Synthesis and Characterization of Methylcellulose-Poly(ethylenimine)2k for Gene Delivery System

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Abstract: In this study, methylcellulose-poly(ethylenimine)2k (MC-PEI2k) was synthesized for gene delivery system. First, MC was oxidized by periodate oxidation and PEI2k molecules were attached to oxidized MC via reductive amination reaction between aldehyde moieties of oxidized MC and amines of PEI2k. Chemical structures and molecular weights of MC-PEI2k were confirmed by 1H NMR and GPC, respectively. MC-PEI2k could condense pDNA into positively charged and nano-sized polyplex particles. MC-PEI2k showed concentration-dependent but lower cytotoxicity than PEI25k. Transfection efficiency of MC-PEI2k was found to be lower than PEI25k in HeLa cells in serum-free condition but higher in serum condition, due to its good serum-compatibility. These results suggested the potential of MC-PEI2k for gene delivery system.

Key words: Gene delivery, methylcellulose, poly(ethylenimine), serum-compatibility.

1. Introduction

For several decades, many polymeric gene delivery carriers have been developed for efficient gene delivery systems because of beneficial properties such as non-immunogenicity, low cytotoxicity, ease of manufacturing, and ability to transfer large size of genes into cells [1]. Methylcellulose is a cellulose ether derivative having a structure in which several hydroxyl groups are substituted with a methyl group (Degree of substitution: 1.4-2.5) [2], [3]. Methylcellulose has been widely used in the field of emulsifiers, thickeners, and drug delivery due to its excellent biocompatibility and unique sol-gel transition property [4]-[6]. Recently, cationic methylcellulose derivative (MC-PEI) containing low molecular weight PEI (PEI0.8k) has been reported as a non-toxic and efficient gene delivery carrier [7]. MC-PEI0.8k showed a potential for gene delivery systems due to its good serum compatibility and low cytotoxicity, possessing endosome buffering ability. However, high amount of the polymer was required for efficient transfection, because of its low charge density. Therefore, in this study, PEI with higher molecular weight, PEI2k was grafted to methylcellulose and characterized for gene delivery systems in order to improve this weakness.

2. Materials and Methods

2.1. Materials

Methyl cellulose (15 cP), polyethylenimine (PEI, molecular weight 2 kDa and 25 kDa), agarose, ethidium

bromide, ethylenediaminetetraacetic acid (EDTA), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazalium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagles' Medium (DMEM), Dulbecco's Phosphate Buffered Saline (DPBS), Fetal Bovine Serum (FBS), and 0.25 % Trypsin-EDTA were purchased from Invitrogen-Gibco (Carlsbad, CA). Sodium periodate and sodium tetrahydroborate were purchased from Junsei (Japan). HPLC grade Water was purchased from Duksan (Korea). The plasmid DNA, pCN-Luci containing a firefly luciferase reporter gene was amplified in *Escherichia coli,* DH5α and isolated by Nucleobond Xtra Midi kit (Macherey-Nagel, Germany). Luciferase assay system and reporter lysis buffer were pruchased from Promega (Madison, WI). 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-PEI2k

MC-PEI2k was synthesized according to the previous report with slight changes [7]. First, MC was dissolved in water (80 °C). Then, About 2 times more volume of cool water was added and the solution was cooled to 4 °C. After agitation for complete solubilization of MC, MC solution was added by dropwise to sodium periodate solution (water). The reaction was maintained for 24 hours (nitrogen atmosphere, dark condition, and room temperature). The reaction mixture was dialyzed against ultrapure water with a dialysis membrane (MWCO = 6-8000) for 1 day and lyophilized to obtain oxidized MC (OXMC). Secondly, PEI2k solution (1 molar equivalent to glucose units of OXMC, water) was added by dropwise to OXMC solution (water) for grafting PEI2k to OXMC. After the reaction for 24 hours (nitrogen atmosphere, dark condition, and room temperature), sodium tetrahydroborate solution (water) was mixed subsequently for reduction of imine bonds between OXMC and PEI2k. After further reaction for 24 hours, the reaction mixture was dialyzed against ultrapure water with a dialysis membrane (MWCO = 10k) for 6 days and lyophilized to obtain a final product, MC-PEI2k.

The each step of polymer synthesis was confirmed by ^{1}H NMR (D_2O, 400 MHz JEOL JNM-LA400, Japan). 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) using polyethyleneglycols with various molecular weights as standards. The assay was run on Ultrahydrogel 250 column (Waters, Milford, MA) with 1% formic acid as an eluent. The concentration of the polymer solutions was set to 10 mg/mL and the flow rate to 0.6 mL/min.

2.3. pDNA Condensation Ability Measurement of MC-PEI2k

Agarose gel electrophoresis was performed to examine the pDNA condensation ability of MC-PEI2k. Agarose gel (0.7%, w/v) containing 0.5 μg/mL of ethidium bromide was prepared in Tris-Acetate-EDTA (TAE) buffer. The polyplex solutions (0.5 μg pDNA) were prepared in Hepes buffer (pH 7.4) at various weight ratios ranging from 0.25 to 2.0 (polymer/pDNA) for 30 min. After loading the samples into the gel, the electrophoresis was run for 15 min at 100 V (Mupid-2plus, Takara Bio Inc., Japan). The locations of pDNA bands were observed by using a UV illuminator (GelDoc™ XR+ gel documentation system, Bio-Rad, Hercules, CA).

2.4. Characterization of MC-PEI2k Polyplex

The Z-average sizes and zeta-potential values of the polyplexes were measured by using a Zetasizer Nano ZS (Malvern Instruments, UK) with He-Ne laser beam (633 nm) at 25 °C. 4 μg of pDNA was used to prepare polyplex solutions at weight ratios ranging from 0.1 to 25. After 30 min of incubation, the polyplex solutions were diluted by ultrapure water to 1 mL before measurements. In addition, serum stability of MC-PEI2k polyplexes was also examined by measuring their average sizes in serum condition. MC-PEI2k polyplex

(weight ratio = 20) solutions were prepared and diluted by DMEM (serum-free or serum (10% FBS) condition). After incubation at 37 °C, Z-average sizes of the polyplexes were measured at predetermined times (0, 30, and 60 min). Average particle sizes and zeta-potential values were measured 3 times.

2.5. Cell Culture

Human cervical cancer cells (HeLa cells) were used for cell experiments of MC-PEI2k. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in humidified atmosphere (5% CO_2) at 37 °C.

2.6. Cytotoxicity of MC-PEI2k

Cytotoxicity of MC-PEI2k was identified by MTT assay. PEI2k and PEI25k were used as controls. Cells were seeded on 96-well plates at 1 x 10^4 cells/well. After achievement of 70-80% confluency (~24 hours), cells were exposed to serum-free DMEM containing various concentration of the polymers ranging from 0 to 100 μg/mL for 4 hours. Then, the medium was changed with fresh DMEM containing 10% FBS. After 24 hours of incubation, cells were treated with 25 μL of MTT (2 mg/mL, DPBS) for 2 hours and the medium was carefully removed. 150 μL of DMSO was added to dissolve formazan crystals formed by proliferating cells and the absorbance was measured at 570 nm using a microplate reader. The results were presented as relative cell viabilities (RCV, %) to cell only values. All experiments were performed in triplicate.

2.7. Transfection Efficiency of MC-PEI2k

Transfection efficiency of MC-PEI2k was analyzed by luciferase transgene expression assay. Cells were seeded on 24-well plates at a density of 5 x 10^4 cells/well and incubated for 24 hours. Before transfection, the media were exchanged with serum-free DMEM for the assay in non-serum condition and with fresh DMEM (10% FBS) for the assay in serum condition, respectively. Then, cells were treated with the polyplexes (0.5 μg pDNA) formed at various weight ratios for 4 hours. PEI25k (weight ratio=1) were used as a control. After incubation for 4 hours, the cells were rinsed with DPBS and shaken for 30 min at room temperature with reporter lysis buffer. The cell lysates were centrifuged and luciferase activities of the supernatants were measured by using luciferase assay reagents on a microplate reader. A protein quantification assay was also performed using a BCA™ protein assay 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.

3. Results and Discussion

3.1. Synthesis of MC-PEI2k

MC-PEI2k was synthesized by reductive amination of oxidized MC (OXMC) with PEI2k. First, MC was oxidized by periodate oxidation reaction, where periodate ions can cleave C-C bonds between C2 and C3 carbons of glucose units in methylcellulose, forming dialdehydes [8]. As shown in Fig. 1(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). Methylcellulose oxidization was confirmed by the disappearance of H-2 proton peak at 3.3-3.4 ppm, meaning H-2 protons were converted to aldehyde protons after periodate oxidation (Fig. 1(b)). After reaction of PEI2k with OXMC via imine formation and its reduction to stable C-N bond by sodium tetraborohydrate, proton peaks from PEI2k (-NHC$\mathbf{H_2}$C$\mathbf{H_2}$) were observed (2.5-3.0 ppm) (Fig. 1(c)). Comparing the proton peaks of MC and PEI2k, it was calculated that one molecule of PEI2k was grafted to every 14.4 glucose units of MC. The synthetic yield of MC-PEI2k from OXMC was about 60%.

Molecular weights of MC-PEI2k were measured by GPC. Weight average molecular weight (Mw) of MC-PEI2k was 21.6 kDa and number average molecular weight (Mn) of MC-PEI2k was 13.2 kDa.

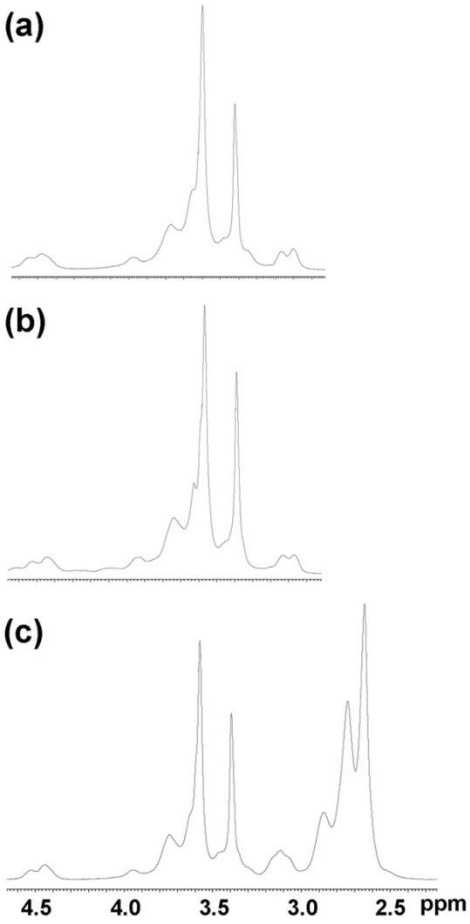

Fig. 1. ^{1}H NMR spectra of (a) MC, (b) OXMC, and (c) MC-PEI2k.

3.2. pDNA Condensation Ability of MC-PEI2k

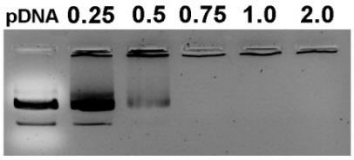

Fig. 2. Agarose gel electrophoresis result of MC-PEI2k polyplex. Numbers mean the weight ratios of the polyplexes.

pDNA condensation ability of MC-PEI2k was examined by agarose gel electrophoresis (Fig. 2). Uncondensed pDNA migration was observed even at a weight ratio of 0.5 but MC-PEI2k could condense pDNA via electrostatic interactions from a low weight ratio of 0.75 completely. Therefore, it is confirmed that MC-PEI2k can condense pDNA efficiently due to the cationic PEI moieties.

3.3. Z-Average Sizes and Zeta-Potential Values of MC-PEI2k Polyplexes

Z-average sizes and Zeta-potential values of MC-PEI2k polyplexes were measured by Zeta-sizer. As shown in Fig. 3(a), MC-PEI2k polyplexes could form 250 nm or less sized nanoparticles at higher ratio weights than 0.1. Zeta-potential values of MC-PEI2k polyplexes increased from -16.6 mV at a weight ratio of 0.1 to 38.5 mV at a weight ratio of 10, exhibiting the formation of positively charged and stable polyplexes (Fig. 3(b)). This results well corresponded with the previous agarose gel electrophoresis result and showed that

MC-PEI2k could form positively charged polyplex nanoparticles with pDNA, which are necessary for effective endocytosis [9].

Serum stability of MC-PEI2k polyplexes was also examined by measuring the average sizes in serum condition (10% FBS). In general, positively charged polyplexes can form large aggregates with negatively charged serum proteins, which may decrease their cellular uptake and transfection efficiency. In serum-free condition, MC-PEI2k polyplexes showed about 230 nm sizes regardless of incubation time (Table 1). In serum condition, the sizes of the polyplexes were not changed significantly, maintaining 223-237 nm sizes even after 60 min of incubation. These results mean that MC-PEI2k possesses a high serum stability, which can benefit its gene delivery in serum condition.

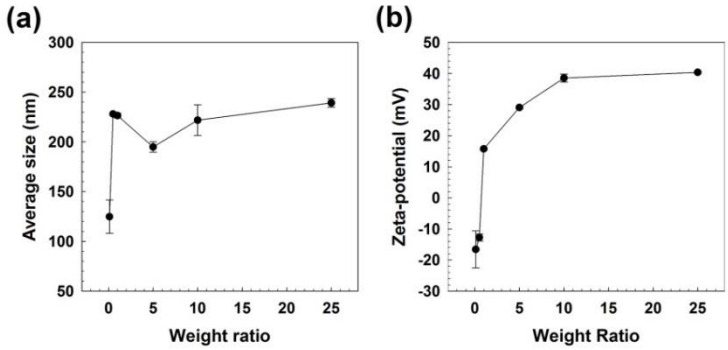

Fig. 3. (a) Z-average sizes and (b) Zeta-potential values of MC-PEI2k polyplexes.

Table 1. Z-Average Sizes of MC-PEI2k Polyplexes in Serum-Free or Serum Condition

	Incubation time (0 min)	30 min	60 min
wo 10% FBS	230.4 ± 13.1 nm	225.7 ± 14.1 nm	228.7 ± 11.9 nm
w 10% FBS	236.8 ± 48.8 nm	236.8 ± 59.4 nm	223.0 ± 33.6 nm

3.4. Cytotoxicity of MC-PEI2k

Cytotoxicity of MC-PEI2k was evaluated by MTT assay (Fig. 4). Viabilities of PEI25k-treated cells decreased to less than 10% from 10 μg/mL, displaying its severe cytotoxicity, as expected. On the contrary, in the case of PEI2k, cell viabilities were almost 100% at all concentrations examined, meaning its marginal cytotoxicity due to the low molecular weight. MC-PEI2k showed high cell viabilities at low concentrations but they decreased to less than 90% from 40 μg/mL in a concentration-dependent manner. Considering the previous study reporting minimal cytotoxicity of MC-PEI0.8k [7], it is thought that somewhat cytotoxicity of MC-PEI2k would be induced by its higher molecular weight and charge density than MC-PEI0.8k.

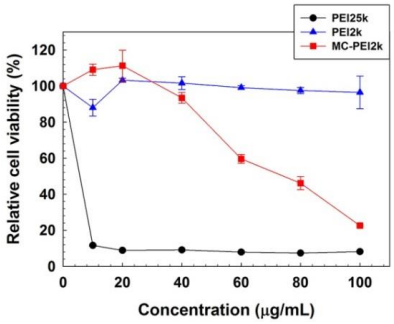

Fig. 4. MTT assay result of MC-PEI2k in HeLa cells.

3.5. Transfection Efficiency of MC-PEI2k

Transfection efficiency of MC-PEI2k was examined by luciferase transgene expression assay. Weight ratios ranging from 5 to 30 were used for MC-PEI25k polyplexes due to the cytotoxicity of MC-PEI25k. As shown in Fig. 5(a), transfection efficiency of MC-PEI2k increased along with the increase of the weight ratios in both serum-free and serum conditions. In serum-free condition, MC-PEI2k showed about 25% transfection efficiency at a weight ratio of 30, compared to PEI25k. In general, it was reported that transfection efficiency of cationic polymers is inhibited by non-specific interaction with serum proteins [10]. However, transfection efficiency of MC-PEI2k was less inhibited and 2.26 times higher than that of PEI25k at the same weight ratio in serum condition (Fig. 5(b)). These results mean that MC-PEI2k possesses a high serum stability, as already elucidated in section 3.3, probably due to the shielding effect of hydroxyl and methoxy functional groups of MC, which can inhibit non-specific interaction of serum proteins [7].

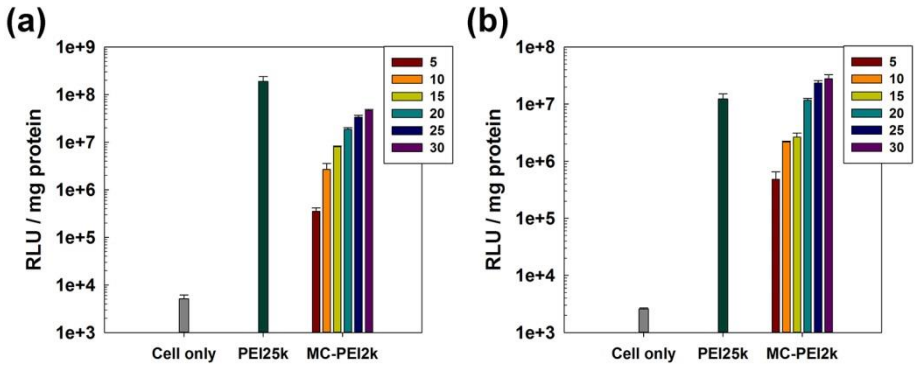

Fig. 5. Transfection results of MC-PEI2k in HeLa cells.

4. Conclusion

MC-PEI2k was synthesized for gene delivery systems. MC-PEI2k could retard pDNA even at a low weight ratio of 0.75, showing high pDNA condensation ability. It could form positively charged and nano-sized polyplex particles, which makes them interact with anionic cell surface and be internalized easily. Cytotoxicity of MC-PEI2k was lower than PEI25k but somewhat high at elevated concentrations because of the high charge density. Transfection efficiency of MC-PEI2k was lower than PEI25k in serum-free condition but higher in serum condition, which means MC-PEI2k possessed a high serum compatibility. Therefore, these results suggest a potential of MC-PEI2k for efficient gene delivery systems.

Acknowledgment

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