Carbohydrate Polymers 110 (2014) 268–277

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Cationic methylcellulose derivative with serum-compatibility and endosome buffering ability for gene delivery systems

Kyunghwan Kim[a,1], Kitae Ryu[a,1], Tae-il Kim[a,b,*]

[a] Department of Biosystems and Biomaterials Science and Engineering, College of Agriculture and Life Sciences, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 151-921, Republic of Korea
[b] Research Institute of Agriculture and Life Sciences, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 151-921, Republic of Korea

ARTICLE INFO

Article history:
Received 22 November 2013
Received in revised form 17 March 2014
Accepted 20 March 2014
Available online 2 April 2014

Keywords:
Gene delivery systems
Methylcellulose
Low molecular weight PEI
Low cytotoxicity
Endosome buffering ability
Serum-compatibility

ABSTRACT

In this work, methylcellulose was employed as a template polymer with graft of polyethylenimine 0.8 kDa (PEI0.8k) for gene delivery systems. Synthesized PEI-grafted oxidized methylcellulose (MC-PEI) could condense pDNA into positively charged and nano-sized particles, which could protect pDNA from serum nuclease. The cytotoxicity of MC-PEI was minimal in both serum-free and serum condition due to the biocompatibility of methylcellulose and low cytotoxicity of PEI0.8k. MC-PEI polyplex also showed low cytotoxicity in serum condition. In serum condition, MC-PEI showed less decreased transfection efficiency than PEI25k, meaning good serum-compatibility of MC-PEI. Bafilomycin A1-treated transfection results indicate that the transfection of MC-PEI is mediated via endosomal escape by endosome buffering ability. Flow cytometry results suggest that MC-PEI polyplex could be internalized into cells and efficiently deliver pDNA to cells due to its serum-compatibility. These results demonstrate that MC-PEI possesses a potential for efficient gene delivery systems.

1. Introduction

Gene delivery is to deliver genetic materials such as pDNA, oligonucleotides, or siRNA to cells for the induction, enhancement, or inhibition of gene expression. In order to deliver genetic materials to the targeted cells with high efficiency and low toxicity, tremendous gene delivery carriers have been developed, including viral vectors and non-viral vectors (Giacca & Zacchigna, 2012; Huang & Kamihira, 2013; Wang, Li, Ma, & Steinhoff, 2013).

Among them, polymeric gene delivery carriers have attracted a lot of attention due to their non-immunogenicity, low cytotoxicity, non-integration of exogenous genes into host chromosomes, ease of manufacturing, and the ability to transfer large size of genes (Luo & Saltzman, 2000). In addition, their capability to possess multiple bioactive functionalities via chemical modification makes them potential gene delivery carriers with high transfection efficiency (Jeong, Kim, & Park, 2007; Kang, Huh, & Bae, 2012; Liu & Huang, 2002).

Polysaccharides, long carbohydrate molecules of repeated monomer units joined together by glycosidic bonds, have been utilized as template polymers for gene delivery systems due to their advantages such as availability from replenishable resources, biocompatibility and biodegradability (Khan et al., 2012). In general, cationic moieties or other functionalities have been introduced to polysaccharides for gene delivery systems by chemical modifications of hydroxyl or carboxylate groups because they usually lack pDNA condensing ability except chitosan which has primary amines. For example, chitosan has been further modified to succinated chitosan (Toh, Chen, Lo, Huang, & Wang, 2011), chitosan lactate (Weecharangsan, Opanasopit, Ngawhirunpat, Rojanarata, & Apirakaramwong, 2006), poly(L-lysine)-grafted chitosan (Yu et al., 2007), or chitosan-graft-polyethylenimine (Jiang et al., 2007) for gene delivery systems. Cyclodextrin was also utilized for gene delivery systems as β-cyclodextrin containing polycations (Popielarski, Mishra, & Davis, 2003), cyclodextrin–polyethyleneimine conjugates (Forrest, Gabrielson, & Pack, 2005), or cationic polymers containing α-cyclodextrin and oligoethylenimine (Yang, Li, Goh, & Li, 2007). In the case of dextran, dextran–glycidyltrimethylammonium chloride conjugate (Thomas, Rekha, & Sharma, 2010), dextran-grafted polyethyleneimine (Tseng, Tang, & Fang, 2004), or

* Corresponding author at: Department of Biosystems and Biomaterials Science and Engineering, College of Agriculture and Life Sciences, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 151-921, Republic of Korea. Tel.: +82 2 880 4636; fax: +82 2 873 2285.
E-mail addresses: st_kyunghwan@hanmail.net (K. Kim), kitaefriend@snu.ac.kr (K. Ryu), seal1004@snu.ac.kr (T.-i. Kim).
[1] These authors contributed to this work equally.

http://dx.doi.org/10.1016/j.carbpol.2014.03.073
0144-8617/

dextran–spermine conjugate (Hosseinkhani, Azzam, Tabata, & Domb, 2004) has been developed for gene delivery systems. Other polysaccharides including pullulan (Rekha & Sharma, 2009), alginate (Krebs, Salter, Chen, Sutter, & Alsberg, 2010), or hyaluronan (Yun, Goetz, Yellen, & Chen, 2004) also has been utilized as template polymers for gene delivery.

Methylcellulose (MC) is one of the cellulose ether derivatives in which some hydroxyl groups of cellulose are methylated (1.4 < substitution degree < 2.5), which possesses an amphiphilic character. Its water solubility is known to depend on the distribution of methyl substituents (Hirrien, Desbrieres, & Rinaudo, 1996). MC has been used as a food additive or in cosmetics for thickening and emulsifier properties. Recently, applications of MC for hydrogel or drug delivery systems have been reported (Liang et al., 2004; Liu et al., 2004; Wang, Lapitsky, Kang, & Shoichet, 2009) due to its biocompatibility and unique sol–gel transition property (Hirrien, Chevillard, Desbrieres, Axelos, & Rinaudo, 1998). However, to our knowledge, chemical modification of MC itself has not been reported for gene delivery systems so far, although other cellulose derivatives such as hydroxypropyl cellulose (Xu et al., 2009) or carboxymethyl cellulose (Griesenbach et al., 2010) has been examined as gene delivery carriers and methylcellulose has been physically formulated for gene delivery (Griesenbach et al., 2010; Sinn, Burnight, Hickey, Blissard, & McCray, 2005).

In this study, methylcellulose was utilized as a template polymer for gene delivery systems due to its biocompatibility for the first time. MC-PEI was synthesized by grafting low molecular weight PEI (PEI0.8k) to periodate-oxidized MC (OXMC). PEI0.8k was employed to provide cationic property, maintaining low cytotoxicity. Then, the properties of MC-PEI for gene delivery systems were characterized to identify the potential as non-toxic and efficient gene delivery carriers.

2. Materials and methods

2.1. Materials

Methylcellulose (15 cP, 2% in H_2O), polyethylenimine (PEI, molecular weight 0.8k and 25 kDa), poly-L-lysine (PLL, molecular weight 70–150 kDa), agarose, ethylenediaminetetraacetic acid (EDTA), ethidium bromide, heparin sodium salt, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO). Sodium periodate and sodium tetrahydroborate were purchased from Junsei (Tokyo, Japan). The plasmid DNA, pCN-Luci containing a firefly luciferase reporter gene was amplified in *Escherichia coli* DH5α and isolated by PureLink HiPure Plasmid Filter Purification kit (Invitrogen, Carlsbad, CA). Luciferase assay system and reporter lysis buffer were purchased from Promega (Madison, WI). Fetal bovine serum (FBS), 0.25% Trypsin–EDTA, Dulbecco's phosphate buffered saline (DPBS), and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Carlsbad, CA). BCA™ protein assay kit was purchased from PIERCE (Rockford, IL). All other chemicals were purchased and used without any further purification.

2.2. Synthesis and characterization of MC-PEI

First, MC and sodium periodate were dissolved in water, respectively. In the case of MC, MC powder was added to the about 1/3 of the required volume of hot water (80 °C) with agitation and the remainder of the water was added as cold water to lower the temperature. After cooling to 0–5 °C for 30 min, the solution was agitated further for at least 30 min at room temperature for complete solubilization of MC. Then, MC solution was added by dropwise to the sodium periodate solution while stirring, in order to oxidize MC. After 24 h of oxidation reaction (room temperature, dark condition, under nitrogen environment), the reaction mixture was dialyzed against ultrapure water with dialysis membrane (MWCO = 3.5k) for 2 days. The oxidized MC (OXMC) was obtained by following lyophilization. Secondly, PEI0.8k (5 molar equivalent to glucose units of OXMC) water solution was added to the OXMC solution for the conjugation of PEI to OXMC and the reaction was conducted for 24 h at room temperature (dark condition, under nitrogen environment). Sodium tetrahydroborate water solution was then subsequently mixed with the solution. After 24 h of further reaction (room temperature, dark condition, under nitrogen environment), the reaction mixture was dialyzed for 3 days. The final product, MC-PEI was obtained as a white solid after following lyophilization. The each step of polymer synthesis was confirmed by ^{1}H NMR and ^{13}C NMR (D_2O, 400 MHz JEOL JNM-LA400 and 600 MHz AVANCE 600). FT-IR spectra of the polymers were also recorded with a FT-IR spectrometer (Nicolet iS5, Thermo Scientific). The polymer samples were analyzed with the range of 600–4000 cm^{-1} using KBr pellets containing the prepared materials. The synthetic scheme of MC-PEI was shown in Fig. 1. The molecular weights of polymers were determined by gel permeation chromatography (GPC: YL-9100, Young Lin Instrument, Korea). Polyethyleneglycols with various molecular weights were used as standards. The assay was run on Ultrahydrogel 250 column with 1% formic acid as an eluent. The concentration of the polymer solutions was set to 10 mg/mL and the flow rate to 1 mL/min.

2.3. Agarose gel electrophoresis

Agarose gel electrophoresis was performed to examine the pDNA condensation ability of polymers. Agarose gel (0.7%, w/v) containing ethidium bromide (0.5 μg/mL) was prepared in Tris–Acetate–EDTA (TAE) buffer. The polyplexes (0.5 μg pDNA) were prepared in Hepes buffer (pH 7.4) at various weight ratios (polymer/pDNA) for 30 min of incubation at room temperature. After loading of samples, the electrophoresis was run for 15 min at 100 V (Mupid-2plus, Takara Bio Inc., Japan). The locations of pDNA bands were observed by UV illuminator (ChemiDoc XRS+ gel documentation system, Bio-Rad, Hercules, CA).

2.4. Protection ability of polyplex from serum

The protection ability of polyplex from serum was investigated by agarose gel electrophoresis. Polyplex solutions (0.5 μg pDNA) prepared at various weight ratios were incubated with 50% FBS for 30 min at 37 °C. Then, heparin sodium salt solutions were added to the polyplex solutions to 10 mg/mL of final concentration for the dissociation of pDNA from polyplex. After 30 min of further incubations, the polyplex solutions were electrophoresed and the locations of released pDNA bands were identified by UV illuminator. PEI25k polyplex was used as a control.

2.5. Average particle size and Zeta-potential measurements

Average particle sizes and Zeta-potential values of polyplexes were measured by Zeta-sizer Nano ZS (Malvern Instruments, UK) with He–Ne laser beam (633 nm) at 25 °C. The polyplex solutions (5 μg pDNA in 0.5 mL) were prepared in ultrapure water at various weight ratios ranging from 0.5 to 100. After 30 min of incubation, the polyplex solutions were diluted to 1 mL before measurements. Average particle sized and Zeta-potential values were measured 3 times.

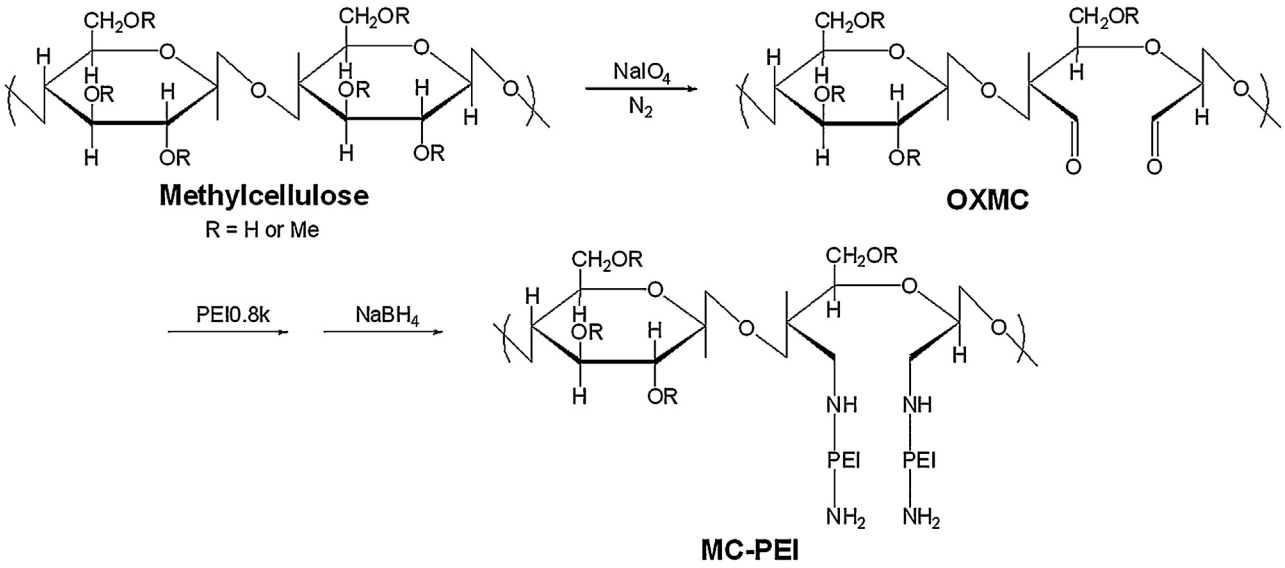

Fig. 1. Synthetic scheme of MC-PEI.

2.6. Transmission electron microscopy (TEM)

10 μL of MC-PEI polyplex solutions (0.5 μg pDNA) were prepared and deposited on TEM copper grid plates. The samples were then stained with filtered uranium acetate solutions for 30 s. After absorption of residual solutions, polyplex images were visualized using a TEM (JEM1010, JEOL, Japan) with an accelerating voltage of 80 kV.

2.7. Cell culture

C2C12 mouse myoblast cells and HeLa human cervical adenocarcinoma cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a 5% CO_2 incubator at 37 °C.

2.8. MTT assay

MTT assay was performed to examine the cytotoxicity of polymers and polyplexes. C2C12 cells and HeLa cells were seeded in a 96-well plate in 100 μL DMEM (10% FBS) at a density of 1×10^4 cells/well, respectively. Having achieved 70–80% confluency after 24 h, the cells were exposed to polymer solutions (20–100 μg/mL) in serum-free DMEM or serum-containing DMEM for 4 h. Polyplexes (0.5 μg pDNA) solutions were also prepared and treated to the cells in serum condition for MTT assay. bPEI25k (branched PEI 25 kDa) and PEI0.8k were used as controls. Subsequently, the media were changed with fresh DMEM (10% FBS). After 24 h, the cells were treated with 25 μL of MTT stock solution (2 mg/mL in DPBS) and incubated for 2 h at 37 °C. After removing each medium carefully, 150 μL of DMSO was added to each well to dissolve the formazan crystal formed by proliferating cells. The absorbance was measured at 570 nm using a microplate reader (Synergy H1, BioTek, USA). Results were presented as relative cell viabilities (RCV, percentage values relative to value of untreated control cells). All experiments were performed in quadruplicate.

2.9. In vitro transfection experiments in serum-free or serum condition

The transfection efficiencies of polymers were examined by measuring luciferase reporter gene expression in serum-free or serum condition in C2C12 cells and HeLa cells, respectively. Cells were seeded in a 24-well plate at a density of 5×10^4 cells/well in DMEM (10% FBS) and were grown to reach 70–80% confluency. Before transfection, the media were exchanged with serum-free DMEM for the assay in non-serum condition and with freshly prepared DMEM (10% FBS) for the assay in serum condition, respectively. The cells were treated with polyplex solutions (0.5 μg pDNA) with various weight ratios ranging from 25 to 100. PEI25k (weight ratio = 1) and PEI0.8k polyplexes (weight ratio = 10, 20 and 30) were used as controls. After 4 h of incubation, the media were exchanged with fresh DMEM (10% FBS) and the cells were incubated for further 2 days at 37 °C in 5% CO_2. Then, the media were removed and the cells were rinsed with 240 μL of DPBS and shaken for 30 min at room temperature with 120 μL of reporter lysis buffer. The collected cell lysates were centrifuged and 20 μL of supernatants were used for assay. Luciferase activities were measured by using 100 μL of luciferase assay reagent on a microplate reader (Synergy H1, BioTek, USA). A protein quantification assay was performed using a BCA™ Protein Assay Reagent Kit to measure the total amount of cellular proteins. The final results were presented in terms of RLU/mg cellular protein. All experiments were performed in triplicate.

2.10. Transfection with bafilomycin A1

Transfection experiments were performed with bafilomycin A1 in order to examine the effect of endosomal buffering ability of polymers. HeLa cells were seeded with the identical method to the above experiments. Before transfection, bafilomycin A1 solutions (200 nM) were treated to cells for 10 min. After 4 h of incubation with polyplex solutions, the media were exchanged with fresh DMEM (10% FBS). PEI25k polyplex and PLL polyplex (weight ratio = 5) were used as controls. The cells were incubated for further 2 days and the following assays were performed with method to the above experiments.

2.11. Flow cytometry

Flow cytometry was performed to investigate the cellular uptake of polyplexes. HeLa cells were seeded at a density of 2×10^5 cells/well in a 12-well plate in DMEM medium containing 10% FBS and grown to reach 70–80% confluency prior to transfection. Before transfection, medium of each well was exchanged for

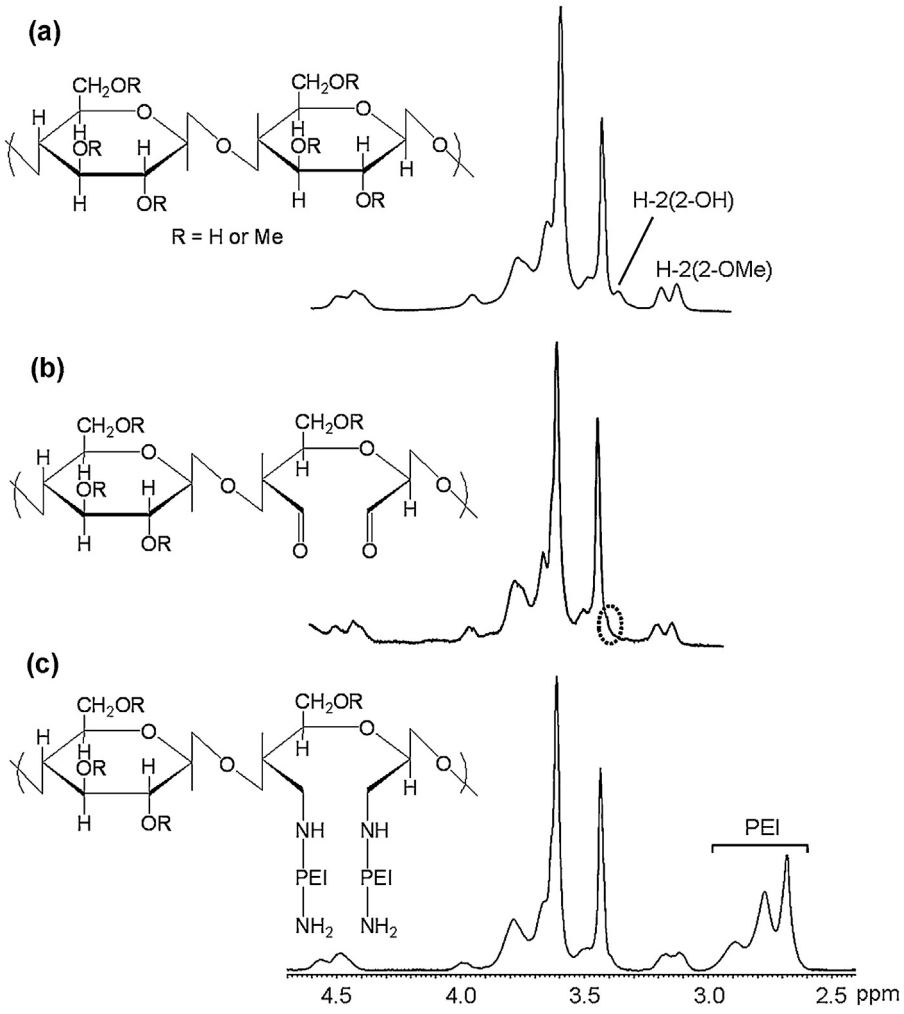

Fig. 2. ^{1}H NMR spectra of polymers in D_2O. (a) Methylcellulose (MC), (b) oxidized methylcellulose (OXMC), and (c) PEI-grafted oxidized methylcellulose (MC-PEI).

fresh serum-free medium or serum-containing medium. pDNA was labeled with YOYO-1 iodide (1 molecule of the dye per 50 base pairs of the nucleotide). The cells were treated with polyplex solutions (0.5 μg pDNA) at different weight ratios for 4 h at 37 °C. Then, the media were aspirated off from the wells and the cells were washed two times with ice-cold DPBS. After trypsinization, the cells were suspended in 250 μL DPBS. The cellular uptake of fluorescence-labeled polyplexes was examined by using the BD FACScan analyzer or BD FACSCalibur (Becton Dickinson, San Jose, CA) at a minimum of 1×10^4 cells gated per sample. Analysis was performed by using Becton Dickinson CellQuest software. The data were processed by using Flowing software.

3. Results and discussion

3.1. Synthesis and characterization of polymers

First, methylcellulose was oxidized by using periodate oxidation for conjugation of PEI. It was reported that periodate ions, IO_4^- can react with vicinal diols to cleave carbon–carbon bonds, leading to the formation of dialdehyde which can be further modified (Vold & Christensen, 2005). In a similar way, periodate ions could cleave bonds between C2 and C3 carbon of glucose units in methylcellulose. Here, the degree of substitution of methylcellulose was given as 1.7 from the manufacturer. Based on this value, 0.5 molar equivalent periodates to glucose units of methylcellulose was used for oxidation.

In Fig. 2(a), H-2 protons of methylcellulose were identified (H-2(2-OH): 3.3–3.4 ppm, H-2(2-OMe): 3.05–3.2 ppm). Proton peaks from H-2(2-OH) disappeared in Fig. 2(b) (dashed oval), meaning H-2 protons were converted to aldehyde protons after periodate oxidation. H-2(2-OMe) proton peaks were observed to show no significant differences during oxidation reaction, indicating that participation of C2 with —OMe group to the reaction is minimal. It was reported that only the unsubstituted glycol groups could react with periodate ions but overoxidation of 2,3, 2,6, or 3,6-dimethylglucoses were also possible (Gibbons, 1956; Nadzhimutdinov, Sarymsakov, & Usmanov, 1981). Oxidation degree of methylcellulose was calculated as 56% based on the decrease of the integral of H-2(2-OH) protons. After dialysis followed by lyophilization, OXMC was reacted with PEI0.8k by reductive amination. Aldehydes of OXMC can react with primary amines of PEI0.8k via imine formation and sodium tetrahydroborate can reduce imine to stable C—N bond. Fig. 2(c) shows the proton peaks of the final product, MC-PEI. Proton peaks from PEI0.8k (—NHC**H$_2$**C**H$_2$**—) appeared in region ranging from 2.6 ppm to 3.0 ppm. Comparing all the proton peaks of methylcellulose and the proton peaks of PEI, the degree of PEI graft was calculated as 6.9% and it means that one molecule of PEI was grafted to every 14.5 glucose units of methycellulose.

Fig. S1 shows ^{13}C NMR results of MC and MC-PEI. The spectra of OXMC could not be obtained due to the high viscosity of OXMC NMR sample with high concentration (10 mg/mL). The specific carbon peaks of MC (C_1: 105.1 ppm, $C_{2,3,4}$: 76.7–86.0 ppm, C_5:

76.4 ppm, C_6: 72.8 ppm, and $-OCH_3$: 62.1 ppm) and PEI (carbon next to 3° amine: 54.0 ppm, carbon next to 2° amine: 48.5 ppm, and carbon next to 1° amine: 41.4 ppm) were identified, which means that this cationic methylcellulose derivative containing PEI moieties was successfully synthesized. FT-IR analysis results of the polymers were also presented in Fig. S2. The characteristic absorption bands for MC were observed at 1050 cm^{-1} (C—O—C stretching vibration), 2880 cm^{-1} (C—H stretching vibration), and 3450 cm^{-1} (O—H stretching vibrations). OXMC result showed another weak C=O stretch band at 1720 cm^{-1}, which stands for aldehyde formation. After PEI graft reaction, new bands for N—H bending and N—H wagging appeared at 1570 cm^{-1} and 810 cm^{-1}, respectively in MC-PEI result, meaning that PEI was successfully grafted to OXMC.

Molecular weights of the polymers were measured by GPC. It was found that methylcellulose has M_w of 35.5 kDa (PDI = 5.06). OXMC showed the decreased M_w of 14.6 kDa (PDI = 5.04), which means the depolymerizaion of MC by overoxidation. M_w of MC-PEI was measured to be 10.9 kDa (PDI = 2.19). The underestimation of MC-PEI molecular weight after the PEI graft to OXMC is thought to be mainly due to the structural difference between MC-PEI (comb-type shape) and OXMC (linear shape). The decreased PDI value is probably due to the removal of small molecular weight fragments of MC.

Endosome buffering capacities of polymers were also examined by acid–base titration (Fig. S3 in supplementary). In general, endosome buffering capacity of polymeric vector is thought to induce efficient endosomal escape of polyplexes and high transfection efficiency. But it was also reported that the transfection efficiency after the introduction of endosome buffering moieties could be decreased due to the change in hydrophobicity and pDNA condensing ability of the polymers (Kim, Rothmund, Kissel, & Kim, 2011). Endosome buffering capacities of PEI25k and PEI0.8k were measured to be 23.2% and 32.2%, respectively. MC-PEI showed the endosome buffering capacity of 31.3%. These results demonstrate the potential of MC-PEI with high endosome buffering ability, which may lead to efficient endosomal escape of the polyplexes and transfection.

3.2. Agarose gel electrophoresis

pDNA condensing ability is one of the prerequisites for gene delivery carriers. pDNA condensing ability of MC-PEI was examined by agarose gel electrophoresis. As shown in Fig. 3(a), weak migration of pDNA was observed at a weight ratio of 0.5. However, MC-PEI could retard pDNA from a weight ratio of 0.75 completely, meaning pDNA was totally condensed by MC-PEI at the low ratio. Therefore, it is confirmed that MC-PEI can condense pDNA efficiently due to the cationic moieties from grafted PEI molecules.

3.3. Protection of pDNA from serum

Gene delivery carriers should protect genetic materials from hostile environment such as serum nuclease during gene delivery. Therefore, protection ability of MC-PEI polyplex from serum was examined by agarose gel electrophoresis (Fig. 3(b)). After incubation in 50% FBS condition, heparin sodium salt was added to polyplex solution for dissociation of pDNA from the polyplex and then the solution was electrophoresed.pDNA itself was found to be degraded into small fragments in serum condition (lane 2). However, no degraded pDNA fragments were observed from MC-PEI polyplex at weight ratios ranging from 25 to 100 (lanes 3–6). As a control, PEI25k also showed similar pDNA protection ability. Supercoiled pDNA bands (lane 1, bottom) disappeared probably due to the nicking by serum in the case of both MC-PEI and PEI polyplex. These results indicate that MC-PEI could protect pDNA from serum

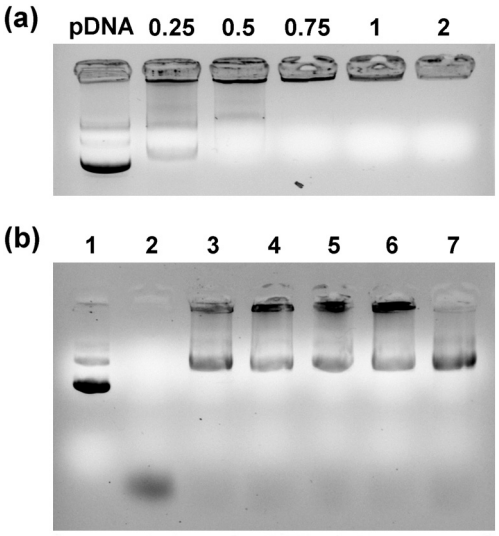

Fig. 3. (a) Agarose gel electrophoresis result of MC-PEI. Numbers mean weight ratios of MC-PEI polyplexes. (b) Result of pDNA protection from serum (50%). 1: pDNA only, 2: pDNA + serum, 3: MC-PEI polyplex (weight ratio (WR) = 25) + serum, 4: MC-PEI polyplex (WR = 50) + serum, 5: MC-PEI polyplex (WR = 75) + serum, 6: MC-PEI polyplex (WR = 100) + serum, 7: PEI25k polyplex (WR = 1) + serum.

attack by strong complexation similarly with PEI25k and suggest a high stability of MC-PEI polyplex in blood plasma.

We also examined the effect of heparin on serum activity. In Fig. S4, it was observed that pDNA degradation by serum is strongly inhibited in the presence of heparin. Therefore, it was found that pDNA could remain intact even in the serum condition after dissociation from polyplexes when pDNA exists in the presence of heparin.

3.4. Average sizes and Zeta-potential measurements of polyplexes

Average sizes and Zeta-potential values of MC-PEI polyplexes were measured by Zeta-sizer. It was observed that MC-PEI polyplexes have sizes ranging from 100 to 150 nm at all weight ratios in number-average size measurement, as shown in Fig. 4(a). Although the sizes showed some variations, they were all below 150 nm, meaning the formation of polyplexes with the proper sizes for efficient cellular uptake (Zauner, Ogris, & Wagner, 1998).

In the case of Zeta-potential (Fig. 4(b)), about −20 mV value was observed for MC-PEI polyplex at a weight ratio of 0.5, indicating incomplete pDNA condensation by MC-PEI. However, 20 mV value at a weight ratio of 1 shows the formation of positively charged polyplexes. This result is well correlated with the above pDNA retardation assay result. At high weight ratios over 10, MC-PEI polyplex showed consistent values ranging from 30 to 40 mV, which means the formation of stable polyplexes. These results demonstrate that MC-PEI can form positively charged polyplexes with pDNA, which may be easily adsorbed onto plasma membrane for facilitation of polyplex cellular uptake.

3.5. Morphology of polyplexes

The sizes and shapes of MC-PEI polyplexes were observed by TEM. As shown in Fig. 4(c), MC-PEI polyplexes with compact sphere shapes were identified at all weight ratios ranging from 25 to 75, displaying about 100 nm or less diameters. There were no significant differences between them, which is consistent with the tendency of the size measurement. However, these size values were less than the values measured by Zeta-sizer, which may be due to the shrinkage of polyplexes examined in dry condition for TEM

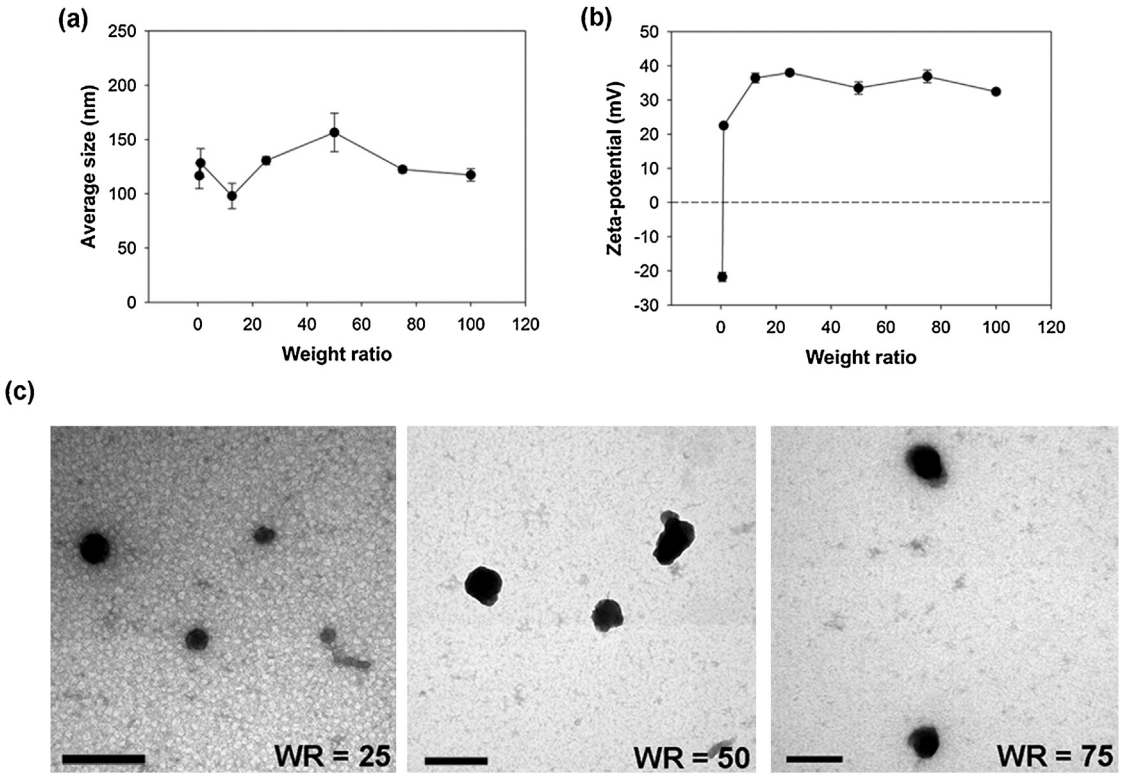

Fig. 4. (a) Average size measurement result of MC-PEI polyplexes. (b) Zeta-potential value measurement result of MC-PEI polyplexes. (c) TEM images of MC-PEI polyplexes. Black scale bars represent 200 nm. WR means weight ratio of polyplex.

observation in contrast to the hydration condition for Zeta-sizer measurement.

3.6. Cytotoxicity

The cytotoxicity of MC-PEI was investigated by MTT assay in C2C12 cells and HeLa cells. In Fig. 5(a), MC-PEI showed high cell viability (~90%) even at high concentration of 100 μg/mL in C2C12 cells. PEI25k exhibited low cell viability below 20% even at low concentration (20 μg/mL), which means high cytotoxicity of PEI25k. Interestingly, high cell viability of PEI0.8k (~90%) at low concentration was decreased to about 40% at higher concentration than 80 μg/mL. It is notable that PEI0.8k amount of MC-PEI at 100 μg/mL is regarded to be equivalent to the amount at 25 μg/mL of PEI0.8k because MC-PEI contains about 25% weight portion of PEI0.8k. Fig. 5(b) and (c) present MTT assay results of polymers and polyplexes in serum condition in C2C12 cells, respectively. Similarly with serum-free condition, cytotoxicity of both MC-PEI and MC-PEI polyplexes was found to be low even in serum condition. In HeLa cells, MC-PEI and PEI0.8k displayed high cell viability over 90% even at high concentration of 100 μg/mL (Fig. 5(d)). PEI25k still showed severe cytotoxicity in similar with C2C12 cell result. However, MC-PEI and MC-PEI polyplexes displayed high cell viability over 80% even at high concentration in serum condition in Fig. 5(e) and (f), respectively. These results demonstrate the minimal cytotoxicity of MC-PEI and MC-PEI polyplex due to the biocompatibility of MC and low cytotoxicity of grafted PEI0.8k.

3.7. Transfection experiments

Transfection efficiency of MC-PEI was evaluated by measuring luciferase transgene expression. Fig. 6(a) shows the transfection results in C2C12 cells in the absence of serum. PEI0.8k was also used as a control. Amounts of PEI0.8k used for polyplex formation were determined based on the amount of PEI0.8k grafted to MC-PEI. MC-PEI showed about 300–2200 times higher transfection efficiency than PEI0.8k, which means the importance of proper molecular weight of polymeric gene carriers for efficient gene delivery. It was reported that low molecular weight PEI showed low transfection efficiency due to easy dissociation of pDNA from polyplex (Godbey, Wu, & Mikos, 1999). However, transfection efficiency of MC-PEI was lower than that of PEI25k. MC-PEI polyplex at a weight ratio of 75 displayed about 8 times lower transfection efficiency than PEI25k polyplex. In the case of HeLa cell results (Fig. 6(b)), MC-PEI also showed the highest transfection efficiency at a weight ratio of 75, which is still about 3 times lower than that of PEI25k. The transfection efficiency of MC-PEI was found to be about 400–1300 times higher than that of PEI0.8k. These results indicate that MC-PEI didn't show enough transfection efficiency as high as PEI25k but displayed significantly improved transfection efficiency in comparison with PEI0.8k in serum-free condition.

Transfection efficiency of MC-PEI was also examined in serum condition for investigation of potential to in vivo gene delivery (Fig. 6(c) and (d)). In general, it has been reported that transfection efficiency of cationic polymers is inhibited by interaction with serum (Goldman et al., 1997). As expected, the transfection efficiencies of both PEI25k and MC-PEI were decreased in serum condition in comparison with serum-free condition. However, MC-PEI showed high transfection efficiency which is even about 3–5 times higher than PEI25k transfection efficiency in C2C12 cells (Fig. 6(c)). In similar with results in C2C12 cells, the transfection efficiency of MC-PEI (weight ratio = 100) in HeLa cells was found to be as high as that of PEI25k (Fig. 6(d)). These results demonstrate that the transfection efficiency of MC-PEI could be much less inhibited than that of PEI25k by serum interaction. It is thought to be due to the good serum-compatibility of MC-PEI polyplex, which

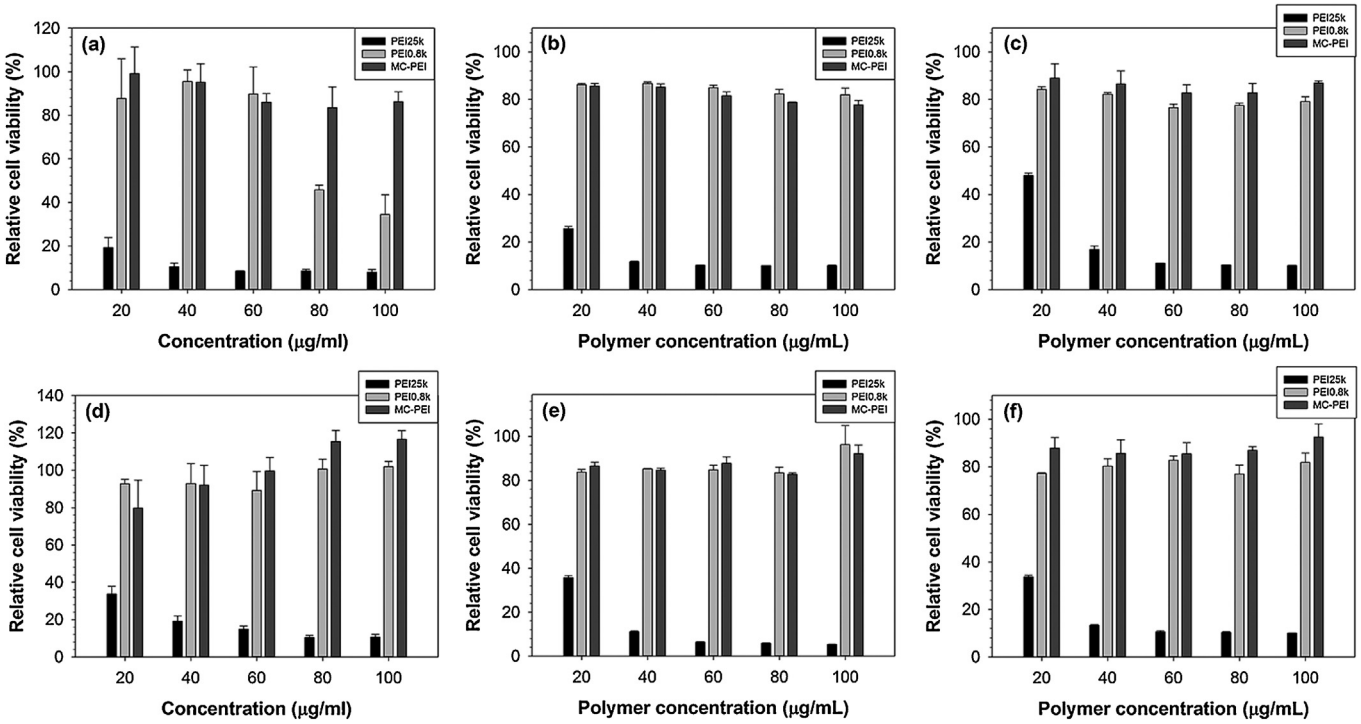

Fig. 5. MTT assay results in C2C12 cells (a, b, and c) and HeLa cells (d, e, and f). (a, d) Polymer results in serum-free condition, (b, e) polymer results in serum condition, and (c, f) polyplex results in serum condition.

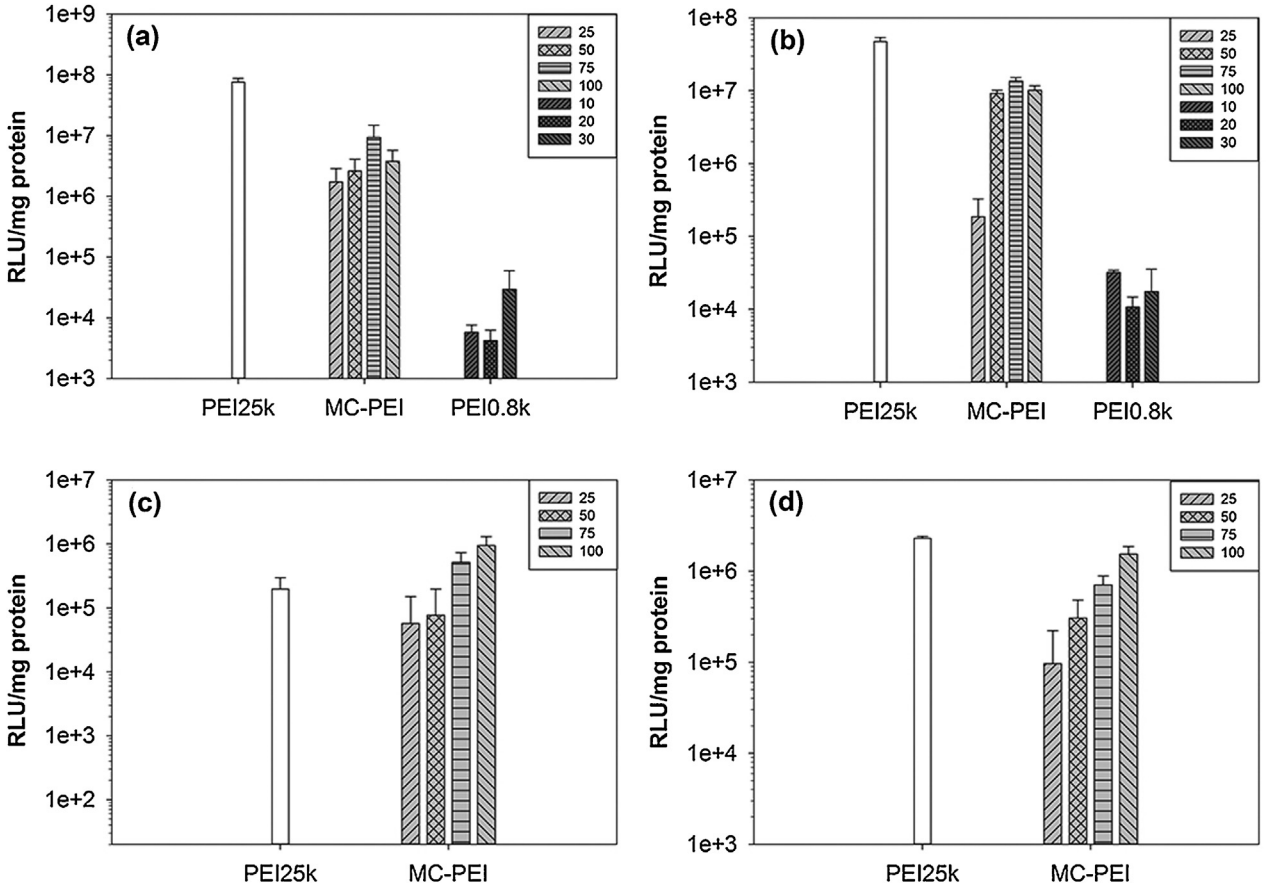

Fig. 6. Transfection efficiencies of polymers in serum-free condition in (a) C2C12 cells, (b) HeLa cells and transfection efficiencies of polymers in serum condition in (c) C2C12 cells, (d) HeLa cells. Numbers in box represent weight ratios of polyplexes (WR of PEI25k = 1, weak gray: MC-PEI, dark gray: PEI0.8k).

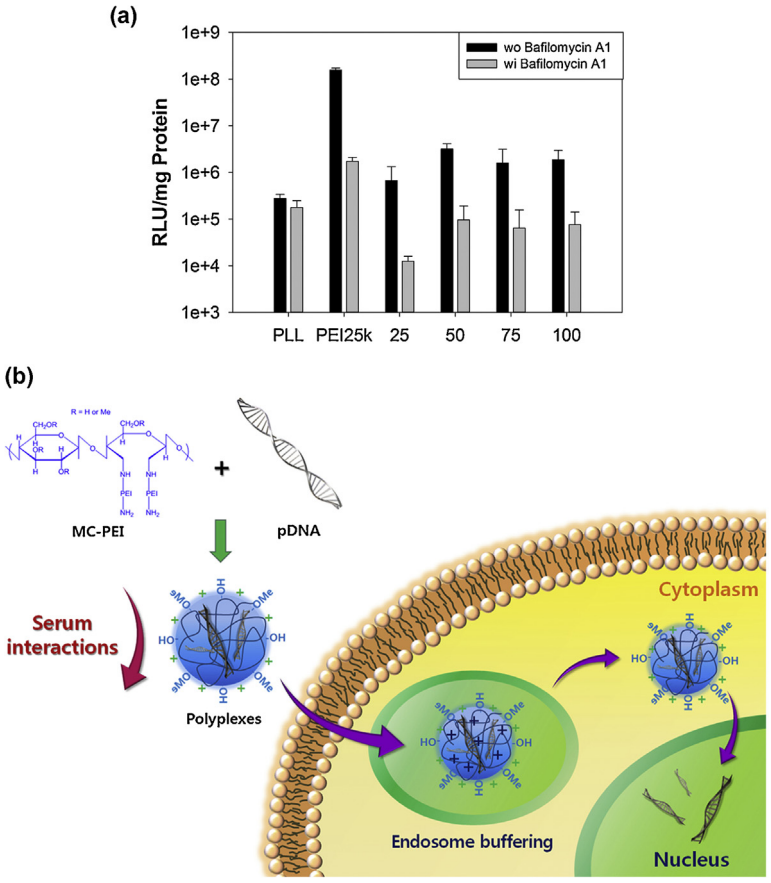

Fig. 7. (a) Bafilomycin A1-treated transfection efficiencies of polymers. Numbers mean weight ratios of MC-PEI polyplexes. PLL and PEI25k polyplexes were prepared at a weight ratio of 5 and 1, respectively. (b) Schematic diagram of MC-PEI polyplex transfection mechanism.

may be induced by shielding effect of hydroxyl or methoxy groups in methycellulose backbone from serum proteins, although MC-PEI polyplex also showed strong positive Zeta-potential values. These results suggest the potential of MC-PEI for in vivo gene delivery.

3.8. Transfection mechanism

Transfection experiments were performed with the treatment of bafilomycin A1 in order to investigate the effect of endosome buffering ability on transfection. Bafilomycin A1 is an inhibitor of vacuolar type ATPase which has been known to decrease the transfection efficiency of PEI by inhibiting the proton pump of endosome (Kichler, Leborgne, Coeytaux, & Danos, 2001). PLL was used as a control because PLL has no endosome buffering moieties such as tertiary amines. In Fig. 7(a), the transfection efficiency of PLL shows no significant differences between without and with Bafilomycin A1 conditions, which means the acidification of endosome by proton pump has no crucial effects to endosomal escape of PLL polyplex, as anticipated. However, the transfection efficiency of MC-PEI was dramatically decreased in Bafilomycin A1 condition in similar with PEI25k result. These results strongly demonstrate that the transfection of MC-PEI polyplex is mediated via endosomal escape by endosome buffering ability.

Based on the previous findings, the transfection mechanism of MC-PEI is presented in Fig. 7(b). It is supposed that nano-sized polyplex particles of MC-PEI possess good serum-compatibility due to the surficial methoxy and hydroxyl groups and they can escape from endosome by endosome buffering ability after cellular uptake, leading to the efficient gene delivery.

3.9. Cellular uptake of polyplexes

In order to examine of cellular uptake efficiency of the polyplexes, flow cytometry assay was performed. YOYO-1 iodide labeled pDNA was used for detection of internalized polyplex into cells. Each cellular uptake efficiency (%) was calculated by setting up M1 region as a gate. In Fig. 8(a), each cellular uptake efficiency in serum-free condition was as follows (PEI25k: 53.9%, MC-PEI (weight ratio (WR) = 25): 47.7%, MC-PEI (WR = 50): 63.3%, MC-PEI (WR = 75): 63.6%, and MC-PEI (WR = 100): 59.4%). In Fig. 8(b), each cellular uptake efficiency in serum condition was as follows (PEI25k: 97.8%, MC-PEI (WR = 25): 97.4%, MC-PEI (WR = 50): 91.8%, MC-PEI (WR = 75): 91.5%, and MC-PEI (WR = 100): 90.4%). MC-PEI polyplex showed similar cellular uptake to PEI25k polyplex in both serum-free condition and serum condition.

However, we could not find a close correlation between cellular uptake efficiency and transfection efficiency, which means other factors (e.g. efficient pDNA dissociation or pDNA localization to cell nucleus) may play an important role for efficient gene delivery. One possible explanation for this is as follows. Usually, positively charged polyplexes can interact with negatively charged serum proteins. This serum protein-adsorbed polyplex aggregates may be internalized well into cells by fast sedimentation in vitro condition. In the case of PEI25k polyplex, difficulty of serum protein desorption by strong electrostatic interaction with PEI25k may disturb pDNA dissociation from the polyplex after cellular uptake, leading to the decreased transfection efficiency in comparison with serum-free result. However, in the case of MC-PEI, adsorbed serum protein may be desorbed easily from the polyplex after cellular uptake due to the weak interaction by serum-compatibility of MC-PEI.

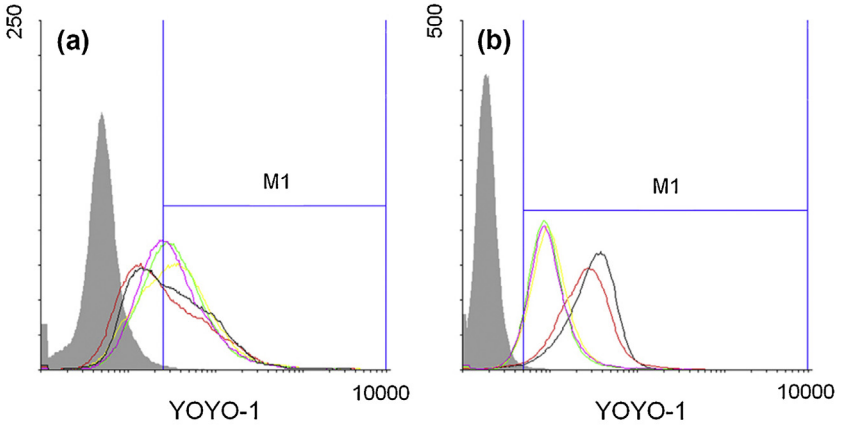

Fig. 8. Flow cytometry results of polyplexes in (a) serum-free condition and (b) serum condition. Gray histogram: cell only, black line: PEI25k polyplex, red line: MC-PEI polyplex (WR = 25), yellow line: MC-PEI polyplex (WR = 50), green line: MC-PEI polyplex (WR = 75), and purple line: MC-PEI polyplex (WR = 100). Each cellular uptake efficiency (%) of polyplex was calculated by setting up M1 region as a gate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Therefore, pDNA release from MC-PEI polyplex may be relatively less disturbed, which could lead to less decrease of MC-PEI transfection efficiency than PEI25k transfection efficiency by serum interaction. In addition, with the increase of weight ratios, increased amount of co-internalized MC-PEI can facilitate endosomal escape of the polyplexes by endosome buffering effect.

Therefore, these results suggest that MC-PEI polyplex could be internalized into cells and efficiently deliver pDNA to cells due to its serum-compatibility. Further modifications would be required in order to overcome hurdles (e.g. dissociation of pDNA from polyplexes or nuclear localization of delivered pDNA) for efficient gene delivery and the strategies may include the introduction of biodegradable moieties or nuclear localization signals, variation of conjugated PEI molecular weights or degree of PEI conjugation.

4. Conclusions

In this work, methylcellulose was utilized as a template polymer for gene delivery systems for the first time. Low molecular weight PEI0.8k was grafted to oxidized methylcellulose in order to provide pDNA condensing ability and endosome buffering ability. The synthesized MC-PEI could condense pDNA into positively charged and nano-sized polyplex and show high protection ability from serum nuclease. The cytotoxicity of MC-PEI and MC-PEI polyplex was found to be minimal in serum-free condition or serum condition probably due to the biocompatibility of MC and low cytotoxicity of PEI0.8k. Flow cytometry results for cellular uptake of the polyplexes show that the cellular uptake efficiency of MC-PEI polyplex was similar to that of PEI25k polyplex in both serum-free condition and serum condition. It was also suggested that in the case of MC-PEI polyplex, adsorbed serum protein may be desorbed easily from the polyplex after cellular uptake due to the weak interaction by serum-compatibility of MC-PEI and that pDNA release from the polyplex may be relatively less disturbed, which could lead to less inhibition of MC-PEI transfection than PEI25k transfection by serum interaction. This relatively less decreased transfection efficiency of MC-PEI in serum condition shows a potential for efficient gene delivery in vivo system due to the serum-compatibility of MC. In addition, it was revealed that the transfection of MC-PEI is mediated via endosomal escape by endosome buffering ability. In conclusion, these results demonstrate that MC-PEI possesses a potential for gene delivery systems and this work suggests a guide for further modifications of methylcellulose for efficient gene delivery systems.

Acknowledgements

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0015045). We also acknowledge the National Instrumentation Center for Environmental Management (NICEM) for permission to take ^{1}H NMR, ^{13}C NMR and flow cytometry measurements, Prof. Cheol-Heui Yun for permission to take flow cytometry measurements, and Prof. Yan Lee for permission to take Zeta-sizer measurements.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.03.073.

References

Forrest, M. L., Gabrielson, N., & Pack, D. W. (2005). Cyclodextrin–polyethylenimine conjugates for targeted in vitro gene delivery. *Biotechnology and Bioengineering, 89*(4), 416–423.

Giacca, M., & Zacchigna, S. (2012). Virus-mediated gene delivery for human gene therapy. *Journal of Controlled Release, 161*(2), 377–388.

Gibbons, G. C. (1956). Periodate oxidation of water-soluble methylcellulose and its constituent methylglucoses. *Journal of the Textile Institute Transactions, 47*, T511–T529.

Godbey, W. T., Wu, K. K., & Mikos, A. G. (1999). Size matters: Molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. *Journal of Biomedical Materials Research, 45*(3), 268–275.

Goldman, C. K., Soroceanu, L., Smith, N., Gillespie, G. Y., Shaw, W., Burgess, S., et al. (1997). In vitro and in vivo gene delivery mediated by a synthetic polycationic amino polymer. *Nature Biotechnology, 15*(5), 462–466.

Griesenbach, U., Meng, C., Farley, R., Wasowicz, M. Y., Munkonge, F. M., Chan, M., et al. (2010). The use of carboxymethylcellulose gel to increase non-viral gene transfer in mouse airways. *Biomaterials, 31*(9), 2665–2672.

Hirrien, M., Chevillard, C., Desbrieres, J., Axelos, M. A. V., & Rinaudo, M. (1998). Thermogelation of methylcellulose: New evidence for understanding the gelation mechanism. *Polymer, 39*(25), 6251–6259.

Hirrien, M., Desbrieres, J., & Rinaudo, M. (1996). Physical properties of methylcelluloses in relation with the conditions for cellulose modification. *Carbohydrate Polymers, 31*(4), 243–252.

Hosseinkhani, H., Azzam, T., Tabata, Y., & Domb, A. J. (2004). Dextran–spermine polycation: An efficient nonviral vector for in vitro and in vivo gene transfection. *Gene Therapy, 11*(2), 194–203.

Huang, S., & Kamihira, M. (2013). Development of hybrid viral vectors for gene therapy. *Biotechnology Advances, 31*(2), 208–223.

Jeong, J. H., Kim, S. W., & Park, T. G. (2007). Molecular design of functional polymers for gene therapy. *Progress in Polymer Science, 32*(11), 1239–1274.

Jiang, H.-L., Kim, Y.-K., Arote, R., Nah, J.-W., Cho, M.-H., Choi, Y.-J., et al. (2007). Chitosan-graft-polyethylenimine as a gene carrier. *Journal of Controlled Release*, *117*(2), 273–280.

Kang, H. C., Huh, K. M., & Bae, Y. H. (2012). Polymeric nucleic acid carriers: Current issues and novel design approaches. *Journal of Controlled Release*, *164*(3), 256–264.

Khan, W., Hosseinkhani, H., Ickowicz, D., Hong, P.-D., Yu, D.-S., & Domb, A. J. (2012). Polysaccharide gene transfection agents. *Acta Biomaterialia*, *8*(12), 4224–4232.

Kichler, A., Leborgne, C., Coeytaux, E., & Danos, O. (2001). Polyethylenimine-mediated gene delivery: A mechanistic study. *The Journal of Gene Medicine*, *3*(2), 135–144.

Kim, T.-i., Rothmund, T., Kissel, T., & Kim, S. W. (2011). Bioreducible polymers with cell penetrating and endosome buffering functionally for gene delivery systems. *Journal of Controlled Release*, *152*(1), 110–119.

Krebs, M. D., Salter, E., Chen, E., Sutter, K. A., & Alsberg, E. (2010). Calcium alginate phosphate-DNA nanoparticle gene delivery from hydrogels induces in vivo osteogenesis. *Journal of Biomedical Materials Research Part A*, *92*(3), 1131–1138.

Liang, H.-F., Hong, M.-H., Ho, R.-M., Chung, C.-K., Lin, Y.-H., Chen, C.-H., et al. (2004). Novel method using a temperature-sensitive polymer (methylcellulose) to thermally gel aqueous alginate as a pH-sensitive hydrogel. *Biomacromolecules*, *5*(5), 1917–1925.

Liu, F., & Huang, L. (2002). Development of non-viral vectors for systemic gene delivery. *Journal of Controlled Release*, *78*, 259–266.

Liu, W., Zhang, B., Lu, W. W., Li, X., Zhu, D., Yao, K. D., et al. (2004). A rapid temperature-responsive sol–gel reversible poly(N-isopropylacrylamide)-g-methylcellulose copolymer hydrogel. *Biomaterials*, *25*(15), 3005–3012.

Luo, D., & Saltzman, W. M. (2000). Synthetic DNA delivery systems. *Nature Biotechnology*, *18*(1), 33–37.

Nadzhimutdinov, S., Sarymsakov, A. A., & Usmanov, K. U. (1981). Regularities in the synthesis of cellulose dialdehyde and its ethers. *Cellulose Chemistry and Technology*, *15*, 613–628.

Popielarski, S. R., Mishra, S., & Davis, M. E. (2003). Structural effects of carbohydrate-containing polycations on gene delivery. 3. Cyclodextrin type and functionalization. *Bioconjugate Chemistry*, *14*(3), 672–678.

Rekha, M. R., & Sharma, C. P. (2009). Blood compatibility and in vitro transfection studies on cationically modified pullulan for liver cell targeted gene delivery. *Biomaterials*, *30*(34), 6655–6664.

Sinn, P. L., Burnight, E. R., Hickey, M. A., Blissard, G. W., & McCray, P. B., Jr. (2005). Persistent gene expression in mouse nasal epithelia following feline immunodeficiency virus-based vector gene transfer. *Journal of Virology*, *79*, 12818–12827.

Thomas, J. J., Rekha, M. R., & Sharma, C. P. (2010). Dextran–glycidyltrimethylammonium chloride conjugate/DNA nanoplex: A potential non-viral and haemocompatible gene delivery systems. *International Journal of Pharmaceutics*, *389*, 195–206.

Toh, E. K., Chen, H.-Y., Lo, Y.-L., Huang, S.-J., & Wang, L.-F. (2011). Succinated chitosan as a gene carrier for improved chitosan solubility and gene transfection. *Nanomedicine*, *7*(2), 174–183.

Tseng, W.-C., Tang, C.-H., & Fang, T.-Y. (2004). The role of dextran conjugation in transfection mediated by dextran-grafted polyethylenimine. *The Journal of Gene Medicine*, *6*(8), 895–905.

Vold, I. M. N., & Christensen, B. E. (2005). Periodate oxidation of chitosans with different chemical compositions. *Carbohydrate Research*, *340*(4), 679–684.

Wang, W., Li, W., Ma, N., & Steinhoff, G. (2013). Non-viral gene delivery methods. *Current Pharmaceutical Biotechnology*, *14*(1), 46–60.

Wang, Y., Lapitsky, Y., Kang, C. E., & Shoichet, M. S. (2009). Accelerated release of a sparingly soluble drug from an injectable hyaluronan–methylcellulose gel. *Journal of Controlled Release*, *140*(3), 218–223.

Weecharangsan, W., Opanasopit, P., Ngawhirunpat, T., Rojanarata, T., & Apirakaramwong, A. (2006). Chitosan lactate as a nonviral gene delivery vector in COS-1 cells. *AAPS PharmSciTech*, *7*(3), E1–E6.

Xu, F. J., Ping, Y., Ma, J., Tang, G. P., Yang, W. T., Li, J., et al. (2009). Comb-shaped copolymers composed of hydroxypropyl cellulose backbones and cationic poly((2-dimethyl amino)ethyl methacrylate) side chains for gene delivery. *Bioconjugate Chemistry*, *20*(8), 1449–1458.

Yang, C., Li, H., Goh, S. H., & Li, J. (2007). Cationic star polymers consisting of α-cyclodextrin core and oligoethylenimine arms as nonviral gene delivery vectors. *Biomaterials*, *28*(21), 3245–3254.

Yu, H., Chen, X., Lu, T., Sun, J., Tian, H., Hu, J., et al. (2007). Poly(L-lysine)-graft-chitosan copolymers: Synthesis, characterization, and gene transfection effect. *Biomacromolecules*, *8*(5), 1425–1435.

Yun, Y. H., Goetz, D. J., Yellen, P., & Chen, W. (2004). Hyaluronan microspheres for sustained gene delivery and site-specific targeting. *Biomaterials*, *25*(1), 147–157.

Zauner, W., Ogris, M., & Wagner, E. (1998). Polylysine-based transfection systems utilizing receptor-mediated delivery. *Advanced Drug Delivery Reviews*, *30*(1-3), 97–113.