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Insights into the mechanism of magnetofection using MNPs-PEI/pDNA/free PEI magnetofectins

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ABSTRACT

Magnetofection is an efficient new physical gene transfection technology. Despite its effective gene delivery capability, till now relatively little work has been conducted on the mechanism of magnetofection, especially the intracellular fates of the components of magnetofectins and their effects on magnetofection. In this study, we investigated the mechanism of magnetofection using magnetofectins that were prepared via electrostatic self-assembly of the three components: polyethyleneimine (PEI)-coated magnetic nanoparticles (MNPs-PEI), plasmid DNA (pDNA) and PEI in the free form (free PEI). TEM observation and agarose gel electrophoresis assays have indicated MNPs play the role of driving magnetofectins to the cell surface without entering into the nucleus. Confocal microscopic tracking of fluorescence-labeled PEI has shown that the free PEI (green) can be found in the nucleus but almost all of the MNPs-PEI (red) are confined in the cytoplasm in COS-7 cells 30 min post-transfection or in SPC-A1 cells 90 min post-transfection, implying that the pDNA/PEI complex must separate from MNPs-PEI before entering into the nucleus. In addition, reporter gene assays showed the magnetofectins, in which the free PEI was absent, failed to transfect SPC-A1 or COS-7 cell lines; and there was an optimal ratio of the constituents of magnectofectins to achieve optimal transfection efficiency by balancing stable complex formation and facile release of PEI/pDNA from the complex. In summary, our findings further the knowledge of magnetofection and can be helpful for the design and preparation of gene delivery vehicles for effective magnetofection.

1. Introduction

Gene delivery is a major challenge in gene therapy. The effectiveness of gene expression is dependent on multiple factors involved in the processes of cellular uptake, intracellular release and nuclear entry (Luo and Saltzman, 2000; Mintzer and Simanek, 2009). Polyethyleneimine (PEI) is often considered the gold standard of gene transfection (Mintzer and Simanek, 2009). The DNA/PEI complexes are capable of escaping from endosomes or preventing endolysosome formation by the proton sponge effect (Bieber et al., 2002; Godbey et al., 2000; Merdan et al., 2002; Moret et al., 2001), and protecting the DNA from degradation by DNase or low pH (Bertschinger et al., 2006; Boussif et al., 1995). By complexing with and condensing the DNA, PEI facilitates the DNA transport into the nucleus (Kircheis et al., 2001; Merdan et al., 2002; Pollard et al., 1998). However, the major challenges for successful gene delivery by PEI are reducing its cytotoxicity and improving its transfection efficiency (Mintzer and Simanek, 2009). In order to improve the transfection efficiency, Plank and co-workers pioneered a new method to combine magnetic nanoparticles (MNPs) and PEI with pDNA. This technique, termed magnetofection, combines the advantages of the vector and MNPs (Mykhaylyk et al., 2007; Plank and Rosenecker, 2009; Scherer et al., 2002; Schillinger et al., 2005). In this way, the complexes of DNA and their vectors combined with MNPs (termed magnetofectins) could be concentrated to the target cells within minutes by applying a magnetic field and the transfection efficiency could be improved up to several thousand fold compared with experiments that were carried out with nonmagnetic gene vectors (Mykhaylyk et al., 2007).

The assembly of each component of the magnetofectins has significant effect on the level of gene expression (Arsianti et al., 2010). In the previous studies on the assembly of MNPs with PEI, there are two main types of combining methods, namely, covalent bounding and the electrostatic interaction. McBain and his colleagues developed a novel, efficient DNA delivery and transfection agent through covalent attachment of PEI onto the surface of MNPs (McBain et al., 2007). Similarly, Yiu and co-workers demonstrated that PEI-coated iron oxide-silica particles could also act as an efficient transfection agent (Yiu et al., 2007). Plank and others prepared magnetofectins via the electrostatic interaction between

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PEI-precoated MNPs (MNPs-PEI), pDNA and free PEI. They demonstrated as-prepared complexes could act as effective agents for gene delivery (Huth et al., 2004; Mykhaylyk et al., 2007; Scherer et al., 2002), and the free PEI had a great effect on transfection efficiency (Plank et al., 2003). However, the roles and intracellular fates of the free PEI and the coated PEI *were not clear*. Our group have used charged MNPs (both positive and negative), replacing MNPs-PEI to evaluate the gene expression; and the results have showed as-prepared magnetofectins are effective agents for magnetofection (Wang et al., 2009a,b). These facts suggest the free PEI, instead of the coated PEI on the MNPs, is crucial to magnetofection. However, the direct evidence is inadequate. To our knowledge, there is very little information in the literature regarding the effects of the combining-manners of PEI with MNPs on magnetofection efficiency, and in particular, how their intracellular fates will affect gene delivery. Therefore, studies along this line are highly needed to optimize the vector complexes design for magnetofection.

The aim of this paper is to elucidate the roles and the intracellular fates of MNPs and PEI in magnetofection and to gain a fundamental understanding on the design of *magnetofectins*. We prepared electrostatically self-assembled magnetofectins by mixing MNPs-PEI with pDNA followed by adding free PEI to the MNPs-PEI/pDNA complexes. Their magnetic responsiveness *and stability in culture medium w*ere investigated. We observed the intracellular trafficking of *magnetofectins* by TEM, and the intracellular fates of free PEI and MNPs-PEI by fluorescence labeling methods. In addition, we investigated the effect of free PEI on transfection efficiency by varying the N/P ratio (molar ratio of PEI nitrogen to DNA phosphate) using various cell lines. Our results would help gain new insights into the mechanism of magnetofection and contribute to the better design of *magnetofectins.*

2. Experimental

2.1. Materials

PEI (25 kD) and Glutaraldehyde solution were from Sigma–Aldrich. Oregon Green 488 carboxylic acid, succinimidyl ester; Rhodamine Red™-X, succinimidyl ester-5-isomer and penicillin/streptomycin were from Invitrogen. Luciferase assay substrate solution was purchased from Promega. The plasmid expressing enhanced green fluorescent protein (pEGFP-C1 plasmid, Clontech) and plasmid expressing luciferase (pGL3-control vector, Promega) were amplified in *Escherichia coli* (strain DH5α) and purified using an endotoxin-free Maxi-prep plasmid kit (Qiagen). BCA™ protein kit was from Thermo Scientific. COS-7 (SV 40 transformed kidney cells of African green monkey), SPC-A1 (Human lung adenocarcinoma cell line) and SPC-A1 (Human lung adenocarcinoma cell line) cell lines were purchased from the Cell Bank of Chinese Academy of Science. DMEM culture medium (high glucose) and fetal calf serum were obtained from Hyclone. Six-well (about 2000 mT) and 96-well (about 1200 mT) Nd–Fe–B magnetic plates were purchased from Allrun. All other reagents were obtained from Shanghai Reagent Company.

2.2. Preparation of MNPs-PEI and magnetofectins

The MNPs were prepared by standard coprecipitation of ferric and ferrous salts in alkaline solution (Jolivet et al., 2004) and the MNPs were coated with PEI according to our previous work (Wang et al., 2009a,b). MNPs-PEI were observed with TEM (*JEM-1020*) and their zeta potentials were measured by a Zetasizer 2000 (Malvern). To prepare PEI-based *magnetofectins*, 3 μg of pEGFP-C1 or pGL3-control vector was suspended in 100 μl serum-free DMEM buffer followed by adding 100 μl physiological salt solution containing 1.2 μg MNPs-PEI and incubated at room temperature for 10 min. Free PEI solution at 0.26 mg/ml (1 μg of DNA corresponds to 3 nmol of phosphate, and 1 μl of PEI corresponds to 6 nmol of amine nitrogen) was added according to the ratios of amine nitrogen of PEI to phosphate of DNA (N/P = 0, 5, 10, 20, and 30) and incubated at room temperature for another 15 min. In the case of N/P ratio of zero, the free PEI was absent, when the magnetic nano binary complexes (MNPs-PEI/pDNA) were formed. The ternary complex of MNPs-PEI/pDNA (pEGFP-C1)/free PEI (N/P = 20) was observed with TEM (*JEM-1020*).

About the above magnetofection formulation, 3 μg of pDNA and 1.2 μg of MNPs were required for each well of a 6-well plate, and 0.2 μg of pDNA and 0.15 μg of MNPs were required for each well of a 96-well plate.

2.3. Agarose gel electrophoresis

Standard agarose gel electrophoresis was performed as described in previous studies (Bertschinger et al.). To determine if pDNA in *magnetofectins* (N/P = 0, 5, 10, 20, or 30) could be precipitated in the sediment after magnetic separation for 15 min with a 6-well Nd–Fe–B magnetic plate, the level of pDNA in the supernatant was analyzed by agarose gel electrophoresis after magnetic separation. Naked pDNA was used as a control.

To determine whether the culture medium has an influence on the stability of magnetofectins, one volume of magnetofectins (N/P = 5, 10, 20 or 30; M/M = 0.4) in 0.9% physiological saline was mixed with 3 volumes of culture medium (serum-free medium or serum-supplemented medium), and then incubated at 37 °C for 2 h as described in reference (Bertschinger et al., 2006). The supernatants were analyzed by agarose gel electrophoresis after magnetic separation for 15 min with the magnetic plate. Different PEI/pDNA complexes (N/P = 5, 10, 20, or 30) were analyzed by agarose gel electrophoresis. In all cases 3 μg of pEGFP-C1 was diluted in 100 μl 0.9% physiological saline. The solution of pDNA/MNPs or PEI/pDNA was incubated for 10 min at room temperature.

2.4. Cell culture and magnetofection protocol

In all cases COS-7, and SPC-A1 cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin, and cultured at 37 °C in a humidified 5% CO_2 atmosphere. For magnetofection, cells (10^5 cells/well in a 6-well plate or 10^4 cells/well in a 96-well plate) were seeded one day before magnetofection and grown in DMEM containing 10% FBS. At approximately 80% of confluency, cells were washed with PBS and the culture medium were changed to serum-free DMEM medium (0.6 ml/well in a 6-well plate or 50 μl/well in a 96-well plate). Magnetofectins (400 μl/well in a 6-well plate or 50 μl/well in a 96-well plate) were added, followed by a 15 min incubation (37 °C, 5% CO_2) on a 6-well or 96-well magnetic plate. After magnetofection, the medium was removed from each well and replaced with DMEM medium supplemented with 10% FBS followed by further incubation.

2.5. Confocal and TEM analysis

In order to determine the different fates of coated PEI and free PEI in magnetofection, complexes were labeled fluorescently and tracked intracellularly via confocal microscopy using the methods described in elsewhere (Godbey et al., 1999). Free PEI was labeled by Oregon Green 488 carboxylic acid-succinimidyl ester and coated-PEI was labeled by Rhodamine Red-X-succinimidyl ester-5-isomer. Complexes were formed via electrostatic self-assembly by mixing labeled MNPs-PEI, pDNA and labeled free PEI.

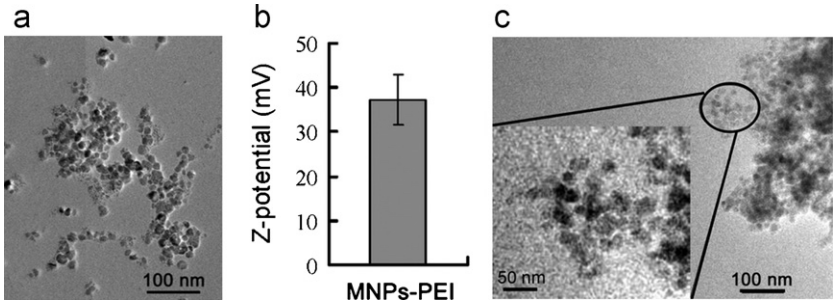

Fig. 1. Characterization of MNPs-PEI. (a) TEM image of MNPs-PEI; (b) zeta potential of MNPs-PEI; (c) TEM image of ternary complex of MNPs-PEI/pDNA/free PEI.

The mass ratio of labeled MNPs to pDNA was 0.4 and the ratio of amine nitrogen of PEI to phosphate of pDNA was 20. And then 0.4 ml of labeled complexes was added to each well of a 6-well plate with 0.6 ml serum-free medium added beforehand. The 6-well plate was placed directly over a 6-well magnetic plate for 15 min, and then the medium was changed to 2 ml of complete medium after transfection. COS-7 or SPC-A1 cells were incubated at 37 °C until confocal imaging was performed at 30 min or 90 min post-transfection. The free PEI was excited at 488 nm, with an emission spectrum which peaks at 520 nm (green). The MNPs-PEI marker was excited at 560 nm, with an emission spectrum which peaks at 580 nm (red).

To determine the intracellular localization of *magnetofectins*, COS-7 cells were observed with TEM after transfection. The magnetic field was applied for 15 min, and the culture medium was changed to complete medium. COS-7 cells were cultured for further 24 h after magnetofection without application of a magnetic field. The cell samples were collected immediately after transfection, 30 min, 2 h and 24 h post-transfection, respectively. The cells was trypsinized and washed 3 times in PBS, centrifuged and fixed with 4% glutaraldehyde solution. Cells were then post-fixed in 1% OsO_4 for 2 h at room temperature, and then dehydrated in graded ethanol. After being embedded in Epon 812 epoxy and thin sectioned using a diamond knife to a maximum thickness of 100 nm, the sections were stained with lead citrate and uranyl acetate, and observed in a Philips CM 120 transmission electron microscope.

2.6. Magnetofection analysis

COS-7 and SPC-A1 cells were transfected by different *magnetofectins* with pEGFP-C1 or pGL3-control vector pDNA. Magnetofectins were prepared at the same M/M ratio of 0.4 but with different N/P ratios (0, 5, 10, 20 or 30). In all cases, the amount of pDNA (3 or 0.2 μg for each well of a 6 or 96-well plate) and MNPs-PEI (1.2 or 0.08 μg for each well of a 6 or 96-well plate) were consistent. The amount of free PEI changed based on the N/P ratios. For N/P ratio of zero, no free PEI was used. After 48 h incubation post-magnetofection, the cells were washed with PBS, and observed using an inverted *microscope* (Leica DMI 3000B) with a 20× objective to determine the percentage of EGFP positive cells. In parallel experiments, cells were washed three times with PBS, and lysed in 100 μl lysis buffers for 20 min at room temperature. Fifty μl of the samples were measured in a Multilabel Counter (Victor-3, Perkin Elmer) that injects 100 μl luciferase assay substrate solution to each sample. The light emission parameter was set to be 10 s. Protein content of another 50 μl sample was determined by a BCA™ protein assay. Luciferase protein expression levels or activity were expressed as relative light units (RLU)/mg of protein.

3. Results

3.1. Characterization of MNPs-PEI

We synthesized MNPs-PEI as described in Section 2.1, which were well-dispersed (Fig. 1a) and positively charged (Fig. 1b). The complexing state of MNPs-PEI/pDNA/free PEI can be clearly seen in Fig. 1c, and the size of the ternary complex was about 20 nm. There were more characterizations of the surface modification can be found in our previously published article (Wang et al., 2009b).

3.2. Magnetic response of magnetofectins and their stability in culture medium

We then complexed MNPs-PEI, plasmid DNA (pDNA, pEGFP-C1) and free PEI to make the magnetofectins, and their association/dissocation under magnetic field and stability in the cell culture medium were studied by electrophoresis, as detailed in Section 2.1. Fig. 2a depicted the electrophoresis results of ternary pDNA/PEI/MNPs complexes after applying of magnetic field. When

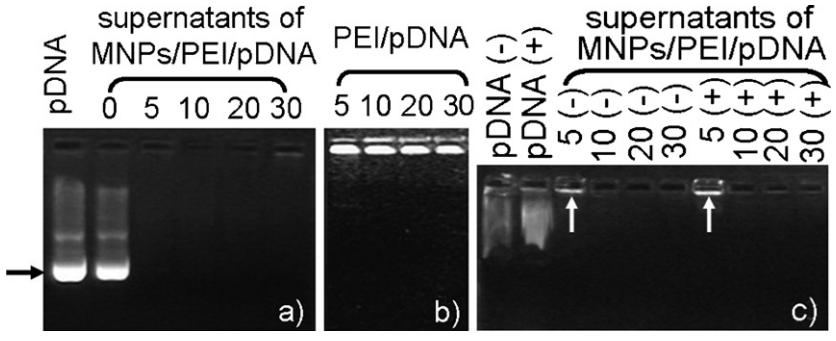

Fig. 2. Characterization of magnetic response and stability in culture medium of magnetofectins. (a) *Magnetofectins were prepared at different N/P ratios* (0, 5, 10, 20, or 30). After magnetic separation of magnetofectins, the level of pDNA in the supernatant was analyzed by electrophoresis. Naked pDNA was used as a control. The black arrow indicates the position of the supercoiled pDNA; (b) agarose gel electrophoresis of PEI/pDNA complexes, *prepared at different N/P ratios*; (c) the effects of culture medium on the stability of magnetofectins. The white arrows indicate the positions of pDNA/PEI complexes. (−) refers to serum-free culture medium and (+) refers to serum-supplemented medium.

the free PEI was present, no plasmid DNA remained in the supernatant after magnetic separation, which implied that all the pDNAs were sedimented along with the MNPs in the form of pDNA/PEI/MNPs ternary complexes (Fig. 2a). Interestingly, sufficient complexing took place when the N/P ratio was as low as 5 (Fig. 2a). Moreover, when there was no free PEI (N/P ratio = 0), the coated PEI on the MNPs (MNPs-PEI) was incapable of complexing with pDNA, almost all of which remained in the supernatant after magnetic separation (Fig. 2a). This was also corroborated by results shown in Fig. 2b where free PEI could strongly complex with pDNA and completely retard its migration under electric force.

pDNA in serum-free and serum-supplemented culture medium (Fig. 2c) were both degraded after incubation at 37 °C for 2 h. PEI/pDNA complexes only accumulated in the supernatant at the lower N/P ratios (Fig. 2c, white arrows). When the N/P was 10, 20 and 30, the pDNA was absent from the gel indicating pDNA was completely removed by magnetic separation. These results also suggested the ternary magnetofectins were stable in the culture medium with or without serum.

3.3. Cellular uptake and intracellular trafficking of magnetofectins

To follow the route of PEI and MNPs-PEI uptake into the cell, the free PEI was labeled by Oregon Green 488 (green) and coated-PEI on MNPs-PEI was labeled by Rhodamine Red (red). We then complexed the labeled-MNPs-PEI, the pDNA (pEGFP-C1) and the labeled-free PEI to prepare the magnetofectins, and their intracellular trafficking in COS-7 cells and SPC-A1cells were studied by confocal microscopy. The confocal micrographs in Fig. 3 represent the optical sections (*x*–*y* axis) of COS-7 cells after 30 min incubation (Fig. 3a–d) or SPC-A1 cells after 90 min incubation (Fig. 3e–h) with serum-complemented DMEM medium at 37 °C post-magnetofection. In Fig. 3a,b,e, and f, a fluorescent layer coinciding with the nucleus outline could be observed. These images showed distinctive patterns of free PEI and coated PEI in their nucleus entry in COS-7 cells and SPC-A1 cells. Much of free PEI was aggregated in clumps in the nucleus (Fig. 3a and e). In contrast, coated PEI (MNPs-PEI) could hardly be observed in the nucleus (Fig. 3b and f). Typically, much of coated PEI was found to be dispersed in the perinuclear region and scattered throughout the cytoplasm. We observed an unobvious overlap of the coated PEI signal (red) and the free PEI signal (green) as revealed by merging both images (yellow staining in Fig. 3c).

To further determine the intracellular localization of magnetofectins, COS-7 cells were incubated with the ternary complexes in an external magnetic field. The intracellular uptake and trafficking of magnetofectins at different time points was visualized using TEM (Fig. 4). There were a few complexes attached to the cell surface after 15 min of exposure to the magnetic field and none of them were observed in the cytoplasm (Fig. 4a, white arrows). Exposure to the magnetic field for 15 min and further incubation of the cells for 30 min led to aggregation of the complexes entered the cell (Fig. 4b, white arrows). Further incubation of the cells for 2 h post-transfection, it was found that multiple clusters of complexes were distributed throughout the cytoplasm surrounding the nucleus but none of them was observed in the nucleus (white arrows, Fig. 4c). Up to 24 h post-transfection, the complexes were still confined to the cytoplasm and none of them was found in the nucleus (Fig. 4d).

3.4. Roles of free PEI and coated PEI in magnetofection

In order to further evaluate the roles of free PEI and coated PEI in magnetofection, COS-7 and SPC-A1 cells were transfected with magnetofectins at the same w/w (weight to weight) ratio (same amount of MNPs-PEI), but different N/P ratios (different amount of free PEI). We used pEGFP-C1 for efficient GFP expression that can be easily determined by fluorescence microscopy, and pGL3-control plasmid for luciferase expression that can be readily measured in a plate reader. The transfection efficiency, i.e., the percentage of GFP-positive cell for transfection with pEGFP-C1 (Fig. 5a and c) or the luciferase activity for transfection with pGL3-control vector (Fig. 5b and d) were determined, respectively. When the free PEI was absent from the complexes (N/P = 0), neither GFP expression

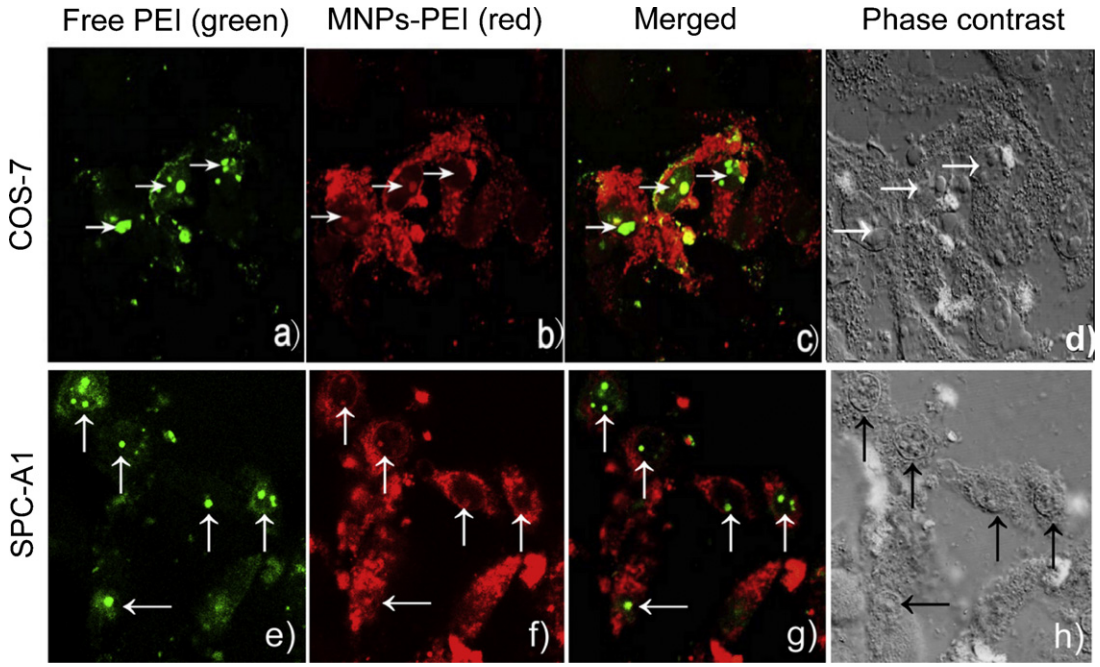

Fig. 3. Tracking of labeled magnetofectins in COS-7 cells 30 min post-transfection (a–d) or SPC-A1 cells 90 min post-transfection (e–h) by confocal microscopy. Free PEI was labeled by Oregon Green 488 (a, e, green) and coated-PEI to MNPs-PEI was labeled by Rhodamine Red (b, f, red). Magnetofectins, formed by mixing labeled PEI, labeled MNPs-PEI and pDNA, were used to transfect cells. Yellow color indicates close proximity of the green and red fluorophores (c). Arrows indicate the positions of nucleus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

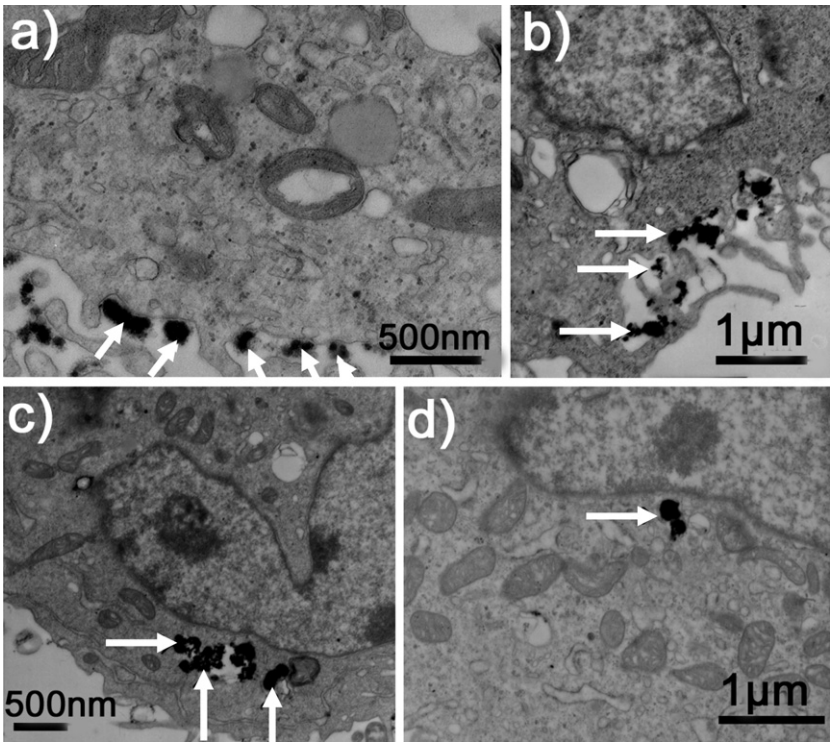

Fig. 4. TEM images displaying cellular trafficking of magnetofectins inside COS-7 cells. COS-7 cells were transfected with magnetofectins. The cells were exposed to a magnetic field for 15 min followed by further incubation up to 24 h without the application of the magnetic field. COS-7 cells were observed with TEM at 0 min (a), 30 min (b), 2 h (c) and 24 h (d) post-magnetofection. Arrows indicate magnetofectins (or MNPs-PEI).

nor luciferase activity was detected in both cell lines, which was indistinguishable from the control group that was transfected with free pDNA alone (Fig. 5, control). With the increase of free PEI, especially when the N/P ratio was above 5, the transfection rates and protein expression levels of both cell lines apparently increased. Although the optimized transfection conditions of the magnetofectins were cell line-dependant and gene-dependant as shown in Fig. 5, the magnetic ternary complexes exhibited higher gene transfer activity when compared to the binary ones where N/P ratio was over 5 (but not when the N/P ratio became to high)

4. Discussion

In magnetofection, the complexes of pDNA and their vectors combined with MNPs can be concentrated to the target cells by applying a magnetic field. In this process MNPs play the role of driving magnetofectins to cell surface (Huth et al., 2004). However, the role of MNPs after their cellular uptake and the differences between the roles of free PEI and coated PEI were not clear. In our study we have attempted to answer these unsolved but important questions.

First we prepared the magnetofectin and studied its magnetic responsiveness and stability. In our ternary magnetofectin system, pDNA may have four possible combining forms: pDNA alone, pDNA/MNPs, pDNA/PEI and pDNA/PEI/MNPs complexes. The agarose gel electrophoresis assay demonstrated that pDNA could only be efficiently sedimented by the magnetic force when it was present in the pDNA/PEI/MNPs ternary complexes. No naked pDNA was detected in supernatants when the free PEI was present as shown in Fig. 2a, implying that the stability of PEI/pDNA complexes was not influenced by combining MNPs with them. Electrophoretic mobility experimental results of magnetofectins in culture medium also showed that the complexes were stable in culture medium at N/P ratio over 10, indicating that the PEI protection of the pDNA was not influenced by MNPs-PEI. However, when the N/P ratio was 5, the electrophoretic experiment was able to visualize pDNA freed from magnetic complexes. At this N/P ratio (5 or below), the positively charged PEI was likely to be insufficient compared to the negatively charged pDNA, and all the positive charges of PEI was neutralized by negative charges of pDNA. Therefore, only part of the pDNA was combined with MNPs, and the binding force between PEI/pDNA with MNPs was not strong enough to bear the magnetic force during separation.

We then studied the cellular uptake and intracellular trafficking of magnetofectins by TEM and confocal microscopy. The cellular uptake can be regarded as a two-step process: first, a binding step on the cell membrane and second, the internalization step (Wilhelm et al., 2003). TEM observations have demonstrated that MNPs-PEI plays the role of driving magnetofectins to cell surface and do not directly draw the magnetofectins into COS-7 cells, which is in accordance with the analysis by Huth et al. (2004). The observation suggested that the magnetofectins crossed cell membranes and were internalized into cells. TEM observation also demonstrated at 24 h post-magnetofection, the electron dense MNPs containing complexes were confined to the cytoplasm and not found in the nucleus, suggesting that MNPs were incapable of traversing the nuclear envelope. Because of the lower electron density of pDNA and PEI, the pDNA and PEI associated to the MNPs-PEI could not be visualized. Thus the TEM method could not provide direct evidence for determining whether PEI/pDNA had detached from magnetofectins (MNP-PEI) before entering the nucleus. *However, for gene transfection, the pDNA indeed entered the nucleus. Thus it* must detach from MNPs while remain complexed with PEI before nuclear entry. Confocal microscopy results corroborated this conclusion. As shown in Fig. 3, almost all of the coated PEI (MNPs-PEI) was found to be in the cytoplasm in both cell lines, whereas much of the free PEI was also found in the nucleus of COS-7 cells within 30 min or SPC-A1 cells within 90 min post-magnetofection. Therefore, MNPs-PEI was likely to detach from magnetofectins before entering the nucleus. In other words, it is the free PEI but not the MNPs-PEI that could enter nucleus.

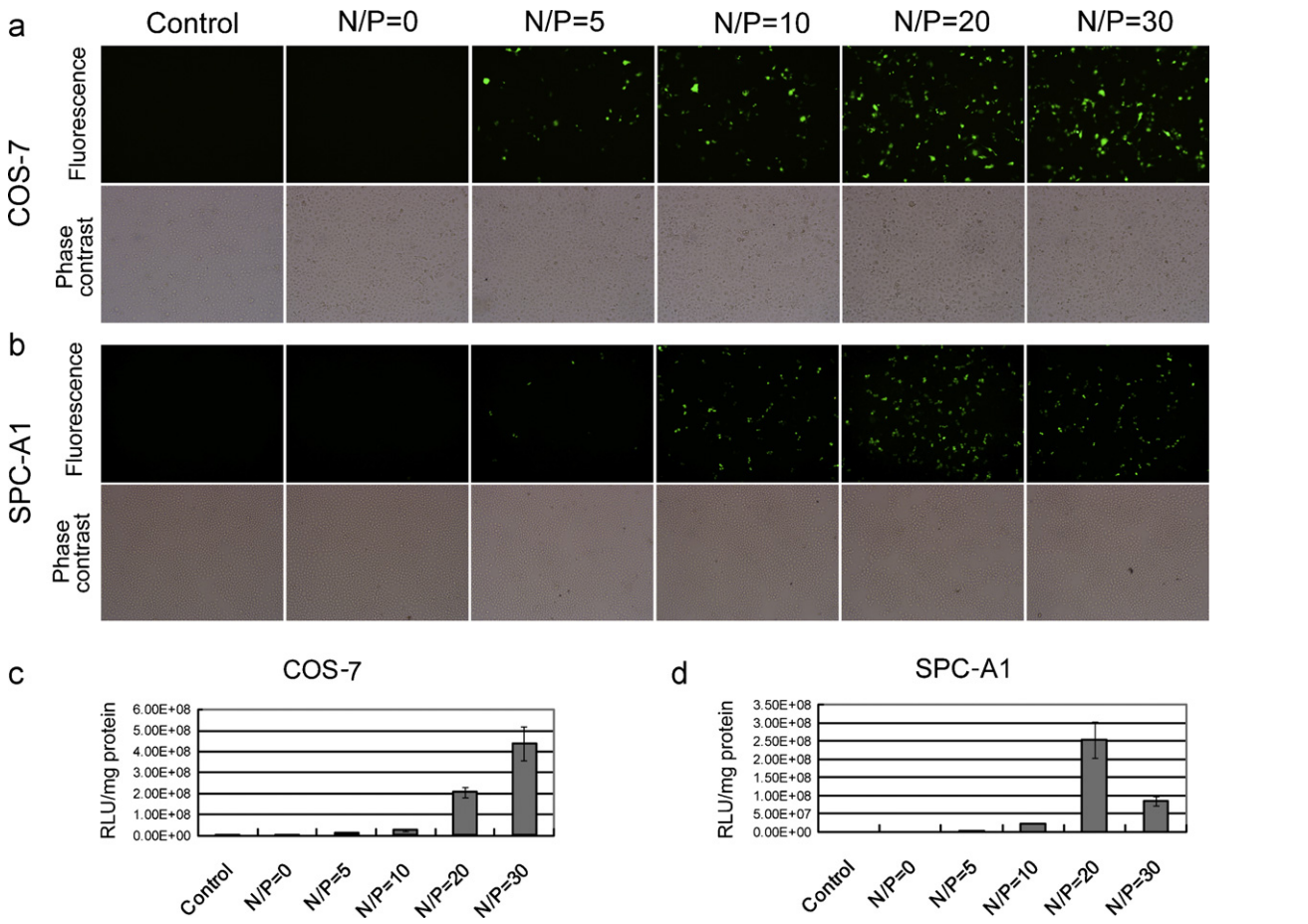

Fig. 5. Evaluation the role of free PEI in magnetofection. Figure represent qualitative analysis of pEGFP-C1 transfection to COS-7 (a) or SPC-A1 cells (c) by fluorescent microscopy, and quantitative analysis of pGL3-control transfection to COS-7 (b) or SPC-A1 cells (d) using a plate reader (mean ± SD, N = 3), respectively. Complexes were formed by mixing MNPs-PEI with pDNA at M/M ratio of 0.4 and followed by mixing PEI with pDNA at different N/P ratios (0, 5, 10, 20 and 30). In (a) and (c) 3 μg of pEGFP-C1 was used for each well of a 6-well plate, and in (b) and (d) 0.2 μg of pGL3-control vector pDNA was used for each well of a 96-well plate. Transfection of the cells with free pDNA alone was used as controls.

Furthermore, our magnetofection assays also demonstrated the pDNA, in complex with the free PEI, had entered the nucleus. We further evaluated the different roles of free PEI and coated PEI. In the transfection assays, the mass of MNPs-PEI and pDNA were the same, so the only variable was the mass of free PEI. When the free PEI was absent (N/P = 0) from the magnetofectins, neither GFP nor luciferase expression was detected. With the increase of free PEI, especially when the N/P ratio was over 5, the transfection rates and protein expression levels of both cell lines apparently increased. Therefore, it is the free PEI instead of coated PEI to the MNPs that is crucial to magnetofection. Our previous work has demonstrated it was not necessary to prepare PEI-coated MNPs for magnetofection (Wang et al., 2009a,b). In addition, we used charged MNPs replacing the MNPs-PEI in the magnetofectins to conduct transfection experiments, and the results showed that the complexes of simply charged MNPs (both positively charged and negatively charged), PEI and pDNA are effective agents for magnetofection (Wang et al., 2009a,b). For this reason, we consider that the only role of coated PEI here is providing charges to MNPs to form the magnetofectins. Not surprisingly, our transfection results were in accordance with the electrophoresis results. The N/P ratio affects the stability of the complexes (Bertschinger et al., 2006), which in turn, affects gene expression (Arsianti et al., 2010). However, the relationship between N/P ratios and gene expression, and the optimal N/P ratio need to be further elucidated.

We summarize the possible steps of magnetofection in Fig. 6 which also show the roles and the fates of magnetofectins: (1) cellular uptake, (2) intracellular release, (3) unpackaging of the MNPs/PEI–pDNA complex, and (4) nuclear entry. We prepared magnetofectins via electrostatic self-assembly by mixing MNPs-PEI with pDNA followed by adding free PEI to MNPs-PEI/pDNA complexes. Magnetofectins were manipulated and sedimented to cell membranes by magnetic force, and then internalized via endocytosis (Huth et al., 2004). Before nuclear entry, MNPs detach from pDNA/PEI complex. The free PEI, complexed with pDNA, was transported into the nucleus. The mechanisms of how MNPs detach from pDNA/PEI complex remains to be elucidated.

Based on our findings that there are some optimal ratios between the components in the magnetofectins for the transfected cells, we propose some critical factors for the design and assembly of magnetofectins that could yield successful transfection. It is important to keep the combination strength between the charged MNPs and the PEI/pDNA complexes at a moderate level, i.e., the ratio of MNPs and PEI/pDNA must be tested. As such, in the magnetofectin complexes, there will be: (i) sufficient amount of MNPs to allow controllable magnetic manipulation to pull the complex to the cells; (ii) balanced amount of free PEI not only to stabilize the formed complex, but also to allow facile release of PEI/pDNA from the ternary complex; (iii) proper amount of pDNA that is determined by the number of cells for transfection.

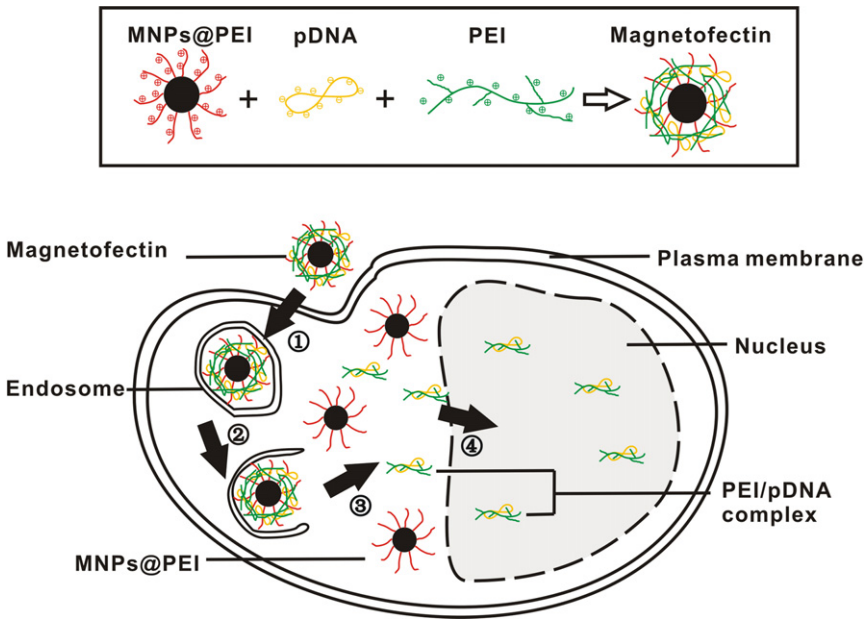

Fig. 6. Scheme of the preparation of magnetofectins and their intracellular fate. MNPs-PEI play the role of driving magnetofectins to cell surface and separate from pDNA/PEI complex before the complexes entering nucleus. Free PEI, complexed with pDNA, was transported into the nucleus. Four main steps for DNA delivery: (1) cellular uptake, (2) intracellular release, (3) unpackaging of the MNPs/PEI–pDNA complex, (4) nuclear entry.

The ultimate optimal ratio shall be determined experimentally for each cell type, but our findings provide a good guideline to start with.

5. Conclusions

In this study, we have shown that the role of the MNPs in magnetofection is to drive the complexes to the cell surface, and before entering nucleus, MNPs must detach from vector/pDNA complexes. Moreover, we have demonstrated that *it is* the *free PEI* instead of *the coated PEI* to the MNPs *that is* critical to magnetofection. It is crucial to modulate the combination strength between the charged MNPs and the PEI/pDNA complexes to achieve controllable DNA magnetic manipulation. By balancing stable complex formation versus facile release of PEI/pDNA from the complex, higher magnetic gene transfer rate can be realized in mammalian cell culture systems. Our study may be helpful for the design of *magnetofectins*, particularly those composed of MNPs and cationic polymer vector components.

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