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High mobility group box 1 protein enhances polyethylenimine mediated gene delivery in vitro

Yuan Shen[a], Hui Peng[b], Jingjing Deng[a], Yuting Wen[a], Xin Luo[a], Shirong Pan[c], Chuanbin Wu[a], Min Feng[a,*]

[a] School of Pharmaceutical Sciences, Sun Yat-sen University, Zhongshan II Road 74, Guangzhou 510080, PR China
[b] School of Zhongshan Medicine, Sun Yat-sen University, 74 Zhongshan Road II, Guangzhou 510080, PR China
[c] Cardiovascular Laboratory, The First Affiliated Hospital, Sun Yat-sen University, 80 Zhongshan Road II, Guangzhou 510080, PR China

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ABSTRACT

The relatively low efficiency of transgene expression is a major drawback of non-viral gene delivery systems despite it being a safer alternative for gene therapy. Modifications of non-viral carriers with peptides have been utilized to improve their gene transfer efficiency. In this study, we described a new combined carrier, which was comprised of a cationic polymer, polyethylenimine (PEI), and a nuclear protein, high mobility group box 1 (HMGB1) containing nuclear localization sequences (NLS). The HMGB1/branched or linear PEI (b-PEI or l-PEI) combined carriers have been investigated for their cytotoxicity and ability to condense and deliver plasmid DNA (pDNA) to mammalian cells in vitro. Both HMGB1 and PEI formed complexes with pDNA are revealed by a gel-retardation assay. Compared with pDNA/l-PEI complexes, a reduction in particle size was observed for pDNA/HMGB1/l-PEI ternary complexes. In MTT assay the results of cell viability suggested lower cytotoxicity for HMGB1/PEI combined carriers. Transfection efficiencies of pDNA/HMGB1/PEI ternary complexes were evaluated by green fluorescent protein expression level measured by fluorescence spectroscopy and flow cytometry. Transfection efficiencies for pDNA/HMGB1/l-PEI ternary complexes and pDNA/HMGB1/b-PEI ternary complexes showed more than 2.9-fold and 4.0-fold greater than that for pDNA/l-PEI complexes and pDNA/b-PEI complexes, respectively. Thus, our results showed that HMGB1/PEI combined carriers may be a versatile non-viral carrier for pDNA with high transfection efficiency.

1. Introduction

Development of safe and effective gene delivery carriers is critical to the ultimate success of gene therapy. Non-viral gene delivery carriers, such as polyethlenimines (PEIs) (Kunath et al., 2003), poly(L-lysine) (PLL) (Curiel et al., 1991) and polyamidoamine (Bielinska et al., 1996), have been widely investigated. They exhibit a superior safety profile compared with viral carriers, and they also have potential advantages of ease of production and usage, low immune response, and unrestricted DNA size (Kircheis et al., 2001; Choosakoonkriang et al., 2003; Park et al., 2006). However, their relatively low efficiency of transgene expression is a major drawback. Non-targeting delivery of plasmid DNA (pDNA) to specific cells, poor release of the polyplexes from the endosome into the cytoplasm and subsequently inefficient transfer into the nucleus are thought to contribute to their low gene transfer efficiency (Ogris et al., 2001; Bremner et al., 2004). Increase in the target specificity for non-viral carriers can be shown by attaching specific ligands on carriers, such as transferring, which resulted in up to a several hundred-fold increase in transfection efficiency (Kircheis et al., 1997). Similarly, the enhancement of endosomal escape can be achieved by adding endosomolytic agents (Zuber et al., 2001) or fusogenic peptides (Lee et al., 2001) in polyplexes. After the pDNA complexes have been taken up by cells and are released from endosome into the cytosol, the polyplexes still have to enter the nucleus. The transport of the transfected DNA from the cytosol into the nucleus is certainly one of the major limitations for efficient gene transfer of non-viral carriers. Inefficient nuclear transport can be addressed by coupling nuclear localization sequences (NLS) to pDNA and the results have indicated the potential of this approach to improve pDNA nuclear delivery (Escriou et al., 2003). Another approach to surmount poor nuclear transfer is using endogenous nuclear proteins such as histones or high mobility group proteins (HMGs) as gene delivery carriers, which can lead to improvement of pDNA nuclear importation as well as decreases of cytotoxicity (Kaouass et al., 2006). High mobility group box 1 (HMGB1) is one of HMG groups and the abundant nuclear proteins that regulate and facilitate various DNA-related activities such as transcription,

* Corresponding author. Tel.: +86 20 87331215; fax: +86 20 87331215.
E-mail address: fengmin@mail.sysu.edu.cn (M. Feng).

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replication and recombination (Bustin, 1999). HMGB1 is composed of high mobility box A, box B, and C-terminal acidic regions. Positively charged residues of lysine and arginine in box A and box B domain can bind to pDNA via electrostatic interactions (Palau et al., 1977). HMGB1 is also known to have NLS that can facilitate their nuclear import. The presence of both NLS and the DNA binding box makes HMGB1 a good candidate for gene delivery. However, using these nuclear proteins alone as gene transfer carriers showed much lower transfection efficiency than cationic polymers (Kim et al., 2008).

In this study, we described a new combined carrier, which was comprised of a cationic polymer, PEI, and a nuclear protein, HMGB1. The rationales of using HMGB1/PEI combined carriers for non-viral gene delivery are firstly, the main intracellular barriers for gene delivery may be overcome by using these combined carriers. PEI moiety in combined carriers can deliver pDNA into cells by endocytosis, and then release pDNA into cytosol by polycationic "proton sponges". Another moiety, HMGB1 containing NLS, can sequentially transfer pDNA into nuclei of cells. Overcoming these barriers may result in higher transfection efficiency. Secondly, it is a concise approach for the formation of pDNA/HMGB1/PEI ternary complexes. As the amphiphilic macromolecule, HMGB1 contains several positively charged residues such as lysine and arginine, and negatively charged carboxyl terminal acids. Thus, HMGB1 can interact both with pDNA and PEI via electrostatic interactions, resulting in self-assemble formation of pDNA/HMGB1/PEI ternary complexes. Thirdly, compared with using PEI alone, the cytotoxicity of these HMGB1/PEI combined carriers may be lower. HMGB1 is non-cytotoxic and also has the ability to interact with pDNA (Palau et al., 1977). So in the combined carriers, HMGB1 moiety is able to condense pDNA, which may lead to the decrease of PEI concentration in the gene delivery system. Fourthly, compared with using HMGB1 alone, the combined carriers based on synthetic polymer as major component are low-cost carriers. Thus, here the HMGB1/PEI non-viral carriers have been investigated specifically for their cytotoxicity and ability to condense pDNA. To address the efficiency of these combined carriers for gene delivery, transfection efficiencies of pDNA/HMGB1/PEI ternary complexes were evaluated by green fluorescent protein expression level in mammalian cells in vitro.

2. Experiment

2.1. Materials

Branched polyethylenimine (b-PEI Mw 25,000) and linear polyethylenimine (l-PEI Mw 25,000) were purchased from Sigma–Aldrich and Polysciences, respectively. High mobility group box 1 (HMGB1) was obtained from Sigma–Aldrich. RPMI Medium 1640 medium, fetal bovine serum (FBS) and penicillin–streptomycin were acquired from Gibco. pEGFP-C1 was a gift from West China University of Medical Sciences. EndoFree Plasmid Kit was purchased from Tiangen. The HeLa cell line (human cervix epithelial carcinoma cells) was obtained from the American Type Culture Collection (ATCC). Agarose was purchased from Biowest. Ethidium bromide was purchased from Invitrogen. Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma–Aldrich. All the other reagents were of analytical grade.

2.2. Preparation of plasmid pEGFP-C1

The plasmid pEGFP-C1 (4.8 kbp) encoding the enhanced green fluorescent protein gene under the control of the human cytomegalovirus (CMV) was replicated in competent high-copy DH5-α *Escherichia coli* strain grown in kanamycin (50 μg/mL) Luria–Bertani medium and endotoxin-free pDNA was purified by EndoFree Plasmid Kit according to the manufacturer's protocol. The concentration of pDNA was determined by UV absorbance at 260 nm using an extinction coefficient of 0.02 $mg^{-1}\,cm^{-1}$ mL, and its purity was evaluated by UV spectroscopy (E 260/280 nm ratio). pDNA size and homogeneity were assayed by 1.0% agarose gel electrophoresis.

2.3. pDNA/HMGB1/PEI ternary complex formation

Plasmid pEGFP-C1 was condensed with both PEI and HMGB1 to form ternary complexes. The pDNA/HMGB1/PEI ternary complexes were prepared at a final pDNA concentration of 20 μg/mL. Since HMGB1 was designed as the reagent to transfer pDNA from cytosol to nuclei, HMGB1 was firstly added to complexes with pEGFP-C1. Briefly, pEGFP-C1 was mixed with HMGB1 solution based on weight ratio of HMGB1 to pDNA from 0.1 to 100 by gentle vortex and incubated at room temperature for 15 min to allow spontaneous pDNA/HMGB1 complex formation. Then for adjustment of N/P ratio (molar ratio of PEI-nitrogen to DNA-phosphate), various amounts of b-PEI or l-PEI were added to pEGFP-C1/HMGB1 mixture and rapidly mixed by pipetting. The mixture was incubated for 30 min at ambient temperature yielding the pDNA/HMGB1/PEI ternary complexes before use.

2.4. Transmission electron microscopy

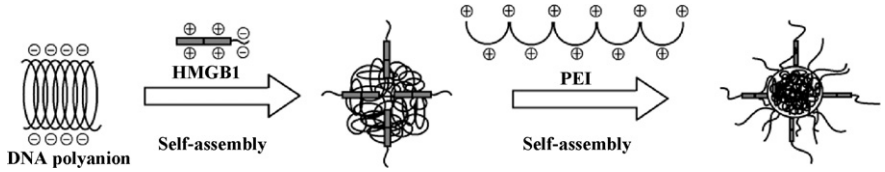
The pEGFP-C1/HMGB1/PEI ternary complexes were prepared according to the aforementioned formation protocol. 5-μL drops of fresh prepared complexes were placed on a 300-mesh formvar carbon coated copper grid and allowed to equilibrate for 3 min. Solution was wiped off with filter paper and the grids were then stained with 2% phosphotungstic acid and allowed to air dry. Images were taken using a JEM-2010HR transmission electron microscope (TEM) set to an accelerating voltage of 80 kV.

2.5. Measurement of the particle size

The particle size and size distribution of pEGFP-C1/HMGB1/PEI ternary complexes were measured by using a photon correlation spectroscopy (PCS) on a Malvern Zetasizer NS90 (Malvern Instruments, Malvern, UK) equipped with a 10-mW helium neon laser producing light at a wavelength of 633 nm and the Malvern PCS version 2.41 (2002) software. Ternary complexes were prepared as described in the previous section and measured in glass cuvettes at 25 °C and a fixed scattering angle of 90° through a 400 μm pinhole. For data analysis, the viscosity (0.88 mPa s) and the refractive index (1.33) of distilled water were used. Each data was determined at least in three independent experiments with each measurement averaging the data of 10 subruns to obtain average volume diameters (Dv) and number diameters (Dn). Polydispersity index (PI) of particle size distribution was calculated based on the ratio of Dv/Dn.

2.6. pDNA binding assay

To determine whether HMGB1 complex with pEGFP-C1 and PEI, a series of gel retardation assays were performed by electrophoresis. The pEGFP-C1/HMGB1/PEI ternary complexes formulated at a range of N/P ratios and pDNA/HMGB1 weight ratios were prepared. 10 μL of each complex sample was loaded into wells of a 1.0% agarose gel prepared in TAE buffer containing 0.5 μg/mL ethidium bromide. The samples were subsequently electrophoresed in an electric field of 80 V for 60 min, and bands corresponding to pDNA were visualized on a UV transilluminator (Benchtop UV Transilluminator GDS 8000, USA) and photographed.



Scheme 1. Formation of self-assembling pDNA/HMGB1/PEI ternary complexes.

2.7. Cell culture

HeLa cells were cultured in RPMI Medium 1640 supplemented with 10% defined fetal bovine serum (FBS) and 100 μg/mL penicillin and streptomycin sulfate. Cells were cultured in a culture incubator at 37 °C and in a 90% humidified atmosphere containing 5% CO_2.

2.8. Cytotoxicity assay

Cytotoxicity of PEI, HMGB1/PEI combined carriers and pEGFP-C1/HMGB1/PEI ternary complexes were evaluated by MTT colorimetric assay. Briefly, HeLa cells were seeded in 96-well plates at a density of 8000 cells/well. After 24 h, the culture medium was replaced with serial dilutions of samples in full medium at the same end concentrations used for the transfection experiments and cells were incubated for another 24 h, respectively. Then 20 μL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 5 mg/mL in phosphate buffered saline) solution was added. After 4 h of incubation the supernatant was aspirated, and the formazan crystals were dissolved in 150 μL DMSO. Absorption was measured photometrically at 570 nm with a background correction using a Bio-Tek ELX800 ELISA reader. Values of eight measurements were normalized to 100% for the control group (exposure to full medium). Cells without addition of MTT were used as a blank for calibration of the spectrophotometer to zero absorbance. The turnover of the substrate relative to control cells was expressed as relative cell viability, and was calculated as $A_{(test)}/A_{(control)} \times 100\%$.

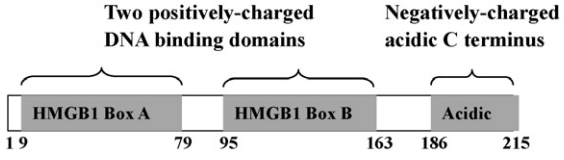
2.9. In vitro transfection study

HeLa cells were seeded 24 h prior to transfection into 12-well plates at a density of 1.5×10^4 cells per well in 1.0 mL of culture medium. Prior to starting the transfection experiment, the cells were rinsed twice with warm phosphate buffered saline (PBS, pH 7.4) and 0.2 mL of pEGFP-C1/HMGB1/PEI ternary complexes as described under 'pDNA/HMGB1/PEI ternary complex formation' and 0.8 mL of serum-free medium were added. The final pDNA concentration was 2 μg/well. After transfection for 4 h at 37 °C 90% humidified atmosphere and 5% CO_2, the cells was rinsed with warm PBS and supplied with 1 mL culture medium (containing 10% FBS). After leaving for 48 h for enhanced green fluorescent protein expression, the cells were rinsed twice with PBS and then observed green fluorescence by fluorescence spectroscopy (Olympus BX51 Fluorescence Spectroscopy, Japan). Subsequently the cells were treated with trypsin/EDTA for 2 min, collected by centrifugation and then resuspended in 0.5 mL PBS and kept on ice until analysis. Percentage of EGFP-expressing cells and mean fluorescence intensity (MFI) were employed to quantify transfection efficiency by flow cytometry using BeckmanCoulter ESP ELITE System (BeckmanCoulter, USA) equipped with an argon laser with an excitation wavelength of 488 nm. The filter setting for emission was 530/30 nm bandpass. Data were acquired in linear mode and visualized on linear mode.

3. Results and discussion

3.1. Formation of pDNA/HMGB1/PEI ternary complexes

Covalent conjugation of targeting moieties or any other bioactive reagent with gene delivery carriers requires chemical reactions, which could be determinate to the bioactivities of sensitive moieties. During the preparation of the pDNA/HMGB1/PEI ternary complexes used in the current study, bioactive nuclear protein HMGB1 was introduced simply through electrostatic interaction, as shown in Scheme 1. HMGB1 is the biomacromolecule containing both negatively and positively charged domains (Bottger et al., 1988). The positively charged residues in HMGB1 could interact with negatively charged phosphate pDNA backbone via electrostatic interactions. In the same way, the negatively charged terminal acidic domain in HMGB1 could interact with positively charged amines of PEI. This straightforward self-assembly approach circumvented the need of aggressive and reagent-wasteful chemical reactions, and would effectively preserve the bioactivities of nuclear protein. HMGB1 used in this study is a human, recombinant protein with N-terminal histidine tag expressed in *E. coli*. It contains 215 amino acids and has a molecular weight of approximately 25 kDa (Lu et al., 1996). It is composed of high mobility box A, box B, and a acidic C terminus, as shown in Scheme 2. It has also been demonstrated as a non-sequence specific DNA binding protein (Stros and Reich, 1998). Here, the formation of pEGFP-C1/HMGB1/PEI ternary complexes was prepared by two-step complex method. First, box A and box B containing lysine and arginine of HMGB1 were bound to pEGFP-C1 formed pEGFP-C1/HMGB1 complexes. Then, both negatively charged acidic C terminus of HMGB1 and part of negatively charged phosphate DNA were self-assembled to interact with PEI to form compact particles. The complexation of pDNA, HMGB1 and PEI led to a significant decrease in pDNA size, resulting in complexes a volume 10^4 to 10^6 times smaller than that of naked pDNA (Neu et al., 2005). TEM images showed that the morphology of both pEGFP-C1/HMGB1/b-PEI ternary complexes and pEGFP-C1/HMGB1/l-PEI ternary complexes were all nearly spherical in nature. Compact homogeneously distributed complexes were observed. The average diameter was approximately 32 ± 5 nm and 51 ± 8 nm as shown in Fig. 1. The size of these ternary complexes observed by TEM was smaller than that of complexes measured by PCS (Fig. 1). It might be reasonable in view of the swelled state of these complexes specimen in solution and the contracted state under dry conditions.



Scheme 2. Schematic structure of HMGB1.

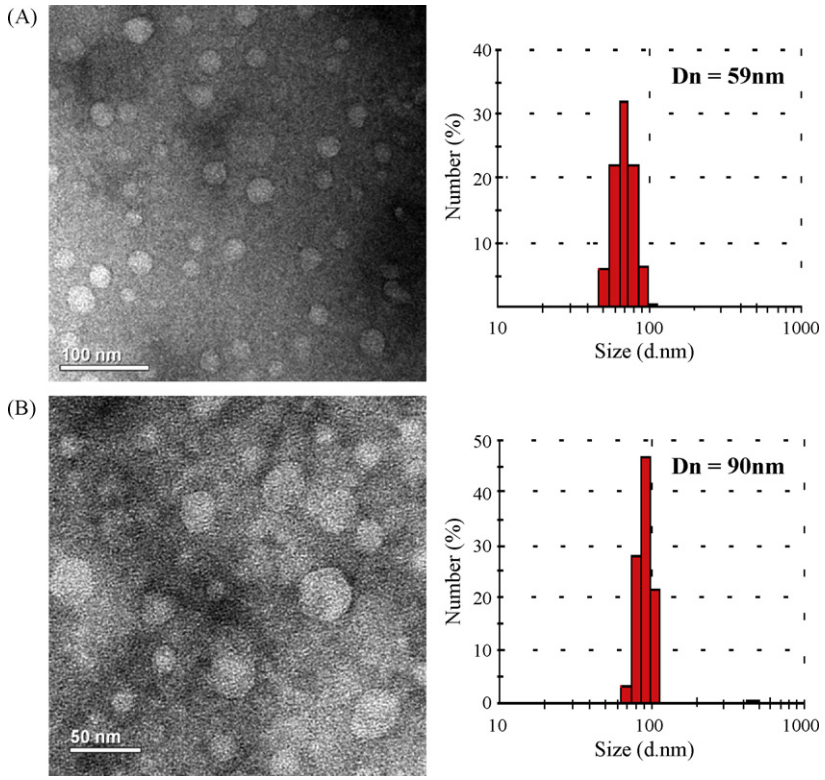


Fig. 1. TEM images and size distribution: (A) pEGFP-C1/HMGB1/b-PEI ternary complexes and (B) pEGFP-C1/HMGB1/l-PEI ternary complexes.

3.2. The effect of varying the HMGB1 concentrations on particle size

The particle size significantly affects the level of cellular uptake, and in some cell lines, only submicron size particles were efficiently taken up (Panyam and Labhasetwar, 2003; Olton et al., 2007). In this respect, to assess the size and size distribution of these ternary complexes was critical. The average particle size of pEGFP-C1/HMGB1/b-PEI ternary complexes and pEGFP-C1/HMGB1/l-PEI ternary complexes in 5% glucose (with no ionic strength) was assessed by PCS and shown in Fig. 2. The average diameter of these ternary complexes at varying weight ratios of HMGB1 to pDNA was all in the nano range. Fig. 2B shows that particle size and PI of pEGFP-C1/HMGB1/l-PEI ternary complexes displayed a dependence on the nuclear protein moiety of the vector. At the N/P ratio of 10, the average number diameter of pEGFP-C1/l-PEI polyplexes without HMGB1 was 224 nm with the PI of 4.87. However, when pEGFP-C1 and l-PEI complexed with HMGB1, the average particle sizes obviously decreased to 198 nm and 70 nm corresponding to the weight ratio of HMGB1 to pDNA of 0.01 and 100, respectively. Furthermore, compared with pDNA/l-PEI polyplexes, the PI of pEGFP-C1/HMGB1/l-PEI particle size distribution based on the ratio of Dv/Dn was in the range of 1.0–1.1, indicating narrow size distribution of these ternary complexes. These results indicated adding HMGB1 in l-PEI complexes resulted in smaller particle size and more homogenous particle formation which could enhance complex stability. It might be concluded that HMGB1 could assist l-PEI to condense pDNA into more compact particles. However, the particle size of ternary complexes using b-PEI did not show significant change at varying weight ratios of HMGB1 to pDNA as showed in Fig. 2A. Some literatures have reported that b-PEI exhibited superior pDNA compaction ability compared to l-PEI (Wightman et al., 2001; Gao et al., 2007). So if the ability of HMGB1 condensed pDNA was much weaker than that of b-PEI, the effect of HMGB1 on the significant diameter change of pEGFP-C1/HMGB1/b-PEI ternary complexes might not be observed.

3.3. The effect of varying the HMGB1 concentrations on pDNA binding

As potential DNA delivery carriers, they must be able to package DNA into small particles capable of being endocytosed (Fichter et al., 2008). To confirm whether these combined carriers, HMGB1/PEI, could form ternary complexes with pDNAs, the gel retardation

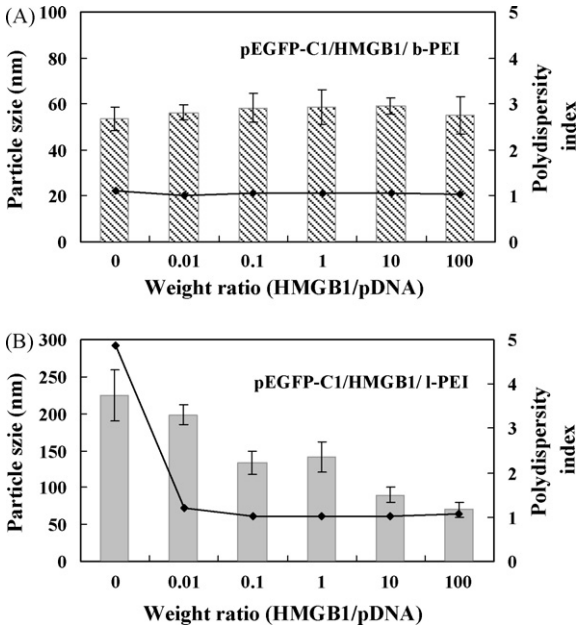


Fig. 2. Particle size (columns) and polydispersity index (lines) assessment of pEGFP-C1/HMGB1/PEI ternary complexes with varying ratio of HMGB1 to pDNA (w/w) based on N/P of 10. (A) pEGFP-C1/HMGB1/b-PEI ternary complexes and (B) pEGFP-C1/HMGB1/l-PEI ternary complexes.

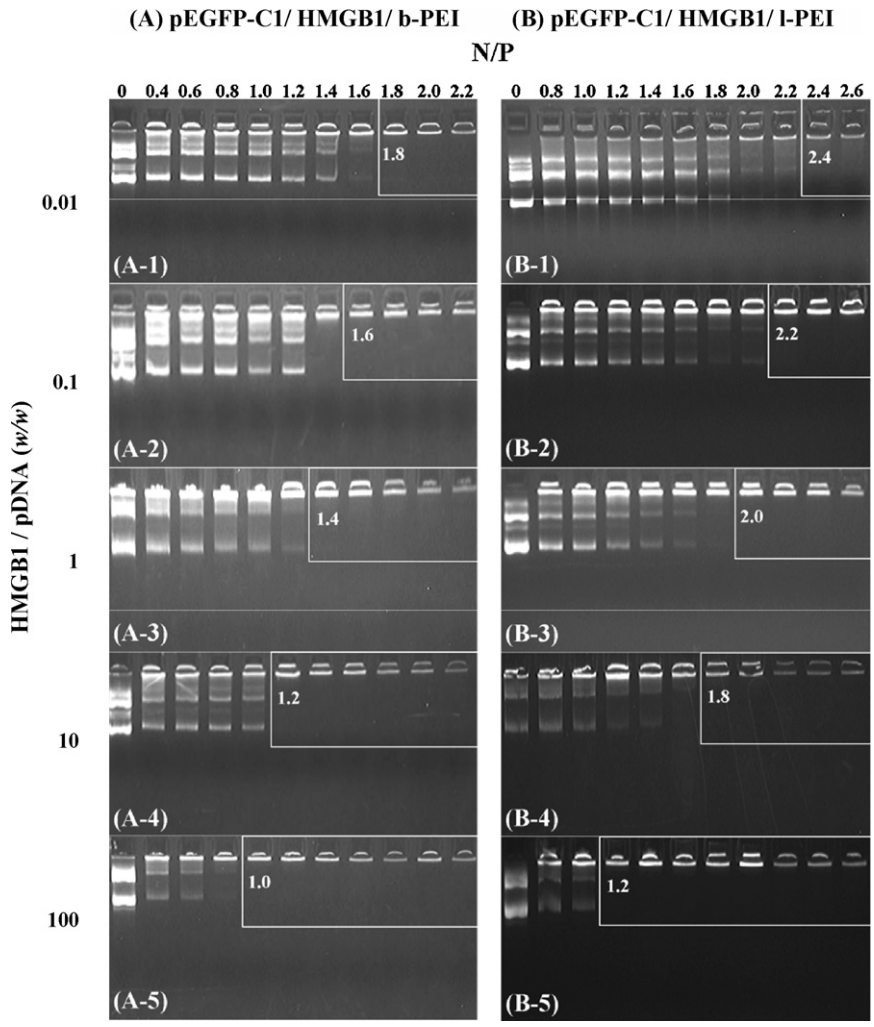


Fig. 3. Agarose gel analysized the effect of varying the weight ratio of HMGB1 to pDNA on pDNA binding. Lane 1 (N/P = 0) as the control for naked pDNA only. (A-1–5) pEGFP-C1/HMGB1/b-PEI ternary complexes; (B-1–5) pEGFP-C1/HMGB1/l-PEI ternary complexes.

assay was performed. Agarose gel images as shown in Fig. 3 revealed that with increase in the HMGB1 concentrations in pEGFP-C1/HMGB1/PEI ternary complexes, the N/P ratio of PEI completely retarded pDNA was decreased from 1.8 to 1.0 for b-PEI complexes and from 2.4 to 1.2 for l-PEI complexes, respectively. These results confirmed that HMGB1 could assist PEI to condense pDNA to form DNA ternary complexes. The interaction between HMGB1 and pDNA was thought that HMGB1 box A and box B have three α-helix structures with high number of positive-charged lysine and arginine residues that contribute to the charge interactions with negative-charged phosphate residues in the DNA backbone (Stott et al., 2006). This assay indicated that the HMGB1 played a role for nucleic acid condensation, which might result in lesser amount of PEI required for completely condensing pDNA.

3.4. Cell viability studies

The cytotoxicity of PEI is known to be concentration-dependent, structure-dependent as well as molecular weight-dependent (Brunot et al., 2007). Compared with using PEI alone as carriers, since HMGB1 was also capable of binding DNA, the concentration of PEI in ternary complexes should be decreased, which might modify its biological properties. The cytotoxicity of the PEI, HMGB1/PEI combined carriers and pEGFP-C1/HMGB1/PEI ternary complexes were estimated by MTT colorimetric assay and presented as the relative cell viability as illustrated in Fig. 4. Both b-PEI and l-PEI caused the decrease of cell viability in a concentration-dependent manner. l-PEI (25 kDa) showed higher cell viability than b-PEI (25 kDa) at all concentrations (Fig. 4A), indicating that l-PEI was less cytotoxic than b-PEI. It was reported that due to the high degree of branching in b-PEI it has a higher charge density compared to equally sized l-PEI with a linear structure (Fischer et al., 2003). The PEI with concentrations of 2.65 μg/mL and 5.30 μg/mL was chosen to investigate the cytotoxicity of combined carriers and pDNA complexes, since these PEI concentrations were used in following transfection study. Compared with using PEI alone, it was noted that the cytotoxicity of both HMGB1/b-PEI combined carriers and HMGB1/l-PEI combined carriers were reduced at all these dose level of HMGB1, as shown in Fig. 4B and C. It might be explained that since HMGB1 is the endogenous protein and is non-cytotoxic with negatively charged C-terminal acidic domain (Kim et al., 2008), the high charge density of 25K PEI might be partially neutralized by HMGB1. The high charge density of PEI was thought to contribute to their high cytotoxicity (Brunot et al., 2007). The cytotoxicity of pEGFP-C1/HMGB1/PEI combined complexes was also investigated. The result showed that at the N/P ratio of 10, cell viability of all pEGFP-C1/HMGB1/b-PEI and pEGFP-C1/HMGB1/l-PEI ternary complexes samples were more than 85%, but not showed a significant increase compared with the pDNA/PEI complexes without HMGB1. At higher N/P ratio of 20, cell viability of pEGFP-C1/HMGB1/l-PEI combined complexes

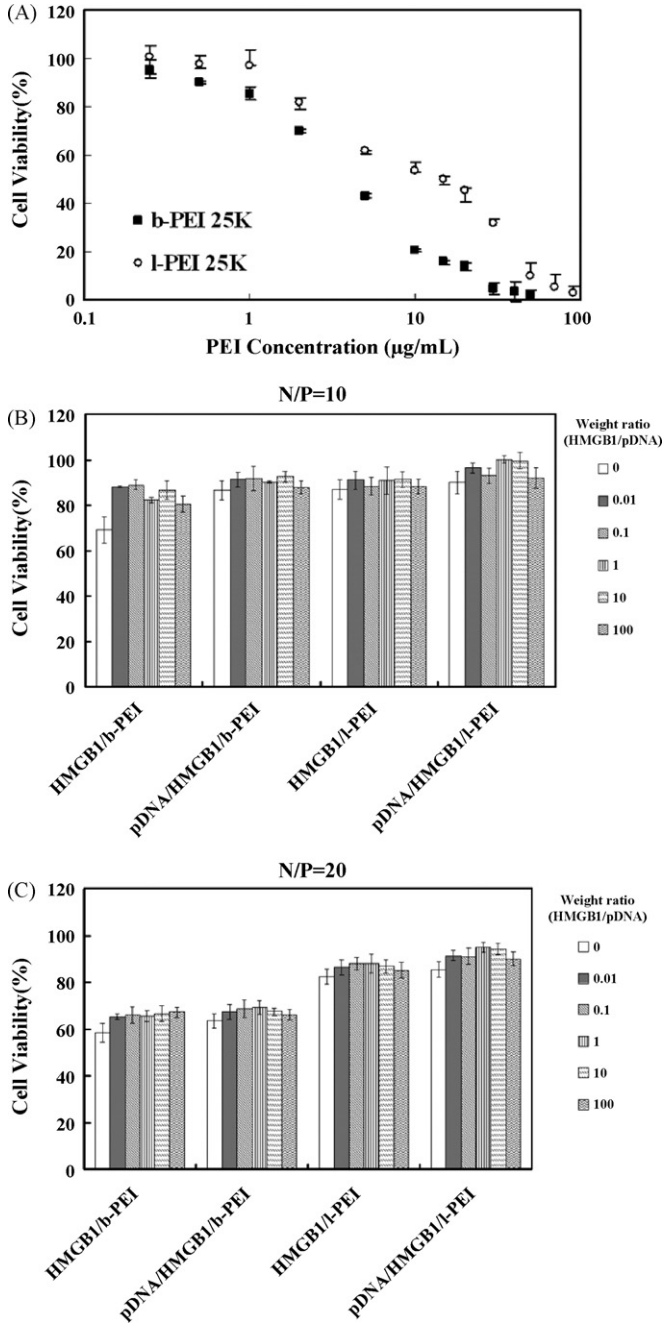


Fig. 4. Cytotoxicity studies. (A) Effect of different concentrations of PEI on the viability of HeLa cells; (B) effect of different weight ratios of HMGB1 to pDNA of ternary complexes at the N/P ratio of 10 and at the PEI concentration of 2.65 μg/mL; (C) effect of different weight ratios of HMGB1 to pDNA of ternary complexes at the N/P ratio of 20 and at the PEI concentration of 5.30 μg/mL.

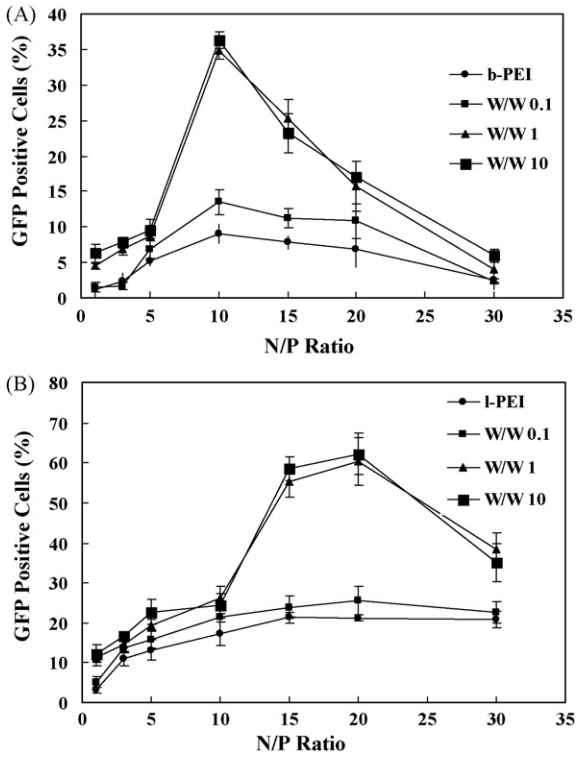


Fig. 5. Comparison of transfection with (A) pEGFP-C1/HMGB1/b-PEI ternary complexes and pEGFP-C1/b-PEI polyplexes, and (B) pEGFP-C1/HMGB1/l-PEI ternary complexes and pEGFP-C1/l-PEI polyplexes at varying N/P ratios and varying pDNA/HMGB1 ratios in HeLa cells.

was significantly higher than that of the pEGFP-C1/HMGB1/b-PEI ternary complexes. It is as expected due to the lower cytotoxicity of l-PEI. However, the cytotoxicity of pEGFP-C1/HMGB1/PEI ternary complexes did not show a significant decrease compared with the pDNA/PEI complexes without HMGB1. It might be explained in the formation of pEGFP-C1/HMGB1/PEI ternary complexes, that HMGB1 was firstly added to complexes with pEGFP-C1 and then added PEI, resulted in the PEI existing on the surface of complexes. So the complex surface of pEGFP-C1/HMGB1/PEI ternary complexes did not differ from that of pEGFP-C1/PEI complexes. These results demonstrated that as compared with PEI, HMGB1/PEI combined carriers have lower cytotoxicity, and suggested their potential as a gene delivery carrier for further applications.

3.5. In vitro transfection efficiency studies

To investigate whether HMGB1 could improve the transfection efficiencies, the pDNA/HMGB1/PEI ternary complexes were assessed for in vitro transfection efficiency in HeLa cells by green fluorescent protein (GFP) assay using pEGFP-C1 at various N/P ratios and various weight ratios of HMGB1 to pDNA as shown in Fig. 5. In parallel, polyplexes consisting of pDNA and PEI alone were prepared having the same N/P ratio and were compared to pDNA/HMGB1/PEI ternary complexes in terms of transfection efficiency. At corresponding N/P ratios, the transfection efficiencies of pDNA/HMGB1/b-PEI and pDNA/HMGB1/l-PEI ternary complexes were higher than pDNA/b-PEI polyplexes (Fig. 5A) and pDNA/l-PEI polyplexes (Fig. 5B), respectively. These results indicated that the nuclear protein HMGB1 could enhance the transfection efficiency of PEI mediated gene delivery. At the HMGB1/pDNA weight ratios of 1 and 10, and N/P ratio of 10, pDNA/HMGB1/b-PEI ternary complexes possessed remarkable transfection efficiency. Their GFP expression level was highly superior by 3.8–4.0 orders of magnitude in comparison to the transfection obtained with pDNA/b-PEI polyplexes. Furthermore at the pDNA/HMGB1 weight ratios of 1 and 10, and N/P ratio of 20, the GFP expression level of pDNA/HMGB1/l-PEI combined complexes was highly superior by 2.9 orders of magnitude compared to the transfection obtained by pDNA/l-PEI polyplexes. Meanwhile, at lower N/P ratio (such as 1, 3 and 5) both the pDNA/HMGB1/b-PEI and pDNA/HMGB1/l-PEI ternary complexes only showed slightly higher transfection activities compared to those of pDNA/PEI polyplexes. These results might suggest that at low N/P ratio, where close-to-neutral complexes were formed (Demeneix and Behr, 2005), charge-mediated cellular uptake of PEI

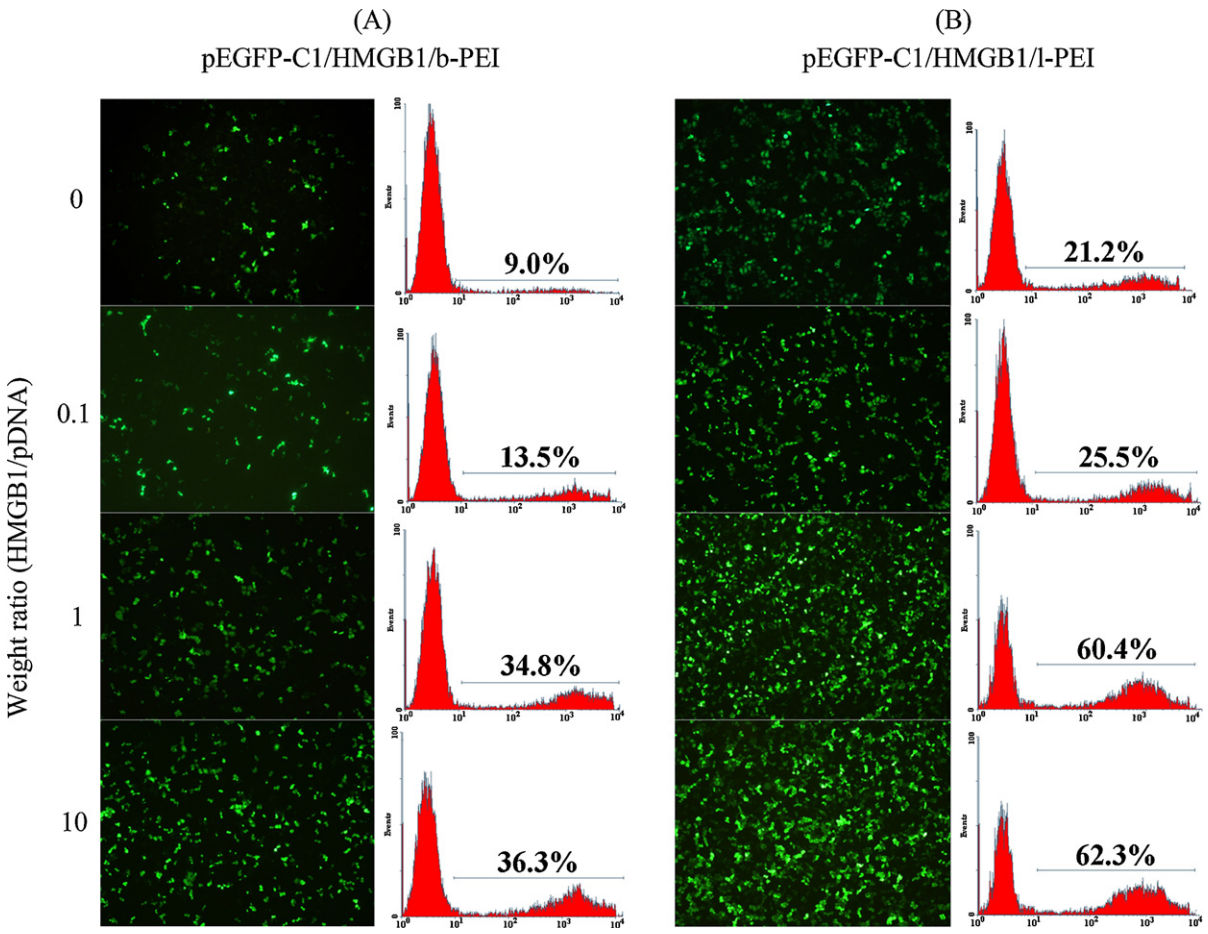


Fig. 6. Fluorescence microscopy and flow cytometry investigation of HMGB1 increasing the proportion of GFP-expressing cells. (A) pEGFP-C1/HMGB1/b-PEI ternary complexes and pEGFP-C1/b-PEI polyplexes at N/P ratio of 10, and (B) pEGFP-C1/HMGB1/l-PEI ternary complexes and pEGFP-C1/l-PEI polyplexes at N/P ratio of 20.

for pDNA/HMGB1/PEI combined complexes was hardly facilitated by electrostatic interaction with cells, resulting in less amount of pDNA/HMGB1/PEI ternary complexes into cell and low GFP expression level. However, at high N/P ratio, charge-mediated cellular uptake was highly efficient for pDNA/HMGB1/PEI ternary complexes due to the presence of net positive surface charge of the ternary complexes that allowed them to attach onto the negatively charged cell membranes. After pDNA/HMGB1/PEI ternary complexes were endocytosized and then pDNA was released into cytosome, HMGB1 could carry pDNA into nucleus, resulting in high GFP expression level (Bottger et al., 1988). Furthermore, the apparent dependence of transfection activities on N/P ratio and HMGB1/pDNA weight ratio for these pDNA ternary complexes indicated that the transfection activities were likely a reflection of the combined effects of charge-mediated cellular uptake and HMGB1 nucleus transfer.

To study the influence of HMGB1 content in the pDNA/HMGB1/PEI ternary complexes on the transfection efficiency, we prepared several pDNA/HMGB1/PEI combined complexes with different contents of HMGB1 according to the protocol described above. Fig. 6 summarizes the GFP expression level observed by fluorescent microscopy and GFP positive cells percentage measured by flow cytometry of these combined complexes with different contents of HMGB1. It should be noted that the number of green fluorescence spots in these images of pDNA/HMGB1/b-PEI combined complexes became increased, and the GFP positive cells percentage was increased from 9.0% to 36.3% with increase in the HMGB1/pDNA weight ratio from 0 to 10, respectively. Same results were obtained from pDNA/HMGB1/l-PEI ternary complexes. In the range of HMGB1/pDNA weight ratio from 0 to 10, the number of green fluorescence spots in these images of pDNA/HMGB1/l-PEI ternary complexes also appeared to increase, and the GFP positive cells percentage was from 21.2% to 62.3%. The higher GFP positive cells percentage with the increase in HMGB1 content might be attributed to the transfer of pDNA into nucleus by HMGB1.

4. Conclusion

This work described the novel pDNA/HMGB1/PEI ternary complexes for gene delivery. These ternary complexes were comprised of a cationic polymer PEI, a nuclear protein HMGB1, and pDNA. We demonstrated that the ternary complexes formed via the electrostatic interactions between pDNA, HMGB1 and PEI. These pDNA/HMGB1/PEI ternary complexes were able to improve transfection activities as compared to the pDNA/PEI polyplexes. The ternary complexes developed in this work possessed the potential to serve as a platform for developing high efficient non-viral carriers medicated gene delivery.

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