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Mannosylated polyethylenimine coupled mesoporous silica nanoparticles for receptor-mediated gene delivery

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ABSTRACT

Organic–inorganic nanohybrids have been studied for their use as non-viral transfection agents. The purpose of this study was to examine the ability of mesoporous silica nanoparticles (MSN) coupled with mannosylated polyethylenimine (MP) to transfect plasmid DNA *in vitro*. Although MSN is biocompatible and has low cytotoxicity, it is not easily transfected into a variety of cell types. To overcome this barrier, MP was coupled to MSN (abbreviated as MPS) to target macrophage cells with mannose receptors and enhance transfection efficiency. The DNA conveyance ability of MPS was examined by evaluating properties such as particle size, zeta potential, complex formation, protection of plasmid DNA against DNase-I, and the release of DNA upon cell entry. Particle sizes of the MPS/DNA complexes decreased with increasing weight ratio of MPS to DNA, while the zeta potential increased. Complete MPS/DNA complexes were formed at a weight ratio of five, and their resistance to DNase-I was evaluated. Cytotoxicity studies showed that MPS/DNA complexes resulted in a high percentage of cell viability, compared with PEI 25K as a vector. The transfection efficiency of MPS/DNA complexes was evaluated on Raw 264.7 and HeLa cell lines. It was found that MPS/DNA complexes showed enhanced transfection efficiency through receptor-mediated endocytosis via mannose receptors. These results indicate that MPS can be employed in the future as a potential gene carrier to antigen presenting cells.

1. Introduction

In the past several years, gene therapy has been studied as a medical treatment due to its potential applications for the replacement of dysfunctional genes and cure of inherited and acquired diseases (Friedmann, 1996; Crystals, 1995a). Essentially, the term gene therapy refers to the transmission of DNA encoding a therapeutic gene of interest into targeted cells or organs with consequent expression of the transgene. However, a primary problem with gene therapy is how safely and efficiently the therapeutic genes are delivered to the targeted cells. In this context, many delivery systems have been developed that utilize a variety of materials. Systems currently under study are classified as either viral or non-viral vectors. Viral vectors are very effective in terms of transfection efficiency, but they have fatal drawbacks such as toxicity, immunogenicity, inflammatory response, and oncogenic effects when used *in vivo* (Crystals, 1995b; Tripathy et al., 1996). These limitations of viral vectors have led to the development of novel synthetic vectors which are non-viral in origin (Han, 2000).

In recent years, organic–inorganic hybrid materials, having properties both of inorganic and organic substances, have been extensively investigated as potential non-viral vectors for gene therapy applications (Chowdhury and Akaike, 2005). As an inorganic material, mesoporous silica nanoparticles (MSN), formed by polymerizing silica source in the presence of surfactants, have many advantages for intracellular delivery, such as large surface area, tunable pore sizes and volumes, and encapsulation of drugs, proteins and biogenic molecules. Moreover, they can be tailored with a variety of surface modifiers in order to increase biocompatibility and targetability. In addition, polyethylenimine (PEI) can be used as an organic modifier to generate efficient and versatile agents for gene delivery. PEI usage leads to high transfection efficiency due to its so-called 'proton sponge effect' allowing endosomal escape and transfer of DNA to the nucleus (Boussif et al., 1995; Yamazaki et al., 2000; Kirchis et al., 2001). However, the cytotoxicity and the transfection efficiency of PEI is affected by its various molecular weights and shapes (Fisher et al., 1999; Godbey et al., 1999). Some studies have demonstrated that high molecular weight PEI exhibits high transfection efficiency and cytotoxicity, while low molecular weight PEI shows reduced transfection efficiency and cytotoxicity (Thomas et al., 2005; Forrest et al., 2003; Tang et al., 2006).

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Macrophages play a major role as effector cells in the immune response to foreign antigens. These cells are specialized for the processing and presentation of antigens on the cell surface, which can lead to activation by T-cell recognition. The activated macrophages secrete various factors that regulate the development of the adaptive immune response and mediate inflammation, such as interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). For effective gene delivery and successful gene expression in macrophages, many strategies have been developed using selective targeting systems (Porgador et al., 1998; Akbari et al., 1999).

To enhance transfection efficiency and avoid non-specific interaction between plasma proteins and the cell membrane, receptor-mediated endocytosis of the DNA complexes is required. It is well known that mannose receptors are abundantly expressed on antigen presenting cells (APCs) such as macrophages (Sallusto et al., 1995; Ferkol et al., 1996; Jiang et al., 1995). The introduction of mannose to a gene carrier can provide selective macrophage targeting for a delivery system. Previously, Kim et al. reported that mannosylated chitosan induced receptor-mediated endocytosis and targeting into APCs, especially dendritic cells having mannose receptors (Kim et al., 2006).

This study describes the coupling of mannosylated polyethylenimine (MP) to the surface of MSN (abbreviated as MPS) as a method to lower cytotoxicity and enhance transfection efficiency through receptor-mediated endocytosis. The physicochemical properties of MPS/DNA complexes were evaluated by gel electrophoresis, particle size measurement and zeta potential measurement. The cytotoxicity and transfection efficiency of the MPS/DNA complexes *in vitro* were also evaluated with respect to the use of these nanohybrids as gene carriers.

2. Materials and methods

2.1. Materials

Tetraethyl orthosilicate (TEOS), hexadecyltrimethylammonium bromide (CTAB), 3-(triethoxysilyl)propylisocyanate, α-D-mannopyranosylphenyl isothiocyanate, and DNA (sodium salt, from calf thymus) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Branched polyethylenimine 1800 and 25 K were purchased from Wako (Osaka, Japan). Cell Titer 96 Aqueous One Solution Reagent for the cell viability assay, the Luciferase Reporter 1000 Assay System for the *in vitro* transfection assay and the Luciferase pGL3-control vector with SV-40 promoter were obtained from Promega (Madison, WI, USA).

2.2. Synthesis of mannosylated polyethylenimine (MP)

MP was synthesized according to a modified method of Diebold et al. (1999). Briefly, branched PEI 1800 dissolved in distilled water was mixed with various amounts of α-D-mannopyranosylphenylisothiocyanate in DMSO. The reaction was performed for 1 day at room temperature. Excess water was then added and lyophilized. This process was repeated several times. The content of mannose in MP was determined by a sulfuric acid micro-method (Monsigny et al., 1998).

2.3. Synthesis of MSN and MPS, PEI/MSN (PS)

MSN was prepared by the method of Radu et al. (2004). Briefly, CTAB (1.00 mg, 2.74 mmol) was first dissolved in 480 mL distilled water with NaOH (2.00 M, 3.5 mL). When the solution temperature was adjusted to 353 K, TEOS (5.00 mL, 22.4 mmol) was introduced dropwise to the solution. The mixture was stirred for 2 h to give rise to white precipitation. The solid product was spun down by centrifugation, washed with distilled water and methanol, and dried under vacuum. The resulting materials (1.50 g) were refluxed for 24 h in a solution of 9.00 mL of HCl (37.4%) in 160 mL methanol to remove the surfactant. The reflux was centrifuged, washed with water and methanol, and the surfactant-free MSN material was placed under high vacuum. MSN (1.00 g) was refluxed for 20 h in 80 mL anhydrous toluene with 3-(triethoxysilyl)propylisocyanate (0.25 mL, 1.00 mmol). The resulting material was mixed with MP in isopropyl alcohol. The product was centrifuged, washed with water and methanol, and dried under vacuum. The PEI was also coupled with MSN in isopropyl alcohol, and then centrifuged, washed and dried. The washing processes were repeated until no more PEI was detected in the supernatant solution. The synthetic scheme of MPS is shown in Fig. 1. The composition of MSP was evaluated by NMR spectroscopy (Bruker, 600 MHz, Germany).

2.4. Determination of grafting percentage

The amount of polymer grafted onto the silica surface was determined by weight loss by thermal gravimetric analysis (TGA Q5000-IR, TA instruments, USA). The polymer-grafted silica was heated at 10 °C/min to 800 °C under N_2. The percentage of grafting was determined by the following equation (Kaneko et al., 2006):

$$\text{Grafting}(\%) = \frac{A}{B}100$$

where *A* is the weight of polymer grafted onto the silica surface and *B* is the weight of MPS used in the reaction.

2.5. DNA retardation assay

The formation of plasmid DNA–nanoparticle complexes was examined by agarose gel electrophoresis. Complex formation was induced at various weight ratios from 1 to 50, and formation reactions were incubated at room temperature for 30 min. The complexes were loaded on 1% agarose gels, stained with ethidium bromide (0.2 μg/mL), and run with Tris–acetate (TAE) buffer at 100 V for 40 min.

2.6. Protection and release assay of DNA

MPS/DNA complexes and naked DNA (0.2 μg) were separately incubated with DNase-I (1 unit) in DNase/Mg^{2+} digestion buffer (50 mM, Tris–Cl, pH 7.6, and 10 mM $MgCl_2$) at 37 °C for 30 min. All samples were subsequently treated with 4 μL 250 mM EDTA for 10 min to inactivate DNase-I digestion and mixed with sodium dodecyl sulfate (SDS) in 0.1 M NaOH (pH 7.2) at final concentration of 1.0%. Finally, all solutions were incubated at room temperature for 2 h and were run on 1% agarose gels in TAE running buffer at 50 V for 1 h.

2.7. Particle size measurement and zeta potential assay

The particle sizes and surface charges of the MPS/DNA complexes were determined according to weight ratios using an electrophoretic light scattering spectrophotometer (ELS 8000, Otsuka electronics, Osaka, Japan) with 90° and 20° scattering angles, respectively, at room temperature.

2.8. Transmission electron microscopy

The morphology of MPS/DNA complexes prepared at weight ratio 20 was observed using TEM (LIBRA 120, Carl Zeiss, Germany). One drop of MPS/DNA complexes was placed on a copper grid and

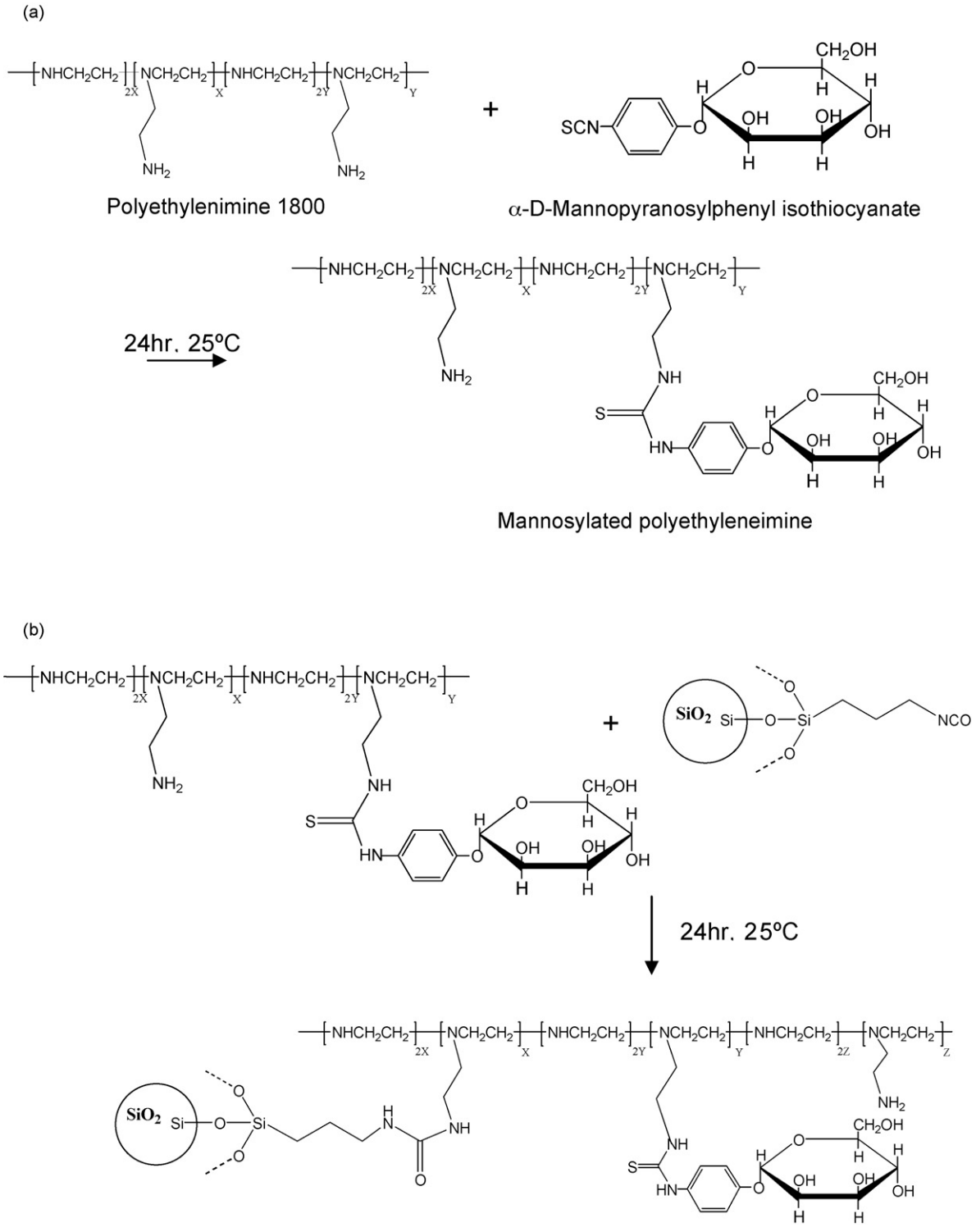

Fig. 1. Synthetic scheme of MP and MPS.

the grid was allowed to dry for 10 min before examination with an electron microscope.

2.9. Cell lines and cell culture

Raw 264.7 murine macrophage cells, HeLa cells (Korean Cell Line Bank, Seoul, Korea) were grown in Dulbecco's modified Eagle medium (HyClone, Utah, USA), supplemented with 10% fetal bovine serum, streptomycin at 100 mg/mL, penicillin at 100 U/mL. All cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere.

2.10. Cytotoxicity

Cytotoxicity was assessed using a CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega). Briefly, cells were seeded with a density of 2×10^4 cells/well onto 96-well microtiter

plates for 24 h. The growth medium was removed and replaced with fresh serum-free medium containing various amounts of complexes. Twenty-four hours later, 20 μL CellTiter 96® AQ_{ueous} One Solution Reagent was added to each well and the plates were incubated for 1–4 h at 37 °C in a humidified, 5% CO_2 incubator. The absorbance was then read at 490 nm using a microplate reader (GLR 1000, Genelabs Diagnostics, Singapore). Non-treated cells were used as a control.

2.11. Transfection assay

For transfection, Raw264.7 macrophage cells and HeLa cells were seeded in 24-well plates at a density of 2×10^5 cells per well and allowed to adhere overnight. The media was replaced with serum-free media containing MPS/DNA complexes at various weight ratios and incubated for 4 h. Serum-free media was replaced with fresh media containing serum and further incubated for 48 h. The luciferase assay was carried out according to the manufacturer's instructions. The relative light units (RLUs) due to luciferase activity were measured with a chemiluminometer (Autolumat LB953, EG and G, Berthold, Germany). RLUs were normalized to protein concentration in the cell extract measured by BCA protein assay kit.

Table 1
Amount of mannose in MP by sugar assay

	Theoretical degree (mol%)	Experimental degree (mol%)
MP-4	5.8	4.17
MP-7	17.4	7.63
MP-10	29	10.77
MP-12	40.6	12.3
MP-15	58	15.8

Furthermore, a competition assay was carried out to confirm the uptake of MPS/DNA complexes mediated with mannose receptor by adding various amount of mannose. Raw 264.7 cells were preincubated for 15 min with various amounts of mannose, and transfection assays were conducted as described above. All transfections were performed in triplicate.

2.12. Statistical analysis

Data were expressed as mean ± standard deviations (S.D.). Data were analyzed using unpaired Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

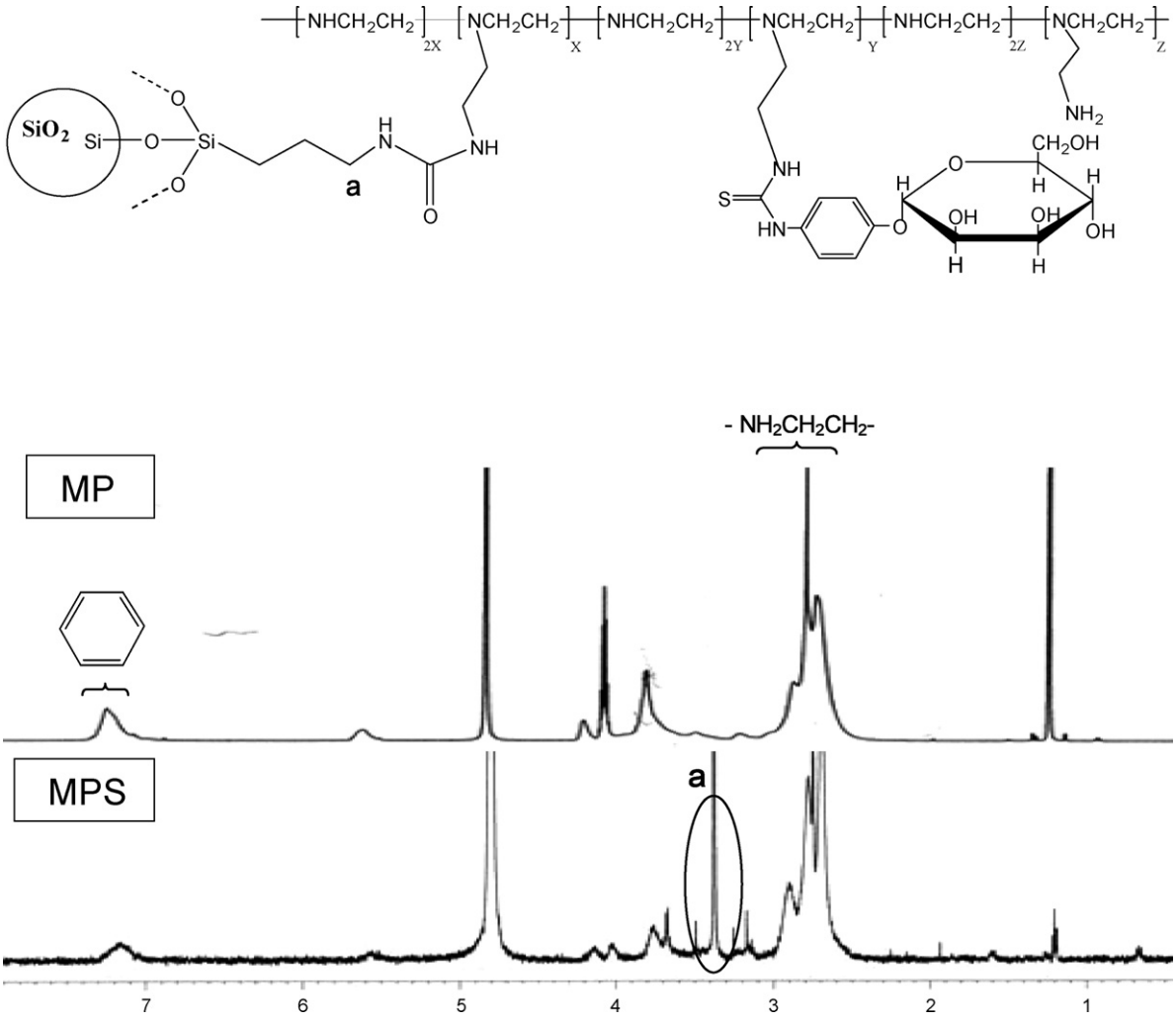

Fig. 2. Representative ^{1}H NMR spectrum (600 MHz) of MPS in D_2O.

Table 2
Amount of MP in MPS by TGA analysis

	Theoretical degree (wt%)	Experimental degree (wt%)
MPS-7	1000	30.61
MPS-15	1000	30.79
MPS-7	2000	33.2
MPS-15	2000	34.29

3. Results and discussion

3.1. Synthesis of MP and MPS

MP was synthesized by a thiolurea linkage reaction between the isothiocyanate group of α-D-mannopyranosylphenylisothiocyanate and the primary amine group of PEI. The chemical composition of mannose in MP was determined by sugar assay, which range from 4.17 to 15.8 mol% on the concentration of mannose (Table 1). MP and MSN functionalized with isocyanatopropyl silanone were coupled through a urea linkage between the amine group of MP and the isocyanate group of MSN. Continuously, the MP-coupled MSN (MPS) was confirmed by NMR spectroscopy (Fig. 2) and then the MP amounts grafted to MSN were determined by TGA analysis. The MP-7 and MP-15 were grafted onto the MSN surfaces for synthesis of MPS, which increased with increasing amounts of MP (Table 2). Also, the substitution value of the mannose group did not affect the extent of MP grafting onto the MSN surface. However, the percent of MP grafting was considerably smaller than the theoretical degree. This may have been due to aggregation of MSN and the steric hindrance by branched PEI chains grafted onto the MSN surface.

3.2. Characterization of MPS/DNA complexes

One important property for a useful gene carrier is the condensation of DNA onto its surface. The cationic charge density of MPS can form complexes with DNA by an ionic interaction with phosphate groups of DNA. The condensing ability of MPS with DNA was confirmed by agarose gel electrophoresis (Fig. 3). DNA migration on the agarose gel was retarded with increasing amounts of MPS. Use of an MPS/DNA weight ratio greater than five resulted in complete complexation of plasmid DNA.

The particle sizes of MPS/DNA complexes were analyzed by dynamic light scattering to investigate the degree of compaction with DNA. It is already known that particle sizes should be limited to less than 150 nm in diameter for endocytosis by various types of mammalian cells (Guy et al., 1995). The sizes of the complexes decreased with an increase in weight ratio and ranged from

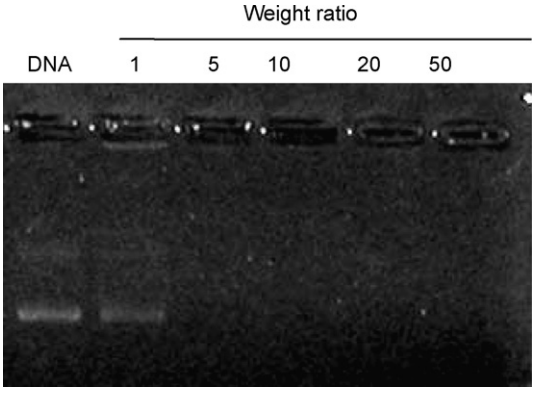

Fig. 3. Agarose gel electrophoresis of MPS/DNA (pGL3-control) complexes at various weight ratios using 1% agarose in Tris–acetate running buffer.

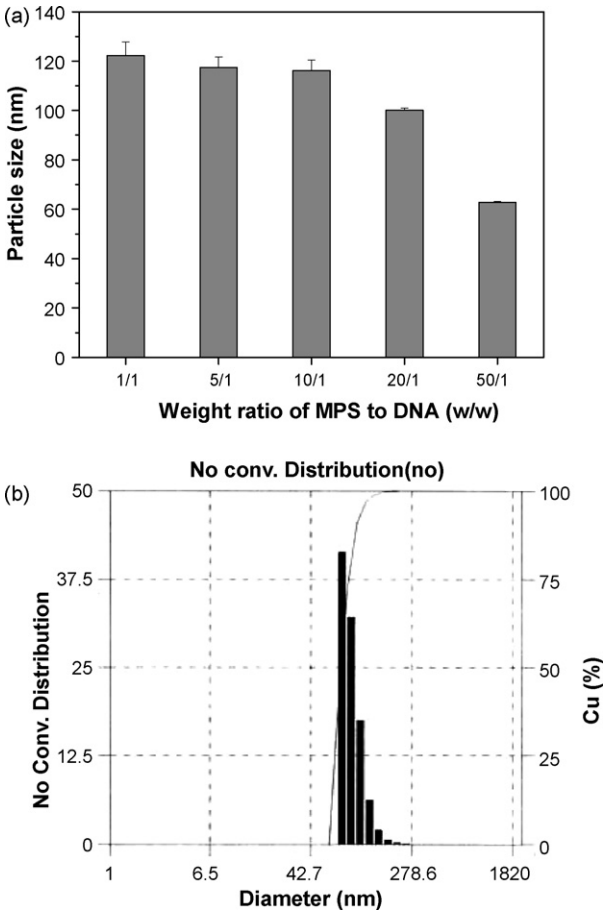

Fig. 4. Particle sizes of MPS-7/DNA complexes at various weight ratios (a) and size distribution profile of representative MPS-7/DNA complexes prepared at weight ratio 20 (b) (mean ± S.D., $n = 3$).

60 to 130 nm (Fig. 4a), suggesting that high weight-ratio complexes can transfect cells through a receptor-mediated mechanism. The MPS/DNA complexes at weight ratio 20 demonstrate a nearly unimodal size distribution (Fig. 4b). Moreover, the morphology of MPS/DNA complexes prepared at weight ratio 20 was observed as spherical in shape with little aggregation (Fig. 5).

Surface charge is also an important property for a gene delivery system as it can influence stability, cell adhesion, and transfection efficiency (Mahato et al., 1997; Nomura et al., 1997). The presence of a positive surface charge on the complex could lead to attachment to cell surfaces having anionic polyelectrolytes at a neutral pH value (Mislick and Maldeschwieler, 1996). The zeta potential analysis of MPS/DNA complexes at various weight ratios are shown in Fig. 6. The MPS/DNA complexes of weight ratio 1 show a negative zeta potential, whereas complexes with a weight ratio from 5 to 50 show increasing positive charges as the weight ratio is increased. These results indicate that MPS are able to facilitate gene transfer due to their advantageous particle sizes and surface charges.

For efficient gene delivery, the DNA combined with the gene carrier should be protected against degradation by nucleases as well as release of DNA for gene expression (Huang et al., 2005; Zabner et al., 1995). The effect of MPS on protection and release of plasmid DNA was examined using DNase-I. MPS could protect DNA against DNase-I, compared with naked plasmid DNA, and released DNA molecules from the MPS/DNA complexes by SDS treatment were nearly intact (Fig. 7). These results indicate that MPS complexes are sufficiently able to deliver DNA inside the target cells.

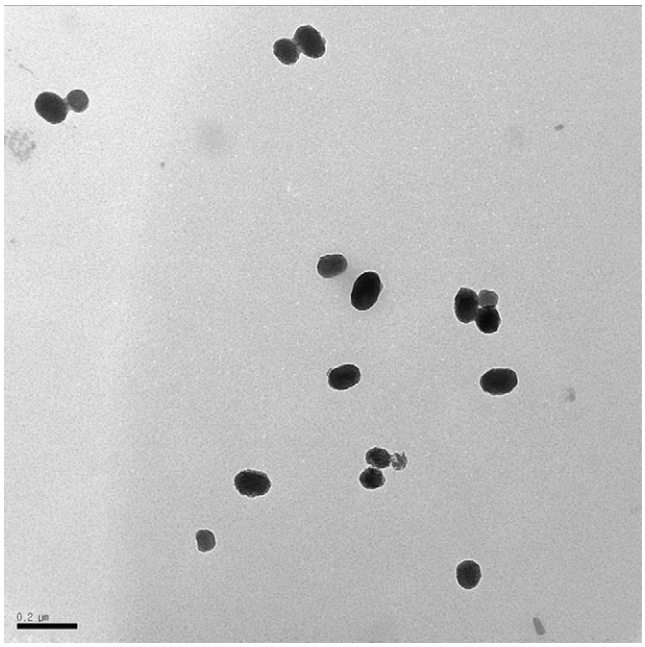

Fig. 5. TEM image of MPS-7/DNA complexes prepared at weight ratio 20. Scale bar: 200 nm.

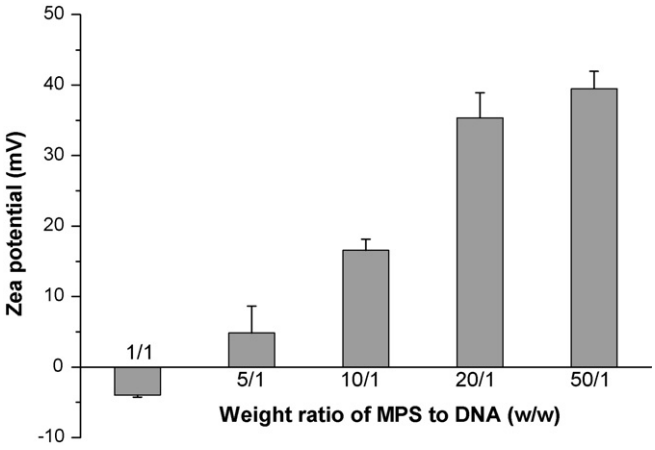

Fig. 6. Zeta potential measurement of MPS-7/DNA complexes in distilled water at various weight ratios (mean ± S.D., $n = 3$).

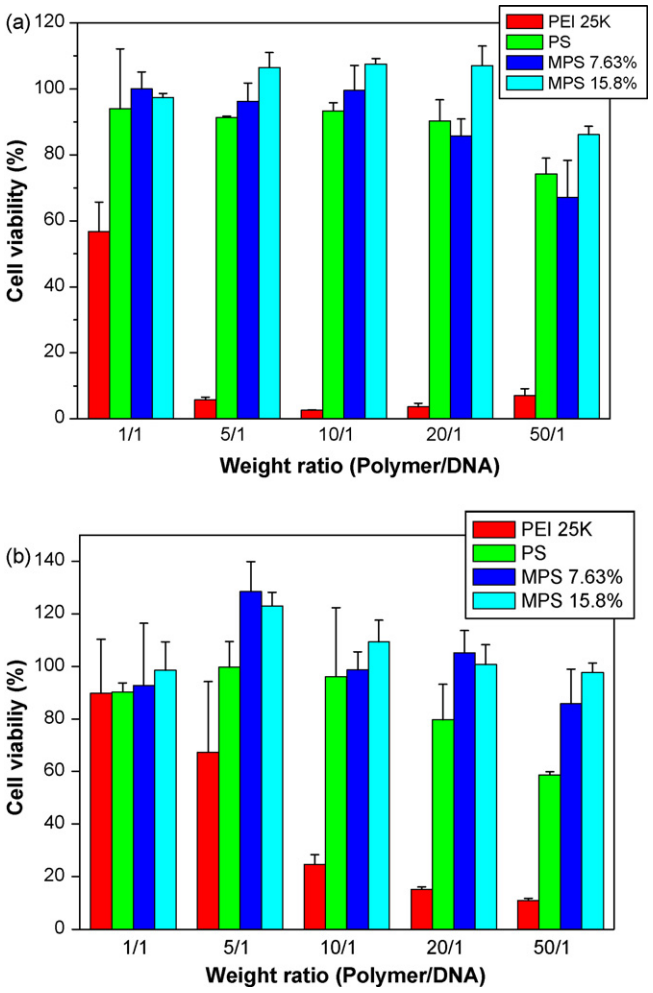

Fig. 8. Cell viability of MPS/DNA complexes at various weight ratios on (a) Raw 264.7 and (b) HeLa cells (mean ± S.D., $n = 3$).

3.3. Cell viability and transfection efficiency of MPS polymer in vitro

To evaluate the cytotoxicity of the MPS/DNA complexes on cells, two cell lines were treated with the complexes, and cell viability tests were performed. The effects of MPS, PS, and PEI on the viability of Raw 264.7 macrophage cells and HeLa cells were examined,

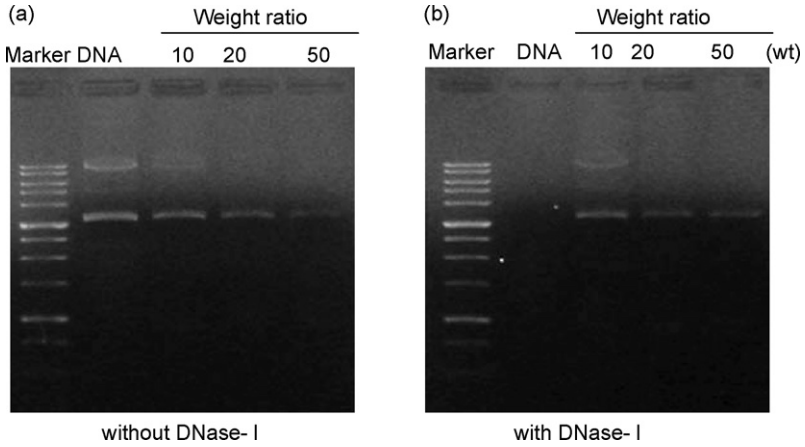

Fig. 7. Protection (a) and release assay (b) of MPS-7/DNA complexes at various weigh ratios using DNase-I. DNA was released by 1% SDS treatment of the MPS/DNA complexes.

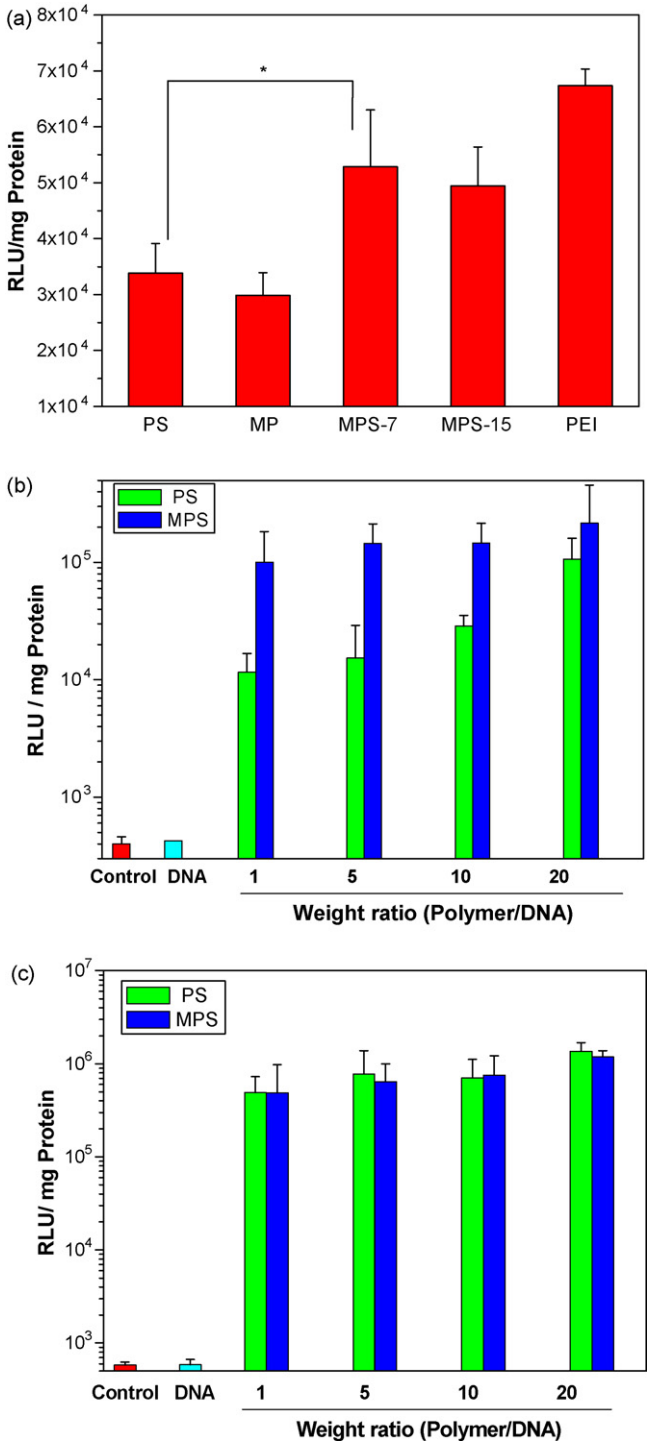

Fig. 9. Transfection efficiency of MPS-7/DNA and MPS-15/DNA complexes on Raw 264.7 (a), and PS/DNA and MPS-7/DNA complexes on Raw 264.7 (b) and HeLa cells (c) at various weight ratios (mean ± S.D., $n = 3$, $^{*}p < 0.05$).

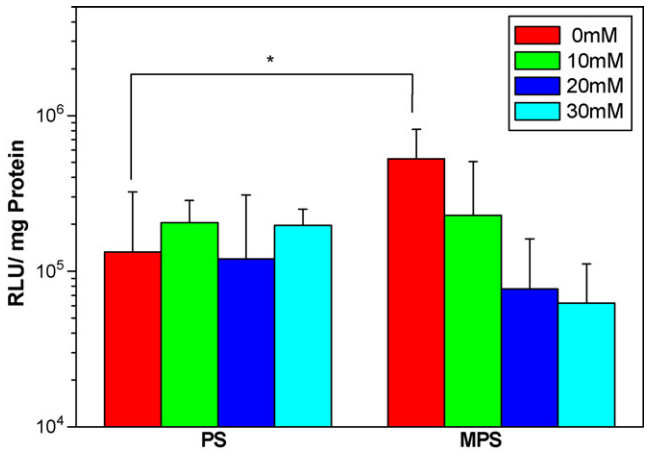

Fig. 10. Competition assay of MPS/DNA complexes prepared at charge ratio 20 by adding mannose (10, 20 or 50 mM) as a competitor of MPS (mean ± S.D., $n = 3$, $^{*}p < 0.05$).

and the results showed that the cell viability was reduced by increasing the weight ratio of complexes (Fig. 8). Cell viabilities associated with treatment with MPS/DNA complexes and PS/DNA complexes were greater than 80% up to a weight ratio of 20. The PEI/DNA complex treatment resulted in high cytotoxicity depending on the concentration, while MPS/DNA complex treatment resulted in reduced cytotoxicity when compared with PS/DNA complexes. This may be due to the hydrophilicity of the MPS surface, whereas polymer aggregation followed by non-specific adhesion to the cell surface may cause high cytotoxicity, impairing important membrane functions (Ogris et al., 1999).

To examine the transfection efficiency of MPS/DNA complexes, luciferase activity assays were performed on Raw 264.7 macrophage cells with abundant mannose receptors and HeLa cells, which lack mannose receptors (Garner et al., 1994). The luciferase activity resulting from transfection with MPS-7/DNA and MPS-15/DNA complexes was compared with that resulting from transfection with PS/DNA and PEI/DNA complexes at weight ratio 5 (Fig. 9a). The MPS/DNA complexes show high transfection efficiency than PS/DNA and MP/DNA complexes, while comparable to PEI complex. Moreover, the transfection efficiency of MPS-7/DNA complex was similar to that of MPS-15/DNA complex. It has been reported that ligand targeting to specific cells, such as that achieved by galactose or mannose, was sufficient at 5 mol% for receptor-mediated gene delivery to the cells (Zanta et al., 1997). Since the MPS-7 complexes which have about 7 mol% mannose group were sufficient for receptor-mediated endocytosis for gene delivery to macrophages, the MPS-7 complexes were selected for subsequent experiments. The transfection efficiency of MPS/DNA complexes was higher than PS/DNA complexes on the macrophage cells (Fig. 9b and c), while MPS/DNA complexes did not show such a pattern on HeLa cells. These results indicate that MPS/DNA complexes are internalized into cells by mannose receptor-mediated endocytosis.

To confirm the receptor-mediated endocytosis of MPS/DNA complexes, the transfection activity of MPS/DNA complexes and PS/DNA complexes prepared at a charge ratio of 20 was investigated in the presence of various amount of mannose as a competitor for the complexes (Fig. 10). The transfection efficiency of MPS/DNA complexes was significantly inhibited in the presence of mannose, while that of PS/DNA complexes was not changed. In other words, the availability of mannose receptors was essential for entry of MPS/DNA complexes into macrophages through a receptor-mediated delivery system.

4. Conclusions

In this study, we demonstrated that MPS was successfully prepared and evaluated its potential as a targeting gene delivery system to macrophages. The evaluation of its ability to form complexes with DNA, particle size, zeta potential, protection against DNase-I, and release of DNA showed that MPS has suitable physicochemical properties as a gene carrier. Furthermore, MPS treatment

resulted in low cytotoxicity of Raw 264.7 macrophage cells and HeLa cells, promoting it as a safe gene carrier. It also enhanced transfection efficiency on Raw 264.7 macrophage cells over that of HeLa cells, exhibiting mannose receptor-mediated gene transfer. Therefore, MPS has potential as a gene delivery system for macrophage-selective targeting.

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