International Journal of Pharmaceutics 358 (2008) 263–270

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Cationic lipid-coated magnetic nanoparticles associated with transferrin for gene delivery

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ARTICLE INFO

Article history:
Received 8 April 2007
Received in revised form 18 February 2008
Accepted 18 February 2008
Available online 4 March 2008

Keywords:
Gene delivery
Magnetic nanoparticles
Transferrin
Cationic lipid
Magnetofection

ABSTRACT

Cationic lipid-coated magnetic nanoparticles (MPs) associated with transferrin were evaluated as gene transfer vectors in the presence of a static magnetic field. MPs were prepared by chemical precipitation and were surface-coated with cationic lipids, composed of DDAB/soy PC (60:40 mole/mole). These cationic MPs were then combined with polyethylenimine (PEI) condensed plasmid DNA, followed by transferrin. The resulting magnetic electrostatic complexes retained relatively compact particle size and showed complete DNA condensation. Their transfection activity in the presence of a static magnetic field was evaluated by luciferase and green fluorescent protein (GFP) reporter genes. The magnetic complexes exhibited up to 300-fold higher transfection activity compared to commonly used cationic liposomes or cationic polymer complexes, based on luciferase assay. The enhancement in transfection activity was maximized when the cells were exposed to the vectors for a relatively short period of time (15 min), or were treated in media containing 10% serum. Incorporation of transferrin further improved transfection efficiency of the cationic MPs. However, when cells were incubated for 4 h in serum-free media, magnetic and non-magnetic vectors showed similar transfection efficiencies. In conclusion, transferrin-associated cationic MPs are excellent gene transfer vectors that can mediate very rapid and efficient gene transfer *in vitro* in the presence of a magnetic field.

1. Introduction

Gene therapy is an emerging therapeutic modality for the treatment of genetic and infectious diseases. However, challenges in developing safe and efficacious gene transfer vectors have limited its clinical application. Synthetic non-viral gene transfer vectors are being developed as alternatives to viral vectors (Glover et al., 2005). Among non-viral gene delivery systems under development are electrostatic complexes derived from cationic liposomes (lipoplexes) (Felgner et al., 1987; Gao and Huang, 1991), cationic polymers (polyplexes) (Boussif et al., 1995; Pack et al., 2005), and lipid–polymer–DNA (LPD) ternary complexes (lipopolyplexes) (Gao and Huang, 1996; Guo et al., 2002; Lee and Huang, 1996). These delivery systems have shown relatively efficient transfection activities *in vitro*. However, low *in vivo* efficiency remains a major drawback of these types of vectors, despite efforts to improve their design (Luo and Saltzman, 2000).

Physical methods have been investigated to facilitate gene transfer. These include projectile delivery (Yang et al., 1990), hydrodynamic delivery (Andrianaivo et al., 2004), electroporation (Mir et al., 1999), sonoporation (Bao et al., 1997), and magnetic force-mediated delivery (Gersting et al., 2004; Huth et al., 2004; Mah et al., 2002; Scherer et al., 2002). Magnetic force-mediated gene delivery, also termed "magnetofection", is related to the concept of magnetically targeted drug delivery (Meyers et al., 1963) and involves the application of a static magnetic field that guides magnetic particle (MP)-associated gene vectors to accumulate on the cell surface (Dobson, 2006; Mah et al., 2002; Scherer et al., 2002). A number of strategies have been explored based on magnetofection. Mah et al. (2002) demonstrated efficient gene delivery both *in vitro* and *in vivo*

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doi:10.1016/j.ijpharm.2008.02.020

in by using magnetic microsphere-bound adeno-associated virus (AAV). Scherer and coworkers have developed polyethylenimine (PEI)-coated MPs, and demonstrated efficient magnetofection in a variety of cell lines, as well as in excised airway epithelium (Huth et al., 2004; Scherer et al., 2002; Schillinger et al., 2005).

In the present study, novel vector formulations, based on cationic lipid-coated MPs, associated with transferrin, were synthesized and evaluated. The roles of lipid-to-DNA ratio, incorporation of transferrin, cellular incubation time, presence of serum in the incubation medium, and presence of a magnetic field were determined.

2. Materials and methods

2.1. Materials

Cholesterol (Chol), dimethyldioctadecylammonium bromide (DDAB), soy phosphatidylcholine (PC), polyethylenimine (PEI) (branched, average MW 25 kDa), and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma–Aldrich Co. (St. Louis, MO). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemical Co. (Rockford, IL). Cell lysis buffer and luciferase assay kit were purchased from Promega (Madison, WI). Culture media, fetal bovine serum (FBS), and antibiotics were purchased from Gibco-BRL (Grand Island, NY). Lipofectin™ and PureLink™ HiPure Plasmid Megaprep kit were purchased from Invitrogen (Carlsbad, CA). Permanent Nd–Fe–B cylinder magnets (1/4 in. diameter × 1/2 in. thick) were purchased from K&J Magnetics (Jamison, PA). All other chemicals were of reagent grade. Particle size was determined by dynamic light scattering (DLS) using Nicomp Particle Sizer Model 370. Zeta potential (ξ) of particles was measured on a Brookhaven 90plus Particle Analyzer (Holtsville, NY).

2.2. Cell culture

KB cell line, which has been identified as being derived from human cervical cancer HeLa cell line (Masters et al., 2001), was obtained as a gift from Dr. Philip Low (Department of Chemistry, Purdue University, West Lafayette, IN), and were cultured in RPMI-1640 medium (without folic acid) supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% FBS. Human EGFR cDNA transfected $F98_{EGFR}$ glioma cell line was kindly provided by Dr. Rolf F. Barth (Department of Pathology, The Ohio State University, Columbus, OH) (Wu et al., 2004), and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented 10% FBS. All cell lines were cultured continuously as a monolayer in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Plasmid DNA preparation

Plasmid *pc*DNA3-CMV-*luc* (*p*DNA) encoding firefly luciferase gene and containing a human cytomegalovirus (CMV) promoter–enhancer was used in this study. The pDNA was propagated in *E. coli* and purified using a Megaprep kit from Invitrogen. Plasmid DNA purity and concentration were confirmed by measuring absorbance at 260 and 280 nm on a Shimadzu UV-160U spectrophotometer and by agarose gel electrophoresis (Chiu et al., 2004; Gosselin et al., 2001).

2.4. Synthesis of cationic lipid-coated MPs (Fig. 1)

MPs were prepared by chemical precipitation, as described previously (Shen et al., 1999). Briefly, 50 mg of $FeCl_2 \cdot 4H_2O$ and 135 mg of $FeCl_3 \cdot 6H_2O$ (molar ratio 2:1) were dissolved in 40 mL degassed water under N_2 with vigorous stirring. Magnetite precipitation was then generated by addition of 2 mL 28% (w/w) NH_4OH and 200 mg oleic acid in 5 mL of acetone at 80 °C for 30 min. After cooling to room temperature, the MPs were isolated by magnetic decantation, followed by acetone washing to remove unbound oleic acid. The resulting oleic acid coated MPs were dried under vacuum.

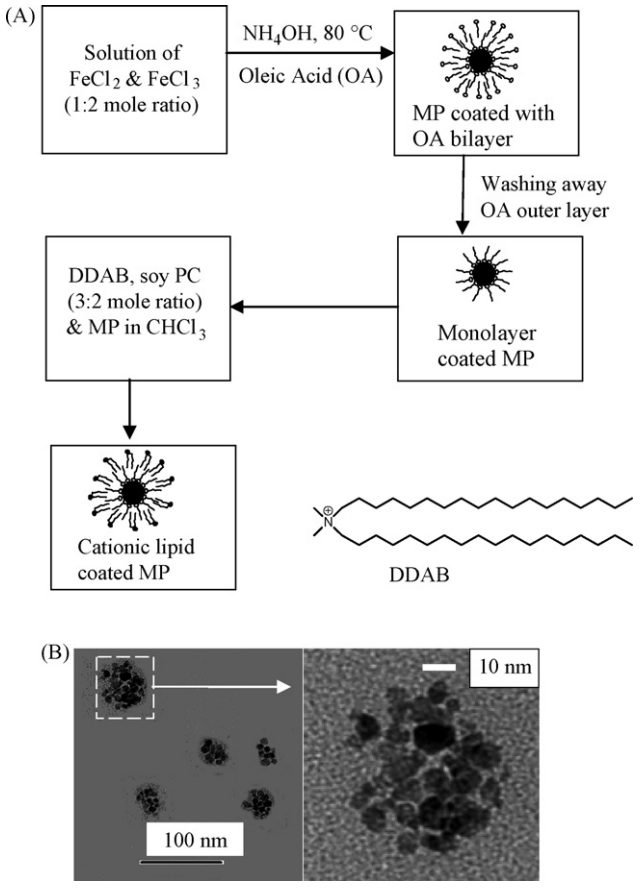

Fig. 1. Synthesis and characterization of MPs. (A) Scheme for preparation of cationic lipid (DDAB)-coated MPs. (B) Transmission electron micrographs (TEM) of DDAB/PC-coated MPs. Picture on the right panel had a higher magnification.

Coating of MPs with cationic lipids was then carried out. Briefly, dry MPs (5 mg) were mixed with lipids (100 mg) consisting of DDAB/soy PC (60:40 mole/mole) dissolved in $CHCl_3$. The suspension was dried into a thin film in a round-bottom flask on a rotary evaporator, and then further dried for 2 h under vacuum. The lipids mixture was hydrated in 2.5 mL PBS (pH 7.4), and sonicated by a probe-type sonicator (Sonics VCX-130-PB, Newtown, CT) for 15 min. Excess DDAB/soy PC liposomes were removed by magnetic decantation. Size and morphology of the particles were characterized by transmission electron microscopy (TEM) and DLS. Zeta potential (ξ) of the cationic lipid-coated MPs was measured on a Brookhaven 90plus Particle Analyzer (Holtsville, NY). The magnetization of the MPs was measured on a SQUID magnetometer (Quantum Design MPMS-5). Briefly, samples were placed in a small plastic tube, and inserted into the SQUID chamber. The magnetization measurements were taken at zero field cooled (ZFC) and field cooled (FC) at a constant field for the temperature range of 5–300 K. The magnetization at different applied magnetic fields ranging from −60,000 to 60,000 H (Oe) was also measured at both 5 and 300 K.

Cationic liposomes, consisting of DDAB/soy PC (60:40 mole/mole) without MPs, were also prepared by the same thin film hydration/sonication method described above.

2.5. Preparation of transferrin-associated MPs carrying plasmid DNA

Transferrin-associated MPs were prepared by complexation of PEI (25 kDa)-condensed pDNA with cationic lipid-coated MPs, followed by addition of transferrin. One μg pDNA was mixed with varying amounts of PEI (25 kDa) in 30 μL HEPES buffer (20 mM, pH 7.4) and incubated for 15 min to yield PEI/pDNA complexes with PEI nitrogen to pDNA phosphate (N/P) ratios of 1, 2 and 3. Then, 0.9, 1.2, or 1.5 μL of MPs (10 μM in DDAB concentration) were added to the PEI/pDNA complexes (containing 1 μg pDNA) to yield MP/(PEI/pDNA) complexes with positive (from DDAB and PEI)/negative (from pDNA) charge (+/−) ratios of 3, 4, and 5. HEPES buffer (20 mM, pH 7.4) was then added to the resulting complexes to yield a final volume of 150 μL. For preparation of transferrin-associated particles, iron-saturated transferrin (2 mg/mL) was added to the complexes, followed by gentle mixing and incubation at room temperature for 15 min. The MPs were characterized for size distribution by DLS and for DNA condensation by agarose gel electrophoresis. The structure of the particles was studied by atomic force microscope (AFM) (Allen et al., 1997; Dunlap et al., 1997). PEI/pDNA complexes formed at varying N/P ratios were visualized on a Nanoscope III Scanning Force Microscope (Digital Instruments Inc., Santa Barbara, CA) in the dry tapping mode.

2.6. In vitro transfection assays

Plasmid DNA, encoding luciferase or green fluorescence protein (GFP) reporter gene, was used in the transfection studies. Approximately 10^5 cells/well were seeded on 24-well plates 24 h prior to transfection in growth media containing 10% FBS. At a confluence level of 70–80%, cells were washed twice with PBS, and incubated with 500 μL media (with or without 10% FBS) containing 1 μg pDNA in transfection vectors at 37 °C. Lipofectin™ lipoplexes were used as a reference control and were synthesized following manufacture's protocols. Cationic liposome/(PEI/pDNA) complexes were prepared as a non-magnetic control by the same procedures as described above for MP/(PEI/pDNA) complexes. The cells were incubated with the vectors for either 15 min or 4 h, in the presence or absence of a magnetic field. The transfection media were then replaced with fresh culture media, and the cells were incubated for an additional 24 h. Magnetic field for *in vitro* transfection study was provided by a 24-well plate containing sintered Nd–Fe–B permanent magnets (one in each well and with a surface magnetic field strength of 5 kGauss) placed directly underneath the 24-well plate containing the cells. Cells transfected with vectors carrying GFP pDNA were visualized and photographed on a Nikon fluorescence microscope. For vectors carrying a luciferase reporter gene, cells were lysed and assayed for luciferase activity using a kit from Promega. Briefly, the cells were washed with ice-cold PBS (pH 7.4), and lysed in 100 μL lysis buffer for 5 min at room temperature. Ten μL lysate was then mixed with 50 μL luciferase assay reagent, and luminescence was measured by integrating for 10 s on a Mini-Lum luminometer (Bioscan Inc., Washington, DC). Protein content was measured by BCA protein assay, using BSA as standard. After background subtraction, relative light unit (RLU) values were normalized to sample protein content. Each 10,000 RLUs equals approximately 1 pg luciferase protein.

2.7. Evaluation of cytotoxicity by the magnetic vectors

Cytotoxicity of magnetic vectors was evaluated by MTT assay. Briefly, KB cells were seeded in 96-well plate at 5×10^3 cells/well, and incubated in triplicates with serial dilutions of the vectors for 4 h at 37 °C, followed by washing with PBS (pH 7.4). Cells were then allowed to grow in fresh RPMI-1640 medium containing 10% FBS for an additional 48 h, and analyzed for viability by the MTT assay (Mosmann, 1983). Untreated cells were used as a reference control and taken as being 100% viable.

2.8. Statistical analysis

The results were reported as means ± standard deviation. A Student's *t*-test was used to determine significance of the differences among treatments groups. A p value of less than 0.05 is considered significant.

3. Results

3.1. Synthesis and characterization of cationic lipid-coated MPs

Cationic lipid-coated MPs were synthesized in a two-step process. High temperature (80 °C) and high concentration of NH_4OH were used because these favor the formation of the Fe_3O_4 (black) over brown-color Fe_2O_3 (brown) (Massart et al., 1995; Shen et al., 1999; Zhang et al., 1997). After acetone washing, the MPs could be resuspended in $CHCl_3$ but not in aqueous medium. This indicated the presence of oleic acid coating on the MPs. Additional coating of the MPs with cationic lipids was then carried out with addition of DDAB/soy PC. Following magnetic decantation, drying and weighing of the sediment, 90% of lipids remained associated with MPs. The size of the cationic lipid-coated MPs was measured by DLS and found to be 78.3 ± 59.5 nm. TEM images showed that MPs consisted of clusters of magnetite cores of <10 nm in diameter (Fig. 1B) with lipid coating. The zeta potential of the particles was 85.54 ± 6.28 mV.

The MPs exhibited superparamagnetism, as evident by zero coercivity and remanence on a hysteresis loop (Fig. 2A). Zero field-cooled (ZFC) versus field-cooled (FC) measurement showed a freezing (or blocking) temperature (T_b) of ~150 K (Fig. 2B). For superparamagnetic particles, T_b is related to the volume of sample and the magnetic anisotropy energy (K), which is sensitive to particle structure and composition. Below T_b, thermal activation is no longer able to overcome the magnetic crystalline anisotropy of the MPs and becomes magnetically frozen showing remanence and coercivity. Above T_b, the noninteracting MPs shows zero remanence and zero coercivity because of the thermal energy that allows magnetization to flip between easy directions surpassing the energy barriers at zero fields. Therefore, dc magnetic responses clearly indicated superparamagnetism of the MPs.

3.2. Physicochemical characterization of magnetic vectors

The proposed mechanism of complex formation is illustrated in Fig. 3A. We found that both N/P and positive (DDAB-coated MP) to negative (pDNA) charge (+/−) ratios are critical in determining the structure of the magnetic gene vectors. AFM images of pDNA/PEI complexes depicted detailed structures of these particles at N/P ratios of 1 and 3. At N/P ratio of 1, pDNA/PEI complexes appeared to be less compact and consisted of looped structures with condensation nodes (Fig. 3B). In contrast, pDNA/PEI at N/P ratio of 3 had compact structures with sizes in the range of 30–50 nm.

MP-associated complexes were further analyzed by agarose gel electrophoresis. As shown in Fig. 4A, MPs alone could not completely condense pDNA, and ethidium bromide staining was detectable even at relatively high DDAB/pDNA (+/−) ratios. However, combining the MPs with PEI resulted in complete condensation of the pDNA at all N/P ratios (1 to 3) tested (Fig. 4B). Upon Triton-X 100 detergent treatment, which released surface bound MP, pDNA could be visualized at N/P ratios of 1 and 2,

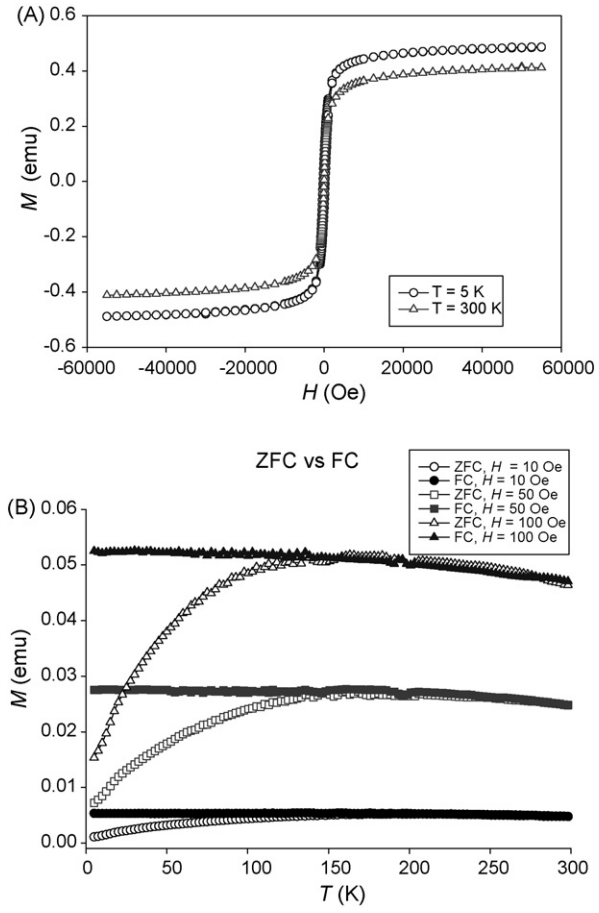

Fig. 2. Magnetization curves of cationic lipid-coated MPs as determined by SQUID.

but not at the N/P ratio of 3. Interestingly, ternary complexes MP/(PEI/pDNA) formed at N/P ratio of 2 yielded large particle size (1373.9 ± 923.2 nm). This was probably due to particle aggregation when zeta potential was near zero. Zeta potential of PEI/pDNA complexes was negative at the N/P ratio of 1, and became positive once N/P ratio exceeded 2 (Table 1). Addition of the slightly negatively charged human transferrin to the complexes resulted in only a slight increase in particle size (Betgovargez et al., 2005). After associating with transferrin, the complexes showed reduced zeta potential (Table 1). The summary of particle sizes is illustrated in Fig. 4C.

Table 1
Zeta potentials of various vector complexes

Complexes	Zeta potential (mV)
a	
PEI/pDNA (N/P = 1)	−37.52 ± 1.42
PEI/pDNA (N/P = 2)	11.39 ± 2.95
PEI/pDNA (N/P = 3)	18.70 ± 2.88
b	
MP/pDNA (+/− = 4/1)	28.19 ± 1.99
MP/pDNA (+/− = 6/1)	43.07 ± 3.60
MP/pDNA (+/− = 10/1)	64.82 ± 5.42
c	
MP/(PEI/pDNA) (+/− = 3/1), N/P = 1	44.09 ± 3.62
MP/(PEI/pDNA) (+/− = 4/1), N/P = 1	66.83 ± 4.68
MP/(PEI/pDNA) (+/− = 6/1), N/P = 1	59.44 ± 6.79
MP/(PEI/pDNA) (+/− = 10/1), N/P = 1	67.33 ± 5.73
d	
MP/(PEI/pDNA) (+/− = 4/1), N/P = 1, plus 5 μg/μg pDNA/transferrin	41.31 ± 6.08
MP/(PEI/pDNA) (+/− = 4/1), N/P = 1, plus 10 μg/μg pDNA/transferrin	23.72 ± 1.08
MP/(PEI/pDNA) (+/− = 4/1), N/P = 1, plus 15 μg/μg pDNA/transferrin	12.63 ± 7.12

In conclusion, a low N/P ratio was necessary to form negatively charged PEI/pDNA complexes that can further interact with cationic MPs. The N/P ratio of 1 was, therefore, chosen for further transfection studies. By pre-condensing pDNA with PEI-25k at N/P ratio of 1, MP/(PEI/pDNA) ternary complexes were then synthesized. These were more compact than binary complexes of MP/pDNA formed at the same DDAB/pDNA (+/−) ratio. Transferrin-associated complexes exhibited a similar pattern in agarose gel electrophoresis to that of MP/(PEI/pDNA) ternary complexes without transferrin (data not shown).

3.3. Magnetic transfection studies

Optimizing formulation parameters, such as DDAB/pDNA (+/−) charge ratio, is essential for enhancing transfection activities of magnetic vectors. The highest transfection activity had a DDAB/pDNA (+/−) charge ratio of 4 (Fig. 5). Further increases in DDAB/pDNA (+/−) charge ratio led to a significant decrease in transfection efficiency in all three types of complexes, which was probably due to increased cytotoxicity. The level of luciferase gene expression mediated by the MP/(PEI/pDNA) ternary complexes was significantly enhanced compared to the MP/pDNA binary complexes at the same DDAB/pDNA (+/−) charge ratio. Association of transferrin to the ternary complexes led to a further increase in the transfection efficacy.

Transfection efficiency was determined using either 15 min or 4 h incubation time. Compared to Lipofectin™, PEI, and cationic liposomes (composed of DDAB/soy PC at 60:40 mole/mole) control groups, the magnetic vectors achieved a much greater (>300-fold) transfection activity in the 15-min incubation group (Fig. 6A). In addition, magnetic vectors mediated transfection showed greater serum resistance compared to other standard transfection agents. However, magnetic complexes did not enhance transfection compared to PEI under optimized conditions for PEI polyplexes, i.e., with a 4-h incubation and in the absence of serum.

The role of the magnetic field on magnetic vectors was investigated by comparing transfection efficiency with or without application of an external magnetic field. Without magnetic field, the transfection efficiency was drastically reduced. Very limited luciferase activities were produced by the magnetic vectors (>200-fold less), if the cells were treated for only 15 min (Fig. 6).

The optimal amount of transferrin for transfection mediated by magnetic field was also evaluated. An enhancement of transfection was observed at 5 or 10 μg transferrin per μg pDNA. However, further increasing the transferrin amount led to a decrease in the level of luciferase gene expression, which indicated that 10 μg transferrin was optimal for the transfection.

The transfection activity of these magnetic gene vectors was further tested on rat glioma F98 cells. Magnetic complexes showed excellent transfection activity with 15 min incubation. And as expected, transferrin-associated magnetic vectors gave the greatest transfection activities among all vectors (Fig. 7). Luciferase gene expression in F98 cell was generally lower than that in KB cells, which may be due to the inherent difference in susceptibility to transfection between these two cell lines.

3.4. Cell viability assay

Cationic lipids usually carry some cytotoxicity. Therefore we performed cell viability assays on these magnetic vectors. The results

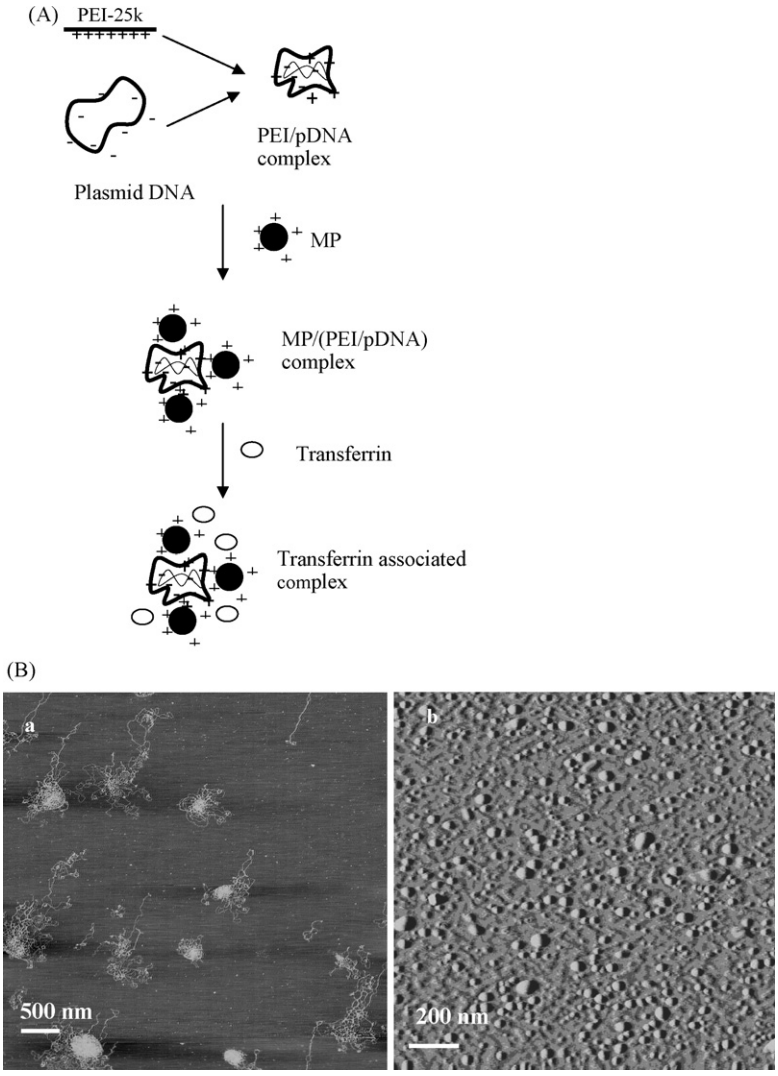

Fig. 3. Synthesis and structure of transferrin-coated magnetic vectors. (A) Schematic representation of transferrin-associated magnetic vectors. pDNA was first condensed by PEI-25k, and then incubated with cationic lipid-coated MP to yield MP/(pDNA/PEI) ternary complexes. Further addition of transferrin resulted in transferrin-associated magnetic vectors, as described in Section 2. (B) AFM images of pDNA/PEI: (a) PEI/pDNA complexes formed at N/P ratio of 1 and (b) PEI/pDNA complexes formed at N/P ratio of 3.

showed relatively low cytotoxicity for complexes formed at low DDAB/pDNA (+/−) charge ratios, and elevated cytotoxicity for vector formed at high charge ratios (Table 2).

4. Discussion

In this report, we investigated transferrin-associated cationic lipid-coated MPs for magnetofection, and demonstrated rapid and efficient gene delivery in the presence of an external magnetic field in KB and F98 cells.

Transfection studies were designed to investigate the optimal formulation parameters, such as DDAB/pDNA (+/−) ratio, PEI/pDNA ratio, and associated transferrin amount, as well as the roles of *in vitro* transfection conditions, such as incubation time, cell culture media, and magnetic field strength.

As illustrated in Fig. 3A, this delivery system is based on rational selection of individual components and composition ratios. Both N/P ratio of the polyplex component and positive (DDAB-coated MP) to negative (pDNA) charge (+/−) ratios are critical to complex size and transfection efficiency of the magnetic gene vectors. PEI was chosen as a pDNA condensation agent, that can, in addition, act as a proton sponge to facilitate endosomal lysis (Boussif et al., 1995; Pack et al., 2005). In our study, under optimal cationic lipid/pDNA ratio, complexes with PEI showed better transfection efficiency (Fig. 5) than complexes without PEI. The optimal PEI amount was essential to the transfection activity. Complexes with N/P ratio of 1 showed superior transfection activities to those formed at N/P ratio of 3 (Fig. 8), which further justified the gel electrophoresis and AFM analysis results (Figs. 3 and 4). At N/P ratio of 3, pDNA was completely condensed by PEI therefore; the PEI/pDNA polyplexes were no longer negatively charged (zeta potential data, Table 1) to enable further complexation with MPs via electrostatic interaction. Based on these observations, we selected the polyplex N/P ratio of 1 for MP complexation. N/P ratio 2 was not chosen because of severe aggregation that occurred at that specific N/P ratio during polyplex formation.

Incorporation of transferrin was shown to further greatly enhance gene delivery, which could be due to transferrin receptor-mediated cellular uptake of the vectors (Chiu et al., 2006; Kircheis et al., 2001). Magnetic force has been previously reported not to alter the cellular uptake mechanisms of magnetic vectors. Magnetic force merely facilitated the particle sedimentation onto the cell surface,

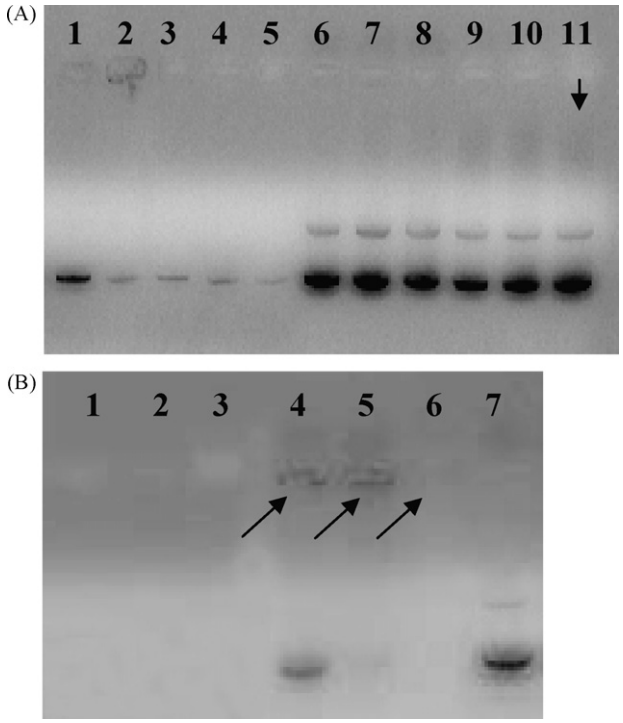

Fig. 4. Mobility of DNA complexes analyzed by agarose gel electrophoresis. (A) Agarose gel electrophoresis of MP/pDNA complexes with ethidium bromide staining. Lane 1–5, particles formed at (+/−) ratios of 3, 4, 5, 6, 10; Lane 6–10, particles formed at (+/−) ratio of 3, 4, 5, 6, 10 and lysed in 2% Triton X-100; Lane 11, naked pDNA control; (B) Agarose gel electrophoresis of MP/(PEI/pDNA) tertiary complexes at (+/−) ratio of 4; Lane 1–3, particles formed at N/P ratio of 1, 2, 3; Lane 4–6, particles formed at N/P ratio of 1, 2, 3 and lysed in 2% triton X-100; Lane 7, naked pDNA control. Complexes were prepared at varying (+/−) ratio as described in Section 2.

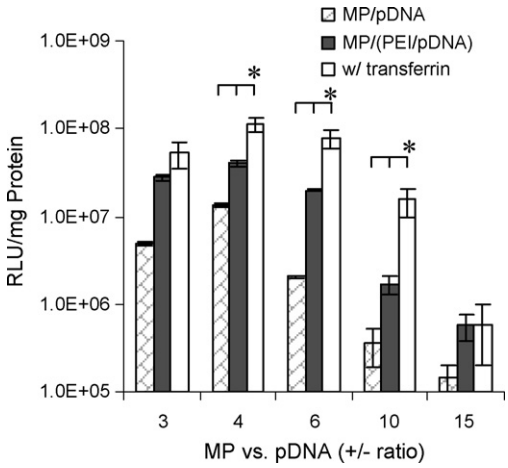

Fig. 5. Effect of DDAB/pDNA (+/−) charge ratio on luciferase gene expression in KB cells. Transfection activities of MP/pDNA complexes (▨), MP/(PEI/pDNA) ternary complexes formed at N/P ratio of 1 (■), and transferrin-associated MP/(PEI/pDNA) complexes formed at 10 μg transferrin/μg pDNA and N/P ratio of 1 (□). Each well was treated with vector complexes containing 1 μg of pDNA in 500 μL of RPMI-1640 medium. After 15-min incubation on top of a magnetic plate, medium was removed and the cells were further incubated for 48 h. The vector complexes were synthesized as described in Section 2. Luciferase expression was presented as RLU per mg cellular protein (mean ± standard deviation, $n = 3$). Asterisk (*) indicates significant differences ($p < 0.05$) compared to MP/pDNA binary and MP/(PEI/pDNA) ternary complexes.

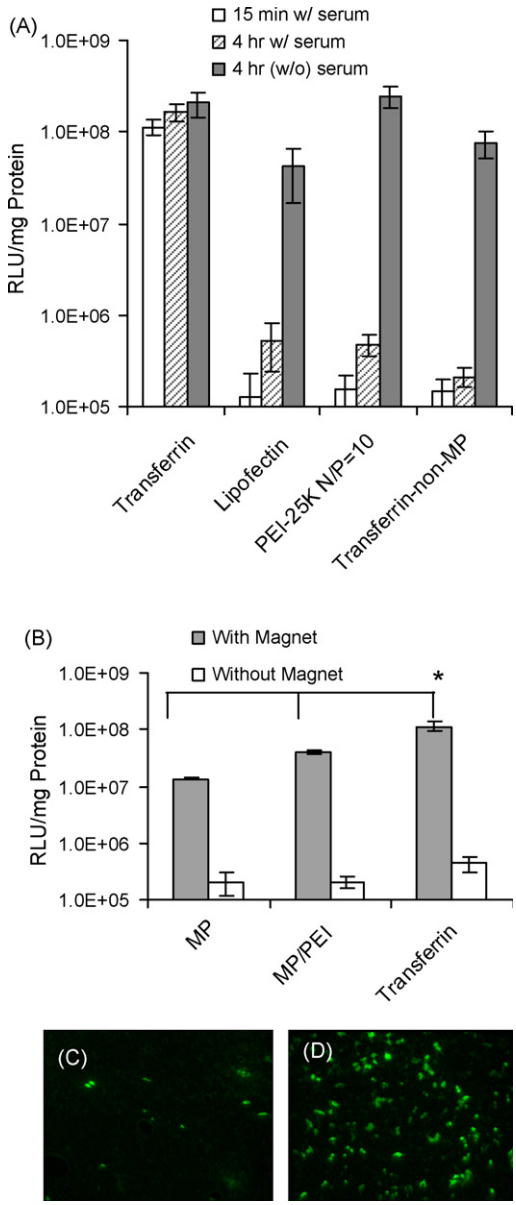

Fig. 6. Efficacy of various transfection systems and impact of incubation time and external magnetic field on transfection efficiency in KB cells. (A) Transfection was carried out using two different incubation times, 15 min (□) and 4 h (▨,■). Incubation was carried out in the presence (□,▨) or absence (■) of 10% FBS in a magnetic field. The cells were incubated with 1 μg of pDNA in various vector formulations on top of a magnetic plate, followed by 48 h further incubation. "Transferrin" group stands for MP/(PEI/pDNA) ternary complexes associated with additional transferrin at (+/−) charge ratio of 4, N/P ratio of 1, and 10 μg transferrin/μg pDNA. "Transferrin-non-MP" group is the complexes formed by DDAB/PC/PEI/ transferrin without MP. Asterisks (*) indicated significant differences ($p < 0.05$) compared with other groups with 15-min incubation. (B) The cells were incubated with 1 μg of pDNA in various vector formulations, either without the presence of magnetic field or on top of a magnetic plate, for duration of 15 min, followed by 48 h further incubation; (C) fluorescence images of cells transfected with GFP plasmid. Cells were transfected with 1 μg pDNA transferrin-associated MP/(PEI/pDNA) using 15 min incubation time in the absence of magnetic field; (D) the same as (C), except that the transfection was performed in the presence of a magnetic field. "MP" stands for MP/pDNA complexes, MP/PEI" stands for MP/(PEI/pDNA) ternary complexes, and "Transferrin" stands for ternary complexes associated with additional transferrin (at (+/−) charge ratio of 4, N/P ratio of 1, and 10 μg transferrin/μg pDNA). Asterisk (*) indicates significance ($p < 0.05$) compared with transfection activities of MP/pDNA complexes and MP/(PEI/pDNA) ternary complexes with external magnetic field. Vector preparation and reporter gene assay were described in Section 2.

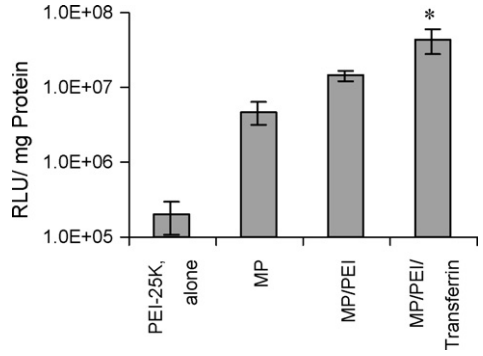

Fig. 7. Transfection activity of magnetic vectors in F98 cells in the presence of an external magnetic field. The cells were incubated with 1 μg of pDNA in various vector formulations on top of a magnetic plate for 15 min, the medium was removed and the cells were further incubated for 48 h. Vector preparation and reporter gene assay were described in Section 2. Luciferase expression was presented as RLU per mg cellular protein (mean ± standard deviation, $n = 3$). "MP" stands for MP/pDNA complexes, MP/PEI" stands for MP/(PEI/pDNA) ternary complexes, and "Transferrin" stands for ternary complexes associated with transferrin (formed at (+/−) charge ratio of 4, N/P ratio of 1, and 10 μg transferrin/μg pDNA). Asterisk (*) indicates significant differences ($p < 0.05$) compared with non-transferrin associated vectors.

Table 2
Effect of magnetic vectors on the viability of KB cells

(+/−) Ratio	Complexes	Viability (%)
(+/−) = 4	MP/pDNA	90 ± 7
	MP/(PEI/pDNA)	87 ± 10
	MP/(PEI/pDNA) with transferrin	92 ± 6
(+/−) = 6	MP/pDNA	82 ± 7
	MP/(PEI/pDNA)	87 ± 6
	MP/(PEI/pDNA) with transferrin	90 ± 4
(+/−) = 10	MP/pDNA	63 ± 7
	MP/(PEI/pDNA)	63 ± 5
	MP/(PEI/pDNA) with transferrin	74 ± 5
(+/−) = 15	MP/pDNA	57 ± 4
	MP/(PEI/pDNA)	55 ± 5
	MP/(PEI/pDNA) with transferrin	71 ± 9

Cells were rinsed twice with PBS, and then incubated with magnetic complexes containing 0.5 μg pDNA in RPMI-1640 growth medium containing 10% FBS. After 4 h incubation, the cells were further incubated for 2 days. Cells viability was measured by MTT assay. The data are expressed as the percentage of OD relative to untreated cells (mean ± standard deviation, $n = 3$). Transferrin group contained 10 μg transferrin/μg pDNA.

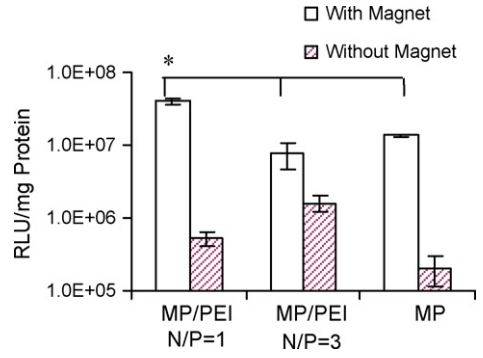

Fig. 8. Effect of PEI-to-pDNA N/P ratio on transfection efficiency in KB cells. The complexes were formed at a lipid-to-DNA (+/−) charge ratio of 4. Data are shown as mean ± standard deviation ($n = 3$). Asterisk (*) indicates significance ($p < 0.05$).

but did not actually "drive" them into the cells (Huth et al., 2004). Instead, non-specific electrostatic or receptor-based mechanisms may be responsible for cellular uptake of magnetic vectors. In the case of transferrin-associated magnetic vectors, better gene transfer efficiency was shown than vectors without targeting. Excessive amount of transferrin lead to a decrease in transfection efficiency. This was probably due to competition between with free transferrin and transferring-coated magnetic vectors for binding to the transferrin receptor on the cellular surface. These findings are consistent with previous reports on non-covalent transferrin-complexed vectors (Simoes et al., 1998).

Transfection efficiency of our magnetic vectors was compared to other well-established non-viral gene transfer systems, such as PEI polyplexes and Lipofectin™ lipoplexes (Fig. 6A). Transfection activities of the magnetic vectors were comparable to these systems under serum-free and long incubation (4 h) conditions. The magnetic vectors were much superior to all other standard transfection systems, by 2–3 orders of magnitude, under the short incubation time (15 min) condition. This is consistent with similar findings of a recent report (Gersting et al., 2004). In the absence of magnetic field, the magnetic vectors produced much less efficient transfection with short 15-min incubation. These results indicated that magnetic field facilitated rapid accumulation of the magnetic vectors on the cells. Another advantage of the magnetic vectors is that they maintained high transfection activity in the presence of serum, while standard cationic lipid-based transfection agents show diminished transfection activities in the presence of 10% serum (Crook et al., 1998). The serum resistance of magnetic vector was possible due to the presence of the magnetic force that moves the magnetic vectors towards the cells. In contrast, high transfection activity was only observed with PEI and lipofectin when the cells were incubated in the absence of serum and for a prolonged period of time. This was because those vectors were taken up by cells through charge interaction, which can be inhibited by serum protein in the medium.

In conclusion, we synthesized cationic lipid-coated magnetic nanoparticles, and prepared transferring-coated MP/(PEI/pDNA) complexes. Transfection using these magnetic vectors required much less incubation time in the presence of an external magnetic field, and transferred gene at high efficiency. This might offer an important advantage for *ex vivo* and *in vivo* gene delivery, where pDNA is subject to nuclease degradation and systemic clearance. Magnetic gene delivery has been explored for *in vivo* application in several proof-of-principle experiments (Galuppo et al., 2006; Scherer et al., 2002; Xenariou et al., 2006). The results showed enhanced gene transfer under influence of a magnetic field. These studies mainly focused on local delivery, and magnetic delivery was shown to be useful as a potential research tool for locally studying the function of genes. Further research is needed to optimize the magnetic gene delivery for *in vivo* application. In addition to plasmid DNA, magnetofection may find utility in the delivery of oligonucleotides, such as siRNA, which, in the absence of chemical modification, display very limited serum stability. Rapidity in delivery could prove an important advantage when stability of nucleic acid is a rate-limiting factor.

Acknowledgement

This work was supported in part by NSF grant EEC-0425626.

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