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Assembly of Polyethylenimine-functionalized Iron Oxide Nanoparticles as Agents for DNA Transfection with Magnetofection Technique

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Various kinds of inorganic nanoparticles have been used as non-viral gene carriers. Two fundamental roles of gene carriers are to bind the DNA molecules and to protect them from enzymatic attacks after internalization into the cells. Therefore all the nanoparticles as gene carriers need to be functionalized. Lately, magnetic gene carriers incorporating PEI have been adopted to improve DNA transfection efficiency. Researchers used the PEI-coated MNPs for DNA entrapment, and found out this complex was not able to achieve efficient DNA transfection, but needed the extra free PEI to deliver the DNA to the cell nucleus. In this study, magnetic gene carriers with small size and surface modifications were prepared to explore the magnetofection process. Different methods for PEI immobilization on smaller MNPs were adopted, in order to compare their DNA binding abilities, transfection and transient gene expression efficiencies. Finally, the magnetofection process was studied with confocal microscopy and flow cytometer. These results shed some light on the mechanism of DNA magnetofection, which has not been fully understood yet.

Introduction

Gene therapy has shown great potential in treating human diseases that originate from defective genes, such as Parkinson's disease and cancers.[1,2] Efficient gene therapy depends on the delivery of therapeutic genes to cells, alternating gene expression by replacing or silencing defective genes.[3] In this process, gene or DNA carrier, or commonly called transfectant, is needed for transportation of genes construct of DNA to cellular compartment. However, although viral vectors are currently the most effective (80%-90%) method in gene delivery, safety and immunogenicity concerns limited their clinical applications.[4-6] Therefore, the use of non-viral vectors has been considered to be an alternative approach for gene delivery. Except for overcoming the major viral delivery toxicity issues,[7] they also have other advantages such as stability *in vivo*, low cost, ease of synthesis and modification.[8] Gene or DNA delivery meditated by non-viral route into the cells is referred as gene or DNA transfection.[9] Despite the biocompatibility and ease of synthesis, transfectants could be toxic and do not deliver nucleic acids into the cells very effectively (20%-30%).[10]

Magnetic nanoparticles (MNPs) have attracted attentions of researchers, mainly because of their low toxicity, ability to be biologically degraded, low cost of production, ease of surface modification, and the potential for magnetic resonance imaging (MRI). Also, external magnetic force can be applied when using magnetic materials[10] to enhance gene transfection efficiency. Normally, the transfection process mediated by magnetic nanoparticles is called "magnetofection". A lot of researchers use more than two non-viral components to combine plasmid DNA (pDNA). The most common design of nanotransfectants is coating MNPs with cationic synthetic polymers, leading to their high cationic charge density at physiological pH. Thus, magnetic vectors can form complex with anionic pDNA and interact with negatively charged cell membrane.[11] This interaction can lead to endocytosis and entrapment of nanovectors within cellular endosomal vesicles.[12] After that, the nanovectors were released into cytoplasm through a so-called "endosomal escape" process before expression.[13] Non-viral magnetic vector incorporating MNPs and polyethylenemine, PEI, has been adopted to improve transfection efficiency.[14-16]

Except the use as gene or DNA transfectants, MNPs can also be used as drug carrier, which is a new strategy for the specific delivery of drugs to targeted cells. To date, different methods for MNP surface modification have already been well developed, aiming to reduce the toxicity, increase water solubility and permeability of cells.[17-19] For example, cyclodextrin, poly ethylene glycol (PEG) and folic acid can be used to modify MNPs to enhance biocompatibility and cellular uptake of nanomaterials. However, for drug delivery, structural morphology plays a more important role than surface modification. Mesoporous,[20] hollow[21, 22] and core-shell[23] structures have already been adopted to increase drug loading

amount and high magnetic saturations. MNPs that are too small may not be suitable for efficient cellular uptake, for they are difficult to be separated or controlled in blood by moderate magnetic field.[24] Thus, in order to be applied in both gene and drug delivery, MNPs should have high surface area, water solubility, biocompatibility and rapid magnetic response.

In this study, magnetic iron oxide nanoparticles were synthesized and modified with PEI. Despite the commonly used method of coating PEI on MNPs, a novel treatment was introduced as conjugating PEI to MNPs. The ternary complex for magnetofection was assembled with modified MNPs, pDNA and free PEI. The results indicated that PEI-conjugated MNPs had higher DNA binding ability and gene transfection efficiency in both HeLa and HepG2 cell lines. To explain it, the magnetofection process was studied with confocal microscopy and flow cytometer. Both the DNA condensation ability with different amount of free PEI and cellular uptake of the magnetic complex were explored. These results suggested that different N/P ratios affected the formation of the complex, the cellular uptake and gene expressions in the DNA transfected cells.

Experimental

Materials

Ferric chloride hexahydrate ($FeCl_3$•$6H_2O$), sodium hydroxide (NaOH), sodium citrate, PEI (25 kD, branched), Oregon Green 488 carboxylic acid, succinimidyl ester; Rhodamine Red™ –X succinimidyl ester 5-isomer; and Hoechst 33342, Trihydrochloride were purchased from Sigma-Aldrich. Dual-luciferase assay substrate solution was from Promega. The pEGFP-N1 plasmid (Clontech) expressing enhanced green fluorescent and pRL-CMV (Clontech) expressed renilla luciferase were amplified in *Escherichia coli* (strain DH 5α) and purified using an endotoxin-free Medi-prep plasmid kit (Qiagen). Plasmid pRL-CMV was covalently labeled by fluorophore Cy5 using a Label IT nucleic acid labeling kit (Mirus). BCA™ protein kit was purchased from Thermo Scientific. HepG2 (Human hepatocellular liver carcinoma) and HeLa (human cervical adenocarcinoma) cell lines were purchased from American Tissue Culture Collection (ATCC). The fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and penicillin–streptomycin were obtained from GIBCO.

Characterizations

The size and morphology of MNPs were examined with transmission electron microscopy (TEM) using a Tecnai™ Spirit microscope (FEI, Japan). A commercial Portable Particle Size Analyzer (Jianke, China) was used to characterize the hydrodynamic diameter of MNPs. The PEI content after both conjugating and coating modifications was measured by using thermogravimetric analyszer (TGA) with a heating rate of 10°C/min in a flowing nitrogen atmosphere using TGA6 instrument (Perkin-Elmer, USA).

Methods

Preparation of MNP-PEI and MNP/PEI. Magnetic nanoparticles (MNPs) were synthesized by standard co-precipitation of ferric and ferrous salts in alkaline solution with addition of sodium citrate.[25] The 25 kDa PEI covalently boned MNPs were synthesized using 1,1'-carbonyldiimidazole as the linker. Typically, the as-prepared MNPs (100 mg) were first dispersed homogenously in distilled DMSO using ultrasound sonicator. They were activated by adding CDI (10 mg) and triethylamine (Et_3N) as the catalyst with stirring for 30 min, and the resulting MNP-CDI was washed with the help of magnet and stored at 4°C. PEI (100 mg) was dissolved in DMSO with mechanical stirring. The described MNP-CDI in DMSO and Et_3N were added drop by drop to the PEI solution over 1.5 h with stirring under nitrogen atmosphere, followed by reaction for 24 h. After reaction, the resulting MNP-PEI was washed with deionized water at least five times and dispersed in aqueous solutions. To compare the MNPs covalently linked and surface coated with 25 kDa PEI (MNP-PEI and MNP/PEI, respectively), MNP/PEI nanoparticles was prepared according to previous work.[26] Generally, the MNP dispersion was mixed with 5% (m/m) PEI solution in an orbital shaker overnight. Then the mixture was neutralized with 0.5 mol/L HCl solution and denoted as MNP-PEI.

Acidification of MNP-PEI and MNP/PEI. Before forming magnetofection assembly, MNP-PEI and MNP/PEI nanoparticles were first pre-acidified in order to entrap and protect the DNA molecules.[27] Briefly, the pH of the as-prepared dispersions were adjusted to 2 using 0.5 mol/L HCl and kept at room temperature for 10 min. Afterward, the pH was increased to 5 to reduce aggregation of MNP-PEI and MNP/PEI.

Preparation of PEI-based magnetofectins. The assemblies of MNP-PEI or MNP/PEI, plasmid DNA and free 25kDa PEI were prepared as follows: for the gene transfection in each hole using the 24-well plate, either 0.5 μg pEGFP-N1 or pRL-CMV control vector was suspended in 20 μL serum-free DMEM, followed by addition of 5 μg nanoparticles in the same 20 μL buffer solution. After incubation for 20 min at room temperature, free PEI solution at 0.26 mg/mL was added to the various N/P ratios (the ratio of amine nitrogen in PEI to phosphate in DNA) at 0, 5, 10, 20 and 30 with incubation for another 20 min.

Agarose gel electrophoresis and DNA condensation study. To determine whether the plasmid DNA was precipitated with magnetic nanoparticles or condensed by the free PEI, 0.8% agarose gel electrophoresis was performed. Magnetic magnetofectins for gene transfection were prepared by varying the mixing order of three components: Fe_3O_4-PEI, plasmid DNA (pEGFP-N1) and extra free PEI. Plasmid DNA construct pRL-CMV (500 ng) was first mixed with Fe_3O_4-PEI in PBS (0.01M, pH=7.4) and incubated for 20 min. Then, free PEI with N/P ratio of 0 to 30 in PBS was added to the mixture and incubated for another 20 min. After that, a magnet was put under the assembly solution for 15 min. Part of the supernatants were taken out and mixed with loading buffer before applying electrophoresis under 120 V for 40 min. To determine whether the DNA was condensed in the ternary complex, the concentration of DNA in the supernatant was determined with UV-Visable spectrophotometer (NanoDrop 2000c, Thermo Scientific). Naked plasmid DNA was used as a control. The DNA leftover in the supernatant was interpreted as the percentage of the control one.

Cell culture and *in vitro* magnetofection. In all cases, HepG2 and HeLa cells were grown at 37 °C, 5% CO_2 in DMEM supplemented with 10% FBS, penicillin at 100 units/mL and streptomycin at 100 μg/mL. For magnetofection, cells were seeded in a 24-well plate at an initial density of 2×10^4 cells/well 24 h before magnetofection and grown in DMEM containing 10% FBS. The medium was removed and replaced with serum-free DMEM medium, each containing the as-prepared magnetofectins with different N/P ratios. The cell culture plates were then put on a magnetic plate for 1 h to facilitate

the MNPs to the adhered cells. Meanwhile, the same procedures were conducted in the absence of magnetic field. Six hours after the magnetofection, the medium was removed and replaced with DMEM supplemented with 10% FBS. For control groups, Lipofectamine 2000™ was performed following the manufacturer's protocols, and the polyplex of plasmid DNA and free PEI with an N/P=10 was formed according to previous work.[28]

GFP reporter expression and Luciferase assay. After 48h incubation after magnetofection, the cell culture medium was removed and washed with PBS twice. The cells were detached using trypsin-EDTA and harvested for analysis of GFP expression by flow cytometry (FACSCanto, BD Biosciences, USA), with 10^4 cells being examined for each sample. Luciferase assay was performed according to the recommended protocol (Dual-Luciferase® Reporter Assay System Protocol, Promega, USA). Briefly, cells were washed with PBS twice and lysed with the addition of 100 μL lysis buffer. Part of cell lysates from each well was used for measuring the luciferase activity with a GloMax 96 microplate luminometer (Promega, USA). The protein assay kit (Bio-Rad, USA) was used to determine the protein concentrations of all the lysates for normalization.

Confocal microscopy. To track the intracellular fate of the different component in the magnetofection process, plasmid DNA, MNP and free PEI were labeled with different fluorescent molecules and tracked with the confocal microscopy as mentioned in previous work.[29] Plasmid pRL-CMV was covalently labeled to Cy5 using the Label IT kit (Mirus, USA) according to the manufacturer's instructions. Coated PEI and covalently bonded PEI were labeled with Rhodamine Red-X-succinimidyl ester-5-isoner, and free PEI was labeled with Oregon Green 488 carboxylic acid-succinimidyl ester. About 6×10^5 cells were seeded in a confocal dish 34.3 mm × 9.3 mm (SPL, Korea). After 24 h, the cellular membrane was stained with Hoechst 33342 for 15 min and then washed with PBS three times. After the formation of magnetofectins as described previously, the complex was added in the confocal dish containing serum-free DMEM. Live cell imaging was performed using Olympus FV1000-IX81 Confocal Microscope equipped with an INU stage-top incubator (Tokai Hit, Japan). HepG2 cells were incubated at 37°C until confocal imaging was performed at 1 h to 6 h post-magnetofection. Hochest 33342, Rhodamine Red-X, Oregon Green and Cy 5 were excited at 405 nm, 488 nm, 532 nm and 635 nm, and the corresponding emission spectrum were 425-475 nm, 500-530 nm, 560 -660 nm and 650-750 nm, respectively. All the spectral data was analyzed by using the FV10-ASW software.

Flow cytometer analysis. To explore the effects of magnetic field and free PEI on cellular uptake of the plasmid, magnetic nanoparticles and free PEI during the magnetofection process, the three components were labeled differently and added to the cells in serum-free DMEM as described above. After incubation at 37°C and 5 % CO_2 for different timed, cells were harvested. They were first washed twice with phosphate buffered saline (PBS) containing 0.001 % SDS, and then rinsed with PBS once. After that, cells were detached with 0.05 % trypsin/EDTA supplemented with 20 mM sodium azide to prevent further endocytosis.[30] After final wash with pipetting, cells were then suspended in ice-cold PBS containing 2% FBS. Cellular uptake of different components was assayed by flow cytometry using a FacsCanto flow cytometer (Beckman Coulter, USA). The fluorophores Oregon Green and Rhodamine Red-X were excited at 488 nm, and Cy 5 at 635 nm, with corresponding emissions were at 530/30, 585/42 and 660/20 nm, respectively. To exclude dead cells and debris, the samples were gated by forward/side scattering, and 10^4 events per sample were collected. Experiments were repeated in triplicates. All the data were analyzed with software Flowjo 7.6.1.

Results and discussion

Characterization of MNP-PEI and MNP/PEI

The TEM images shown in Fig. 1 indicated the size and morphology of MNPs before and after PEI modification. The three kinds of MNPs all had an average diameter about 10 nm. After co-precipitation with sodium citrate, the magnetic nanoparticles were highly negatively charged as – 46.85mV, while both MNP-PEI and MNP/PEI had positive zeta potentials as + 19.18 mV and + 23.32 mV. The negatively charged MNPs have good solubility in water due to the mutual electrostatic repulsion and also attract PEI. For MNP-PEI and MNP/PEI, PEI modification made the formation of nanoparticle aggregates, which is consistent with the previous work.[26]

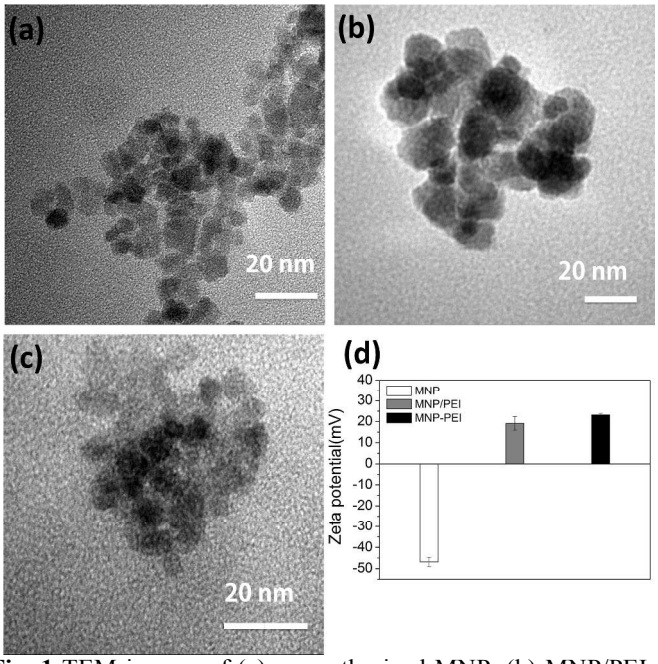

Fig. 1 TEM images of (a) as synthesized MNP; (b) MNP/PEI; (c) MNP-PEI; (d) Zeta potentials of three MNP vectors in PBS buffer (pH=7.4).

The hydrodynamic diameter of MNPs in PBS solution was 250 nm, which was larger than the observed sizes in TEM images. It was due to the effect of sodium citrate as the stabilizing agent and the interactions between nanoparticles and solvent. After PEI coating, the PEI adsorption on the MNPs made the aggregation of nanoparticles.[31] Compared to MNP/PEI, the PEI conjugated MNP (MNP-PEI) was smaller. According to previous work, the increase of PEI on the surface could reduce the hydrodynamic radius.[26, 32] Hence the smaller hydrodynamic diameter of MNP-PEI was due to the higher PEI content, which could be supported by the TGA result.

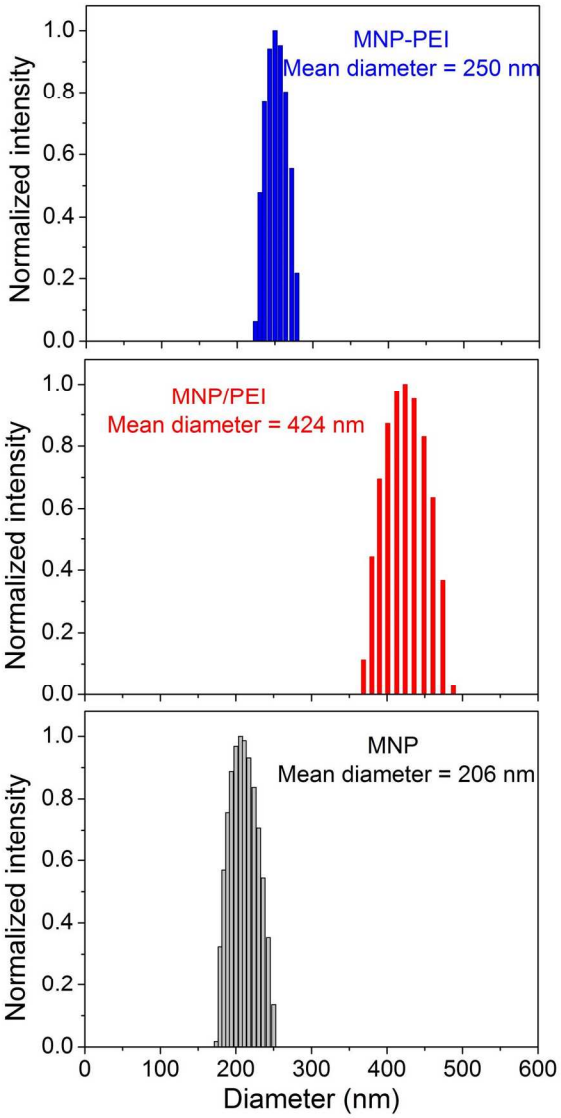

Fig. 2 The distribution of magnetic nanoparticle diameter in PBS (pH=7.4) detected by Dynamic Laser Scattering (DLS).

TGA was used to determine the amount of PEI after modifications as shown in Fig. 3. Both MNP-PEI and MNP/PEI had much larger weight loss than MNP, which indicates successful PEI modification. In addition, there was about 5.3% more PEI in MNP-PEI than MNP/PEI, suggesting PEI covalently bonded magnetic nanoparticles may have stronger DNA condensing ability. It also explained the higher zeta potential value of and smaller hydrodynamic radius of MNP-PEI compared to MNP/PEI.

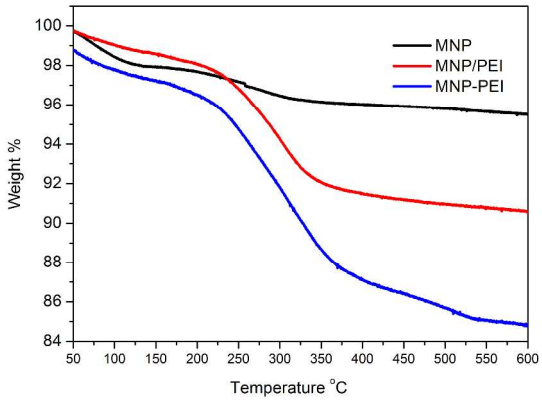

Fig. 3 TGA profile of MNP, MNP/PEI and MNP-PEI, with the weight loss of 3.0%, 8.5% and 13.8%.

Plasmid DNA binding of magnetofectins

Fig. 4 depicted whether there was unbound DNA after precipitation of the complex with applied magnetic field. When N/P =0, *i.e.* in the absence of free PEI, MNP-PEI and MNP/PEI were incapable of condensing all the plasmid DNA. MNP-PEI, however, exhibited better binding ability than MNP/PEI, which could be reflected from the fluorescence intensity under UV-vis light. It was consistent with the TGA result, for it was obvious that the larger amount of PEI on the MNP surface could help bind more DNA. In the presence of free PEI, the percentage of precipitated DNA increased with the increase of N/P ratio. With further increase of N/P ratio (N/P> 4), all plasmid DNA could be precipitated with magnetic nanoparticles in the ternary form of MNP-PEI/pDNA/PEI or MNP/PEI/pDNA/PEI. This was also corroborated by the previous work.[27, 33]

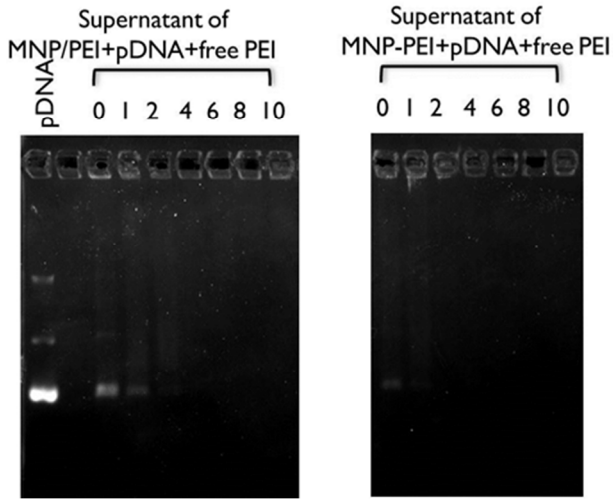

Fig. 4 Magnetofectins were prepared at different N/P ratios. After magnetic separation, the amount of plasmid DNA in the supernatant was analyzed.

After forming the ternary complex and magnetic separation, the amount of unattached PEI molecules was also analyzed. The free PEI was conjugated to FITC according to previous report.[34] Then the FITC fluorescence intensity of the supernatant was read with a microplate reader (SpectraMax).

As shown in Fig. 5, both MNP/PEI and MNP-PEI could successfully form solid ternary form of MNP-PEI/pDNA/PEI or MNP/PEI/pDNA/PEI at N/P ratios from 0 to 10. The fluorescence intensity of FITC was higher at larger N/P ratios (10-40), suggesting some of the free PEI were detached from the magnetic nanostructures. In the layer-by-layer preparation of the complex, DNA was first adsorbed on the MNPs, and then free PEI would cover the outside layer. After DNA adsorption, the surface charge of nanostructures should be highly negative due to the phosphate backbone of DNA. The amount of free PEI adsorbed on the outer layer was determined by the electrostatic interactions between DNA layer and free PEI. So excessive amount of PEI (N/P>10) could not be attached on the magnetic nanostructures but existed in the supernatant.

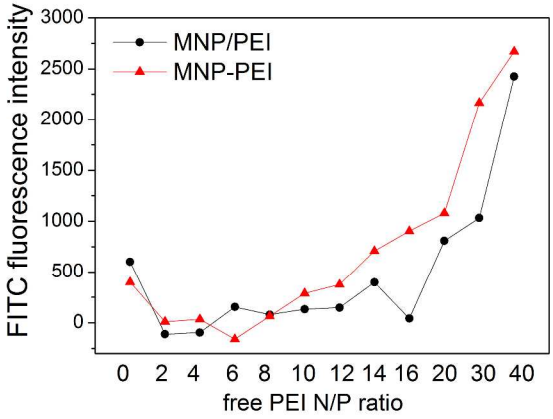

Fig. 5 Ternary magnetofectins were prepared at different N/P ratios. After magnetic separation, part of the supernatant was taken out for analyzing the amount of PEI-FITC.

According to the results of plasmid DNA binding, the as-formed ternary complex of PEI/pDNA/PEI or MNP/PEI/pDNA/PEI at different N/P ratios could be summarized in Fig. 6. MNP/PEI or MNP-PEI (5 μg) could not bind all the 500 ng plasmid DNA but need extra amount of free PEI (N/P>4) to condense all. However, more PEI did not favor the formation of ternary complex. When N/P ratio was larger than 10, some of the PEI could not bind to the nanoparticles but existed as free chains in solution. Finally, the as-formed assembly of magnetofectins was added into the culture medium, with a magnet under the culture dish to attract the magnetic complex to the cell surface. This technique, known as magnetofection, could shorten the time for transfection and enhance the amount of internalized DNA.

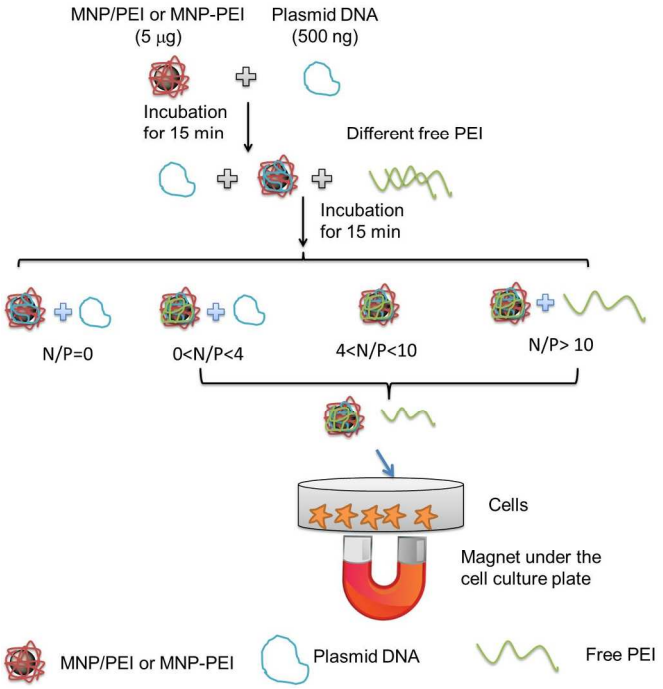

Fig. 6 Schematic illustration of ternary magnetofectins composed of MNP/PEI or MNP-PEI, plasmid DNA and free PEI at different N/P ratios, and the magnetofection procedure.

Effect of Magnetofection on GFP and luciferase expression in HepG2 and HeLa cell lines

To examine the effect of both free PEI and magnetic field on transfection, MNP-PEI and MNP/PEI were mixed with both EGFP and luciferase reporter construct and used to transfect both the human hepatocellular liver carcinoma (HepG2) and the human cervical adenocarcinoma (HeLa) cell lines. To compare the transfection efficiency, the commercially available standard Lipofectamine 2000™ and PEI (N/P=10) were used as control transfection reagents. The results are shown in Fig. 7.

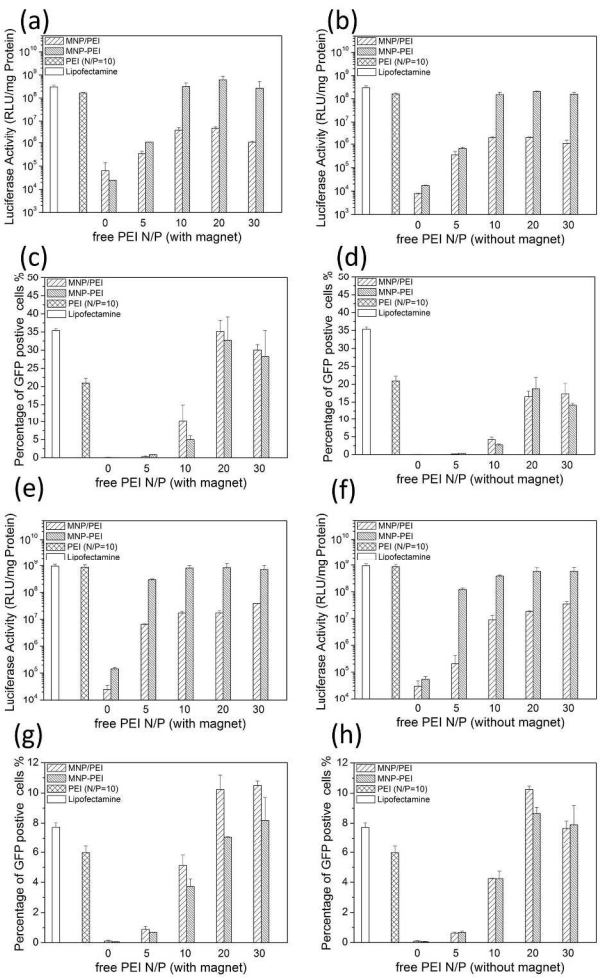

Fig. 7 Evaluation of the roles of free PEI and magnetic field in magnetofection to HepG2 ((a)-(d)) and HeLa ((e)-(h)) cells using luciferase assay: (a) (e) with magnetic field; (b) (f) without magnetic field, and the EGFP expression as in percentage of GFP-positive cells (c) (g) with magnetic field; (d) (h) without magnetic field.

The expression of luciferase and GFP in both cells were successful, with the optimal efficiency equivalent or higher than the standard transfectants. In addition, the efficiency had similar tendency at different N/P ratios. In the transfection assays, in the absence of free PEI (N/P=0), no GFP expression was detected and luciferase expression was very low. With the addition of free PEI, both transfection and gene expression levels were apparently elevated, with N/P=20 being the optimal ratio. In the electrophoresis results, more PEI (N/P>4) could help to precipitate all the plasmid DNA with the magnetic nanoparticles. In the transfection studies with different N/P ratio (5 to 30), the only difference in the composition of the ternary magnetofectin is the amount of PEI. So the free PEI not only provided charges to form the ternary magnetofectin in this study, but also possibly acted as delivery vesicles and protecting agents.[8, 32]

Since the effect of N/P ratio was similar in both cell lines, HepG2 cells could be taken as an example in analyzing the results. As indicated in Fig. 7 (a) and 7 (b), MNP-PEI exhibited increased luciferase expression efficiency than that of MNP/PEI, while in Fig. 7 (c) and 7 (d), the difference in GFP expression of the two magnetofection reagents was not obvious. Moreover, the percentage of pEGFP transfected cells noticeably increased under magnetic field, which was similar in the luciferase expression. When N/P was 20, the transfection efficiencies were no worse than standard Lipofectamine transfection, especially in the luciferase expression. It is possible that the applied magnetic field could help attract more as-formed magnetic ternary complex on the cell surface, rendering to the uptake of more magnetofectins. Therefore, we can conclude that (1) it is the free PEI rather than the PEI on the surface of magnetic nanoparticles that plays the crucial role in the transfection process; (2) however with increasing free PEI, the transfection efficiency for both GFP and luciferase expression with magnetofectins was more effective under magnetic field; (3) MNP-PEI could help increase the luciferase efficiency than MNP/PEI, possibly due to the higher amount of PEI on the surface and enhanced stability.

Intracellular trafficking of magnetofectin pDNA/MNP-PEI/PEI

To better evaluate the gene delivery process of the ternary complex pDNA/MNP-PEI/PEI, the three components were labeled with different fluorescent dyes, and the intracellular trafficking of different components in magnetofectins were studied by confocal microscopy at different time points. Fig. 5 represents the HepG2 cells after 1 h (Fig. 5 a-f) and 6 h (Fig. g-l) incubation at 37°C post magnetofection. Those images indicated the different intracellular fate of MNP/PEI and free PEI in HepG2 cells. At 1 h post-transfection, as indicated by merging two pictures (yellow staining in Fig (f)), the overlap of MNP-PEI signal (red) and some of the PEI (green) could confirm the efficiency of the magnetofection as described earlier. The other PEI, on the other hand, may be detached from the complex. The MNP-PEI was found near the cell membrane, while some the free PEI locates in the perinuclear region and part of them were already in the nucleus.

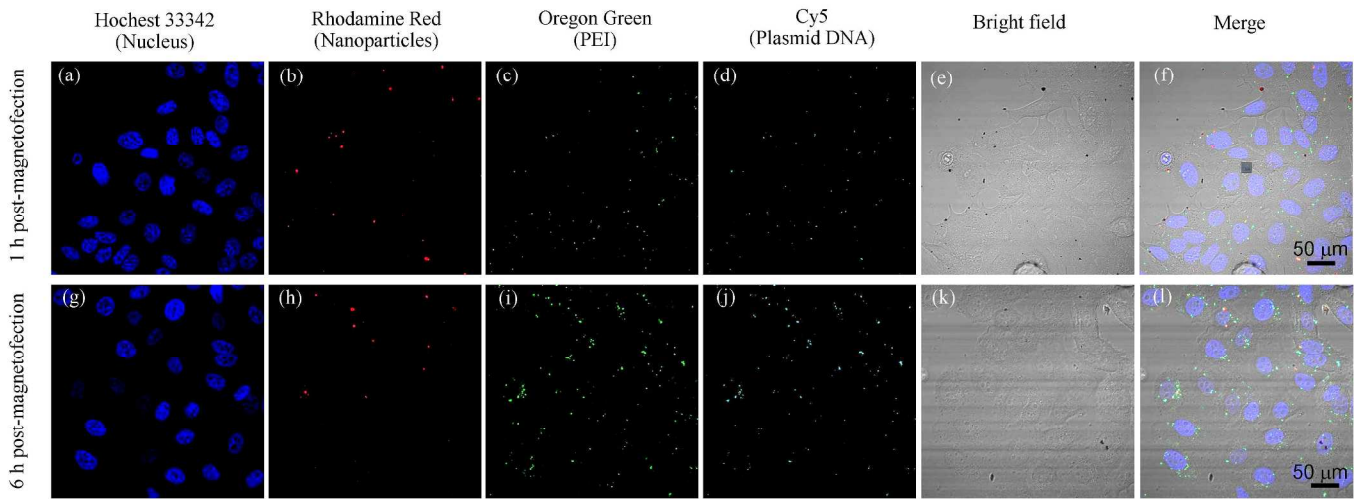

Fig. 8 Tracking of different components in HepG2 cells 1 h post-magnetofection (a to f) and 6 h post-magnetofection (g to l). Nucleus was labeled by Hochest 33342 (a, g), MNP-PEI labeled by Rhodamine Red (b, h), free PEI labeled by Oregon Green 488 (c, i), and plasmid DNA by Cy 5 (d, j). Merged images with bright field were also shown (f, l).

After 6 h incubation post-transfection, the MNP-PEI signal (red) could be found at both near membrane and perinuclear region, while most of the PEI signal (green) was dispersed in the nucleus. This study was consistent with previous studies, in none of which the magnetic nanoparticles were observed in the nucleus.[3, 33, 36, 37] Therefore in the transfection process, it is the free PEI that complexes with plasmid DNA and then delivers it into the nucleus. Hence we could summarize the possible magnetofection process in the following sequence: (1) plasmid DNA, MNP-PEI and free PEI assembled as the ternary complex and were drawn to the surface of the HepG2 cells under magnetic field; (2) After cellular uptake[3, 35, 36] MNP-PEI detached from the ternary complex in the cytoplasm, and PEI/DNA complex formed and entered the nucleus.

Cell viability and cellular uptake of pDNA and free PEI post-magnetofection

In the transfection assay, the optimal N/P ratio was 20 in the magnetofection process. To investigate the critical factors for efficient transfections, both cell toxicity and cell uptake of the magnetofectin were studied. As shown in Fig. 7, at 24 h post-magnetofection, the toxicity of the magnetofectins was related to the amount of free PEI in both cell lines. The toxicity of MNP/PEI nanoparticles was similar to only PEI and less toxic than MNP-PEI nanoparticles. The larger N/P ratio results in the higher cytotoxicity, probably due to the toxic effects caused by the PEI.[29] Since both the PEI content and surface zeta potential in MNP-PEI was higher than those in MNP/PEI, MNP-PEI NPs were more toxic. In both cells lines, all the nanoparticles caused more than 30% cell mortality at the optimal N/P ratio of 20, which was similar to that of Lipofectamine. The results suggested that the cytotoxicity of this magnetofection system was acceptable.

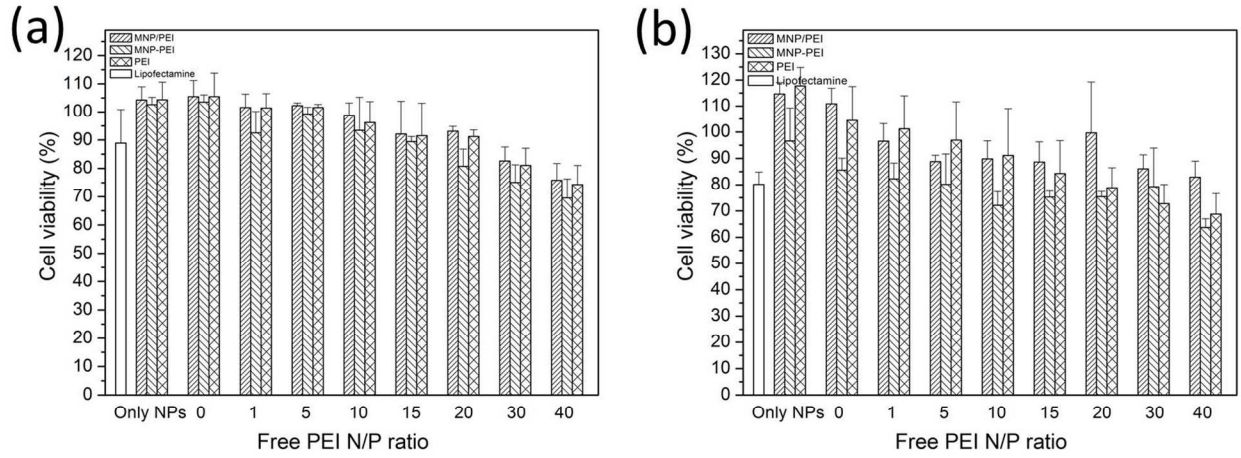

Fig. 9 Viability of (a) HepG2 and (b) HeLa cells after exposed to magnetofectins composed of MNP/PEI and MNP-PEI with free PEI at different N/P ratios for 24 h.

The cell uptake of plasmid pRL-CMV and free PEI 1 h post-magnetofection was indicated in Fig. 10. Two characteristics, namely, the mean fluorescence of Cy5-DNA in Cy5-positive cells and the mean fluorescence of Oregon green-PEI in Oregon

green-positive cells were used to indicate the uptake of the two components. Under magnetic field, HepG2 cells could uptake more than one fold of plasmid DNA than without magnetic field at the same amount of magnetic nanoparticles. This could again confirm the magnet-assisted uptake of nanoparticles, as mentioned in the magnetofection process. The magnetic field could draw more magnetic ternary complex onto the cell surface and lead to higher uptake of the DNA. MNP-PEI could help deliver a little more DNA than MNP/PEI into the cells at N/P ratio 5-10, due to the differences in PEI content and DNA binding ability. When N/P was larger than 20, the DNA uptake was inhibited, while free PEI uptake increased with the increasing N/P. This was due to "saturated" uptake pathways and the competition of free PEI with MNPs in the endocytosis process.[38] Meanwhile, in the case without magnetic field, free PEI uptake increased dramatically when N/P was larger than 20 compared to that under magnetic field. It was obvious that the magnetic field could increase the uptake of magnetic nanoparticles. So the inefficient uptake of MNPs left some pathways for free PEI to enter the cells, which could be confirmed by the increased intensity of free PEI in the case of no magnetic field.

In addition, the magnetofection technique was quite time-saving for taking DNA into the cells. Within one hour post magnetofection, more than 80% percent of the cells were Cy5-positive ones at N/P ratio from 0-20 (Fig 10 e and f). That suggested that plasmid DNA was in most of the cells within one hour after magnetofection. Neither Lipofectamine nor PEI (N/P=10), as the standards, could transfer the DNA into the cells so effectively. Although more than 90% the cells transfected with Lipofectamine were Cy5-psitive, the intensity of the signal was much lower than the magnetofected ones. PEI, on the other hand, may take longer than one hour to take efficient DNA into HepG2 cells. Both the percentage and intensity of those cells were much lower than the others.

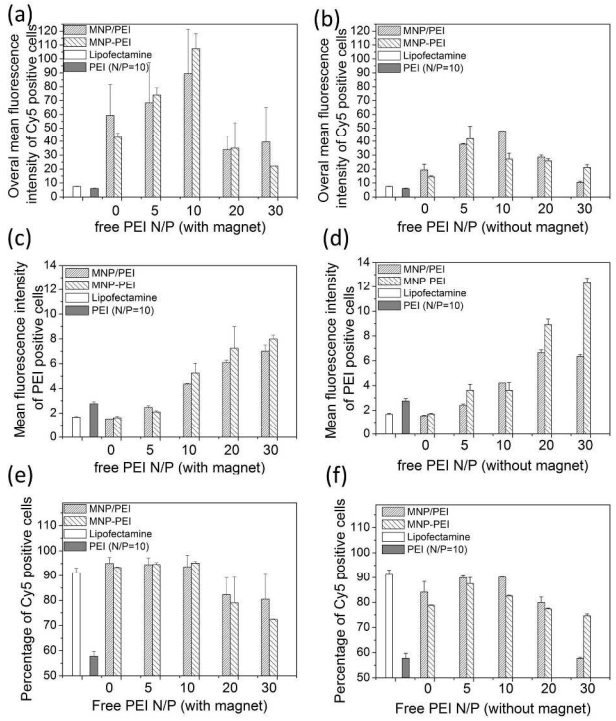

Fig. 10 Cellular uptake of Cy5-labeled plasmid DNA and Oregon Green–labeled free PEI 1 h post transfection in HepG2 cells. The internalization extent is expressed as the median of fluorescence intensity of Cy5-positive cell population (upper), Oregon Green-positive cell population (middle) with magnetic field (a, c) and without magnetic field (b, d), and the percentage of Cy5-positive cells (below) with (e) and without (f) magnetic assistance.

At 6 hours post-magnetofection (Fig. 11), the cellular uptake of PEI was similar to that in Fig. 10. The uptake of plasmid DNA, however, was not different between the ones with and without magnet. It was highly possible that 6 hours was long enough for the MNPs to precipitate on the cells and internalized by them. Since magnetofection took less time, part of the plasmid DNA in the cells was already expressed at this time point. So the signal at 6 hours (Fig. 11 a) was lower than that in Fig 10. In addition, the percentage of Cy5-positive cells transfected with PEI at 6 hours was higher than that at 1 hour post transfection. All of the data suggested that the magnetofection was a more efficient and time-saving method in DNA transfection.

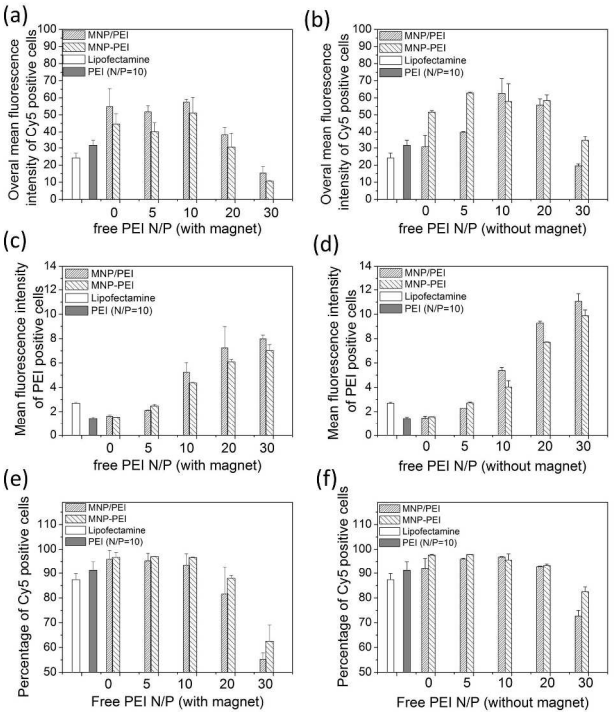

Fig. 11 Cellular uptake of Cy5-labeled plasmid DNA and Oregon Green–labeled free PEI 6 h post transfection in HepG2 cells. The internalization extent is expressed as the median of fluorescence intensity of Cy5-positive cell population (upper), Oregon Green-positive cell population (middle) with magnetic field (a, c) and without magnetic field (b, d), and the percentage of Cy5-positive cells (below) with (e) and without (f) magnetic assistance.

According to the cellular uptake of the magnetofectins, the difference between MNP/PEI and MNP-PEI was not obvious. That is, the MNP-PEI did not assist much more DNA uptake than MNP/PEI. So why is it more efficient in promoting gene expression? It may be largely associated with the structure of MNP-PEI. The PEI in MNP/PEI was attached on MNP only via the electrostatic attraction, so it may not be so stable under physiological conditions, especially in the harsh environment such as the endosomes. The dissociated PEI could destroy the layer-by-layer structure of the ternary complex and greatly inhibited the following steps after cellular uptake. On the other hand, the PEI in MNP-PEI was linked to MNP via the covalent

bonding, which was not affected by the change of environment. It is worth mentioning that the MNP-PEI could keep the same efficiency in DNA transfection even after two-year storage in PBS, while the MNP/PEI did not (data not shown).

The several key factors for successful gene magnetofection are (1) the formation of complex containing both MNP and sufficient plasmid DNA, (2) appropriate amount of free PEI and (3) the magnetic field. The proper amount of free PEI would help forming the complex, and sending plasmid DNA to the nucleus after detach from the magnetofectin. With insufficient PEI, the magnetic nanoparticles cannot draw enough plasmids to the cells, which may suppress the gene transfection,[39] as indicated in the cases with small N/P ratios. Excess PEI aggregated in the cell cytoplasm may show toxic effects to the cells, especially in the lack of magnetic field in the magnetofection process. Although there is no final conclusion about the optimal magnetofection method, this study sheds light on the relationship between plasmid DNA, MNP and free PEI.

Conclusions

In this study, the PEI-conjugated magnetic nanomaterials have been synthesized, with better DNA binding ability and stability than PEI coated ones. With the addition of free PEI, the complex exhibited more efficient transfection under magnetic field. By studying the effects of different N/P ratio, we demonstrated that the free PEI was the key factor in the magnetofection process. The results of confocal microscopy indicated that MNPs were not able to enter the cell nucleus, while the free PEI might detach from the complex and delivered plasmids into the nucleus. Excess PEI might accumulate in the cells without magnetic field, for not enough MNPs were drawn to the cells. An optimal ratio between the MNP, DNA and PEI can be tuned to achieve higher magnetofection efficiency. Our study is helpful in understanding the mechanism of the magnetofection when it comes to the magnetofectin containing polyethylenimine.

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Notes and references

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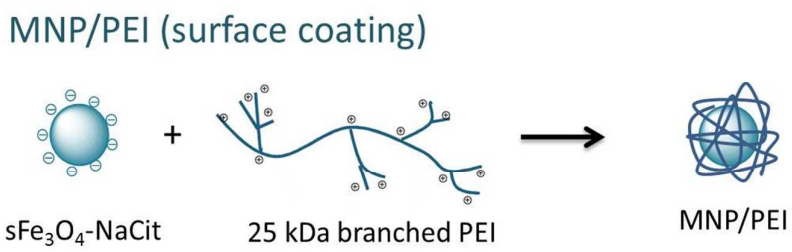

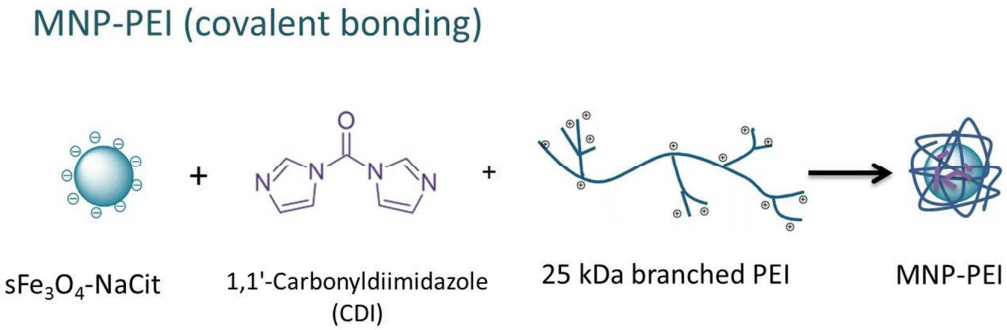

In this work two PEI-functionalized magnetic DNA carriers were prepared for DNA transfection, and the intracellular trafficking of magnetofectins was studied.