

Superparamagnetic Nanoparticles for Effective Delivery of Malaria DNA Vaccine

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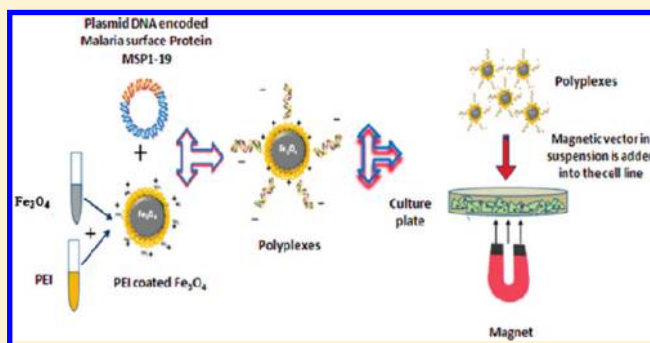
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 Supporting Information

ABSTRACT: Low efficiency is often observed in the delivery of DNA vaccines. The use of superparamagnetic nanoparticles (SPIONs) to deliver genes via magnetofection could improve transfection efficiency and target the vector to its desired locality. Here, magnetofection was used to enhance the delivery of a malaria DNA vaccine encoding *Plasmodium yoelii* merozoite surface protein MSP1₁₉ (VR1020-PyMSP1₁₉) that plays a critical role in *Plasmodium* immunity. The plasmid DNA (pDNA) containing membrane associated 19-kDa carboxyl-terminal fragment of merozoite surface protein 1 (PyMSP1₁₉) was conjugated with superparamagnetic nanoparticles coated with polyethyleneimine (PEI) polymer, with different molar ratio of PEI nitrogen to DNA phosphate. We reported the effects of SPIONs-PEI complexation pH values on the properties of the resulting particles, including their ability to condense DNA and the gene expression *in vitro*. By initially lowering the pH value of SPIONs-PEI complexes to 2.0, the size of the complexes decreased since PEI contained a large number of amino groups that became increasingly protonated under acidic condition, with the electrostatic repulsion inducing less aggregation. Further reaggregation was prevented when the pHs of the complexes were increased to 4.0 and 7.0, respectively, before DNA addition. SPIONs/PEI complexes at pH 4.0 showed better binding capability with PyMSP1₁₉ gene-containing pDNA than those at neutral pH, despite the negligible differences in the size and surface charge of the complexes. This study indicated that the ability to protect DNA molecules due to the structure of the polymer at acidic pH could help improve the transfection efficiency. The transfection efficiency of magnetic nanoparticle as carrier for malaria DNA vaccine *in vitro* into eukaryotic cells, as indicated via PyMSP1₁₉ expression, was significantly enhanced under the application of external magnetic field, while the cytotoxicity was comparable to the benchmark nonviral reagent (Lipofectamine 2000).



1. INTRODUCTION

Malaria is one of the most prevalent and devastating of all human parasitic diseases, exacting a heavy toll of deaths and illnesses particularly on children and pregnant women in the developing countries. According to the World Health Organization, between 300 and 500 million people contract malaria with 1.2 to 2.7 million deaths every year.^{1,2} Methods used to protect individuals living in endemic areas or to prevent the spread of the disease include prophylactic drugs, mosquito eradication, and insecticide-treated bed nets. The prophylactic drugs are relatively expensive for most people in the developing world, while treatment and control have become more difficult with the spread of drug-resistant strains of parasites and insecticide-resistant strains of mosquitoes carrying the parasites. Thus, there is an urgent need for affordable and effective vaccine for malaria that can promote the fight against this deadly disease. Vaccines are unquestionably one of the most cost-effective strategies that complement other strategies for control of the disease.³ DNA

vaccine is a newest means of antigen expression *in vivo* by generation of both humoral and cellular immune responses,⁴ whereas vaccination with traditional protein-based vaccines elicits only antibody-mediated (humoral) immune responses and often requires adjuvant injections. In addition, DNA vaccines are easy to produce and purify.^{5,6}

Only the asexual intraerythrocytic stage of the malaria life cycle is ultimately responsible for all the clinical pathologies associated with the disease, while the number of injected parasites is low at 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 (MSP1₁₉) that plays a crucial role in

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Plasmodium immunity, and is now a leading malaria vaccine candidate.^{7,8} The widely used model system of human malaria is mouse malaria *Plasmodium yoelii*, while the PyMSP1 gene is homologous to *Plasmodium falciparum* PfMSP1 gene. The PyMSP1 protein has a close structure resembling that of PfMSP1 with similar putative signal peptide and glycosylphosphatidylinositol (GPI) anchor. A high level of MSP1 gene expression is required for the development of the current DNA malaria vaccine candidates, to prevent secondary processing of a precursor molecule and hence the formation of MSP1₁₉, so that it can effectively inhibit merozoite invasion of the red blood cells or RBCs.⁹

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 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 nonviral vectors.¹⁰ 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.¹⁰ Thus, magnetofection is an appropriate tool for rapid and specific gene transfection with low dose *in vitro* and site-specific *in vivo* applications.^{11,12}

PEI polymer is known to form cationic complexes that interact nonspecifically with negatively charged DNA and enter the cell via endocytosis,^{13–16} with the ratio of PEI nitrogen to DNA phosphate (N/P) influencing the transfection efficiency and toxicity to transfected cells.¹⁷ 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 through nucleases,^{15,16} while the particles can also be magnetically directed to the specific target site *in vivo*.¹⁸ A main challenge in using nanoparticles is the formation of aggregates, since the transfection functions such as endocytosis rate, cytotoxicity, and velocity of cytoplasmic movement are determined by the size of the gene vector.¹⁹ Rejman et al.²⁰ 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.¹⁹

Recent studies have investigated the effects of size and surface charge of the magnetic vectors with PEI^{21,22} and the arrangement of SPIONs/PEI/DNA vectors on the efficiency of gene delivery.^{15,16} These studies have utilized genes encoding fluorescent proteins to elucidate the cellular entry mechanisms and the uptake of the vectors. Here, we investigate the magnetic targeting of a gene vector *in vitro* to identify approaches to increase the efficiency of delivery of a 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 effect on resulting particle size, surface charge, and ability to condense DNA. Acidification during vector preparation was shown previously to help reduce the extent of particle aggregation,²² an effect we also noted. In this work, we propose that an open polymeric structure at low pH condition is preferable for binding and protecting DNA during

transfection, resulting in significantly higher gene expression with comparable or less cytotoxicity than the leading nonviral reagent.

2. MATERIALS AND METHODS

2.1. Materials. Polyethylenimine with an average molecular weight of 25 kDa (PEI, branched) and trisodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) were purchased from Sigma Aldrich. RPMI 1640 medium (GIBCO), 0.05% trypsin-EDTA, L-glutamine, penicillin/streptomycin, fetal calf serum, and Lipofectamine 2000 (Gibco-BRL, Gaithersburg, MD) were supplied by Invitrogen (Carlsbad, CA). Fe(III) chloride ($FeCl_3 \cdot 6H_2O$) and Fe(II) chloride ($FeCl_2 \cdot 7H_2O$) were purchased from Ajax Finechem and Ajax Chemicals, respectively. Mammalian expression vector VR1020 (Vical Inc., San Diego, CA), plasmid VR1020-PyMSP1₁₉, 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-PyMSP1₁₉ plasmid was amplified in *Escherichia coli* (strain DH5 α) 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 disk Nd–Fe–B magnets (diameter 25 mm, height 5 mm) glued onto the bottom of a 6-well plate.

2.2. Methods. **2.2.1. Preparation of SPIONs/PEI/DNA Polyplexes.** The details of the synthesis and characterization of SPIONs can be found in the Supporting Information. The iron oxide suspension (0.1 mg mL^{-1}) was mixed with different amounts of 10% (w/v) PEI solution (25 kDa branched polyethylenimine), 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 by Wang et al.²² to disperse the aggregates of SPIONs/PEI complexes, each mixture was acidified to pH 2.0 using 0.5 mol L^{-1} 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^{-1} NaOH. The average hydrodynamic diameter and the zeta potential of the samples were determined using a Malvern Zetasizer ZS (Malvern Instruments Ltd., U.K.).

Plasmid VR1020-PyMSP1₁₉ was amplified using *E. Coli* DH5 α . A single colony of *E. Coli* harboring plasmid VR1020-PyMSP1₁₉ 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 $100 \mu\text{g mL}^{-1}$ of Kanamycin). The plasmid VR1020-PyMSP1₁₉ was purified from *E. Coli* cells using an endotoxin-free QIAGEN Mega plasmid purification kit (QIAGEN) according to the manufacturer's protocol. SPIONs/PEI complexes in the mass ratio (R) of 10 were mixed with plasmid DNA encoding VR1020-PyMSP1₁₉ gene at different N/P ratios (i.e., molar ratio of PEI nitrogen to DNA phosphate) using DNA concentration of $10 \mu\text{g mL}^{-1}$ in phosphate buffer (PBS, pH 7.4). The average hydrodynamic diameter, zeta potential, and stability of SPIONs/PEI/DNA polyplexes at PEI: SPIONs ratio of 10 and different N/P ratios were determined for SPIONs/PEI-A/DNA and SPIONs/PEI-N/DNA.

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-PyMSP1₁₉ plasmid were formed at N/P ratios of 0.5 to 30. In each case, the appropriate amount of SPIONs/PEI was mixed with $0.5 \mu\text{g}$ plasmid DNA in $20 \mu\text{L}$ PBS buffer. These solutions were incubated at 37°C for 30 min and mixed with $1 \mu\text{L}$ of the loading dye (bromophenol blue/

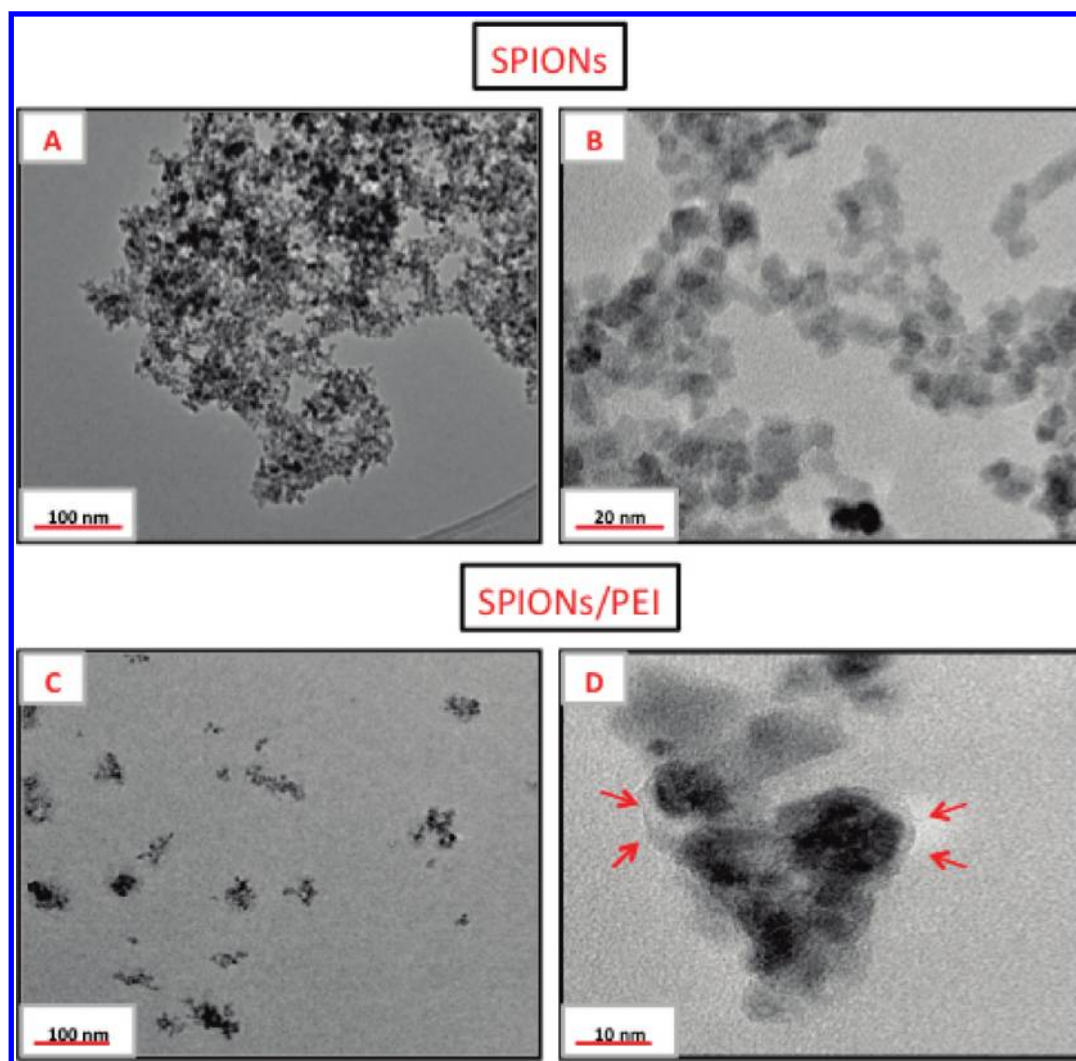


Figure 1. TEM images of (A,B) as-synthesized SPIONs and (C,D) SPIONs/PEI (ratio = 10) at pH 4 displaying better dispersion (arrows indicating layer of adsorbed PEI).

xylene cyanol) solution before loading into agarose gel (0.8% agarose in Tris-borate EDTA buffer containing $5 \mu\text{L}$ ethidium bromide). Electrophoresis was carried out at 60 V for 90 min. The DNA bands were visualized with a UV illuminator.

2.2.2. Cell Cultures and Treatments. COS-7 cell lines were cultivated in the complete RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM of L-glutamine, $100 \mu\text{g mL}^{-1}$ streptomycin, and $100 \mu\text{g mL}^{-1}$ 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 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 polyplexes were prepared with a fixed amount of plasmid DNA encoding VR1020-PyMSP1₁₉ gene ($1 \mu\text{g}$) 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 was used as a positive control according to the manufacturer's instructions. After 5 h, the serum-free media containing nanoparticles were removed from each well and 2 mL of fresh medium containing 20% serum was added and incubated for 48 h at 37°C under 5% CO_2 . Gene delivery mediated by SPIONs/PEI/DNA polyplexes in this work was

examined with or without the application of magnetic field during transfection process. After 48 h, COS-7 cells were washed with PBS buffer, and trypsin was added for collection of the cells to evaluate the transfection efficiency using the Western blot technique. The harvested COS-7 cells 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 traveled 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.

2.2.3. Evaluation of Cell Viability. Evaluation of the cytotoxicity was performed via MTT assay in COS-7 cells. The cytotoxicity of SPIONs/PEI/DNA polyplexes was evaluated in comparison with the lipofectamine–DNA complex and naked SPIONs solutions of 0.1, 0.5, and 1 mg mL^{-1} . Cells were seeded at a density of 2×10^4 cells/well on a 96-well

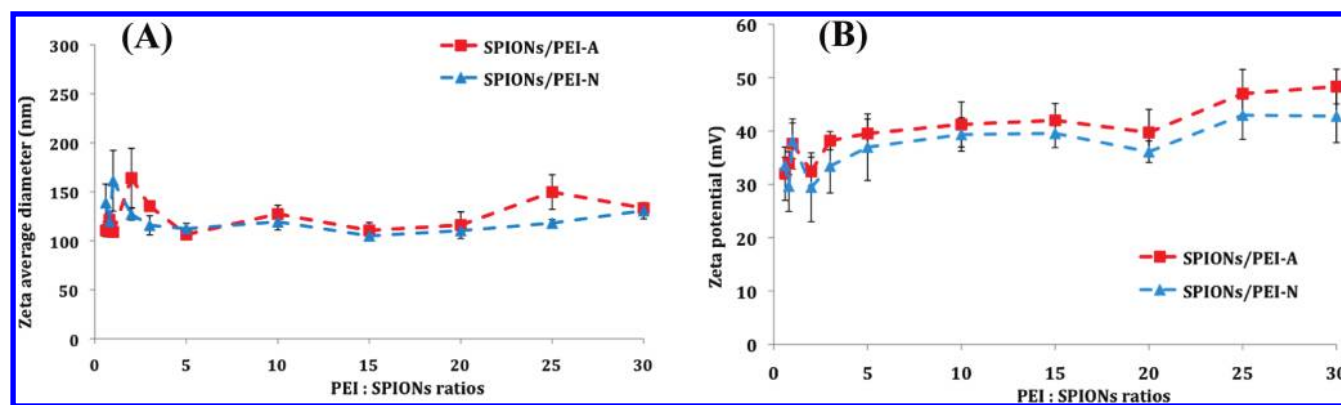


Figure 2. (A) Hydrodynamic diameter (nm) of SPIONs/PEI-A and SPIONs/PEI-N complexes. (B) Zeta potential (mV) of SPIONs/PEI-A and SPIONs/PEI-N complexes with different PEI:SPIONs mass ratios. All data measured at least in triplicate.

microtiter plate at 37 °C in 5% CO₂ atmosphere overnight. SPIONs/PEI/DNA polyplexes, DNA/Lipofectamine 2000 complex, and naked SPIONs solutions were added for a further 24 h incubation. The control well was a culture medium with no particles. 5 μ L of MTT dye solution at a concentration of 5 mg mL⁻¹ in phosphate buffer was added to each well plate. The plate was incubated for a further 4 h at 37 °C in 5% CO₂. After 4 h incubation, the medium was removed from each well and the cells were rinsed with phosphate buffer. 100 μ L of dimethyl sulfoxide (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 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) \times 100%.

3. RESULTS AND DISCUSSION

3.1. Characterization of SPIONs/PEI/DNA Polyplexes. The details of the properties of as-synthesized SPIONs can be found in the Supporting Information (Figure S1). The average diameter of the SPIONs was around 10 \pm 3 nm, while the measured hydrodynamic diameter indicating particle size in suspension was predominantly around 85 \pm 5 nm. The SPIONs were negatively charged with zeta potential of around -42 \pm 2 mV. Both SPIONs and SPIONs/PEI particles showed magnetization of >65 emu g⁻¹ under 15 kOe applied magnetic field at room temperature with 0.01 emu g⁻¹ remanance, indicating superparamagnetic behavior (Supporting Information, Figure S2), while X-ray diffraction pattern indicated the magnetite (Fe₃O₄) phase (Supporting Information, Figure S3).

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 \pm 2 mV) to positive (16 \pm 2 mV), while the particle size increased to approximately 555 \pm 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 positively 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.^{22,23} After reducing the pH to 2.0, the complexes were resuspended 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.^{23,24} As proposed by Vonzelewsky et al.,²³ 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 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 \pm 20 nm to around 100–150 nm at different mass ratios of PEI to SPIONs (Figure 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 deaggregation 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.²² The zeta potentials of SPIONs/PEI complexes were measured under both pH conditions at SPIONs concentration of 0.1 mg mL⁻¹. The SPIONs/PEI-A complexes showed slightly higher positive charges than SPIONs/PEI-N complexes (Figure 2B) because of the protonation of amine groups under 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 3A) were relatively similar to the size of complexes prior to DNA addition, indicating the stability of the vectors. At N/P \leq 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 higher N/P ratio, the charge became increasingly positive, providing enough repulsion to prevent further aggregation. The naked VR1020-PyMSP1₁₉ molecules with negative zeta potential of -59 mV were condensed onto the SPIONs/PEI to form complexes by increasing N/P ratio to reach a maximum zeta potential value of around +30 mV at N/P ratio of \geq 15 (Figure 3B). Despite the fact that SPIONs/PEI complexes at pH

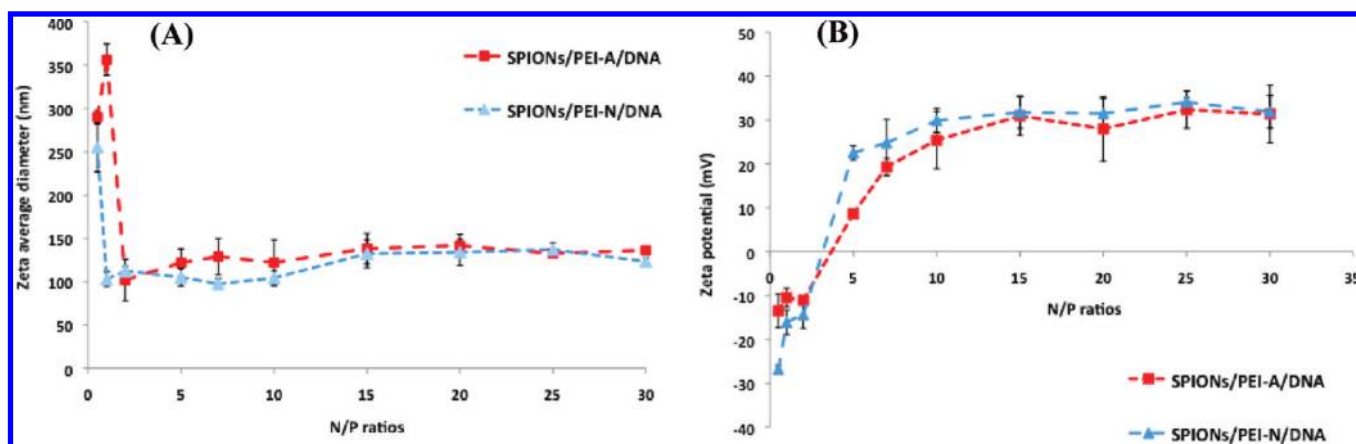


Figure 3. (A) Effects of N/P ratios on hydrodynamic diameter (nm) and (B) zeta potential (mV) of SPIONs/PEI/DNA polyplexes. All data measured at least in triplicate.

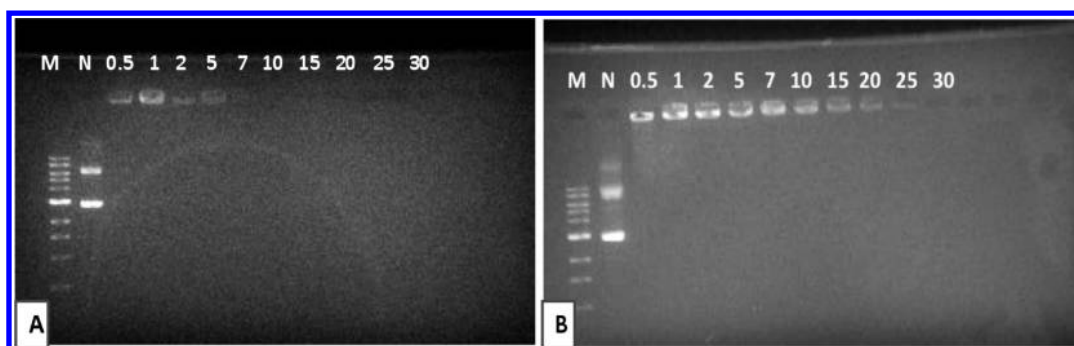


Figure 4. Effects of N/P ratios on the stability of (A) SPIONs/PEI-A/DNA; (B) SPIONs/PEI-N/DNA. Lane M: λ H/E molecular weight size marker. Lane N: plasmid VR1020-PyMSP1₁₉. Lanes 0.5–30 correspond to N/P ratios.

4.0 should have more capability to condense DNA than the complexes at pH 7.0 because of their greater positive charge, there was no significant difference between the zeta potentials of the polyplexes especially at $N/P \geq 15$ (Figure 3B). This implied that, at N/P ratio of ≥ 15 , the complexes have reached the saturated charge ratios of the cationic polymer to DNA.

3.2. DNA Binding Assay. The complexation degree of DNA with cationic PEI was confirmed via agarose gel electrophoresis (Figure 4). SPIONs/PEI-A/DNA polyplexes 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 4A). This indicated incomplete condensation of DNA with PEI at low N/P because of insufficient amount of PEI to condense DNA completely. Further increase of PEI at N/P ratio of >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 Scheme 1.²³ The expansion of the PEI molecular chain at acidic condition probably enabled the polyplexes 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.²⁵

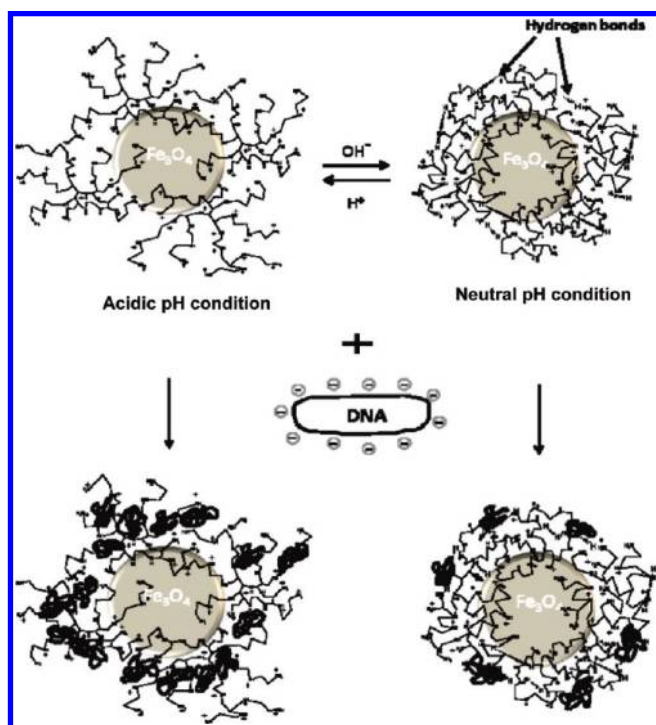
On the other hand, SPIONs/PEI-N/DNA showed relatively higher intensity of fluorescence for N/P ratios of <10 (Figure 4B). Decreasing fluorescence intensity was observed in the slots with increasing N/P ratios, although the intensity only completely disappeared at $N/P \geq 25$. Hence, for this system, 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 uncomplexed 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.²³

3.3. Transfection Efficiency. Western blot analysis of the expression of VR1020-PyMSP1₁₉ in COS7 cells line is shown in Figure 5. Numerical analysis of Western blots detection for VR1020-PyMSP1₁₉ (Figure 6) confirmed that the SPIONs/PEI-A/DNA polyplexes showed dramatically higher gene expression than SPIONs/PEI-N/DNA, especially with magnetofection. The SPIONs/PEI-A/DNA polyplexes at N/P ratios of 10 and 15 had the highest differences in transfection efficiency compared to SPIONs/PEI-N/DNA polyplexes at the same ratios. Gersting et al.²⁶ found that the highest amounts of DNA led to maximum transfection efficacy, while decreasing DNA concentrations led to rapid decrease of the polyplexes'

ability for gene transfection. In this case, the gene expressed at N/P ratios of >10 for SPIONs/PEI-A/DNA polyplexes was of similar magnitude as that 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 greatly improve the transfection efficiency.

Arsianti et al.^{15,16} 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 polyplexes, 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

Scheme 1. Schematic Demonstrating PEI Structures 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,²³ and the Possible Entrapment of DNA in the Respective Structures



DNA molecules within the branched structure of the polymers, because of protonation under acidic condition that expanded the polymeric network (Scheme 1). 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” effect, which protected the DNA from degradation.^{13,14} 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.

3.4. Effect of Magnetofection on Malaria Gene Expression PyMSP1₁₉ in COS-7 Cell Lines. The main concern for insufficient gene concentration on the target tissue for nonviral gene transfection is the insufficient accumulation of gene 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.²⁷ To investigate the impact of magnetofection in the malaria gene delivery, gene transfections of both SPIONs/PEI-A/DNA and SPIONs/PEI-N/DNA polyplexes 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 polyplexes showed noticeably higher transfection efficiency compared to the transfection without magnetic field (Figure 5A). Numerical analysis of Western blots detection for VR1020-PyMSP1₁₉ (Figure 7) confirmed the significant enhancement at almost all N/P ratios, possibly as the magnetic field drew the polyplexes onto the surface of the cells leading to an increase in their cellular uptake. The gene expression of SPIONs/PEI-A/DNA polyplexes without magnetic field was lower than the transfection with Lipofectamine reagent, while the reverse was true when magnetic field was applied. In addition, magnetofection also showed efficient gene delivery with low vector dose for plasmid DNA at shorter incubation time which greatly improved its dose–response profile.^{10,26} In this work, although a relatively low dose of DNA of about 1 μ g 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 polyplexes were similar (~ 150 nm). When N/P ratio was 1, low gene expressions with and without magnetofection were detected, possibly due to the lower DNA binding capability at

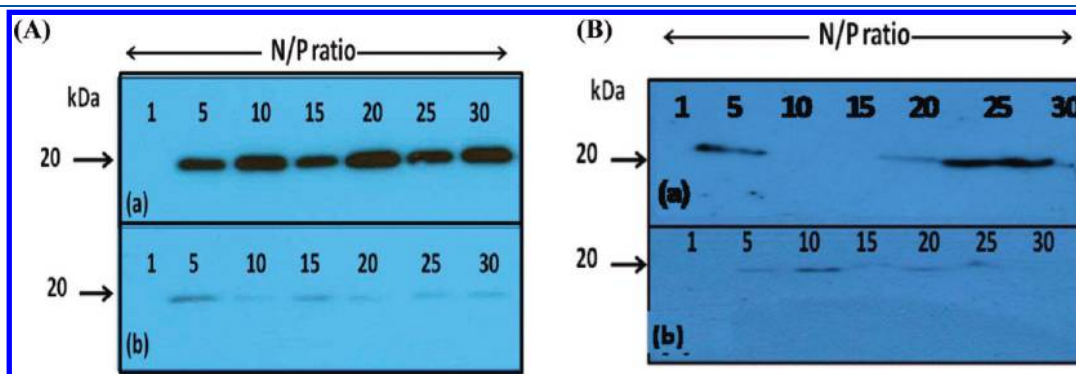


Figure 5. Western blot detection of (A) SPIONs/PEI-A/DNA; (B) SPIONs/PEI-N/DNA. Lanes 1, 5, 10, 15, 20, 25, and 30 correspond to different N/P ratios of SPION/PEI/DNA polyplexes: (a) with magnet; (b) without magnet.

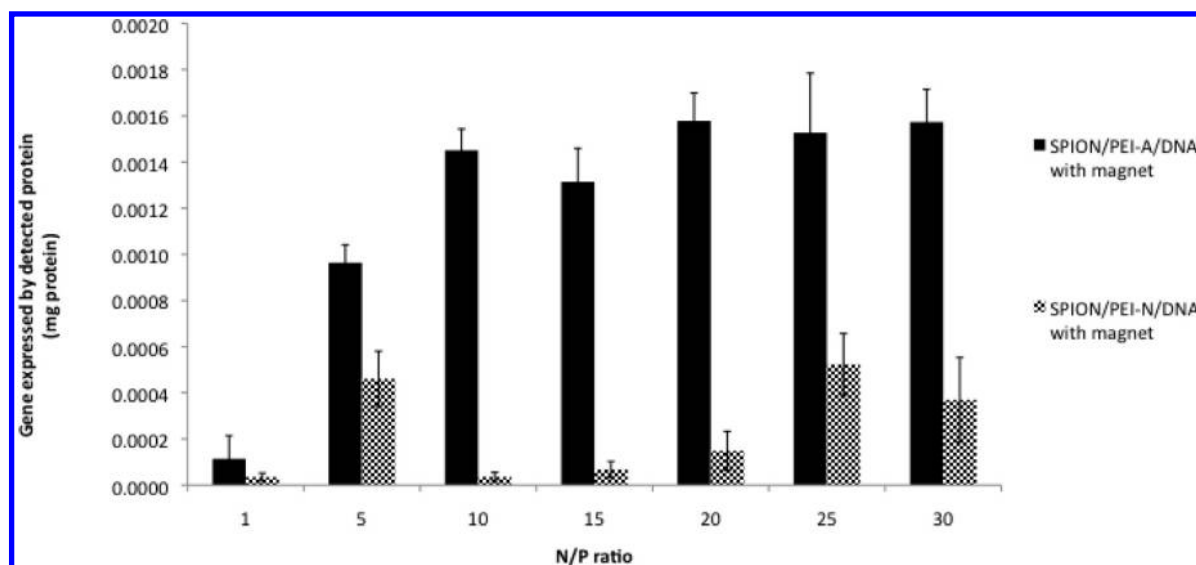


Figure 6. Densitometry results for PyMSP1₁₉ produced by SPIONs/PEI-A/DNA and SPIONs/PEI-N/DNA polyplexes with application of magnetic field during gene transfection process. Experiments were performed at least in triplicate.

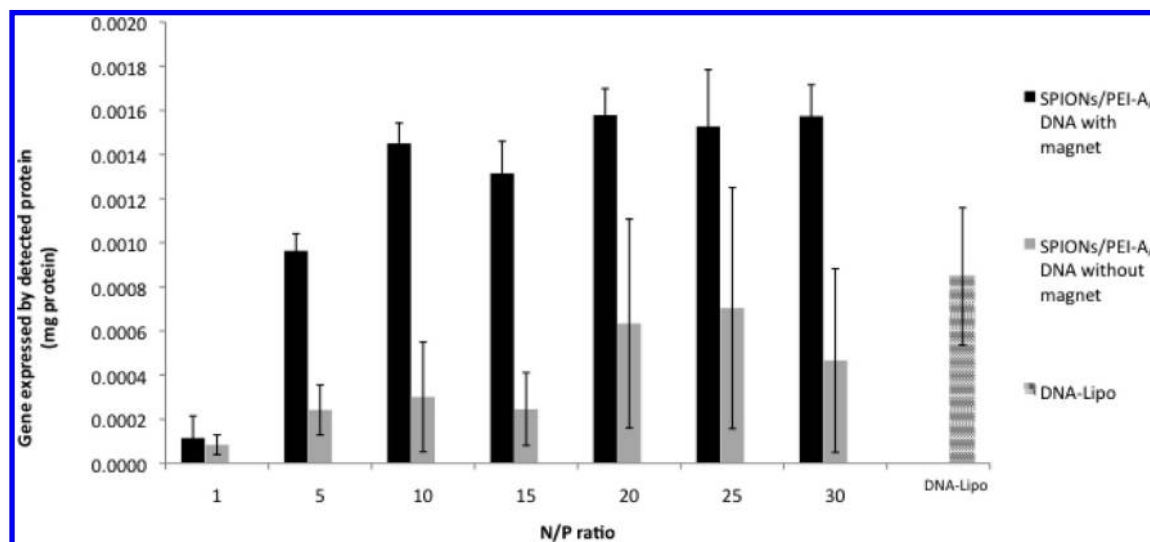


Figure 7. Densitometry results for PyMSP1₁₉ produced by SPIONs/PEI-A/DNA polyplexes with or without application of magnetic field during gene transfection process, and by Lipofectamine 2000 reagent. Experiments were performed at least in triplicate.

this ratio. However with 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 and the application of external magnetic field could play a combined role to achieve elevated gene expression.

Evidence of 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 8) indicated that COS-7 cells transfected with SPIONs/PEI/DNA polyplexes 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.

3.5. In Vitro Cytotoxicity Assay. *In vitro* cytotoxicity of SPIONs/PEI/DNA polyplexes 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 9) showed that there was no significant difference between SPIONs/PEI-A/DNA and SPIONs/PEI-N/DNA polyplexes, with more than 60% of the cells remaining viable. This indicated that the pH of complexation of these polyplexes induced no statistically significant difference in terms of effects on cell viability. The toxic effects of the polyplexes on cell viability were mainly associated with a strong net positive charge of the polyplexes due to presence of PEI polymer, leading to strong interactions of PEI with the cell surface causing disruption to the cellular plasma membrane.^{28–30} Figure 9

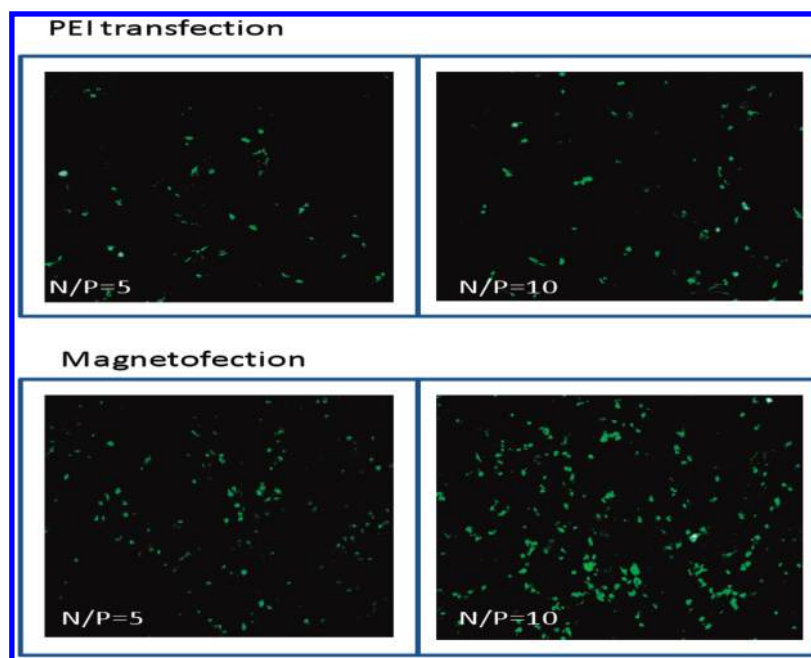


Figure 8. Expression of yellow fluorescent gene (YFP) in COS-7 cells. The upper row shows the effect of PEI polymer transfection alone; the lower row shows the effect of magnetofection with SPIONs/PEI-A/DNA polyplexes.

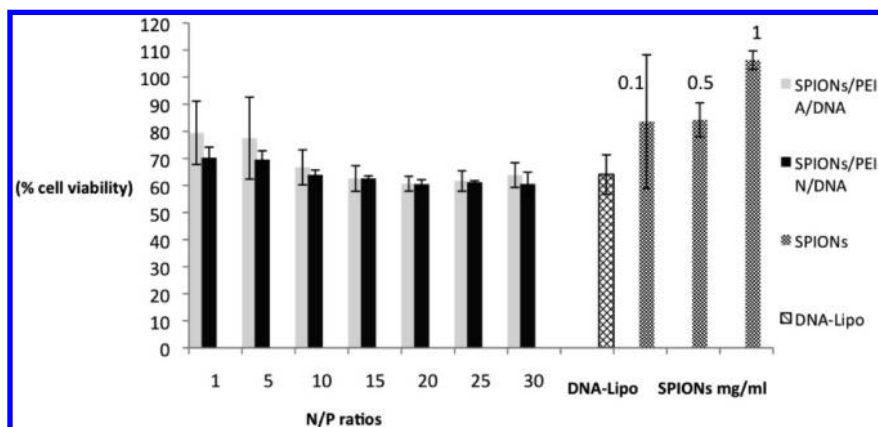


Figure 9. Cell viability from MTT assay results of treated COS-7 cells with different complexes. Experiments were performed at least in triplicate.

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 work.^{15,22} The presence of SPIONs seemed to reduce the toxicity of PEI in comparison to *in vitro* cell viability studies using similar PEI/DNA systems.^{31,32} Notably, more than 83% of cells remained alive when they were incubated with SPIONs solution, reaching 100% viability at 1 mg mL⁻¹ concentration. The protection against cell toxicity could be due to the presence of citrate groups.³³

Lipofectamine 2000 (cationic liposome) is the most effective nonviral reagent examined that yielded consistently high transfection rates accompanied by slightly higher toxicity.³⁴ The cytotoxicity of Lipofectamine is due to the four protonatable amines on its headgroup at physiological pH.³⁵ Here, we observed no obvious difference in cell toxicity between Lipofectamine 2000 and SPIONs/PEI/DNA polyplexes even for N/P

ratios >15. In contrast, SPIONs/PEI/DNA polyplexes showed dramatically higher gene expression than Lipofectamine, indicating that they were more effective gene transfection agents than Lipofectamine, particularly with magnetofection.

4. CONCLUSION

The use of magnetofection for the delivery of malaria DNA vaccine encoded merozoite surface protein MSP1₁₉ 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 gene delivery systems. SPIONs/PEI complexes produced at acidic conditions showed the best DNA binding and gene transfection efficiency compared to 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 application of external

magnetic field during the gene transfection process. In summary, the high transfection potential of these nanoparticles as demonstrated by the expression of MSP1₁₉ protein *in vitro* indicated the possibility of using these vectors with magnetofection technique as an efficient malaria gene MSP1₁₉ delivery carrier, with *in vivo* trials currently underway.

■ ASSOCIATED CONTENT

S Supporting Information. Synthesis and characterization of SPIONs, magnetization plots, and XRD data of SPIONs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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