Polymer-coated magnetic nanoparticles formulation enhanced blood-stage Malaria DNA vaccine delivery with the best PEI/Fe ratio

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ABSTRACT

Superparamagnetic nanoparticles SPIONs with specific sizes and surface characteristics can promote potent immune responses against different parasitic infection by enhancing antigen uptake of proper immune-stimulatory cell types. Magnetite (Fe₃O₄) nanoparticles have shown many potential bio-applications due to their biocompatibility and special characteristics. The use of Polyethylenimine (PEI) polymer-coated superparamagnetic nanoparticles (SPIONs) to deliver malaria genes via magnetofection shows promise in improving the efficiency of gene delivery in vitro application. Here, superparamagnetic nanoparticles with high magnetization value were stabilized with trisodium citrate and successfully coated with Polyethylenimine polymer (PEI) by chemically attaching branched PEI to the surface of iron oxides nanoparticles using two different concentrations of SPIONs in suspension and two different R % mass ratios (w: w) PEI:SPIONs. Plasmid DNA encoding Plasmodium yoelii merozoite surface protein VR1020-PyMSP119 and plasmid DNA encoding a green fluorescent protein (GFP) were added to the PEI-magnetic complexes with different N/P ratios. During particles characteristic experiments, a remarkably small particle hydrodynamic size and highly positive surface charge were shown when iron oxides suspension was 0.1 mg/ml. The magnetic vectors containing R%= 10% mass ratio PEI:SPIONs accompanied by applying permanent magnetic field yielded the highest gene transfection efficiency than others. This phenomenon was attributed to the more effective balanced distribution of the PEI molecules across the surface of the magnetic nanoparticles and to the low range of complex amounts due to the dose effect aiding to colloidal stable beads in the cell culture media.

1. INTRODUCTION

Despite continued efforts to control the malaria disease, it remains a major health problem in many regions of the world, with half of the world’s population at risk and an estimated 243 million cases led to nearly 863,000 deaths in 2008 and most of them were children under 5 years of age (Schwartz et al., 2012). Various strategies have been developed to prevent this burden, such as diagnosis and treatment, and vector control despite considerable efforts to develop a subunit vaccine that offers protective immunity. Because of spreading drug-resistant strains of malaria parasites, attempts to develop an efficient vaccine against this deadly disease have become increasingly demanding. DNA vaccines have emerged as a concrete and viable approach for controlling and preventing infection by malaria and many other pathogenic organisms (Donnelly et al., 1997). Of particular interest, the membrane-associated
19-kD COOH-terminal fragment of merozoite surface protein MSP1 molecule it being a prime candidate for inclusion in a blood-stage malaria vaccine which is generating particular interest as vaccine candidates (Diggs et al., 1993).

The main benefit of DNA vaccine is its ability to induce both humoral and cellular immune responses against antigens encoded by recombinant DNA (Donnelly et al., 1997). The conventional viral gene delivery system for DNA vaccine has made great progress in comparison to non-viral gene delivery system because of its enormously high transfection efficiency and capability 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 large-scale production difficulty with the high-industrialized cost (Patil et al., 2005, Luo and Saltzman, 2000). For these reasons, nanotechnology emerged as a promising strategy to improve the efficiency of gene delivery.

Nanotechnology describes the formation and utilization of materials, devices, and systems through the control of nanometer-sized materials and their application to physics, chemistry, biology, engineering, medicine, and other activities (Hamley, 2003). In particular, intensive efforts in gene delivery technique have been made last few years using magnetic particles to deliver gene of interest using superparamagnetic magnetic nanoparticles SPIONs in order to express its encoded protein in a suitable host or host cell.

Magnetically-guided gene targeting using magnetic nanoparticles (magnetofection) is an innovative, simple and highly effective method to transfected cultured cells or animal model through the use of a magnetic nanoparticles as a gene vector (Dobson, 2006, Al-Deen et al., 2013). Bare magnetic iron oxide nanoparticles have high chemical activity, and they are easily oxidized by contact with air (especially magnetite Fe3O4), which can be resulted in loss of magnetism and colloidal stability. Additionally, magnetic nanoparticles have Nanoparticles have more surface area to volume than larger particles; therefore, they own high surface energies. Hence, they have a tendency to aggregate to reduce their surface energies producing the larger hydrodynamic diameter. Different studies verified that the best particle size for drug/ gene delivery ranges between 10 to 100 nm since they have the longest blood circulation time in vivo due to the reduction of non-specific interactions with serum albumin and cellular components in the bloodstream (Colombo et al., 2012). For that reason, it is important to provide appropriate surface coating and developing some effective protection strategies to keep the stability of magnetic nanoparticles.

Positively charged magnetic nanoparticles allow them to assemble with negatively charged phosphate backbone of nucleic acids due to electrostatic interaction (Harush-Frenkel et al., 2007). Polyethylenimine PEI cationic polymer is one of the most widely used cationic polymers for nucleic acid delivery. However, high transfection efficiencies might be associated with toxicity. In addition, PEI offers a high positive charge density and exhibit a strong proton buffer capacity over a broad pH range (Godbey et al., 2000).

In the present study, we briefly describe the synthesis of polymer coated SPIONs as well as SPION/PEI/DNA complexes and the characterization of the complexes with two concentrations of SPIONs in suspension and two different PEI: SPIONs % mass ratio. PEI-coated SPIONs formulation was used to deliver PyMSP119 and GFP genes via in vitro studies.
The first stage of the study was done to determine the optimum concentrations of SPIONs in suspension during gene complex preparation. While, the second stage was to determine the best ratio \( R \% = (w/w) \% \) PEI: SPIONs mass ratio in terms of particle hydrodynamic size and surface charge using the Malvern Zetasizer Instrument. Polyethylenimine-(PEI) modified magnetic nanoparticles SPIONs were employed to transfer the reporter gene (PyMSP119 and GFP) into mammalian somatic cells (COS-7 cell line), and the gene delivery efficiency with the best R % and different N/P ratios was also examined. In addition, the cytotoxicity profiles of these formulations toward mammalian cells were also evaluated to assess the potential use in biomedical applications. The understanding gained from this work should help in the development of optimal DNA vaccine delivery protocols for diverse diseases where efficient gene delivery plays a protective role, including blood-stage malaria.

2. MATERIALS AND METHODS

2.1. Materials

Mammalian expression vector VR1020 PyMSP119 and DNA encoding a fluorescent protein VR1020-GFP plasmids were amplified in *Escherichia coli* (strain DH5α) and purified using an endotoxin-free Maxi-prep plasmid kit (Qiagen) according to the manufacturer’s instruction. RPMI (Roswell Park Memorial Institute) tissue culture medium and fetal calf serum FCS (GIBCO), 0.05% trypsin-EDTA, L-glutamine, penicillin/streptomycin from were purchased from (Gibco-BRL). DMSO and all reagents for electrophoresis were obtained from Life Technologies. Rabbit anti- mouse polyclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody were from Invitrogen Co.

Branched Polyethylenimine 25 kDa and trisodium citrate dehydrate were purchased from Sigma Aldrich. Fe (III) chloride and Fe (II) chloride were from Ajax Finechem and Ajax Chemicals, respectively. Array 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 consisted of circular disc Nd–Fe–B magnets (diameter (d) 25 mm, height (h) 5 mm) glued onto the bottom of a 6-well plate.

2.2. Methods

2.2.1 Synthesis of SPIONs/PEI complexes

Synthesis and characterization of the Polyethylenimine-coated magnetic nanoparticles is described in detail in our previous paper (Al-Deen et al., 2013, Al-Deen et al., 2014b). Two concentrations of iron oxide suspension were examined in this study (0.1 and 0.5 mg/ml). For PEI coating, the iron oxide suspension was mixed with different amounts of 10% (w/v) PEI solution in water (25 kDa branched Polyethylenimine PEI, the samples were referred to as R % = 1, 2, 3, 5, 10, 15, and 20 according to the PEI: SPIONs mass ratio. During the preparation of these different samples, the mixture was sonicated for 1 min. Following this, each mixture was suspended in RPMI media and neutralized to pH=7.4 by using 0.5 mol/L HCL and 0.5 mol/L NaOH solution and kept it for 30 mins. The size and surface charge of each PEI/SPIONs sample were determined in terms of the average hydrodynamic diameter and zeta potential, respectively, using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) and analyzed with the system software (Dispersion Technology Software, version 4.20, Malvern instrument Ltd.).
2.2.2 Characterizations

The phase purity and crystal structure of the samples were investigated using Philips PW1800 X-ray diffractometer with Cu Kα radiation (λ = 0.154 nm) in the 2θ range of 20-70˚ and 0.04 degree step size. Transmission electron microscope TEM and Scanning electron microscope SEM were also employed for the characterization of the samples. Magnetic measurements were carried out at room temperature (300 K) by means of vibrating sample magnetometer (VSM).

2.2.3 Preparation of plasmids DNA

VR1020-PyMSP119 and VR1020-GFP plasmids were amplified using Escherichia coli DH5α as it is explained in detail in our previous papers (Al-Deen et al., 2014b, Al-Deen et al., 2013). A single colony of E. coli harbouring plasmid VR1020- PyMSP119 and VR1020-GFP were 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-PyMSP119 and VR1020-GFP were purified from E. coli cells using an endotoxin-free QIAGEN Maxi plasmid purification kit (QIAGEN) according to the manufacturer’s protocol.

2.2.4 Preparation of SPIONs/PEI/DNA complexes

In this study, SPIONs/PEI/DNA nanoparticles were prepared by mixing of SPIONs/PEI complexes in the mass ratios of R=10 % with the VR1020-PyMSP119 and VR1020-GFP genes. Briefly, 1µg of DNA was mixed with different amounts of SPIONs coated PEI (SPIONs/PEI) in PBS buffer pH=7.4 according to N/P calculation [N/P= 10, 15, 20]. N/P ratio refers to the molecular ratio of amines (N; cationic groups) in PEI to phosphates (P; anionic groups) in nucleic acids. N/P ratio was calculated using the molar ratio of positive charge to negative charge (Colombo et al., 2012, Gupta and Gupta, 2005, Lacava et al., 1999). The average particle size, size distribution, and zeta potential, of the complexes were determined using Malvern zeta sizer.

2.2.5 Cell cultures and treatments

Cell Culture COS-7 cells were cultured in RPMI supplemented with 10% FBS with antibiotics(2 mM of L-glutamine, 100U ml-1 streptomycin and 100 Uml-1 of penicillin ) and maintained at 37°C in a 5%CO2 humidified atmosphere, as previously described (Al-Deen et al., 2014b).

One day prior to magnetofection, the cells were seeded in a 6-well plate at a density of 2×10^5 cells/ well in 1 mL of supplemented media. The cell culture media was replaced with fresh supplemented media ∼1 h prior to addition of gene vector. The degree of cell confluency in the culture at the time of transfection studies was ∼80% (degree of growth).

Magnetic Vector Transfection: The cells were loaded with 100 µL of the SPIONs/PEI/DNA vector using different N/P ratios (10, 15, and 30) of SPIONs: PEI with a fixed amount of plasmid DNA encoding VR1020-MSP119 and VR1020-GFP genes (1 µg/well) under the influence of magnetic field (magnetofection). For magnetofection, an array of 24 neodymium-iron-boron (Nd-Fe-B) permanent magnets (AMF Magnetics) in the format of a 6-well plate was used. In which a magnet was placed underneath the cells for 2 h to facilitate the localization of SPIONs to the adhered cells during in vitro gene delivery. After 2 h, the cells were rinsed at least thrice to remove the remaining vector with the supplemented cell culture media before finally incubated with fresh supplemented cell culture media for a further 48 h at at 37°C in a 5% CO2 humidified atmosphere. Following 48 h incubation, the cell culture media was
removed, and the cells were washed once with PBS buffer. The cells were detached using 500 μL of trypsin-EDTA and analysed for MSP119 expression using the Western blot technique. The cells subjected to magnetofection was also investigated by fluorescence microscopy to detect VR1020-GFP gene expression. All experiments were performed in triplicate.

2.2.6 Western blot technique

Western blotting for the harvested COS-7 cell pellets was carried out after SDS 10% polyacrylamide gel electrophoresis under reducing conditions and semi-dry blotting on polyvinylidene difluoride (PVDF) membranes. They were incubated sequentially with primary antibody. Later, the membrane was probed with antiserum and horseradish peroxidase-conjugated antibody, respectively, and then visualized by using Lumi-light western blotting substrate.

2.2.7 Evaluation of cell viability

The cytotoxicity is one of the important aspects should be considered for delivery and transfection material. The cytotoxicity of SPIONs/PEI/DNA complexes nanoparticles was evaluated using MTT assay. COS-7 cells at a density of 2×10⁴ cells/well on a 96-well microtiter plate at 37 °C in 5% CO₂ atmosphere prior to the experiment. At treatment time, the medium of cells was replaced with fresh medium supplemented with SPIONs/PEI/DNA complexes prepared previously using two different concentrations of iron oxide suspension (0.1 and 0.5 mg/ml) and plasmid DNA with the different N/P ratios (10, 20, 30), respectively. The control well was a culture medium with no particles. After 24-h incubation, the medium was removed from each well and the cells were washed with phosphate buffer. Consequently, MTT reagent (5 mg/ml) at a final concentration of 0.5 mg/ml was added to each well, and the cells were incubated at 37 °C for 4 h. 100 μL Dimethyl sulfoxide (DMSO) was added to each well in order to solubilize the blue crystal of formazan. The absorbance of different solutions was measured at wavelengths of 570 nm and 690 nm instantaneously using a microplate reader (Magellan, Tecan, Austria).

3. RESULTS AND DISCUSSION

3.1. Description of SPIONs and PEI coated magnetic nanoparticles

Initial physical characterization showed that the magnetite nanoparticles were magnetic, as we previously described in our previous paper (Al-Deen et al., 2011, Al-Deen et al., 2013)(Fig.2A), and showed saturation magnetization values of about of >64 emu/g at room temperature under 15 kOe applied magnetic field with 0.01 emu/g remanance indicating superparamagnetic behavior (Fig.2B). The crystallinity and phase purity of the synthesized SPIONs were determined by X-Ray diffraction (Fig.2C), 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). These results are in agreement with our previous results (Al-Deen et al., 2013, Al-Deen et al., 2011). As seen, all the characteristic peaks corresponding to Bragg diffractions of the crystal planes (220), (311), (222), (400), (422), (511) and (440) agree with standard JCPDS (joint committee on powder diffraction standards) pattern characteristic peaks of the magnetite cubic inverse spinel structure (19-0629) which indicates high phase purity of synthesized nanoparticles.

The morphologies of as-synthesized particles were characterized by TEM and SEM. Core-shell structure of the particles was clearly identified in the magnified image of a single magnetic particle (Fig.1A, C).
Fig. 1A, C showed that the primary nanoparticles have core diameters of below 10nm, which was the optimal particle size for targeting nano-carriers that illustrated in our previous studies (Al-Deen et al., 2011, Al-Deen et al., 2013, Nawwab Al-Deen et al., 2013). The PEI shell could effectively absorb DNA via electrostatic attraction. Specific surface area measurements give approximately 1-2 nm larger diameter representing the deposition of polymeric PEI thin coatings on the SPIONs surface, core phase consisted of Fe₃O₄ nanoparticle showed dark central image comparing with the shells of PEI polymer (Fig. 1B). The hydrodynamic diameter of bare SPIONs using Malvern instruments Zetasizer Nano ZS indicated the hydrodynamic size of 80 nm ± 5 nm in suspension (Table 1, 2).

3.2 Particle size and stability

Two concentrations of iron oxide suspension were examined in this study (0.1 and 0.5 mg/ml) for PEI coating, the iron oxide suspension was mixed with different amounts of 10% (w/v) branched PEI solution. The samples were referred to as R% = 1, 2, 3, 5, 10, 15, and 20 according to the PEI/Fe % mass ratio. The effects of SPIONs with two different concentrations on vector’s stability in the presence of PEI polymer were observed from the change in hydrodynamic size and surface charge. The hydrodynamic diameter of bare SPIONs indicated the size in suspension of 80 nm ± 5 nm (Table 1, 2). The particles were negatively charged with zeta potential of −40.1×2. When polymer was added to the magnetic nanoparticles in suspension with two different concentrations of magnetic solution, 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(due to the presence of amine groups) (Table 1,2). All vectors showed relatively small aggregates sizes when the concentrations of iron oxide suspension was 0.1mg/ml comparing to 0.5 mg/ml suspension. Increasing SPIONs amount to 0.5 mg/ml led to complexes large aggregation (Table 2), suggesting that SPIONs amount at 0.1 mg/ml concentration was sufficient to provide steric stabilization under physiological condition (Table .1). High particle concentrations generally increase non-specific particle– particle and particle–surface interactions, enhance field-induced particle aggregation, cause steric hindrance in particle concentration (van Reenen et al., 2014). Many studies showed that the high concentration of PEI-coated magnetic nanoparticles led to aggregation, which could be further induced by strong magnetic dipole–dipole attractions between the nanoparticles. In bioapplications, large nanoparticles aggregates lead to a blockage of small capillaries within body tissues (Gupta and Gupta, 2005). In our previous study, we showed that high concentration of SPIONs (0.5 mg/ml) in PEI/SPIONs complexes as malaria gene vector in vivo experiment after intravenous injection caused transient shock in Balb/c mice with most animals dying in the first 30 min (Nawwab Al-Deen et al., 2013).

Depending on the amount of added SPIONs, the particles surface charges showed highly
positive charge when SPIONs concentration was 0.1mg/ml at about +29 mV zeta potential especially when SPIONs/PEI % mass ratios were between R=3-15 (Table.1). In addition, SPIONs/PEI vectors with 0.1mg/ml of SPIONs concentration in suspension showed more stability in RPMI media than the SPIONs 0.5 mg/ml vectors, size distributions of these vectors in RPMI media were also presented (Table.2) (Fig.3B). The average sizes of SPIONs/PEI complexes were relatively large when SPIONs concentration was 0.5 mg/mg with a wide size distribution from 200 nm to several microns (in intensity) (Fig. 3B). On the other hand, using 0.1 mg/ml concentration of SPIONs in SPIONs/PEI complexes preparation resulted in reduction of the large aggregates population, to give an average size of <90 nm (in intensity) at PEI: SPIONs % mass ratio of R=10%-15% (Table.1)(Fig 3A). High positive charge is desired as many studies showed that PEI-coated SPIONs with high surface charge would increase the efficiency of gene delivery since the complexation and condensation of negatively charged DNA with high positively charged PEI offer good DNA protection from degradation by nucleases leading to optimal gene transfection results (Al-Deen et al., 2011). Between all vectors SPIONs/PEI complex with SPIONs concentration of 0.1 mg/ml and PEI: SPIONs mass ratio R% =10% showed smallest aggregation size and highly positive charge. Therefore, it is desirable to decrease particle amount to low concentrations at 0.1 mg/ml while maintaining low aggregation rates, high positive surface charge and high cellular capture rates in the bioapplications.

3.3 Transfection efficiency

In aqueous media, magnetite particles can easily form aggregates because of their hydrophobic surfaces with a large surface area to volume ratio. Due to hydrophobic interactions between the particles, they tend to aggregate forming large clusters. The particles aggregation not only affects their magnetic properties, but may also affect their internalization in their target cells due to the loss of the specific activity of an individual nanoparticle. Different studies proved that the magnetic properties and internalization of particles depend strongly on the size of the particles and the surrounding magnetic field strength (Wahajuddin, 2012). Such colloidal stability affects particle size, which should be sufficiently small (<100 nm) to increase cellular uptake and blood circulation time within body (Wiogo et al., 2010). Here we demonstrate that SPIONs/PEI/DNA vector with 0.1 mg/ml SPIONs concentration and PEI: SPIONs % mass ratio of R% =10% showed the best colloidal stability. For optimal gene delivery, we compared gene transfection efficiency of SPIONs/PEI/DNA complexes at PEI/Fe mass ratios R% of 10% between two
different iron oxide suspensions (0.1 mg/ml and 0.5 mg/ml) in the complexes.

![Intensity-based size distributions of SPIONs/PEI vectors incubated in RPMI media for 30 mins with pH of 7.0](image1)

Table 1. Size and Zeta potential of PEI/SPIONs complexes with 0.1 mg/ml concentration of iron oxide and different %PEI: Fe mass ratios incubated in RPMI media for 30 mins (error bars represented means ± for n= 3).

| % PEI/Fe | Size (nm) | Zeta Potential |
|----------|-----------|----------------|
| 0        | 80.1 ± 5  | -40.1 ±2       |
| 1        | 92.3 ± 2  | +25.1 ±3       |
| 2        | 87.7 ± 4  | +25.5 ±4       |
| 3        | 86.9 ± 3  | +29.8 ±4       |
| 5        | 86.3 ± 2  | +29.4 ±4       |
| 10       | 85.5 ± 5  | +29.8 ±3       |
| 15       | 86.5 ± 3  | +26.8 ±3       |
| 20       | 99.5 ± 2  | +22.4 ±6       |

Table 2. Size and Zeta potential of PEI/SPIONs complexes with 0.5 mg/ml concentration of iron oxide and different %PEI: Fe mass ratios incubated in RPMI media for 30 mins (error bars represented means ± for n= 3).

| % PEI/Fe | Size (nm) | Zeta Potential |
|----------|-----------|----------------|
| 0        | 80.1 ± 5  | -40.1 ±2       |
| 1        | 190 ± 4   | +23.1 ±4       |
| 2        | 167 ± 8   | +23.5 ±3       |
| 3        | 159 ± 2   | +24.8 ±4       |
| 5        | 156 ± 4   | +25.4 ±4       |
| 10       | 153 ± 5   | +26.8 ±3       |
| 15       | 151 ± 2   | +26.8 ±3       |
| 20       | 142 ± 2   | +22.4 ±6       |

Figure 3. Intensity-based size distributions of SPIONs/PEI vectors incubated in RPMI media for 30 mins with pH of 7.0: (A) SPIONs concentration of 0.1 mg/ml. (B) SPIONs concentration of 0.5 mg/ml. (1, 2, 3, 5, 10, 15, and 20) refer to PEI/SPIONs % mass ratio.
Among non-viral gene delivery agents, polyethylenimine (PEI) has emerged as a widely used agent for gene delivery. PEI has some advantages over other polycations in that it non-specifically interacts with DNA molecules accompanied with an intrinsic endosomolytic activity (Godbey et al., 1999). The influence of the N/P ratio of PEI amine groups to DNA phosphate one might be an important factor affecting the cytotoxicity and transfection efficiency. To address this idea, we examined the effects of N/P ratio in the malaria gene delivery. Gene transfections efficiency of SPIONs/PEI/DNA complexes prepared by two different concentration of iron oxide suspensions (0.1 mg/ml and 0.5 mg/ml) and R% of 10% were tested using three different N/P ratios (Colombo et al., 2012, Lacava et al., 1999).

DNA encoding a fluorescent protein (GFP) mixed at 1:1 ratio with the VR1020-PyMSP119 expressing vector was used to allow reading out the efficiency of DNA uptake. Western blot analysis using anti-PyMSP119 antibodies was further performed on transfected COS-7 cells pellets to identify the recombinant proteins produced by PyMSP119 plasmid in COS-7.

Western blot analysis of COS-7 cells transfected with VR1020-PyMSP119 plasmid in SPIONs/PEI/DNA complexes can be seen in Fig.4. COS-7 cells transfected by SPIONs/PEI/DNA complexes prepared using 0.1 mg/ml concentration of iron oxide in suspension and N/P ratio of 10 under external magnetic field showed significantly the highest gene transfection efficiency compared to the transfection with the other gene complexes. This result might belong to stability and narrow size distribution of the gene complexes (Table 1), in agreement with our previous study (Al-Deen et al., 2013). Using iron oxide suspensions at 0.1 mg/ml concentration in SPIONs/PEI/DNA complexes preparation with N/P of 10, in particular, played a critical role in producing vectors with narrow size distributions and high stability in RPMI media via electrostatic and steric effects (Fig.3). Interestingly, at low dose (0.1 mg/ml) of magnetic nanoparticles in SPIONs/PEI/DNA vector demonstrated transfection efficiency higher than high dose (0.5 mg/ml) (Fig.4).

The previous result is consistent with the results from fluorescent microscopy (Fig. 5). The observation of fluorescent microscopy indicate that the COS-7 cells transfected with SPIONs/PEI/DNA complexes prepared using SPIONs suspension of 0.1 mg/ml and N/P ratios of 10 have significantly the highest fluorescence expression of plasmid VR1020-GFP under external magnetic field compared to SPIONs/PEI/DNA complexes prepared by 0.5 mg/ml SPIONs suspensions and different N/P ratios. The efficiency of gene transfection increased in the low range of complex amounts due to the dose effect. As the amounts of the complexes increased, extra complexes could lead to aggregation and increase the size of the magnetic gene complexes that lead to decrease the cellular endocytosis in vitro application. Moreover, low cell viability due to high cytotoxicity caused by the excess PEI in the nanoparticles was also the reason of decreased gene transfection efficiency (Fig 6). In vivo application the predominant hypothesis in the same manner is that the aggregation of SPIONs/PEI/DNA complexes could cause a physical blockage of lung capillaries and airflow obstruction resulting in death of the animals, in agreement with our earlier in vivo study (Nawwab Al-Deen et al., 2013).
Figure 4. Densitometry results for PyMSP119 produced by SPION/PEI/DNA complexes with % mass ratio PEI:SPION R=10 and two different iron oxide in suspensions (0.1 mg/ml and 0.5 mg /ml) and three different N/P ratios of (10, 20, 30) with application of magnetic field during gene transfection process.

Figure 5. Expression of yellow fluorescent gene (GFP) in COS-7 cells. The upper row shows the effect of 0.1mg/ml SPIONs concentration in SPIONs/PEI/DNA complexes on gene transfection; the lower row shows the effect of 0.5mg/ml SPIONs concentration in SPIONs/PEI/DNA complexes on gene transfection.

3.4 Evaluation of cell viability

In general, surface characteristics of magnetic particles play an important role in magnetic nanoparticle cytotoxicity and blood circulation time. The results from this study shows that almost all cells stayed alive when they were incubated with bare SPIONs solution with almost 97% cell showed viability when the concentration of magnetic suspension was 0.1 mg/ml which is in agreement with our early result (Al-Deen et al., 2011) (Fig.6). This result could be due to the presence of negatively charged citrate groups on the surface of particles (Lacava et al., 1999). Many previous studies showed that magnetic nanoparticles with cationic surfaces are being more toxic than anionic ones, and neutral surfaces being the most biocompatible (Goodman et al., 2004, Al-Deen et al., 2014a).

Generally, the cell viability decreased drastically to around 50-80% when it treated with SPIONs/PEI/DNA complexe (Fig.6).

With the exception to the other complexes, the SPIONs/PEI/DNA complex with N/P ratio of 10 and 0.1 mg/ml concentration of SPIONs suspension showed higher cell viability close to the cell viability of untreated cells (control).

This result is due to the low concentration of PEI in the SPIONs/PEI/DNA complexes at N/P ratio of 10 that reduced the cell toxicity. The toxic effect of the magnetic gene complexes is mainly associated with the surface interaction of nanoparticles with cell. Strong net positive charge of the complexes due to existence of PEI polymer, lead to strong interactions of PEI with the cell surface causing disruption of the cellular plasma membrane (Florea et al., 2002).

The efficiency of gene transfection with SPIONs/PEI/DNA complex at (N/P10, 0.1 mg/ml SPIONs) increased in the low range of complex amounts due to the dose effect and low cytotoxicity.

The high gene transfection efficiency induced by SPIONs/PEI/DNA gene complexes prepared by (0.1 mg/ml) concentration of SPIONs suspensions and 10% mass ratio of PEI: SPIONs accompanied with low N/P ratio of 10 under magnetic field offer a potential way forward to explore more progress in DNA vaccine vector. Altogether, our study suggests that the use of magnetic gene complexes with
low magnetic nanoparticle dose considerably improve the gene transfection efficiency and minimize the cell toxicity.

Figure.6. Cell viability from MTT assay results of treated COS-7 cells with different complexes. Experiments were performed at least in triplicate. N/P (10, 20, 30) represented PEI nitrogen N/P DNA phosphate ratios, while (0.1, 0.5) mg/ml represented SPIONs suspension concentration alone or in the SPIONs/PEI/DNA complexes.

4. CONCLUSIONS

Overall, our findings suggested that SPIONs/PEI/DNA nanoparticle size, surface charge, gene transfection, and even cell toxicity were affected by the SPIONs suspension concentration during nanoparticles preparation. Moreover, % mass ratio of PEI to SPION in the complexes also significantly affects on particles size and surface charge.

REFERENCES

AL-DEEN, F. N., HO, J., SELOMULYA, C., MA, C. & COPPEL, R. 2011. Superparamagnetic Nanoparticles for Effective Delivery of Malaria DNA Vaccine. Langmuir, 27, 3703-3712.

AL-DEEN, F. N., SELOMULYA, C., KONG, Y. Y., XIANG, S., MA, C., COPPEL, R. & PLEBANSKI, M. 2014a. Design of magnetic polyplexes taken up efficiently by dendritic cell for enhanced DNA vaccine delivery. Gene therapy, 21, 212-218.

AL-DEEN, F. N., SELOMULYA, C., MA, C. & COPPEL, R. L. 2014b. Superparamagnetic Nanoparticle Delivery of DNA Vaccine. In: RINALDI, M., FIORETTI, D. & IURESCIA, S. (eds.) DNA Vaccines: Methods and Protocols. New York, NY: Springer New York.

AL-DEEN, F. N., SELOMULYA, C. & WILLIAMS, T. 2013. On designing stable magnetic vectors as carriers for malaria DNA vaccine. Colloids and Surfaces B: Biointerfaces, 102, 492-503.

COLOMBO, M., CARREGAL-ROMERO, S., CASULA, M. F., GUTIERREZ, L., MORALES, M. P., BOHM, I. B., HEVERHAGEN, J. T., PROSPERI, D. & PARAK, W. J. 2012. Biological applications of magnetic nanoparticles. Chemical Society Reviews, 41, 4306-4334.

DIGGS, C. L., BALLOU, W. R. & MILLER, L. H. 1993. The major merozoite surface protein as a malaria vaccine target Parasitology Today, 9, 300-302.

DOBSON, J. 2006. Gene therapy progress and prospects: magnetic nanoparticle-based gene delivery. Gene therapy, 13, 283-287.

DONNELLY, J. J., ULMER, J. B., SHIVER, J. W. & LIU, M. A. 1997. DNA vaccines. Annual Review of Immunology, 15, 617-648.

FLOREA, B. I., MEANEY, C., JUNGINGER, H. E. & BORCHARD, G. 2002. Transfection efficiency and toxicity of polyethylenimine in differentiated Calu-3 and nondifferentiated COS-1 cell cultures. Aps Pharm sci, 4.

GODBHEY, W., WU, K. K. & MIKOS, A. G. 1999. Poly (ethyleneimine) and its role in gene delivery. Journal of Controlled Release, 60, 149-160.

GODBHEY, W. T., BARRY, M. A., SAGGAU, P., WU, K. K. & MIKOS, A. G. 2000. Poly(ethyleneimine)-mediated transfection: A new paradigm for gene delivery. Journal of Biomedical Materials Research, 51, 321-328.

GOODMAN, C. M., MCCUSKER, C. D., YILMAZ, T. & ROTELLO, V. M. 2004. Toxicity of gold nanoparticles functionalized with cationic and anionic side chains. Bioconjugate chemistry, 15, 897-900.

GUPTA, A. K. & GUPTA, M. 2005. Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. Biomaterials, 26, 3995-4021.
HAMLEY, I. W. 2003. Nanotechnology with soft materials. Angewandte Chemie-International Edition, 42, 1692-1712.

HARUSH-FRENKEL, O., DEBOTTON, N., BENITA, S. & ALTSCHULER, Y. 2007. Targeting of nanoparticles to the clathrin-mediated endocytic pathway. Biochemical and biophysical research communications, 353, 26-32.

LACAVA, Z. G. M., AZEVEDO, R. B., MARTINS, E. V., LACAVA, L. M., FREITAS, M. L. L., GARCIA, V. A. P., REBULA, C. A., LEMOS, A. P. C., SOUSA, M. H., TOURINHO, F. A., DA SILVA, M. F. & MORAIS, P. C. 1999. Biological effects of magnetic fluids: toxicity studies. Journal of Magnetism and Magnetic Materials, 201, 431-434.

Luo, D. & Saltzman, W. M. 2000. Enhancement of transfection by physical concentration of DNA at the cell surface. Nature Biotechnology, 18, 893-895.

NAWWAB AL-DEEN, F., MA, C., XIANG, S. D., SELOMULYA, C., PLEBANSKI, M. & COPPEL, R. L. 2013. On the efficacy of malaria DNA vaccination with magnetic gene vectors. Journal of Controlled Release, 168, 10-17.

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

SCHWARTZ, L., BROWN, G. V., GENTON, B. & MOORTHY, V. S. 2012. A review of malaria vaccine clinical projects based on the WHO rainbow table. Malaria Journal, 11.

VAN REENEN, A., DE JONG, A. M., DEN TOONDER, J. M. & PRINS, M. W. 2014. Integrated lab-on-chip biosensing systems based on magnetic particle actuation—a comprehensive review. Lab on a Chip, 14, 1966-1986.

VOIGT, K., IZSVAK, Z. & IVICS, Z. 2008. Targeted gene insertion for molecular medicine. Journal of Molecular Medicine-Jmm, 86, 1205-1219.

WAHAJUDDIN, S. A. 2012. Superparamagnetic iron oxide nanoparticles: magnetic nanoplastforms as drug carriers. International journal of nanomedicine, 7, 3445.

WIOGO, H. T., LIM, M., BULMUS, V., YUN, J. & AMAL, R. 2010. Stabilization of magnetic iron oxide nanoparticles in biological media by fetal bovine serum (FBS). Langmuir, 27, 843-850.