Water-Soluble and Low Molecular Weight Chitosan-Based Plasmid DNA Delivery

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Purpose. Chitosan, a natural cationic polysaccharide, is a candidate non-viral vector for gene delivery because of its high positive charges and low cytotoxicity. In this study, low molecular weight chitosan (LMWC, molecular weight of 22 kDa) was characterized and evaluated as a gene carrier.

Methods. Plasmid/LMWC complex was analyzed in 1% agarose gel electrophoresis. To confirm that the LMWC protected plasmids from nuclease, DNase I protection assays were performed. pSV-β-galactosidase plasmid/LMWC complex was transfected into 293T cells and transfection efficiency was evaluated by β-galactosidase assay. Cytotoxicity of LMWC was determined by MTT assay.

Results. Unlike high molecular weight chitosan (HMWC), LMWC is highly water soluble, and can form complex with plasmids in physiological buffer. The plasmid DNA was completely retarded at a weight ratio of 1:2 (plasmid:LMWC) in 1% agarose gel. DNase I protection assay showed that plasmids were protected from DNase I over 60 min. The most efficient transfection was obtained at a weight ratio of 1:3 (plasmid:LMWC). The transfection efficiency of LMWC was significantly higher than naked DNA and higher than poly-L-lysine (PLL). MTT assay showed that LMWC was less cytotoxic than PLL.

Conclusions. LMWC is non-toxic and has higher transfection efficiency than PLL. Therefore, LMWC will be useful in the development of safe gene carriers.

KEY WORDS: DNase I; gel retardation; gene delivery; *in vitro* transfection; low molecular weight chitosan (LMWC); MTT assay.

INTRODUCTION

Several methods that enhance the transfer of DNA into eukaryotic cells have been developed for gene therapy. Viral-based gene delivery is currently the most effective way to transfer genes to cells (1). However, it introduces serious concerns about endogenous virus recombinations, oncogenic effects, and immunological reactions (2,3). Such concerns have limited the use of viral vectors for human gene therapy (4). These limitations prompted the development of non-viral delivery systems (5,6). Non-viral delivery systems have several advantages to viral systems. They can accommodate large-size DNA, be modified with appropriate ligands for specific cell targeting, and be administered repeatedly. Non-viral delivery systems generally consist of cationic liposomes, linear cationic polymers, and its conjugate. Cationic polymer-mediated transfection has become a well-established system. Macromolecules such as poly-L-lysine (PLL), cationic dendrimers, and polyethylenimine, interact electrostatically with plasmids to form toroidal structures. However, non-viral gene delivery has its limitations such as toxicity and low transfection efficiency. To reduce the cytotoxicity and increase transfection efficiency, several kinds of derivatives of PLL have been synthesized. These modifications include conjugation of asialoglycoprotein (7–10), transferrin (11,12), antibody (13), lactose (14,15), and mannose (16). Recently, PEG-*g*-PLL showed higher transfection efficiency and lower cytotoxicity than PLL (17).

Chitosan has also been attractive gene carrier, because of its high positive charges and low toxicity to cells. Chitosan is a natural product, and its low toxicity was well-established (18). Chitosan is a biodegradable polysaccharide composed of two subunits, D-glucosamin and *N*-acetyl-D-glucosamine linked together by β(1,4)-glycosidic bonds. General lysozymes in the body degrade chitosan into a common amino sugar, *N*-acetyl glucosamine, which is incorporated into the synthetic pathway of glycoproteins, and is subsequently excreted as carbon dioxide (19). Chitosan was first described as a delivery system for plasmids by Mumper *et al.* (20). So far, several gene delivery trials have been made with chitosan (21–23). Oral gene delivery with chitosan-DNA nanoparticles was also tried (24). However, these trials used relatively high molecular weight chitosan (HMWC, 70 kDa). However, it was hard for this chitosan to be soluble in water, and it was dissolved in acidic solution. Also, water insoluble low molecular weight chitosan (<10 kDa) was evaluated in biocompatibility, body distribution, and ability to complex DNA (25). However, transfection efficiency of this low molecular chitosan has not been reported. In this study, water-soluble low molecular weight chitosan (LMWC, molecular weight of 22 kDa) was characterized and evaluated as a gene carrier. LMWC is highly water soluble and can form complex with plasmids in physiological buffer. The formation of plasmid/LMWC complex was proved and transfection efficiency in 293T cells was evaluated. The cytotoxicity of LMWC also was determined by MTT assay. This study showed that LMWC is one of the candidates for DNA delivery.

METHODS

Preparation of LMWC

Water-soluble LMWC with a molecular weight of 22 kDa, an average degree of polymerization of 136, and a degree of deacetylation of 72.5% was supplied by KITTOLIFE Co., Seoul, Korea. One milligram of LMWC was dissolved in 1 ml of phosphate-buffered saline (PBS, pH 7.3).

Preparation of pSV-β-Galactosidase Plasmid

pSV-β-galactosidase plasmid (Promega, Madison, WI) was introduced into *Escherichia coli* strain DH5α (Gibco-BRL, Gaithersburg, MD), and purified by Qiagen Plasmids Maxi Kits (Qiagen, Valencia, CA). Purity of plasmid DNA

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was certified by OD_{260}/OD_{280} ratio, and by distinctive bands of DNA fragments at corresponding base pairs in gel electrophoresis after restriction enzyme treatment of the DNA. The concentration of plasmid DNA was determined using 1 (OD_{260}) = 50 μg of DNA. Plasmid DNA was stored at –20°C until use.

Preparation of Plasmid/LMWC Complex and Gel Retardation Assay

Plasmid/LMWC complexes were prepared by self-assembly. Various amounts of LMWC was slowly dropped into 1 μg of the plasmid DNA and left for 30 minutes at room temperature for complex formation. The complexes were electrophoresed on 1% (w/v) agarose gel for 60 minutes at 80 V. The gel was stained with ethidium bromide (0.5 μg/ml) for 30 minutes and illuminated on an UV illuminator to show the location of the DNA.

DNase I Protection Assay of Plasmid/LMWC Complex

DNase I protection assay of the pSV-β-galactosidase/LMWC complex was performed on a Perkin-Elmer Lambda 19 spectrophotometer. A temperature-controlled UV quartz cuvette (1.0 cm path, Wilmad, Buena, NJ) was used. The change in absorbance at 260 nm was continuously monitored at 37°C up to 1 hour. For example, the complex with the DNA/LMWC weight ratio of 1:2 was formed by adding a 0.5 ml of LMWC in water (contained 20 μg of polymer) dropwise onto a 0.5 ml of water containing 10 μg of DNA. After 15 minutes, a 20-μl aliquot of DNase I (20 units) was spiked into the mixture. Assays on the naked plasmid and complexes with the DNA/polymer weight ratio of 1:0.5 and 1:1 were also carried out in the same manner. For agarose gel electrophoresis, pSV-β-galactosidase/LMWC complex was formed at 1:2 of weight ratio in 1 × PBS. Naked DNA (10 μg) or pSV-β-galactosidase/LMWC complex (10 μg of plasmid/20 μg of LMWC) was incubated with DNase I (5 units). A sample of the complex suspension (100 μl) was taken at 0, 20, 40, and 60 minutes post-incubation with DNase I and mixed with 100 μl of stop solution (200 mM sodium chloride, 20 mM EDTA, and 1% SDS). To dissociate the plasmid DNA from LMWC, the reaction mixtures were incubated at 60°C overnight. After phenol/chloroform extraction, the DNA was precipitated with ethanol. The pellets were dissolved in 10 μl of TE buffer, and applied to a 1.0% agarose gel electrophoresis.

In Vitro Transfection

293T cells, a human kidney cell line, were maintained in DMEM medium supplemented with 10% FBS in a 5% CO_2 incubator. For the transfection studies, 293T cells were seeded at a density of 2×10^6 cells/dish in 100-mm culture dishes, and incubated for 24 hours before the addition of the plasmid/polymer complex. Plasmid DNA/LMWC complexes or plasmid DNA/PLL complexes were prepared by mixing 10 μg of pSV-β-galactosidase and various amounts of LMWC or PLL in 500 μl of serum-free DMEM medium and incubated for 30 minutes at room temperature. The molecular weight of PLL for transfection and cytotoxicity assays was 20 kDa. Medium from each dish was replaced with 10 ml of serum-free DMEM medium. Five-hundred microliters of plasmid/polymer complex was added to each dish. The cells were then incubated for 4 hours at 37°C in a 5% CO_2 incubator. After 4 hours, the transfection mixtures were removed and 10 ml of fresh DMEM medium containing 10% FBS was added to each dish. Cells were incubated for an additional 44 hours at 37°C.

β-Galactosidase Assay

Cells in each dish were washed with 1 × PBS twice, and 900 μl of 1 × reporter lysis buffer (Promega, Madison, WI) was added to cover the cells. After 15 minutes of incubation at room temperature, the cells were harvested and transferred to a microcentrifuge tube. After 15 seconds of vortexing, the cells were centrifuged at 11k rpm for 2 min. The extracts were transferred to a fresh tube and stored at –70°C until use. The protein concentrations of the extracts were determined by using BCA protein assay kit (Pierce, Iselin, NJ). β-Galactosidase assay mixtures included 100 μg of cell extracts and 150 μl of assay 2 × buffer (200 mM sodium phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, 1.33 mg/ml ONPG) in 300 μl of volume. The mixtures were incubated at 37°C for 90 minutes. After the reaction, 500 μl of 1 M sodium carbonate was added to the mixtures and the absorbance at 420 nm was measured.

MTT Assay

Evaluation of cytotoxicity was performed by the MTT assay. 293T cells were seeded at a density of 5.0×10^4 cells/well, in 24-well flat-bottomed microassay plates (Falcon Co., Becton Dickinson, Franklin Lakes, NJ), and incubated for 24 hours. Plasmid/polymer complex (5 μg of plasmid/10 μg of polymer) was added and incubated for 4 hours at 37°C. At the end of the transfection experiment, the transfection mixture was replaced with 500 μl of fresh DMEM medium without serum. One-hundred and twenty microliters of 2 mg/ml MTT solution in 1 × PBS was then added. Plates were incubated for an additional 4 hours at 37°C. MTT-containing medium was removed and 750 μl of DMSO was added to dissolve the formazan crystal formed by live cells. Absorbance was measured at 570 nm. The cell viability (%) was calculated according to the following equation:

$$\text{Cell viability (\%)} = (OD_{570(\text{sample})}/OD_{570(\text{control})}) \times 100$$

where the $OD_{570(\text{sample})}$ represents the measurement from the wells treated with polymer and the $OD_{570(\text{control})}$represents the measurement from the wells treated with PBS buffer only.

RESULTS

Formulation and Gel Retardation Assay

One milligram of LMWC was dissolved in 1 ml of PBS (pH 7.3). The LMWC was highly water-soluble and completely dissolved in PBS. A gel retardation study was performed to confirm self-assembling complexes of plasmid/LMWC. One microgram of pSV-β-galactosidase plasmid was mixed with various amounts of LMWC, ranging from 1 to 8 μg, in PBS. Plasmid/LMWC complexes were analyzed in 1.0% agarose gel electrophoresis (Fig. 1). The plasmid/LMWC complexes were completely retarded above a 1:2 weight ratio of plasmid: LMWC (Fig. 1, lanes 3–9). The re-

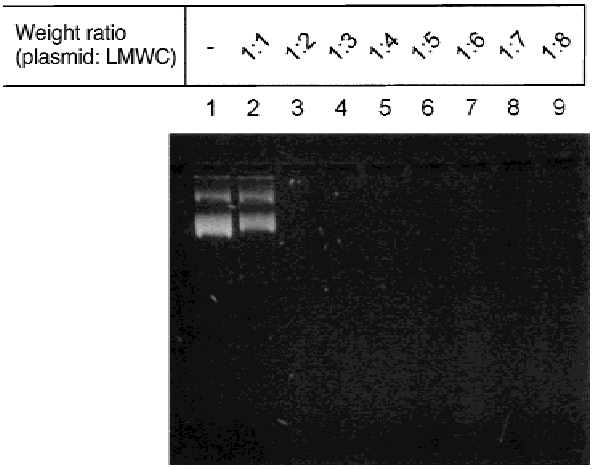

Fig. 1. Gel retardation assay. Plasmid DNA/LMWC complexes were analyzed on a 1% agarose gel electrophoreses. The band retardation showed that complete retardation was achieved at and above a 1:2 weight ratio of plasmid DNA:LMWC.

sults suggest that LMWC was able to condense plasmid DNA into complex at weight ratio of 1:2 (DNA: LMWC).

DNase I Protection Assay

The plasmid DNA was easily degraded by endonucleases such as DNase I (26), which is one of the obstacles for the delivery of plasmid DNA *in vitro* or *in vivo*. Therefore, the stability in the presence of DNase I is one of the essential parameters of systemic gene delivery. To confirm that LMWC protects plasmid from nuclease, DNase I protection assay was carried out. The results from the DNase I stability test are demonstrated in Fig. 2A. This assay utilizes the fact that intact DNA molecules possess hypochromicity and the absorbance at 260 nm thus increases by about 30% upon enzymatic digestion or heat-induced denaturation (27). The absorbance of naked DNA or complex at weight ratio of 1:0.5 (plasmid: LMWC) increased in the course of DNase I reaction. However, the absorbance of complexes at weight ratio of 1:1 or 1:2 (plasmid: LMWC) were not increased by DNase I digestion. This means that plasmid was protected from DNase I at a ratio of 1:1 or 1:2 (plasmid: LMWC). These results were confirmed again by agarose gel electrophoresis in Fig. 2B. After DNase I treatment, DNA was precipitated and analyzed by 1% agarose gel electrophoresis. Naked DNA was destroyed at 60 min after incubation with DNase I (Fig. 2B, lane 4). However, DNA at a 1:2 weight ratio was not degraded more than 60 min (Fig. 2B, lane 8). Therefore, it demonstrated that the chitosan protected the plasmid DNA effectively.

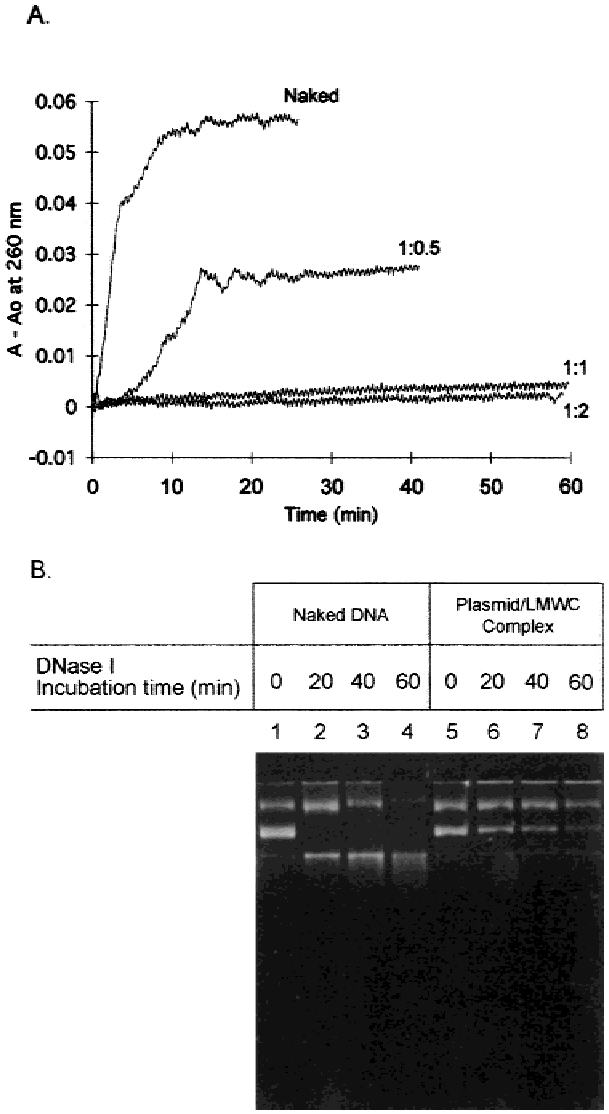

Fig. 2. DNase I protection assay. (A) Profile of absorbance at 260 nm. The complexes with the DNA/LMWC at the indicated weight ratio (plasmid:LMWC) were formed by adding LMWC dropwise onto 10 μg of plasmid DNA. After 15 min, a 20-μl aliquot of DNase I (20 units) was spiked into the mixture. The change in absorbance at 260 nm was continuously monitored at 37°C up to 1 hour. (B) Agarose gel electrophoresis. Naked DNA (10 μg of plasmid) or plasmid DNA/LMWC complex at a weight ratio of 1:2 (10 μg of plasmid:20 μg of LMWC) was digested with DNase I (5 units). One hundred microliters of the reaction mixtures was taken at 0, 20, 40, and 60 minutes post-incubation and mixed with 100 μl of stop solution (200 mM sodium chloride, 20 mM EDTA, and 1% SDS). To dissociate the plasmid DNA from LMWC, the reaction mixtures were incubated at 60°C overnight. After phenol/chloroform extraction, the DNA was precipitated and applied to the 1% agarose gel electrophoresis.

In Vitro Transfection Assay

The most effective weight ratio of plasmid DNA/LMWC in transfection was determined by transfection assay into 293T cells with pSV-β-galactosidase. At a weight ratio of 1:1, 1:2, 1:3, or 1:4, the transfection efficiency of the plasmid/LMWC complex was evaluated. In Fig. 3, at a weight ratio of 1:3, the most effective transfection efficiency was obtained. At weight ratio of 1:1, 1:2, and 1:4, the transfection efficiencies were lower than that at a weight ratio of 1:3.

We evaluated whether the LMWC had an enhanced effect on plasmid delivery into the 293T cells. PLL is a water soluble and cationic gene carrier and has been used often for gene delivery. Therefore, PLL was used as a control carrier to determine transfection efficiency of LMWC. To optimize the weight ratio, various weight ratio of plasmid/PLL complexes were transfected into 293T cells. At a weight ratio of 1:2, plasmid/PLL showed the best transfection efficiency (Fig. 4).

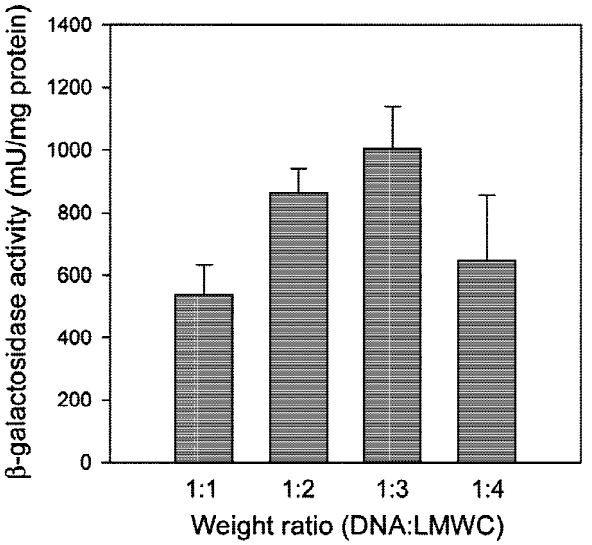

Fig. 3. Effect of plasmid DNA:LMWC ratio on transfection of 293T cells. Various plasmid/LMWC complexes were formulated with a fixed amount of plasmid DNA (10 μg) and increasing amounts of LMWC (10–40 μg). Transfection efficiency was measured by β-galactosidase assay. The highest transfection efficiency was obtained at a 1:3 weight ratio of plasmid: LMWC. The data are expressed as mean values (± standard deviation) of three experiments.

The efficiency of LMWC mediated transfection to 293T cells was significantly higher than that of naked DNA (Fig. 4). Also, LMWC showed 37% higher transfection efficiency than PLL (Fig. 4).

Cytotoxicity

MTT assay was performed to determine the cytotoxicity of LMWC. Plasmid/polymer complex (plasmid/PLL or plasmid/LMWC, 5 μg of plasmid/10 μg of polymer) was added to 293T cells and incubated for 4 hours. After the incubation, cell viability was determined by MTT assay. As a result, only approximately 40% of cells were viable after the incubation with plasmid/PLL complex. However, plasmid/LMWC complex showed negligible cytotoxicity for 293T cells (Fig. 5).

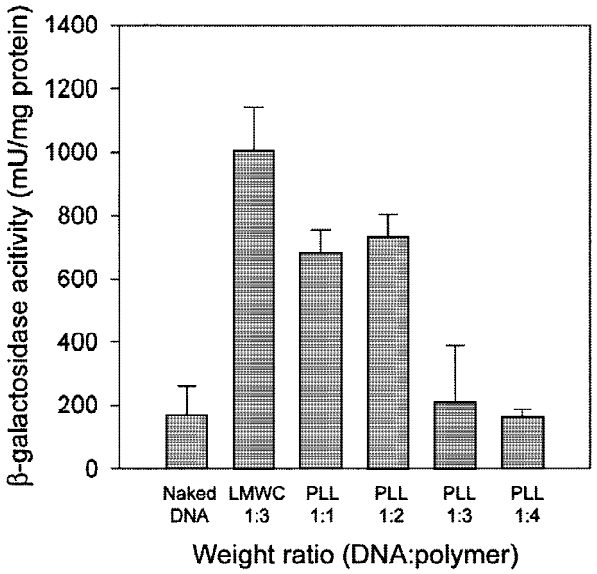

Fig. 4. Comparison of poly-L-lysine and LMWC in transfection of 293T cells. Various plasmid/polymer complexes were formulated with a fixed amount of plasmid DNA (10 μg) and LMWC (30 μg) or increasing amounts of PLL (10–40 μg). Transfection efficiency was measured by β-galactosidase assay. Transfection efficiency was measured by β-galactosidase assay. The data are expressed as mean values (± standard deviation) of three experiments.

DISCUSSION

Chitosan is a natural cationic polysaccharide and a candidate non-viral vector for gene delivery. LMWC has advantages as a gene carrier. First, chitosan is a natural product and it is well established that the chitosan has low toxicity (18). In this study, chitosan shows negligible cytotoxicity for 293T cells. Second, it is highly water-soluble and can form complexes with plasmids in physiological buffer. Generally HMWC is hard to dissolve in water. Therefore, HMWC should be dissolved in acidic solution such as 1% acetic acid. Previously, water insoluble low molecular weight chitosan (<10 kDa) was evaluated in biocompatibility, body distribution, and ability to complex DNA. However, transfection efficiency of this low molecular weight chitosan has not been studied. Third, chitosan interacts electrostatically with many negatively charged polyions such as domethacin (28), sodium hyaluronate (29), pectin, and acacia polysaccharides (30). Previous reports showed that HMWC formed small complexes with plasmids (21–23). Oral gene delivery with chitosan-DNA nanoparticles was also tried (24). However, the surface area of nanoparticles is limited. Therefore, gene delivery with nanoparticles requires a large number of particles, which is unrealistic. In our study, it was proved that LMWC formed plasmid/LMWC complex at a weight ratio of 1:2 (Fig.1). When plasmid is mixed with LMWC, the electrostatic interaction drives the formation of complexes. Plasmid on an agarose gel is completely retarded because of charge neutralization and increase in size of the complexes. This result revealed

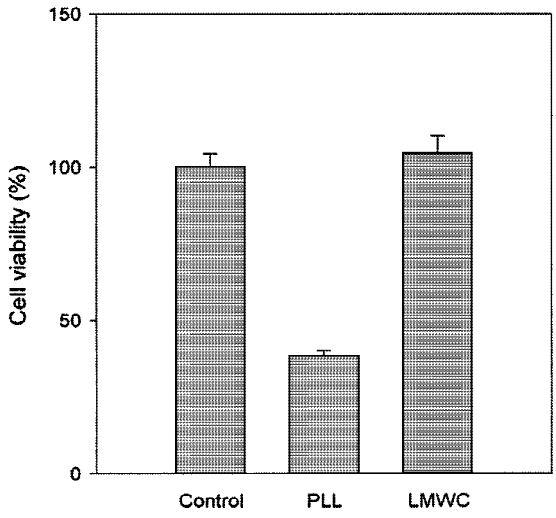

Fig. 5. Cytotoxicity of LMWC for 293T cells. Fifty thousands 293 T cells were plated on 24-well flat-bottomed microassay plates. Plasmid/polymer complex (5 μg of plasmid/10 μg of polymer) was added and incubated for 4 hours at 37°C. After 4 hours, the mixture was replaced with 500 μl of fresh DMEM medium and 120 μl of 2 mg/ml MTT solution. LMWC showed a lower cytotoxicity than PLL. The data are expressed as mean values (± standard deviation) of four experiments.

that LMWC formed plasmid/LMWC complex as effectively as HMWC.

The protective effect of LMWC was evaluated by DNase I protection assay. The possible nuclease that can degrade the plasmids in serum was previously identified (26). DNase I was demonstrated as a major nuclease existing in serum (26). Therefore, gene carriers should protect plasmid DNA in the presence of DNase I. After DNase I treatment, the naked DNA was completely degraded at 60 min (Fig. 2A and 2B, lane 4). However, plasmid DNA in plasmid/LMWC complex was protected from DNase I (Fig. 2B) and the absorbance of the complex at a 1:1 or 1:2 weight ratio was not increased in the course of DNase I treatment (Fig. 2A). These results showed that LMWC protected the DNA efficiently from DNase I. Transfection assay of LMWC showed the most effective level at a weight ratio of 1:3 (Fig. 3). At higher weight ratio, LMWC showed low transfection efficiency. At a weight ratio of 1:3, LMWC shows higher transfection efficiency than PLL (Fig. 4). However, unlike PLL, LMWC is a natural polymer and has low cytotoxicity (Fig. 5).

In conclusion, this study revealed that water soluble LMWC formed complex with plasmid DNA. In addition, LMWC protected plasmid from nuclease effectively. LMWC showed higher transfection efficiency and lower cytotoxicity than PLL in 293T cells. Therefore, LMWC will be useful in the development of safe gene carriers.

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