DNA Delivery Using Low Molecular Water-Soluble Chitosan Nanocomplex as a Biomedical Device

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ABSTRACT: We investigate the potential of low molecular water-soluble chitosan (LMWSC), LMWSC–methoxy polyethylene glycol (MPEG) [LCP], and LMWSC–MPEG–cholesterol (LCP–Ch) as a gene carrier. LMWSC with free-amine group was formed at the low weight ratio, above a 1:2 weight ratio of plasmid DNA:LMWSC. LCP and LCP–Ch achieved complex formation of above 1:8 and 1:24 by reacting with MPEG and cholesterol (Ch) on the amine-group, respectively. Particle sizes at the pH 6.5 and 7.0 were about 100–120 nm and 120–160 nm, respectively. The surface charge of the complex also depended on the pH. At pH 6.5, the surface charge of the complex was higher than that at pH 7.0. The zeta potential of the LCP modified with MPEG or of the LCP-Ch modified with cholesterol has lower positive value than that of LMWSC because of the decrease of the positive charge. The morphologies of the complexes by transmission electron microscope were of spherelike shape with the average diameters of about 100–150 nm. Among the three gene carriers, LCP–Ch/plasmid DNA showed the highest gene expression owing to the hydrophobic interaction between cell surface and cholesterol. No cytotoxicity was observed in the gene carriers investigated in this study at various concentrations. J Appl Polym Sci 102: 3545–3551, 2006

Key words: biocompatibility; chitosan; dynamic light scattering; nanocomplex; TEM

INTRODUCTION

Recently, gene therapy has attracted much attention as a promising therapeutic strategy, because it provides important opportunities to treat various kinds of life-threatening and gene related-diseases by producing biologically active agents or stopping abnormal functions of the cells such as genetic failure or uncontrollable proliferation of cells.[1] However, the actual application of genes to human therapy is limited by several problems, including their instability in body fluids, nonspecificity to the desired cells, degradation by nucleases, and low transfection efficiency. One of the solutions to overcome these problems is to develop an efficient and safe gene delivery system. Generally, gene delivery systems include viral vectors and nonviral vectors. Although viral vectors are currently the most efficient way to deliver genes to cells, nonviral vectors have many advantages such as low cytotoxicity, low immunogenecity, no size limit, low cost, and reproducibility.[2] Until now, gene delivery systems have been investigated in an attempt to enhance the gene expression and reduce the cytotoxicity.[3]

As nonviral vectors, liposomal carriers[4,5] and cationic polymers[6,7] have been extensively investigated for a decade for their advantages of safety and a relatively low cost. Although higher transfection efficiency has been reported by liposomal gene carriers *in vitro*[4], some liposomal gene carriers are unstable in aqueous solution and aggregate in blood.[5] Cationic polymers including poly(L-lysine) (PLL)[8–10] and polyethylenimine (PEI) were able to condense plasmid DNA and protect it from enzymatic degradation, which resulted in the enhancement of the transfection efficiency. However, there still remain such drawbacks as biocompatibility and cytotoxicity in the body. To overcome the biocompatibility problem, nontoxic biodegradable polymeric gene carrier has been developed as a promising gene delivery material.[11,12] A biodegradable polysaccharide composed of two subunits, D-glucosamine and *N*-acetyl-D-glucosamine, linked together by β(1,4)-glycosidic bonds, chitosan has also been applied as an attractive gene carrier, because of its high positive charges and low toxicity to cells. General lysozymes in the body degrade

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chitosan into a common amino sugar, *N*-acetyl glucosamine, which is incorporated into the synthetic pathway of glycoproteins and subsequently excreted as carbon dioxide.[13]

Amine group of glucosamine unit in the structure of chitosan has several advantages as a gene carrier such as noncytotoxicity and strong positive charge, which facilitates formation of the complex with DNA. The transfection ability of DNA mediated by chitosan is different according to the degree of deacetylation and molecular weight.[3,14–16] Since chitosan was first described as a delivery system for plasmids by Mumper et al.,[17] many studies have been conducted for delivery of genes safely in the body. However, application of chitosan as a gene carrier has been limited by poor water-solubility and salts were introduced to solve this problem, resulting in the blocking positive charge of amine group. To overcome these drawbacks of chitosan, we have already prepared low molecular water-soluble chitosan (LMWSC) having free-amine group and was characterized by spectroscopic evaluation.[18] In the previous study,[19] we already demonstrated the potential as a efficient gene delivery of water-soluble chitosan coupled with urocanic acid. Using LMWSC, nanoparticles modified with hydrophobic and hydrophilic group were prepared[20] to solve low transfection efficiency and give targeting moiety for delivery of genes into specific cells. As a hydrophobic group, cholesterol (Ch) was introduced to the position of amine group, which facilitates formation of nanoparticle and cell uptake via endocytosis by LDL receptor. Methoxy polyethylene glycol (MPEG) was modified in the chain of LMWSC to introduce the hydrophobic group and circulate it for a long time in the blood stream. In this study, we evaluated the ability of LMWSC, LMWSC-MPEG (LCP), and LMWSC-MPEG-cholesterol (LCP-Ch) prepared in the previous study[20] as a gene delivery *in vitro*.

METHODS

Materials

Low molecular water-soluble chitosan (LMWSC, 10 KDa, 18 KDa) was supplied by KITTOLIFE, Seoul, Korea. Methoxy polyethylene glycol-*p*-nitrophenyl carbonate (MPEG-*p*-NP, 5 KDa) and cholesteryl chloroformate were purchased from Sigma and Aldrich, respectively. pEGFP-N1 (Clontech, Palo Alto, CA) was introduced into *Escherichia coli* strain DH5 (Gibco-BRI, Gaithersburg, MD), and purified using Qiagen Maxi Kits following the manufacturers directions (Qiagen, Valencia, CA). Ethidium bromide and agarose were purchased from Promega (Madison, WI). Other solvents and chemicals are used in reagent grade.

Gel retardation assay

To confirm the complex formation of the LMWSC, LCP, and LCP-Ch with the plasmid DNA, the gel retardation assay was performed. LMWSC/pEGFP-N1, LCP/pEGFP-N1, and LCP-Ch/pEGFP-N1 complexes were prepared at various weight ratios ranging from 1/1 to 20/1 (carrier/DNA) in PBS (pH 7.4) and left at room temperature for 30 min for complex formation. Then, the complexes were electrophoresed on 1% (w/v) agarose gel for 60 min at 80 V. The gel was stained with ethidium bromide (0.5 μg/mL) for 30 min and illuminated on an UV illuminator to show the location of the DNA.

Dynamic light scattering measurements

The particle size and size distribution of LMWSC/DNA, LCP/DNA, and LCP-Ch/DNA complexes in aqueous environment were investigated by using dynamic light scattering (DLS). The carriers/plasmid DNA complexes were prepared with carriers/DNA weight ratios of 5 and 20 (10 μg/mL DNA in H_2O). Before measurements, the prepared complexes were left at room temperature for 30 min for complex formation. The DLS measurements were carried out using an ELS-800 electrophoretic LS spectrophotometer (NICOMP 380 ZLS zeta potential/particle sizer), equipped with a He–Ne laser operating at 632.8 nm at 25°C and at a fixed scattering angle of 90°.

Morphologies measurement of complexes by TEM

Morphologies of LMWSC/DNA, LCP/DNA, and LCP-Ch complexes were examined with a JEOL JEM-2000 FX-II transmission electron microscope (TEM). A drop of complexes suspended in 0.01% of phosphotungstic acid was placed on a carbon film coated on a copper grid for TEM.

Cell culture and *in vitro* transfection

For transfection studies, HCT 116 cells derived from human colorectal cancer were cultivated in McCoy's

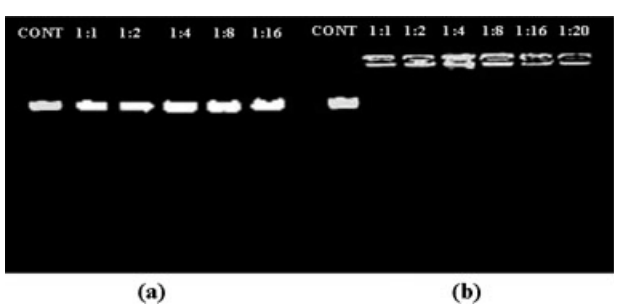

Figure 1 Gel retardation assay I: (a) LMWSC 10 KDa/plasmid DNA and (b) LMWSC 18 KDa/plasmid DNA complexes with various weight ratios.

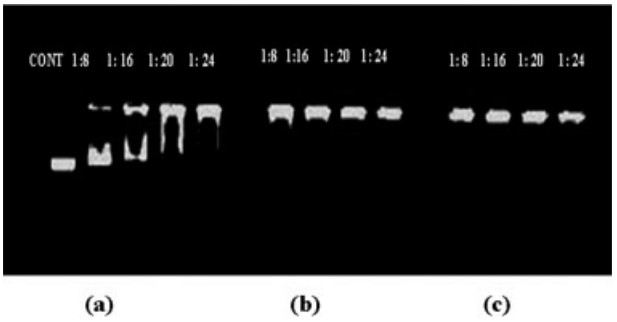

Figure 2 Gel retardation assay of LMWSC derivatives II: (a) LCP-Ch (the substitutive ratio of MPEG : Ch is 2 : 0.6 per 10 glucosamine unit); (b) LCP (the substitutive ratio of MEG is 2 per 10 glucosamine unit); (c) LCP-Ch (the substitutive ratio of MPEG : Ch is 2 : 0.2 per 10 glucosamine unit).

5A supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2. The cells were seeded in 24-well plates and incubated for 24 h before adding the carriers/DNA complexes. LMWSC/DNA, LCP/DNA, and LCP-Ch/DNA complexes were prepared by mixing 5 μg of pEGFP-N1 and various amounts of LMWSC, LCP, and LCP-Ch in a 500 μL of serum-free DMEM medium. Five hundred microliters of plasmid DNA/polymer complex was dropped to each dish. The cells were then incubated for 4 h at 37°C in a 5% CO_2 incubator. The cells were further incubated for 2 days at 37°C.

Cell viability measurement

Cytotoxicity was evaluated by performing the MTT assay. HCT 116 cells were seeded at a density of 1×10^4 cell/well in 96-well cell culture plates and preincubated for 24 h before the LMWSC derivatives treatment. Then, the cells were treated with LMWSC derivatives of various concentrations (1–100 μg/mL in PBS 7.4 with 10% FBS) for 2 h. After treatment, the LMWSC derivative solutions were removed, and fresh cell culture media were added and incubated for 4 h to stabilize the cells. Finally, the cells were incubated with MTT-containing media (0.5 mg/mL MTT in DMEM) for 4 h. Then, the medium was removed, and the formazan crystals formed in living cells were dissolved in 100 μL DMSO. The relative cell viability (%) was calculated according to the following equation based on absorbance at 570 nm:

$$\text{Relative cell viability} = [(OD_{570,\,\text{sample}} - OD_{570,\,\text{blank}})/(OD_{570,\,\text{control}} - OD_{570,\,\text{blank}})] \times 100$$

RESULTS AND DISCUSSION

LMWSC with free-amine group has an excellent potential not only as a gene carrier but also as biomaterials because of its high water solubility, high reactivity by free-amine group, and nontoxicity in the body. Degradation of DNA from DNase I is also one of the obstacles for the delivery of gene *in vitro* or *in vivo*. Therefore, stability in the presence of DNase I is one of the essential parameters of systemic gene delivery. In a previous study,[21] we already confirmed that LMWSC protect the plasmid DNA from DNase I effectively.

In this study, LMWSC and its derivatives, LCP and LCP-Ch, were evaluated as a gene carrier to take advantage of its strong positive charge and target moiety modified at LMWSC. LMWSC derivatives that have been modified with MPEG and cholesterol in our previous study[20] have the potential of a gene carrier that may enhance the transfection efficiency, which is based on targeting into specific cells via endocytosis by LDL receptor.

Gel retardation assay

Gene-condensing compounds such as cationic liposome and cationic polymer were needed for gene delivery in the body. The naked DNA alone is difficult to transfect somatic cells because of its large hydrodynamic volume and negatively charged character. In this study, LMWSC as a cationic polymer was used for complex formation with pEGFP-N1 plasmid DNA. Unlike the high molecular weight chitosan (HMWC) bound salts that dissolve into distilled water, LMWSC is a high water-soluble material, and it also has strong positive charge owing to the free-amine group, which facilitates the complex formation with the negatively charged DNA.

First, we tested the ability of complex formation according to molecular weight of LMWSC. It means

TABLE I
Particle Sizes and Zeta Potential of LMWSC/DNA, LCP/DNA, and LCP-Ch/DNA at Weight Ratio of 40

	Particle size		Zeta potential	
Polymer/DNA complexes	pH 6.5	pH 7.0	pH 6.5	pH 7.0
LMWSC/DNA	128.0 ± 27.8	166.6 ± 37.9	20.23	13.66
LCP/DNA	111.1 ± 26.6	123.6 ± 62.3	2.79	1.95
LCP-Ch/DNA	106.9 ± 23.9	131.2 ± 62.2	4.07	0.49

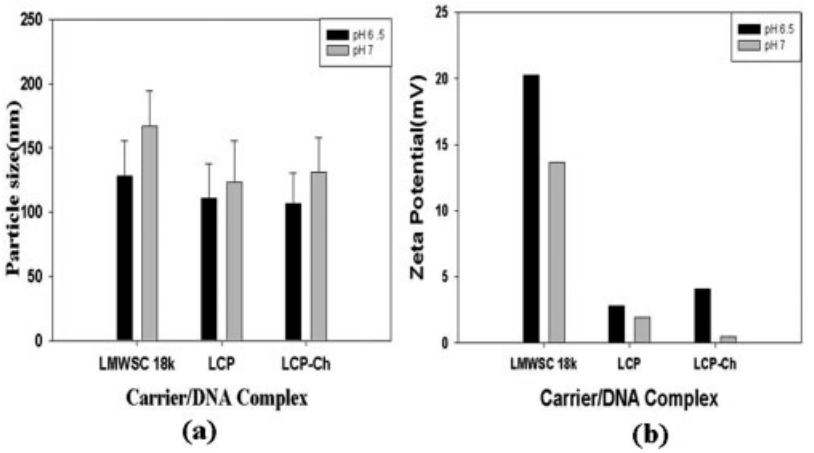

Figure 3 Particle sizes (a) and zeta potential (b) of carrier/DNA complexes at weight ratio of 40 according to the pH of media.

that LMWSC of higher molecular weight has more free-amine group than LMWSC of lower molecular weight. So, the greater the number of free-amine groups, the easier the complex formation at a lower weight ratio.

As shown in Figure 1(a), the LMWSC/plasmid DNA complex prepared with LMWSC of 18 KDa was completely retarded above 1 : 2 the weight ratio of DNA : LMWSC. However, LMWSC of 10 KDa was not able to form the complex with DNA at the weight ratio of 1 : 20 because of weak positive charge [Fig. 1(b)]. These results suggest that the higher the molecular weight of LMWSC, the more the free-amine group results in condensing with DNA easily.

For enhancement of the transfection efficiency of LMWSC, the self-assembled complex formations of LCP/plasmid DNA and LCP-Ch/plasmid DNA were prepared and investigated by the gel retardation assay (Fig. 2). Figure 2 shows the ability of complex formation according to the number of MPEG and cholesterol substituted in LMWSC. The result indicated that an increase of the substitutive group at LMWSC chain induce a higher weight ratio that enables to form the complex with DNA. Because the positively charged amine-group diminishes while reacting with MPEG and cholesterol, LMWSC with free-amine group is superior as a gene carrier owing to its high water solubility, strong positive charge, and high reactivity for modification with target moieties.

Characterization of LMWSC 18 KDa/DNA, LCP/DNA, and LCP-Ch/DNA complexes by DLS and TEM

The particle size of the complex is very important for gene expression. It has already been reported that endocytosis by many types of mammalian cells is limited to particles less than about 150 nm in diameter.[22] The suitable size of the complex for gene delivery is around 100 nm. It has been suggested that the polymer/plasmid DNA complex with a diameter of around 100 nm corresponds to the diameter of the coated pits in endocytosis.[23] To confirm the size of LMWSC/plamid DNA, LCP/plasmid

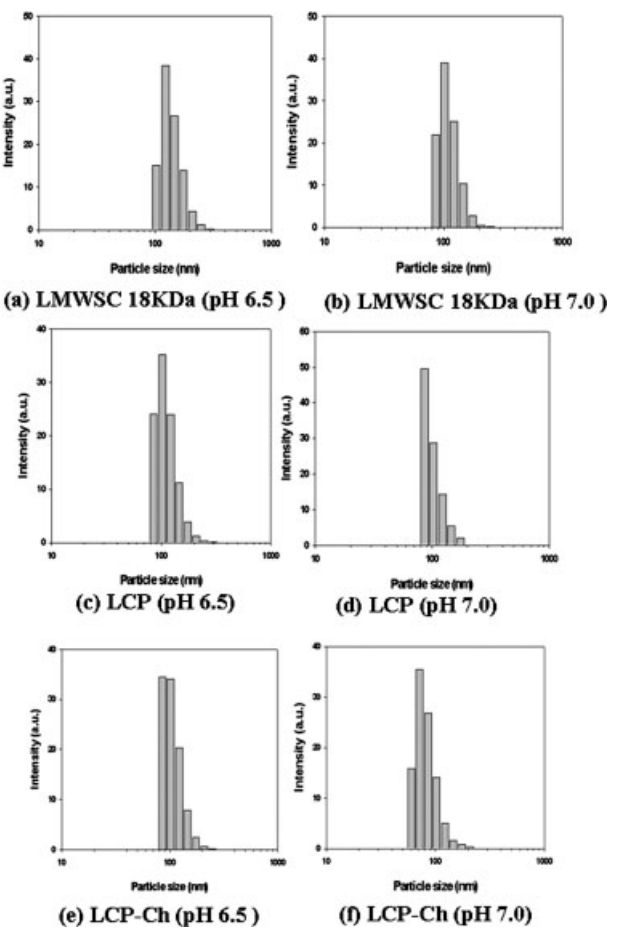

Figure 4 Particle size distribution of carrier/DNA according to pH of media.

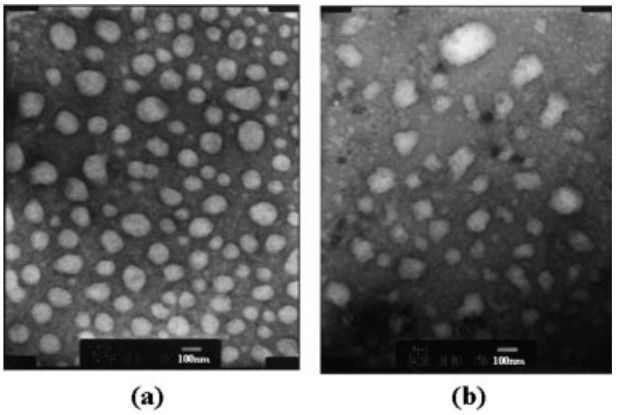

Figure 5 Morphologies of LMWSC 18 KDa/DNA (a) and LCP-Ch/DNA (b) by TEM.

DNA, and LCP-Ch/plasmid DNA complex, the particle size and zeta potential were measured by DLS method. Generally, chitosan is more stable under a weak acidic condition because its pKa value is pH 6.2–6.8.[24,25] Also, the particle size of the complex is dependent on the ratio and concentration of the polymer/plasmid DNA complex.

We investigated the tendency of the complex size according to the pH condition. Table I shows the particle sizes and zeta potential of the complexes at the weight ratio of 40. The result revealed that the particle size and zeta potential of the complexes are affected by pH of media. The particle size of the complexes at the pH 6.5 (100–120 nm) was smaller than at the pH 7.0 (120–160 nm) [Fig. 3(a)]. Since LMWSC has the strong positive charge at the lower pH (around pH 6.5), it can be condensed more compactly than that of lower pH with negatively charged DNA. Figure 3(b) shows the zeta potential of three complexes. At the pH 6.5, the surface charge of complex was higher than that of pH 7.0. From this result, we observed that the zeta potential of LCP modified with MPEG or LCP-Ch modified with cholesterol has lower positive value than that of LMWSC owing to the decrease of the positive charge, because the free-amine group of LMWSC was modified with MPEG or cholesterol.

Figure 4 shows the representative number average size distributions of LMWSC 18 KDa/DNA [Fig. 4(a and b)], LCP/DNA [Fig. 4(c and d)], and LCP-Ch/DNA [Fig. 4(e and f)] complexes at weight ratio of 40 in the PBS of pH 6.5 and 7.0. The size distribution of three complexes was shown to be unimodal, and the particles were uniformly prepared around 100 nm. Furthermore, the size distributions at the pH 6.5 are more regular. The imaging of LMWSC/DNA and LCP-Ch complexes at the weight ratio of 40 is shown in Figure 5. This TEM image indicated that the complexes had spherelike shape, also the average diameters ranged from 100 to 150 nm. This result is consistent with the particle size measured by DLS. This size is suitable for gene delivery.

These results indicate that the particle sizes of LMWSC with free-amine are dependent on pH of media. It means that LMWSC charged with the strong positive at the lower pH is able to form the compact complex with negatively charged DNA. So,

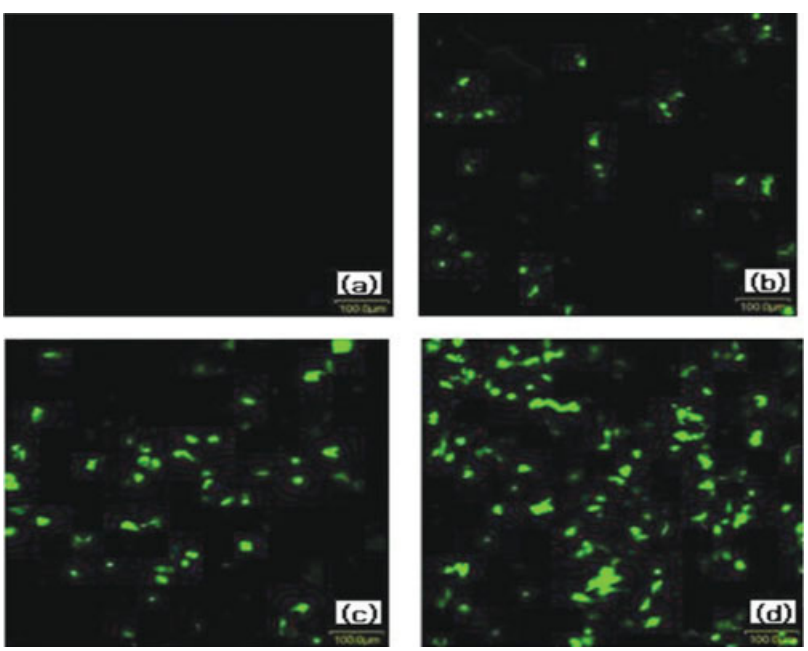

Figure 6 Confocal microscope images of gene-transfected HCT 116 cells at 48 h after transfection: (a) Naked DNA; (b) LMWSC/DNA complex; (c) LCP/DNA complex; (d) LCP-Ch/DNA complex. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

gene-condensing capacity of LMWSC with free-amine group is more excellent than the previously used chitosan.

Transfection efficiency *in vitro* test

Generally, the positive charge of the polymer/DNA complex facilitates cellular uptake of the complex as the positive charge of the complex interacts with negatively charged cell membranes. PLL and PEI have a higher transfection efficiency than chitosan owing to the proton buffering effect. However, the high charge density is closely related to cytotoxicity.[26] In addition, the polymer/DNA complex is rapidly cleared after intravenous injection because of the positive surface charge of the delivery system.[27] Although LMWSC has many advantages as a gene carrier, LMWSC for gene delivery has to overcome such problems as the low transfection efficiency and the rapid clearance in the blood stream. MPEG has an excellent characteristic that it shields excess positive charges of polymer/DNA complex, resulting in long circulation in the blood and reduction of delivery efficiency.[28]

The MPEG and cholesterol at the LMWSC chain that were used in this study have been analyzed and characterized in our previous study.[20] To achieve long circulation of LMWSC/DNA complexes in the blood, the MPEG was modified, and the cholesterol was introduced to the amine group of LMWSC to facilitate the cellular uptake by the LDL receptor. The transfection efficiency was investigated in HCT 116 cell lines using pEGFP-N1 plasmid encoding green fluorescence protein (GFP). The gene expression was observed by the confocal microscopy. As shown in Figure 6, the transfection efficiency of complexes was enhanced by introducing MPEG and cholesterol on the LMWSC. The transfection efficiency of the LCP modified with MPEG was twice as much as that of LMWSC, while the LCP-Ch enhanced the transfection efficiency 50 times more than LMWSC (Fig. 7). This result indicated that the gene transfection efficiency can be enhanced by facilitating the cellular uptake of the carrier/DNA complex that introduces the hydrophobic and hydrophilic moieties.

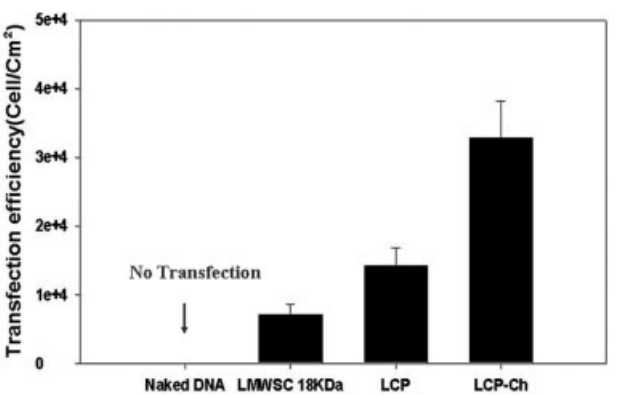

Figure 7 Comparison assay of naked DNA, LMWSC 18 KDa/DNA, LCP/DNA, and LCP-Ch/DNA complexes transfection of HCT 116 prepared at weight ratio of 40.

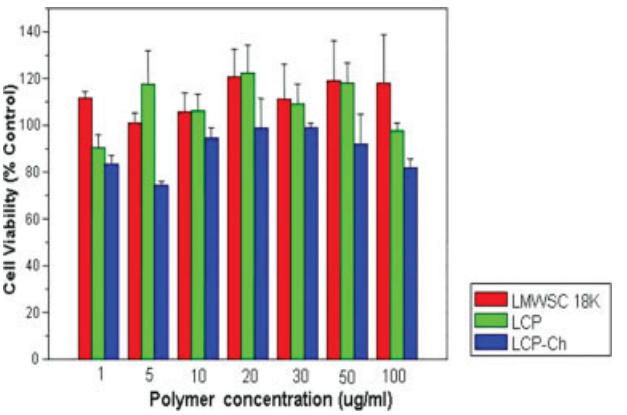

Figure 8 Cytotoxicity of LMWSC 18 KDa, LCP, and LCP-Ch against HCT 116 cell at various concentrations of polymer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Cell viability of the complexes

In designing a delivery vehicle for genes, one of the most important factors is the cytotoxicity. Although the high charge density of PEI and PLL contributes to the formation of complexes and high transfection efficiency, it may also induce cytotoxicity. Effort to solve this problem has been reported by several research groups.[26,29] It is already known that chitosan as a natural polysaccharide is a nontoxic biomaterial.[30] We evaluated the cytotoxicity of LMWSC derivatives, LCP, and LCP-Ch by the MTT assay. The result presented in Figure 8 indicates that in general the cytotoxicity of gene carrier depends on the concentration of polymer. However, no cytotoxicity was ever observed at various concentrations of complexes used in this study. In the case of LCP-Ch to which cholesterol has been introduced, the cell viability decreased about 80%. However, we believe that the carrier sticks to cell membrane through hydrophobic interaction with hydrophobized cell surface by introducing cholesterol to the LMWSC. However, it is negligible cytotoxicity for gene delivery in the body.

CONCLUSIONS

Chitosan, a biodegradable and naturally available material, has emerged as a household name and as an important biomedical material of the 21st century.[30] In spite of its potential as a biomaterial, the application of chitosan that was attempted by sev-

eral research groups has been limited because of its poor water solubility. To increase the water solubility, the amine group of chitosan is modified with salts or water soluble derivatives, resulting in a decrease of the positive charge and reactivity by amine group. To solve these problems, we have prepared LMWSC with free-amine group in our previous study and evaluated its potential as a gene carrier in this study. There is another obstacle in applying LMWSC in the gene delivery system. That is low transfection efficiency compared to PLL and PEI.

In this study, we used LMWSC derivatives modified with MPEG and cholesterol to achieve an enhanced transfection efficiency. First, the complex formation ability of LMWSC and LMWSC derivatives was investigated by gel retardation assay. The LMWSC 10 KDa and 18 KDa formed the complex above 1 : 20 and 1 : 2 weight ratio of plasmid DNA: LMWSC, respectively. This means that the complex formation ability of LMWSC is dependent on molecular weight because of the amount of free-amine group in the glucosamine unit. Also, LCP and LCP-Ch formed the complexes with DNA above 1 : 8 and 1 : 24 weight ratio because of the decrease of amine-group by reacting with MPEG and cholesterol. Particle size is one of the important factors for high gene expression. It depends on the charge density and concentration of polymer/plasmid DNA. We controlled the pH of media to prepare the complex with plasmid DNA. The particle size of the complex prepared in the PBS of pH 6.5 was smaller than that of pH 7.0. It means that the strong positive charge of LMWSC in the PBS of pH 6.5 can be more compactly condensed with negatively charged DNA. The morphologies of complexes by TEM were of spherelike shape, and the average diameter measured by DLS was 100–150 nm. LCP-Ch that introduced the cholesterol showed the highest gene efficiency owing to an easy cell uptake by hydrophobic interaction with cell surface. No cytotoxicity of gene carriers was observed in various concentrations of the carrier. Therefore, LMWSC having a free-amine group and its derivatives will be useful in the development of safe gene carriers.

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