağ, H. and Cuervo, Javier E. (2011). "Molecular Dynamics Simulations of DNA/PEI Complexes: Effect of PEI Branching and Protonation State." Biophysical Journal **100**(11): 2754-2763.

Sun, X. Y., Ma, P., Cao, X. K., Ning, L., Tian, Y. and Ren, C. S. (2009). "Positive hyaluronan/PEI/DNA complexes as a target-specific intracellular delivery to malignant breast cancer." Drug Delivery **16**(7): 357-362.

Sung, S. J., Min, S. H., Cho, K. Y., Lee, S., Min, Y. J., Yeom, Y. I. and Park, J. K. (2003). "Effect of polyethylene glycol on gene delivery of polyethylenimine." Biological & Pharmaceutical Bulletin **26**(4): 492-500.

Surini, S., Akiyama, H., Morishita, M., Nagai, T. and Takayama, K. (2003). "Release phenomena of insulin from an implantable device composed of a polyion complex of chitosan and sodium hyaluronate." Journal of Controlled Release **90**(3): 291-301.

Tang, M. X. and Szoka, F. C. (1997). "The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes." Gene Therapy **4**(8): 823-832.

Tang, R. P., Palumbo, R. N., Nagarajan, L., Krogstad, E. and Wang, C. (2010). "Well-defined block copolymers for gene delivery to dendritic cells: Probing the effect of polycation chain-length." Journal of Controlled Release **142**(2): 229-237.

Tartaj, P., Gonzalez-Carreno, T. and Serna, C. J. (2002). "Synthesis of nanomagnets dispersed in colloidal silica cages with applications in chemical separation." Langmuir **18**(12): 4556-4558.

Tartaj, P., Morales, M. D., Veintemillas-Verdaguer, S., Gonzalez-Carreno, T. and Serna, C. J. (2003). "The preparation of magnetic nanoparticles for applications in biomedicine." Journal of Physics D-Applied Physics **36**(13): R182-R197.

Termeer, C. C., Hennies, J., Voith, U., Ahrens, T., Weiss, J. M., Prehm, P. and Simon, J. C. (2000). "Oligosaccharides of hyaluronan are potent activators of dendritic cells." Journal of Immunology **165**(4): 1863-1870.

Thera, M. A. and Plowe, C. V. (2012). Vaccines for Malaria: How Close Are We? Annual Review of Medicine, Vol 63. C. T. Caskey, C. P. Austin and J. A. Hoxie. Palo Alto, Annual Reviews. **63:** 345-357.

Tian, J. H., Kumar, S., Kaslow, D. C. and Miller, L. H. (1997). "Comparison of protection induced by immunization with recombinant proteins from different regions of merozoite surface protein 1 of Plasmodium yoelii." Infection and Immunity **65**(8): 3032-3036.

Tuteja, R. (2002). "DNA vaccine against malaria: A long way to go." Critical Reviews in Biochemistry and Molecular Biology **37**(1): 29-54.

Varkouhi, A. K., Scholte, M., Storm, G. and Haisma, H. J. (2011). "Endosomal escape pathways for delivery of biologicals." Journal of Controlled Release **151**(3): 220-228.

Veiseh, O., Gunn, J. W. and Zhang, M. Q. (2010). "Design and fabrication of magnetic nanoparticles for targeted drug delivery and imaging." Advanced Drug Delivery Reviews **62**(3): 284-304.

Vijayanathan, V., Thomas, T. and Thomas, T. J. (2002). "DNA nanoparticles and development of DNA delivery vehicles for gene therapy." Biochemistry **41**(48): 14085-14094.

Voigt, K., Izsvak, Z. and Ivics, Z. (2008). "Targeted gene insertion for molecular medicine." Journal of Molecular Medicine-Jmm **86**(11): 1205-1219.

von der Leyen, H. E., Braun-Dullaeus, R., Mann, M. J., Zhang, L. N., Niebauer, J. and Dzau, V. J. (1999). "A pressure-mediated nonviral method for efficient arterial gene and oligonucleotide transfer." Human Gene Therapy **10**(14): 2355-2364.

Vonzelewsky, A., Barbosa, L. and Schlapfer, C. W. (1993). "Poly(ethylenimines) as bronsted bases and as ligands for metal-ions." Coordination Chemistry Reviews **123**(1-2): 229-246.

Wang, R. B., Doolan, D. L., Charoenvit, Y., Hedstrom, R. C., Gardner, M. J., Hobart, P., et al. (1998). "Simultaneous induction of multiple antigen-specific cytotoxic T lymphocytes in nonhuman primates by immunization with a mixture of four Plasmodium falciparum DNA plasmids." Infection and Immunity **66**(9): 4193-4202.

Wang, X., Zhou, L., Ma, Y., Li, X. and Gu, H. (2009). "Control of aggregate size of polyethyleneimine-coated magnetic nanoparticles for magnetofection." Nano Research **2**(5): 365-372.

Wang, X. L., Zhou, L. Z., Ma, Y. J. and Gu, H. C. (2009). "Charged Magnetic Nanoparticles for Enhancing Gene Transfection." Ieee Transactions on Nanotechnology **8**(2): 142-147.

Wang, Y. H., Zheng, M., Meng, F. H., Zhang, J., Peng, R. and Zhong, Z. Y. (2011). "Branched Polyethylenimine Derivatives with Reductively Cleavable Periphery for Safe and Efficient In Vitro Gene Transfer." Biomacromolecules **12**(4): 1032-1040.

Watts, C. (1997). "Capture and processing of exogenous antigens for presentation on MHC molecules." Annual Review of Immunology **15**: 821-850.

Weck, K. E., Kim, S. S., Virgin, H. W. and Speck, S. H. (1999). "Macrophages are the major reservoir of latent murine gammaherpesvirus 68 in peritoneal cells." Journal of Virology **73**(4): 3273-3283.

Weigel, J. A., Raymond, R. C. and Weigel, P. H. (2002). "The hyaluronan receptor for endocytosis (HARE) is not CD44 or CD54 (ICAM-1)." Biochemical and Biophysical Research Communications **294**(4): 918-922.

Weintraub, H., Cheng, P. F. and Conrad, K. (1986). "Expression of transfected DNA depends on DNA toplogy." Cell **46**(1): 115-122.

White, W. I., Evans, C. B. and Taylor, D. W. (1991). "Antimalarial antibodies of the immunoglobulin g2a isotype modulate parasitemias in mice infected with plasmodium-yoelii." Infection and Immunity **59**(10): 3547-3554.

WHO (2010). "World Health Organization.World: Malaria risk areas, 2010. Available from: http://gamapserver.who.int/mapLibrary/Files/Maps/Global_Malaria_2010."

WHO (2011). "World Health Organization.World Malaria Report 2011;World Health Organization: Geneva Switzerland 2011."

Widder, K. J. and Senyei, A. E. (1983). "Magnetic microspheres: A vehicle for selective targeting of drugs." Pharmacology & Therapeutics **20**(3): 377-395.

Wightman, L., Kircheis, R., Rossler, V., Carotta, S., Ruzicka, R., Kursa, M. and Wagner, E. (2001). "Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo." Journal of Gene Medicine **3**(4): 362-372.

Wiogo, H. T. R., Lim, M., Bulmus, V., Yun, J. and Amal, R. (2011). "Stabilization of Magnetic Iron Oxide Nanoparticles in Biological Media by Fetal Bovine Serum (FBS)." Langmuir **27**(2): 843-850.

Wolfert, M. A., Dash, P. R., Nazarova, O., Oupicky, D., Seymour, L. W., Smart, S., Strohalm, J. and Ulbrich, K. (1999). "Polyelectrolyte vectors for gene delivery: Influence of cationic polymer on biophysical properties of complexes formed with DNA." Bioconjugate Chemistry **10**(6): 993-1004.

Wolny, P. M., Banerji, S., Gounou, C., Brisson, A. R., Day, A. J., Jackson, D. G. and Richter, R. P. (2010). " Analysis of CD44-hyaluronan interactions in an artificial membrane system insights into the distinct binding properties of high and low molecular weight hyaluronan." Journal of Biological Chemistry **285**(39): 30170-30180.

Wong, S. Y., Pelet, J. M. and Putnam, D. (2007). "Polymer systems for gene delivery-past, present, and future." Progress in Polymer Science **32**(8-9): 799-837.

Wu, W., He, Q. G. and Jiang, C. Z. (2008). "Magnetic Iron Oxide Nanoparticles: Synthesis and Surface Functionalization Strategies." Nanoscale Research Letters **3**(11): 397-415.

Wu, X. M., He, X. H., Zhong, L., Lin, S. L., Wang, D. L., Zhu, X. Y. and Yan, D. Y. (2011). "Water-soluble dendritic-linear triblock copolymer-modified magnetic nanoparticles: preparation, characterization and drug release properties." Journal of Materials Chemistry **21**(35): 13611-13620.

Xiang, L., Bin, W., Huali, J., Wei, J., Jiesheng, T., Feng, G. and Ying, L. (2007). "Bacterial magnetic particles (BMPs)-PEI as a novel and efficient non-viral gene delivery system." The Journal of Gene Medicine **9**(8): 679-690.

Xiang, S. D., Scalzo-Inguanti, K., Minigo, G., Park, A., Hardy, C. L. and Plebanski, M. (2008). "Promising particle-based vaccines in cancer therapy." Expert Review of Vaccines **7**(7): 1103-1119.

Xiang, S. D., Selomulya, C., Ho, J., Apostolopoulos, V. and Plebanski, M. (2010). "Delivery of DNA vaccines: an overview on the use of biodegradable polymeric and magnetic nanoparticles." Wiley Interdisciplinary Reviews-Nanomedicine and Nanobiotechnology **2**(3): 205-218.

Xu, C., Xie, J., Ho, D., Wang, C., Kohler, N., Walsh, E. G., Morgan, J. R., Chin, Y. E. and Sun, S. (2008). "Au-Fe3O4 dumbbell nanoparticles as dual-functional probes." Angewandte Chemie-International Edition **47**(1): 173-176.

Xu, Z. P., Zeng, Q. H., Lu, G. Q. and Yu, A. B. (2006). "Inorganic nanoparticles as carriers for efficient cellular delivery." Chemical Engineering Science **61**(3): 1027-1040.

Yamazaki, M. and Ito, T. (1990). "Deformation and instability in membrane-structure of phospholipid-vesicles caused by osmophobic association - mechanical-stress model for the mechanism of poly(ethylene glycol)-induced membrane-fusion." Biochemistry **29**(5): 1309-1314.

Yankov, D. S., Trusler, J. P. M., Stateva, R. P. and Cholakov, G. S. (2009). "Influence of pH and acid solutes on the phase behaviour of aqueous solutions containing poly(ethylene glycol) and poly(ethyleneimine)." Biochemical Engineering Journal **48**(1): 104-110.

Yao, J., Fan, Y., Du, R. H., Zhou, J. P., Lu, Y., Wang, W., Ren, J. and Sun, X. J. (2010). "Amphoteric hyaluronic acid derivative for targeting gene delivery." Biomaterials **31**(35): 9357-9365.

Yee, C., Kataby, G., Ulman, A., Prozorov, T., White, H., King, A., Rafailovich, M., Sokolov, J. and Gedanken, A. (1999). "Self-assembled monolayers of alkanesulfonic and -phosphonic acids on amorphous iron oxide nanoparticles." Langmuir **15**(21): 7111-7115.

Zappavigna, V., Falciola, L., Citterich, M. H., Mavilio, F. and Bianchi, M. E. (1996). "HMG1 interacts with HOX proteins and enhances their DNA binding and transcriptional activation." Embo Journal **15**(18): 4981-4991.

Zhang, C., Wangler, B., Morgenstern, B., Zentgraf, H., Eisenhut, M., Untenecker, H., et al. (2007). "Silica- and alkoxysilane-coated ultrasmall superparamagnetic iron oxide particles: A promising tool to label cells for magnetic resonance imaging." Langmuir **23**(3): 1427-1434.

References

Zhang, Y., Kohler, N. and Zhang, M. Q. (2002). "Surface modification of superparamagnetic magnetite nanoparticles and their intracellular uptake." Biomaterials **23**(7): 1553-1561.

Zhao, Q. Q., Chen, J. L., Lv, T. F., He, C. X., Tang, G. P., Liang, W. Q., Tabata, Y. and Gao, J. Q. (2009). "N/P Ratio Significantly Influences the Transfection Efficiency and Cytotoxicity of a Polyethylenimine/Chitosan/DNA Complex." Biological & Pharmaceutical Bulletin **32**(4): 706-710.

Zhao, S. H., Lin, X. L., Zhang, L., Sun, L., Li, J., Yang, W. S. and Sun, Z. W. (2012). "The in vivo investigation of Fe_3O_4-nanoparticles acute toxicity in mice." Biomedical Engineering-Applications Basis Communications **24**(3): 229-235.

Zhou, X. F., Liu, B., Yu, X. H., Zha, X., Zhang, X. Z., Wang, X. Y., et al. (2007). "Using magnetic force to enhance immune response to DNA vaccine." Small **3**(10): 1707-1713.

Zintchenko, A., Philipp, A., Dehshahri, A. and Wagner, E. (2008). "Simple modifications of branched PEI lead to highly efficient siRNA carriers with low toxicity." Bioconjugate Chemistry **19**(7): 1448-1455.

Appendix A: General information about plasmid DNA and magnetic particles

This section provides general details about PyMSP1$_{19}$ plasmid structure and SPIONs properties.

VR1020-PyMSP1$_{19}$ Plasmid

The widely used model system of human malaria is mouse malaria *Plasmodium yoelii* MSP1 (PyMSP1) gene which revealed a homology to *P. falciparum* (PfMSP1) gene. The PyMSP1 protein also has a close structure resembling that of (PfMSP1) with similar putative signal peptide and GPI anchor.

Current malaria DNA vaccine includes PyMSP1$_{19}$ encoded gene was inserted into a mammalian expression vector plasmid VR1020 (Vical Inc., San Diego) via appropriate restriction digestion (*Bam* HI and *Big* II) VR1020 site. PyMSP1$_{19}$ gene was fused at its 5' end to an in-frame tissue plasmiongen activator (TPA) secretion signal. The recombinant plasmid VR1020-PyMSP1$_{19}$ contains a tissue human cytomegalovirus (CMV) immediate early promoter/enhancer elements (CMV promoter and CMV intron A), bovine growth hormone (BGH) terminator and a Kanamycin resistance gene for selection purpose, see Appendix A (Figure A1).

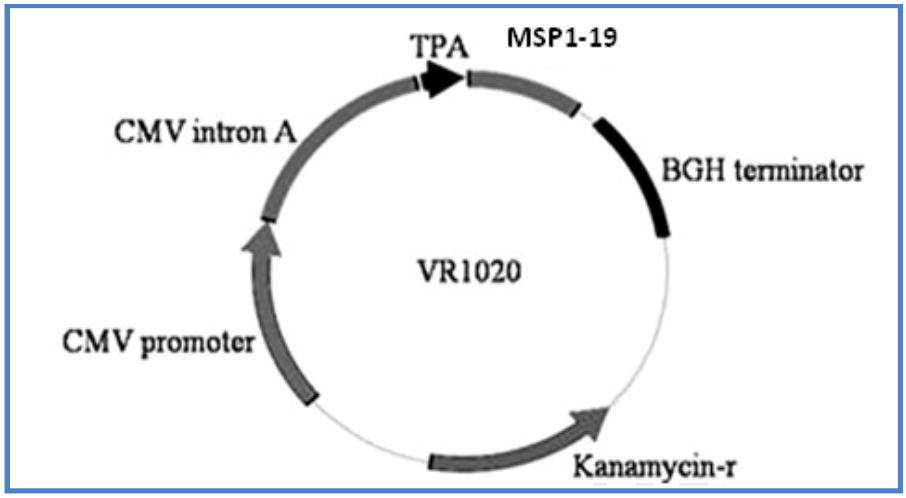

Figure A.1: Schematic figure of plasmid VR1020-PyMSP1$_{19}$

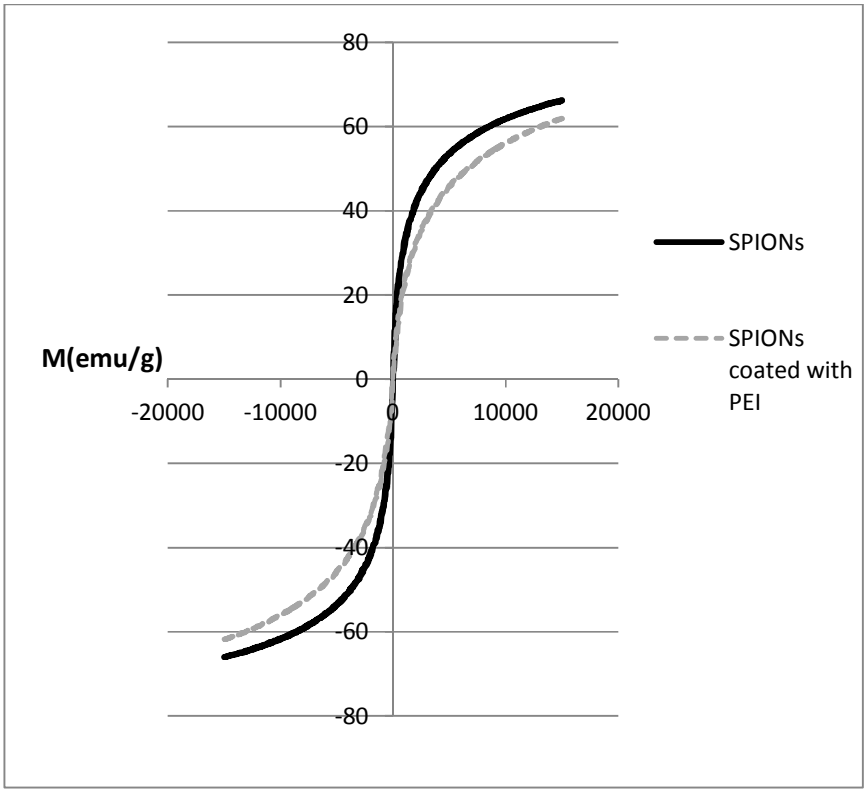

Figure A.2: Magnetisation plots for SPIONs and SPIONs/PEI. The x and y axes indicate applied field (H, kOe) and magnetization (M, emu/g), respectively.

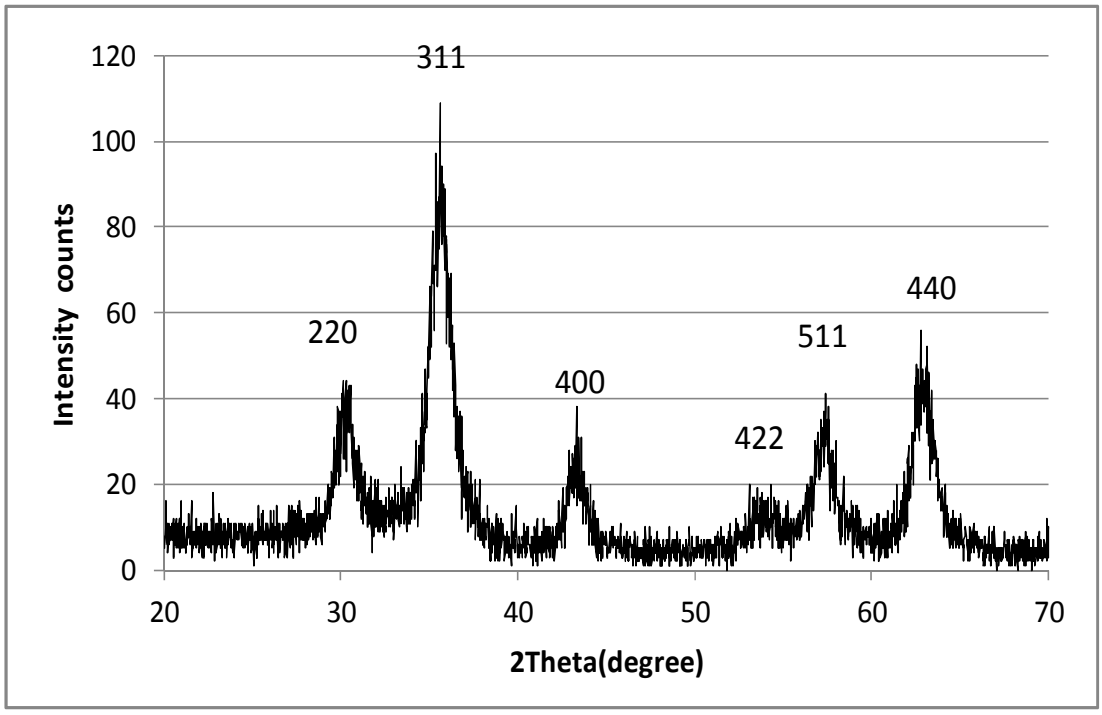

Figure A.3: X-ray diffraction pattern of Fe_3O_4 nanparticles prepared Co-precipitation

Appendix B: The results of antibody responses against PyMSP1$_{19}$ of chapter 4.

The section provides details about the IgG level obtained in BALB/c mice immunized with homologous (DNA vaccination alone) or heterologous (DNA prime-protein boost) regimen.

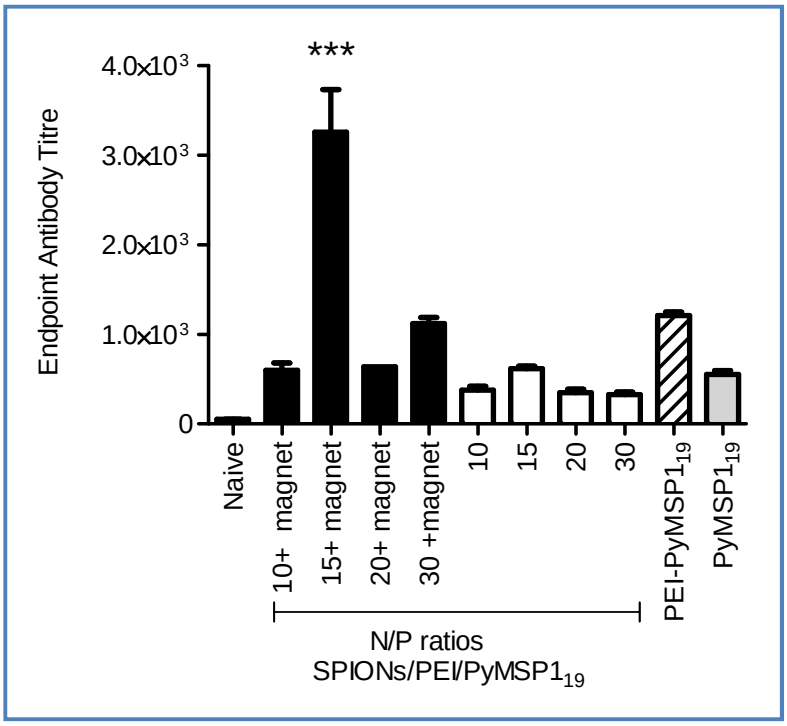

Figure B.1: Total IgG obtained from BALB/c mice immunized intramuscularly with different combinations of PyMSP1$_{19}$ (naked DNA, DNA-PEI, at the N/P ratio of 15 and SPIONs/PEI/DNA at different N/P ratios with or without strong magnetic field). Two weeks after the last immunization (day 42), sera were collected from immunized mice in each group (n=5). Pooled sera were analyzed for the level of IgG antibodies against recombinant protein as a capture antigen. Results shown are the last dilution of sera at which the OD$_{450nm}$ was higher than mean+3SD of control mice. Results are expressed as means ± SD of duplicate. Statistical significance is designated as *** p<0.001.

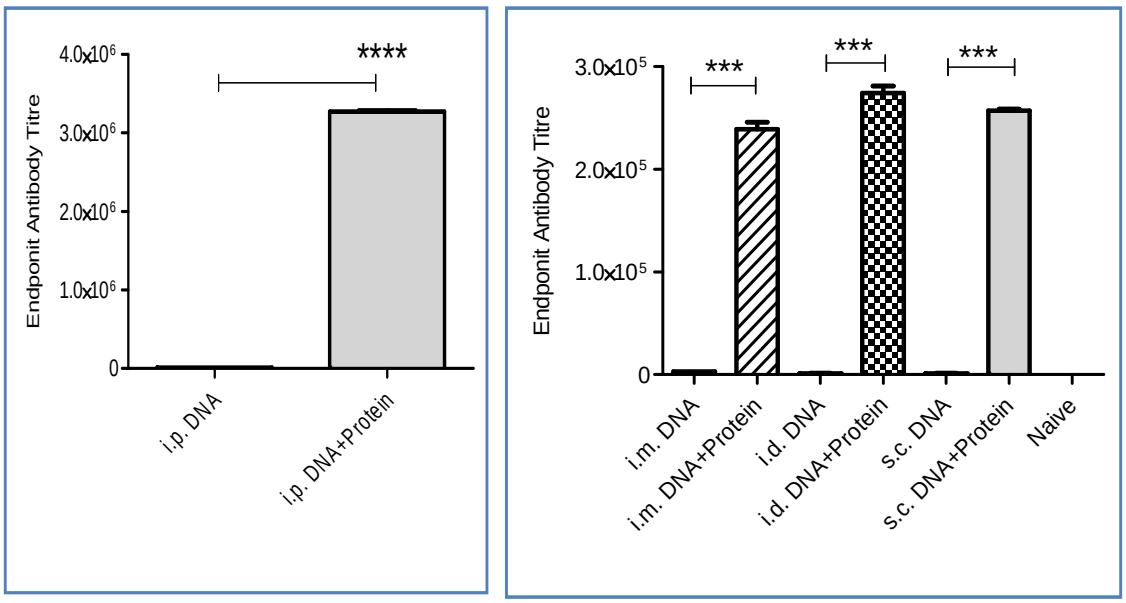

Figure B.2: DNA prime-protein boost effect on total IgG levels against PyMSP1$_{19}$ in mice immunized with SPIONs/PEI/DNA complexes. A group of mice were immunized with three doses of naked DNA or SPIONs/PEI/DNA complexes via different routes of administration with magnet application, followed by a single boost with recombinant protein formulated in incomplete Freund's adjuvant injected intraperitoneally. Sera were obtained two weeks before and after protein boost and pooled for endpoint titre against recombinant protein. Results shown are the last dilution of sera at which the OD$_{450nm}$ was higher than mean + 3SD of control mice. Results are expressed as means ± SD of duplicates. Statistical significance is designed as *** p<0.001.

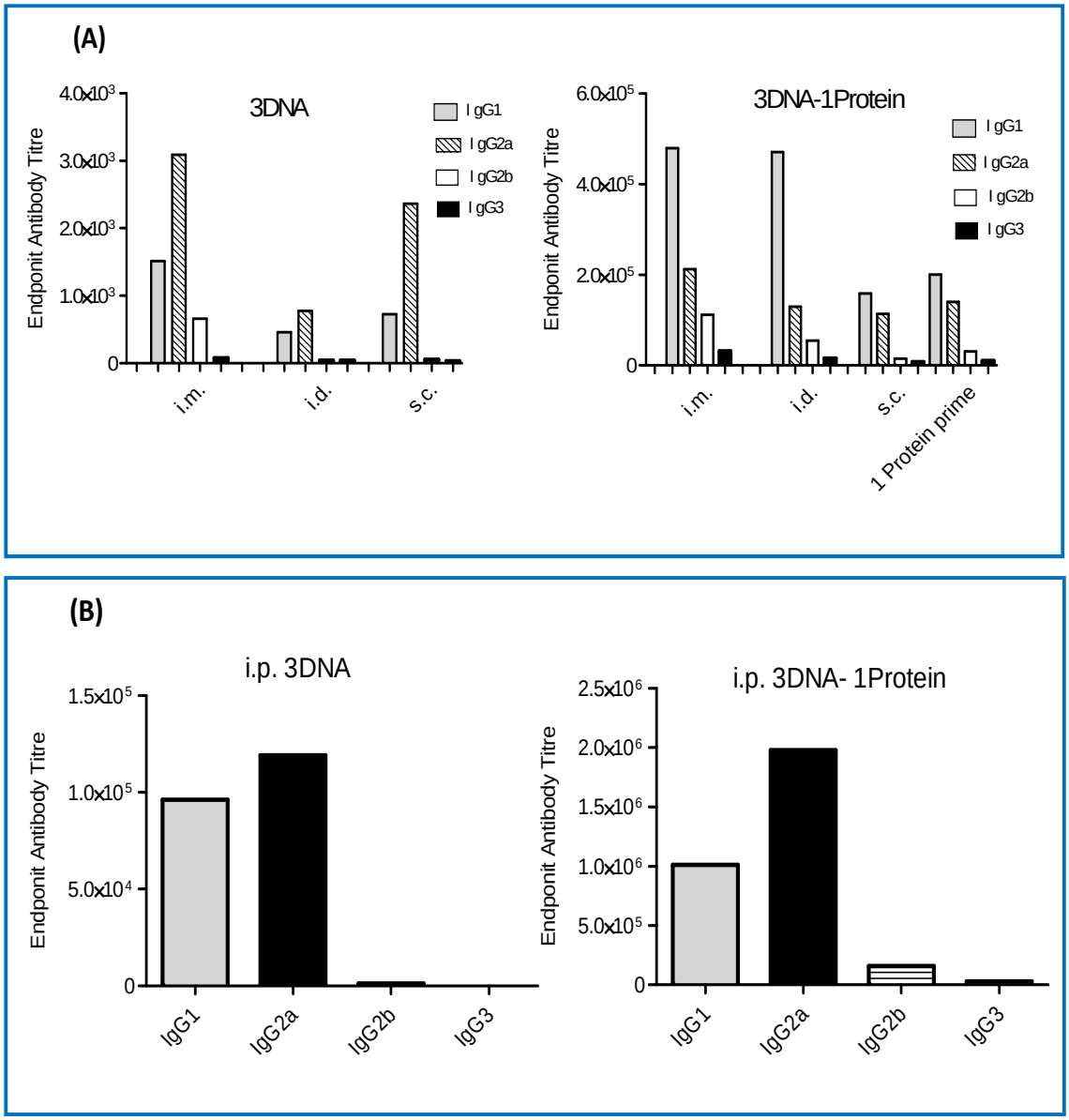

Figure B.3: DNA prime-protein boost regime effects on IgG levels against PyMSP1$_{19}$ in mice immunized with SPIONs/PEI/DNA complexes. A group of mice were immunized with three doses of SPIONs/PEI/DNA complexes via different routes of administration with magnet application followed by a single boost with recombinant protein formulated in incomplete Freund's adjuvant injected intraperitoneally. Sera were obtained two weeks before and after protein boost and pooled for endpoint titre against recombinant protein: (A) IgG antibody subclass titre in the pooled sera collected from i.m., i.d., and s.c. groups before and after single protein boost; (B) IgG antibody subclass titre in the pooled sera collected from i.p. group before and after single protein boost. Results shown are the last dilution of sera at which the OD$_{450nm}$ was higher than mean + 3SD of control mice. Results are expressed as means ± SD of duplicates.

Appendix C: The result of Western blot analysis to detect $PyMSP1_{19}$ expression in dendritic cells DCs.

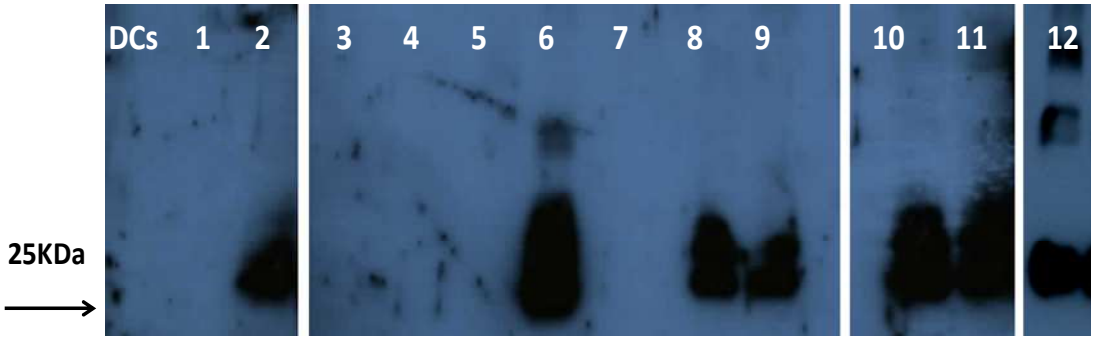

Figure C.1: Western blot detection of $PyMSP1_{19}$ in DCs at 48h post-transfection. Lane DCs, dendritic cells without transfection (control); Lane1, the cells transfected with naked $PyMSP1_{19}$; Lane2, the cells transfected with DNA-Lipofectamine; Lane 3, SPIONs-PEI-DNA with magnet; Lane 4-11 SPIONs/PEI/DNA-HA polyplexes; Lane 4, 5% LMW w/o magnet; Lane 5, 100%LMW w/o magnet; Lane 6, 100% HMAW w/o magnet; Lane 7,5% HMAW w/o magnet; Lane 8, 5% LMW w magnet; Lane 9, 100%LMW w magnet; Lane 10, 100% HMAW w magnet; Lane11, 5% HMAW w magnet; Lane12; $PyMSP1_{19}$ protein expressed in *Escherichia coli.*