저분자량 수용성 키토산을 이용한 동맥벽 표적지향성 유전자 전달체의 합성

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The Synthesis of Gene Carrier to Target Artery Wall Cells using Low Molecular, Water-Soluble Chitosan

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Introduction

Non-viral vectors continue to attract a great deal of interest due to advantageous safety profile. Among the various non-viral gene carriers, chitosan is considered to be an attractive candidate for the gene delivery systems, since cationically charged chitosan interacts with the negatively charged phosphate groups of plasmid DNA and partially protect DNA from nuclease degradation. Chitosan (β-1,4-D-glucosamine) is the second most plentiful biomass and is already known as a biocompatible, biodegradable, and low-toxic polycationic polymer with low immunogenicity[1].

In this study, Low molecular, water-soluble chitosan (LMWSC) was characterized and evaluated as a gene carrier. And artery wall cells targeted gene carrier was synthesized to therapy the disease related with cardiovascular, using LMWSC. AWBP-PEG-*g*-LMWSC was synthesized by conjugation the artery wall binding peptide (AWBP), a specific targeting peptide, to the end of PEGylation LMWSC. Characters of the synthesized AWBP-PEG-*g*-LMWSC were analyzed by measuring FT-IR, ^{1}H-NMR, zeta-potentiometer, and atomic force microscopy (AFM).

Experimental

1. Materials

LMWSC (M_w=22kDa, DDA=84%) was supplied by KITTOLIFE Co., Seoul, Korea. N-Hydroxysuccinimide polyethylene glycol vinylsulfone (NHS-PEG-VS, M_w=3400) was commercially obtained from Shearwater Polymers (Huntsville, AL, USA). Artery wall binding peptide (AWBP) (Sequence 'N'-CGRALVDTLKFVTQAEGAK-'C', M_w=2008) was purchased from Genemed Synthesis (South Sanfrancisco, CA). Dialysis tubing (MWCO 8,000, 12,000) was commercially obtained from Spectrum (Spectrum Medical Industries, Inc., CAL, USA). All other reagents were of analytical grade and used without further purification. pSV-β-galactosidase plasmid (Promega, Madison, WI) was amplified in the *E. coli* strain DH5α (GibcoBRL, Gaithersburg, MD), and purified by Qiagen Plasmids Maxi Kits (Qiagan, Valencia, CA). The purity of the plasmid DNA was

determined by agarose gel electrophoresis and by the ratio of UV absorbance 260/280 ㎚.

2. Plasmid DNA/LMWSC complexes delivery

2.1. Gel retardation assay

Complexes were prepared by adding various amounts of LMWSC in 1㎍ of the plasmid DNA. After incubation for 30 min at room temperature, the complexes were electrophoresed on 1% (w/v) agarose gel for 60 min at 80V. The gel was stained with ethidium bromide (0.5㎍/㎖) for 30 min and illuminated on an UV box.

2.2. *In vitro* transfection

Plasmid DNA/LMWSC complexes or plasmid DNA/PLL complexes were prepared by adding various amounts of LMWSC or PLL into 10㎍ of pSV-β-galactosidase in 500㎕ of serum-free DMEM medium and incubated for 30 min at room temperature. The molecular weight of PLL for transfection and cytotoxicity assays was 20 kDA. 293T cells were seeded 2×10^6 cells per well in 100-㎜ culture dishes, and incubated for 24 h before the addition of the plasmid DNA/polymer complexes. 293T cells, a human kidney cell line, were maintained in DMEM medium supplemented with 10% FBS in a 5% CO_2 incubator. Before adding the complexes to the cells, medium from each dish was replaced with 10 ㎖ of serum-free DMEM medium. Plasmid DNA/polymer complex 500㎕ was added to each dish. After 4 h incubation at 37℃ in a 5% CO_2, the medium was replaced with 10㎖ of fresh DMEM medium containing 10% FBS at 44 h at 37℃ following the start of transfection.

2.3. Cytotoxicity assay

Evaluation of cytotoxicity was performed by the MTT assay. 293T cells were seeded in 24-well flat-bottomed microassy plates (Falcon Co., Becton Dickinson, Franklin Lakes, NJ) at a density of 5.0×10^4 cells/well and incubated for 24 h. The cells were incubated with Plasmid DNA/polymer complex (5㎍ of plasmid/10㎍ of polymer) for 4 h at 37℃. The old medium was replaced with 500㎕ of fresh DMEM medium without serum and then added MTT solution 120㎕ at a concentration of 2㎎/㎖ in 1×PBS. After the plate was incubated for 4 h at 37 ℃, the MTT-containing medium was removed. DMSO (750㎕) was added to each well and mixed thoroughly to dissolve insoluble blue formarzan crystals formed by live cells. Absorbance was measured at 570 ㎚. The cell viability (%) was calculated according to the following equation:

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

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

3. Synthesis of AWBP-PEG-*g*-LMWSC

3.1. Preparation of AWBP-PEG-*g*-LMWSC

PEGylated LMWSC was synthesized by addition of NHS-PEG-VS to the LMWSC solution. The mixture was purified by dialysis against deionized water, followed by lyophilization. AWBP in DMSO was added dropwise into the solution of PEGylated LMWSC and the reaction mixture was dialyzed with the same condition described above and then lyophilized. The each step product was

analyzed by FT-IR (Shimadzu, FT-IR 8700) and ^{1}H-NMR spectrometer (JEOL, JNM-LA 300) and then stored at -20℃ before used.

3.2. Particle size measurement

The particle size of AWBP-PEG-*g*-LMWSC was measured by Part 370+770 (Altamira instruments, USA) at 23℃ and 632 nm wavelength and by AFM (PARK's Science, Autoprobe CP) at room temperature.

Results and Discussion

1. Charaterization of the Plasmid DNA/LMWSC compexes

1.1. Gel retardation assay

A gel retardation study was performed to examine the nature of interactions between the plasmid DNA and LMWSC. Plasmid DNA/LMWSC complexes were analyzed in 1.0% agarose gel electrophoresis (Fig. 1). When a fixed amount of pSV-β-galactosidase was titrated with various amunts of LMWSC, the electrophoretic mobility of plasmid DNA/LMWSC complexes were completely retarded above a 1:2 weight ratio of plasmid DNA:LMWSC (Fig. 1, lanes 3-9). The results suggest that LMWSC was able to condense plasmid DNA into complex at weight ratio of 1:2 (DNA:LMWSC).

1.2. *In vitro* transfection

Fig. 2 represents the results of cell transfection with pSV-β-galactosidase complexed with LMWSC into the 293T cells. At a weight ratio of 1:1, 1:2, 1:3 and 1:4, the transfection efficiency of the plasmid DNA/LMWSC complex was evaluated. The most effective transfection efficiency was obtained at a weight ratio of 1:3. We evaluated whether the LMWSC had an enhanced effect on plasmid delivery into the 293T cells.

PLL is a well-known water soluble and polycation gene carrier, which has been used often for gene delivery. Therefore, PLL was used as a control carrier to determine transfection efficiency of LMWSC. Plasmid DNA/PLL complex showed the best transfection efficiency at a weight ratio of 1:2 into the 293T cells. The transfection efficiency of plasmid DNA/LMWSC to the 293T cells was higher than that of naked DNA and PLL (data not shown).

1.3. Cytotoxicity assay

MTT assay was performed to determine the cytotoxicity of LMWSC. As a result, only approximately 40% of cells were viable after the incubation with plasmid DNA/PLL complex. However, plasmid DNA/LMWSC complex showed negligible cytotoxicity for 293T cells (Fig. 3). It was manifested that LMWSC is a biocompatible material and is capable to apply in vitro and in vivo as DNA carriers for gene delivery as well.

2. Characterization of AWBP-PEG-*g*-LMWSC

2.1. Synthesis of AWBP-PEG-*g*-LMWSC

Fig. 4 shows the synthesis of AWBP-PEG-*g*-LMWSC consisted of two-step reaction. In a fitst reaction, N-hydroxyssucinimide (NHS) group of NHS-PEG-VS was conjugated to the amino group of LMWSC. The product of 1-step was confirmed by FT-IR and ^{1}H-NMR analysis(data not shown). In a two reaction, AWBP was synthesized with the end of vinylsulfone group of VS-PEG-*g*-LMWSC. The final product was identified from the results of ^{1}H-NMR data (data not

shown).

2.2. Particle size measurement

The particle size of AWBP-PEG-*g*-LMWSC was estimated 12.07 nm by zeta potentiometer and AFM (data not shown).

Conclusion

The data from this study provided useful and important information for the future application of *in vitro, in vivo* studies.

References

1. S. Hirano, H. Seino, Y. Akiyama, I. Nonaka, Chitosan : a biocompatible material for oral and intravenous administration, in: C.G. Gebelein, R.L. Dunn (Eds.), Progress in Biomedical Polymers, Plenum Press, New York, 283(1990).

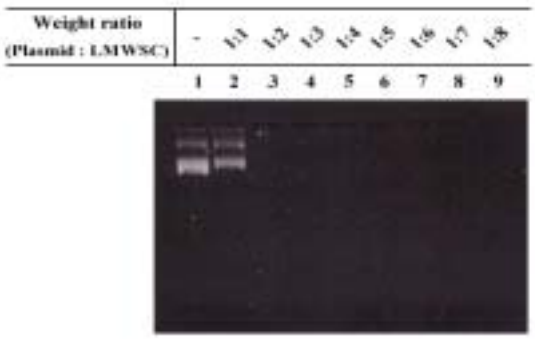

Fig. 1. Gel retardation assay.

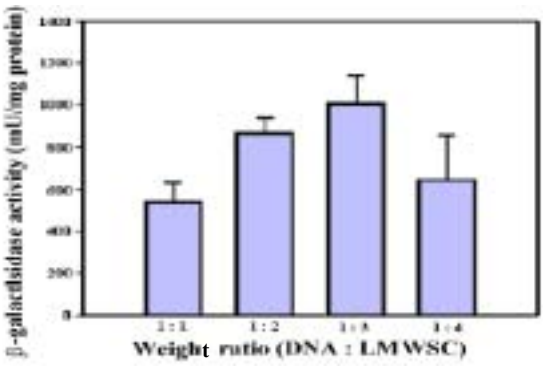

Fig. 2. Effect of plasmid DNA:LMWSC ratio on transfection of 293T cells.

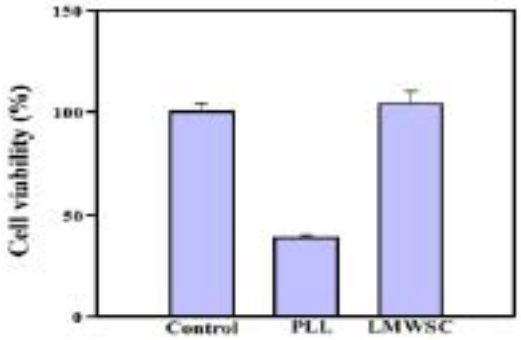

Fig. 3. Cytotoxicity of LMWSC for 293T cells.

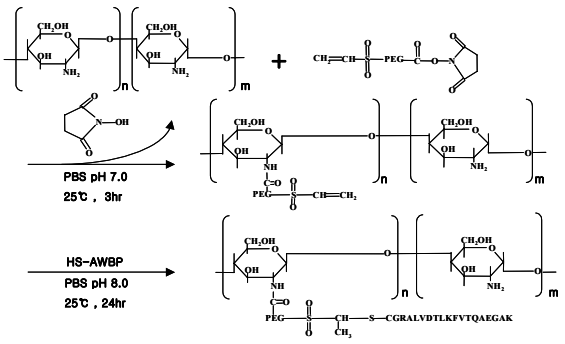

Fig. 4. Synthetic scheme of AWBP-PEG-*g*-LMWSC.