AAPS PharmSciTech 2006; 7 (3) Article 66 (http://www.aapspharmscitech.org).

Chitosan Lactate as a Nonviral Gene Delivery Vector in COS-1 Cells

Submitted: March 8, 2006; Accepted: May 16, 2006; Published: August 4, 2006

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ABSTRACT

The purpose of this research was to evaluate chitosan lactate (CL) of different molecular weights (MWs) as a DNA complexing agent for its efficiency in transfecting COS-1 cells (green monkey fibroblasts) and its effect on cell viability compared with polyethylenimine (PEI), a commercially available cationic polymer. CL and chitosan base dissolved in dilute acetic acid (chitosan acetate [CA]) of different MWs (20, 45, 200, 460 kDa) and N/P ratios (2:1, 4:1, 8:1, 12:1, 24:1) formed complexes with pSV β-galactosidase plasmid DNA. The complexes were characterized by agarose gel electrophoresis and investigated for their ability to transfect COS-1 cells compared with PEI. Additionally, the effect of CL on the viability of COS-1 cells was investigated using 3-(4,5-dimethyliazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The binding of CL/DNA and CA/DNA was dependent on chitosan MWs. The N/P ratio of CL to completely form the complex with the DNA was higher than that of CA. Both CL and CA were comparable in transfection efficiencies at an N/P ratio of 12:1, but less efficient than PEI ($P < .05$). The cell viability in the presence of CL and CA at all MWs was over 90%, whereas that of PEI-treated cells was ~50%. These results suggest the advantage of CL for in vitro gene transfection, with the ease of preparation of polymer/DNA complexes and low cytotoxicity.

KEYWORDS: chitosan, chitosan lactate, transfection efficiency, gene delivery, COS-1 cells.

INTRODUCTION

Gene delivery has been regarded as a powerful tool for curing disease by replacing defective genes, substituting missing genes, or silencing unwanted gene expression. Basically, there are 2 types of gene carriers that deliver foreign DNA into the diseased target cell population. DNA delivery systems are viral and nonviral vectors. Viral vectors are the most effective because of their evolutionary optimization for this purpose. However, recently reported safety issues such as random recombination, oncogenic potential, and immunogenicity[1,2] have set back the rapid development of viral vectors. By contrast, nonviral vectors are safe to use but less efficient. In light of safety concerns, nonviral delivery systems have been developed for gene therapy experiments. Among those, cationic liposomes are widely used for almost all animal cells because they have nonspecific ionic interaction and low toxicity properties.[3-5] However, there are some limitations for cationic liposomes. When they are used for in vivo transfection, they are unstable. Therefore, many polymeric cationic systems such as gelatin, polyethylenimine (PEI), poly(L-lysines), tetraaminofullerene, poly(L-histidine)-graft-poly(L-lysines), DEAE-dextrans, cationic dendrimers, and chitosan have been studied for in vitro as well as in vivo application.[6,7]

Chitosan is a copolymer of N-acetyl-glucosamine and glucosamine. This polymer is a weak base with a pK_a value of the glucosamine residue of ~6.2 to 7.0. Therefore, it is insoluble at neutral and alkaline pH values. In acidic mediums, the amino groups will be positively charged, conferring to the polysaccharide a high charge density.[7] Chitosan has been used in pharmaceutical applications such as film coating, tablets, microparticulate systems, capsules, gel systems, sustained-release systems, and bioadhesion. Chitosan is a nontoxic biodegradable polycationic polymer with low immunogenicity.[7] Possessing positive charges, it can be complexed with negatively charged DNA.[8] Moreover, chitosan protects DNA from nuclease degradation.[9,10] To develop chitosan as a gene carrier, several studies have been conducted to address the preparation of plasmid DNA/chitosan complexes such as nanospheres,[7,9] self-aggregates,[6] and chitosan complexes chemically modified by coupling ligands.[11] Chitosan/DNA complexes have been reported to effectively transfect various cell types, eg, human embryonic kidney cells (HEK293),[12] human lung carcinoma cells (A549),[13,14] B16 melanoma cells,[14,15] African green monkey kidney cells (COS-1),[8,16] HeLa cells,[14,17] Swiss3T3,[17] mesenchymal stem cells or human osteosarcoma cells (MG63),[12] and Caco-2 cells.[16] Several chitosan/DNA complex studies have been conducted to investigate experimental factors such as pH, serum, molecular weight (MW), and degree of deacetylation on in vitro transfection efficiency. MacLaughlin et al[8] found that chitosans having an MW lower than 100 kDa formed small complexes between 100 and 200 nm. The MW of chitosan

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proved to have limited influence on gene expression in vitro. The transfection efficiencies of chitosan/DNA complexes were enhanced in a culture medium of pH 6.9.[14] However, there was much debate about the effect of serum supplement in the medium on transfection efficiencies.[8,14,16,18,19] The degree of chitosan deacetylation is also an important factor in chitosan/DNA formulation, as it affects DNA binding, release, and gene transfection efficiency in vitro and in vivo.[20]

Although it had been suggested that chitosan is a biocompatible and nontoxic polymer, previous studies have revealed that soluble chitosan, like other cationic polymers, displayed concentration-dependent toxicity toward cells in vitro. Cytotoxicity toward B16F10 cells was concentration-dependent and varied according to the salt used and the polymer MW. The ranking of cytotoxicity was chitosan hydrochloride > chitosan glutamate > chitosan lactate (CL).[21] Chitosan and some chitosan salts were reported to be carriers for transfection of DNA,[6,12-15] but each study was separately performed by different investigators. No publication compared the effect of different salt forms on the chitosan/DNA complex and transfection efficiency in the same experiment. Therefore, the aim of this study was to evaluate CL and chitosan base dissolved in dilute acetic acid (chitosan acetate [CA]) of different MWs as a DNA complexing agent for their efficiency in transfecting COS-1 cells (green monkey fibroblasts) and their effect on cell viability compared with PEI, a commercially available cationic polymer.

Materials and Methods

Materials

Chitosan was purchased from Seafresh Chitosan Lab (Bangkok, Thailand) with MWs of 20, 45, 200, and 460 kDa and an 87% degree of deacetylation. Lactic acid and dimethyl sulfoxide (DMSO) were from BDH Laboratories (Poole, England), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Co (St Louis, MO). Agarose was purchased from ISC Bioexpress (Kaysville, UT). High-MW PEI was purchased from Aldrich (Munich, Germany). Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, Trypsin-EDTA, penicillin-streptomycin antibiotics, and fetal bovine serum (FBS) were obtained from GIBCO-Invitrogen (Carlsbad, CA), and 96-well plates were obtained from Costar (Corning, NY). pSVβ-Gal (Promega, Madison, WI) containing bacterial β-galactosidase gene under the control of SV40 promotor was 6820 bp. The β-galactosidase assay kit and λ *Hin*dIII were obtained from Promega. All other chemicals were of cell culture and molecular biology quality. COS-1 (CRL-1650) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD).

Methods

Preparation of Spray-Dried CL

CL was prepared as previously described.[22] Chitosan of different MWs was dissolved in distilled water containing lactic acid in a 1:1.3 molar ratio. The solution was adjusted with distilled water to make a 1% wt/wt solution and stirred for 12 hours. This solution was spray-dried under the following conditions: the inlet temperature was maintained at 125 ± 2°C by using a spray dryer (Minispray Dryer, Büchi 190, Postfach, Switzerland). The obtained powder was collected and stored in a desiccator containing dry silica gel prior to use in each experiment.

Physicochemical Studies of CL

Fourier transform infrared (FTIR) spectroscopy was performed using an FTIR Magna-IR system 750 (Nicolet Biomedical, Madison, WI) (32 scans, Resolution 4.000, wave number range 4000–500 cm^{-1}). The sample was prepared by the KBr pellet method. Differential scanning calorimetry (DSC) of the samples was performed using a differential scanning calorimeter-thermogravimetric analyzer (SDT2960 DSC-TGA, TA Instruments, Inc, New Castle, DE). The scan rate was 10°C/min. Samples of 2 to 4 mg were accurately weighed and sealed in aluminum pans. The samples were heated at a constant rate of 5°C/min over a temperature range of 40 to 300°C. An inert atmosphere was maintained by purging with nitrogen gas. The ^{13}C nuclear magnetic resonance (NMR) spectra of CL were measured using a high-resolution solid-state ^{13}C NMR spectrometer (400 MHz, Bruker, Faellanden, Switzerland). X-ray powder diffraction (XRPD) measurements were performed in the reflection mode on an x-ray diffractometer (Diffractometer D8, Bruker AXS, Karlsruhe, Germany).

pSV β-Galactosidase Plasmid

pSV β-galactosidase (pSVβ-gal) was used to monitor gene transfer and gene expression after transfection. The plasmid was propagated in *Escherichia coli* DH5-α. *E coli* encoding β-galactosidase were grown in Luria Bertani broth and purified by the alkali lysis method.[23] The purity of the plasmid was checked by electrophoresis on a 0.7% agarose gel, and the DNA concentration was determined by measuring the UV absorbance at 260 nm (GeneRay UV-Photometer, Biometra, Goettingen, Germany).

Preparation and Characterization of Chitosan/DNA Complexes

CA was prepared by dissolving chitosan base in 0.2% wt/vol acetic acid solution, whereas CL was dissolved in the sterile water. The chitosan/DNA complexes were prepared at various

charge (N/P) ratios of 2:1, 4:1, 8:1, 12:1, and 24:1 by addition of an aqueous solution containing 5 μg pSVβ-gal to a solution of either CA or CL. The solutions were vortexed for 5 seconds and incubated for 15 minutes at room temperature. The formation of the complexes was determined by electrophoresis on a 0.7% agarose gel compared with a DNA marker, λ *Hin*dIII. UV transillumination of the gel was employed with ethidium bromide to visualize the DNA.

Size and Zeta Potential Measurement

The particle sizes and surface charge of the chitosan/DNA complexes were measured by laser Doppler anemometry using a Zetasizer 3000 (Malvern Instruments, Southborough, MA). The chitosan/DNA complexes were prepared in sterile water (500 μL) at N/P ratios of 2:1, 4:1, 8:1, 12:1, and 24:1 containing a final DNA concentration of 5 μg/mL. Zeta potential measurements were performed using the aqueous flow cell in the automatic mode at 25°C.

In Vitro Transfection Studies Using Cell Culture

COS-1 cells were maintained in DMEM at pH 7.4, supplemented with 10% FBS, 2mM L-glutamine, 1% nonessential amino acid solution, and 0.1% penicillin-streptomycin solution in a humidified atmosphere (5% CO_2, 95% air, 37°C). The cells were grown under standard conditions for 24 hours until 60% to 70% confluency. After cell viability was checked using trypan blue exclusion, 24-well plates were seeded with 5×10^4 cells/cm^2 and grown for 24 hours. Chitosan/DNA complexes were suspended in a low-serum medium, Opti-MEM, and incubated for 15 minutes at room temperature. Prior to transfection, the culture medium was removed and the cells were rinsed with phosphate-buffered saline (PBS, pH 7.4), then supplied with Opti-MEM. The cells were incubated with plasmid DNA alone and chitosan/DNA complexes for 4 hours at 37°C under a 5% CO_2 atmosphere, then washed with PBS twice and grown in culture medium for 24 hours to allow for β-galactosidase expression. PEI was used as a positive control. Each well received 5 μg of PEI complexed with 5 μg of DNA. The cells were harvested by adding 250 μL Reporter Lysis Buffer (Promega, Madison, WI). The β-galactosidase activity was determined using the β-galactosidase assay system kit (Promega) according to the manufacturer's instructions. The protein content was measured using the Bradford protein assay. Results are expressed in milliunits β-galactosidase per milligram protein, as averaged over 3 experiments.

Evaluation of Cell Viability

COS-1 cells were seeded on a 96-well plate at a cell density of 5×10^4 cells/cm^2 and allowed to grow for 24 hours in DMEM with 10% FBS. The cells were then transfected with the same concentrations as in the 24-well plates. Transfection was performed using chitosan/DNA complexes containing an amount of chitosan and DNA equivalent to 70 μg and 5 μg, respectively, and cell viability was compared with either nontreated control cells or PEI/DNA complex–treated positive control cells. After 4 hours of incubation at 37°C, 100 μL of MTT in DMEM (1 mg/mL) was added to each well and incubated for an additional 4 hours under normal growing conditions. Then all media were removed and 100 μL of DMSO was added. Plates were incubated for 30 minutes at 37°C, and the absorbance was measured at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, Meriden, CT). The viability of nontreated control cells was arbitrarily defined as 100%.[12]

Statistical Analysis

All experimental measurements were collected in triplicate. Values are expressed as mean ± SD. The statistical significance of differences in β-galactosidase activity and cell viability was examined using 1-way analysis of variance followed by least significant difference (LSD) post hoc test. The significance level was set at $P < .05$.

RESULTS AND DISCUSSION

Characterization of CL

Characterized by FTIR spectroscopy, CA exhibits the characteristic bands of O-H stretching vibration at 3450 to 3400 cm^{-1}, NH_2 scissioning vibration at 1600 cm^{-1}, and C=O stretching vibrations of the pyranose ring at 1089 to 1031 cm^{-1}; all these values are similar to previously reported values.[24] CL of 4 different MWs (20, 45, 200, 460 kDa) show C=O stretching vibrations at 1734 cm^{-1}, indicating the presence of lactate groups in the chitosan structure. In addition, ^{13}C NMR spectra of CA and CL confirmed the lactate groups in chitosan structure. CA is in a crystalline form with a melting temperature of ~260°C, while CL showed a diffraction pattern of an amorphous state with a melting temperature of ~250°C as investigated by DSC and XRPD (data not shown).

Characterization of Chitosan/DNA Complexes

To determine the optimal complexation conditions, it was necessary to evaluate the degree of binding between either CA or CL and DNA at different MWs and chitosan concentrations. When the concentration of chitosan was changed and the DNA concentration kept constant, the ratio of negatively charged DNA (which is negatively charged because of the phosphate groups) to positively charged chitosan (which is positively charged because of the amine groups)—the N/P

ratio—of the particle formulations was varied (Figure 1; lanes 3-7, chitosan/DNA complexes with an N/P ratio of 2:1, 4:1, 8:1, 12:1, and 24:1). DNA binding is dependent on the MWs. Low-MW CA (20 kDa) formed a complex with DNA completely at an N/P ratio of around 4:1, whereas the N/P ratio for higher-MW CA (45, 200, 460 kDa) was ~2:1. This could also be observed in CL/DNA complexes, with a slight difference. The N/P ratio of 20 and 45 kDa CL/DNA complexes was 8:1, and that of 200 and 460 CL/DNA complexes was 4:1. The lower-MW chitosan required a higher charge ratio to completely bind the DNA, as also indicated by Kiang et al.[20] Compared with CA, CL was observed to require a higher charge ratio to completely form the complex with DNA. This difference might be attributed to the different counterions (acetate ion and lactate ion) present in the medium that could interact with chitosan. To varying degrees, these ions partially neutralize the positive charge of chitosan, thereby decreasing the interaction between chitosan and DNA. Because an acetate ion is smaller than a lactate ion, an acetate ion is less negative than a lactate ion. Therefore, in the case of CL, more chitosan was required to maintain a positive charge for complete interaction with DNA.

The particle size and zeta potential of the complexes at various charge ratios were determined. The particle size of both CA/DNA and CL/DNA complexes increased with an increasing charge ratio (data not shown). This was due to the intermolecular cross-linking between DNA strands by self-aggregates, a phenomenon typically observed with either high DNA concentrations or an excess amount of polycations.[25] Zeta potential is a function of the surface charge that develops when any material is placed in a liquid and is a good index of the electrostatic properties of colloidal particles.[25] An initial negative value of the zeta potential was observed at a low charge ratio (2:1) for both CA/DNA and CL/DNA complexes. This may be due to 2 factors: amino groups in chitosans at physiological conditions have a low effective charge density, and the access of DNA to positive charges on chitosans is sterically hindered by the inherent rigidity of the chitosan backbone.[25] Complexes between self-aggregates and DNA showed an increasing zeta potential in parallel with increasing charge ratios (data not shown). The completely formed complexes of both CA and CL at a charge ratio of 12:1 (Table 1) show an increasing zeta potential in parallel with increasing MW. In comparison with the CL/DNA complex, the CA/DNA complex

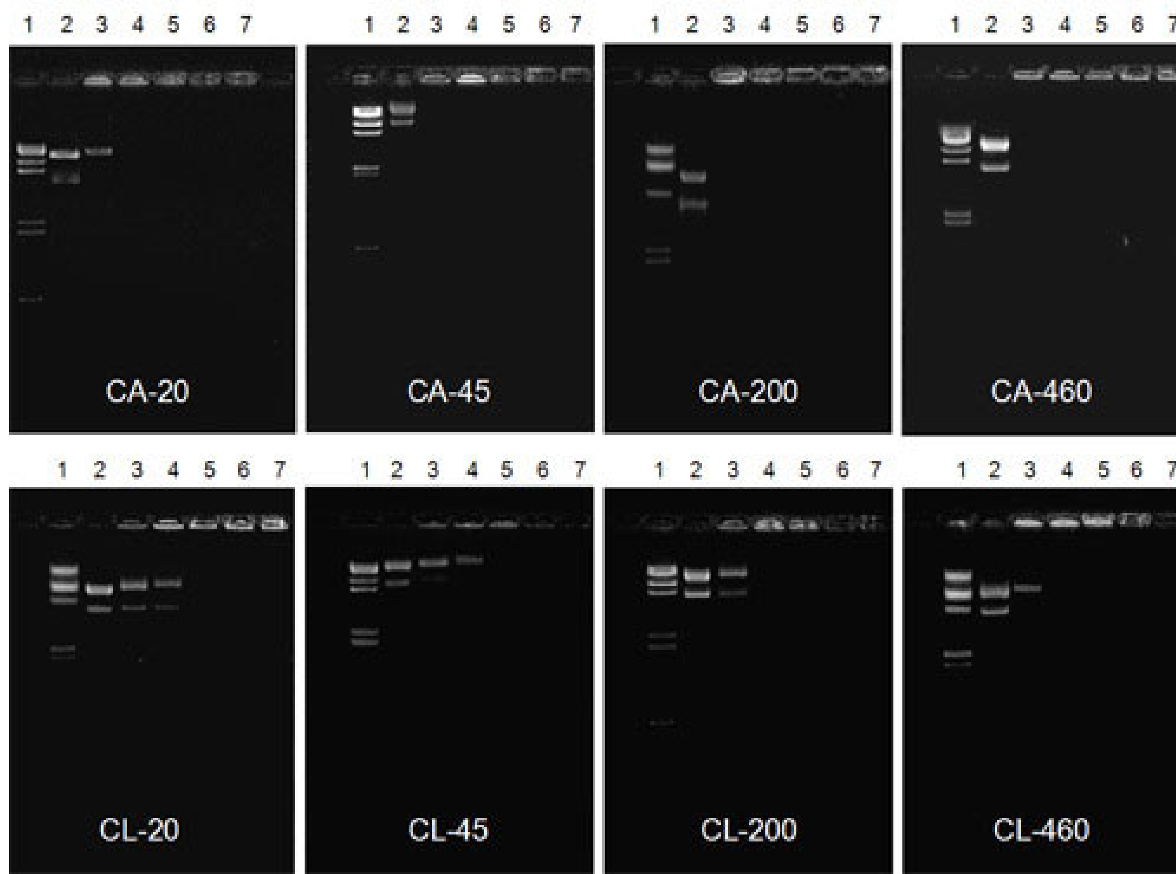

Figure 1. Electrophoresis of self-aggregate chitosan/DNA complexes on 0.7% agarose gel. Lane 1, λ *Hin*dIII DNA marker; lane 2, pSV β-galactosidase plasmid (0.5 μg); lanes 3-7, N/P ratio of 2:1, 4:1, 8:1, 12:1, and 24:1, respectively. CA indicates chitosan acetate; CL, chitosan lactate.

Table 1. Zeta Potential and Particle Size of Self-Aggregate CA/DNA Complexes and CL/DNA Complexes at a Charge Ratio of 12:1 at Various Molecular Weights

Chitosans-Molecular Weight	Zeta Potential, mV	Particle Size, nm
CA-20	44.0 ± 0.17	484.7 ± 10.6
CA-45	44.2 ± 0.70	445.2 ± 27.2
CA-200	48.9 ± 0.90	499.9 ± 26.2
CA-460	47.5 ± 0.26	727.1 ± 97.8
CL-20	28.2 ± 0.30	246.7 ± 6.6
CL-45	30.3 ± 1.10	300.0 ± 3.7
CL-200	35.7 ± 0.80	481.1 ± 12.9
CL-460	35.4 ± 0.10	538.9 ± 15.0

*n = 3; mean ± SD. CA indicates chitosan acetate; CL, chitosan lactate.

showed a higher zeta potential since more chitosan was left after the complete complex formation. CA and CL give complexes in the size range of 445 to 727 nm, and 247 to 539 nm, respectively.

Transfection and Expression of the β-Galactosidase Plasmid Complexed With the Chitosan Particles

To test the ability of CL to transfect COS-1 cells, complexes were made with pSVβ-gal, and these complexes were applied onto cells for 4 hours. After this time the complexes were removed and the cells were allowed to grow for 24 hours in a normal culture medium to express the protein. Figure 2 shows results of cell transfection with CA/pSVβ-gal complexes and CL/pSVβ-gal complexes at a charge ratio of 12:1. The transfection efficiency of chitosan self-aggregate/DNA complexes was higher than that achieved by naked DNA (not shown) but lower than that achieved by the commercially available cationic polymer PEI. Both CA and CL gave appreciable transfection in an equal quantity. Moreover, they showed increasing transfection efficiency in parallel with increasing MW (significant difference, $P < .05$). The exception was for chitosan MW 45 kDa, in that CL showed a slightly greater transfection efficiency than CA/DNA complexes (nonsignificant difference, $P > .05$). In this study, it was evident that the transfection ability of CL is comparable to that of CA at all MWs. The transfection efficiency of CL/DNA and CA/DNA complexes is MW dependent. This result is in agreement with the results reported by Mao et al,[18] MacLaughlin et al,[8] and Huang et al.[26] However, Sato et al[14] found that chitosan/DNA complexes with MWs of more than 100 kDa had lower transfection efficiencies than those with MWs of 15 and 52 kDa in A549, B16, and HeLa cells. The others reported higher transfection efficiencies of chitosan/DNA complexes with MWs of 40 and 84 kDa than of those with MWs of 1 and 110 kDa in an SOJ cell by using a luciferase assay.[19]

Effect of CL/DNA Complexes on Cell Viability of COS-1 Cells

One of the major requirements for cationic polymer vectors for gene delivery is low cytotoxicity. It has been reported that chitosan salts and chitosan derivatives are less toxic than other cationic polymers such as polylysine and PEI in vitro and in vivo.[27-29] CL showed the lowest cytotoxicity, compared with chitosan glutamate and chitosan hydrochloride, toward B16F10 cells.[21] However, no data for this salt in a COS-1 cell line are available. Therefore, the cytotoxicity of the CL/DNA complex was examined in COS-1 cells. Figure 3 shows the effect of CA/DNA and CL/DNA complexes on cell viability, compared with the PEI/DNA complex. When COS-1 cells were incubated with 5 μg of naked DNA, cell viability remained almost the same as that seen in control nontransfected cells (data not shown). There was no significant decrease in cell viability when COS-1 cells were incubated with both CA/DNA and CL/DNA

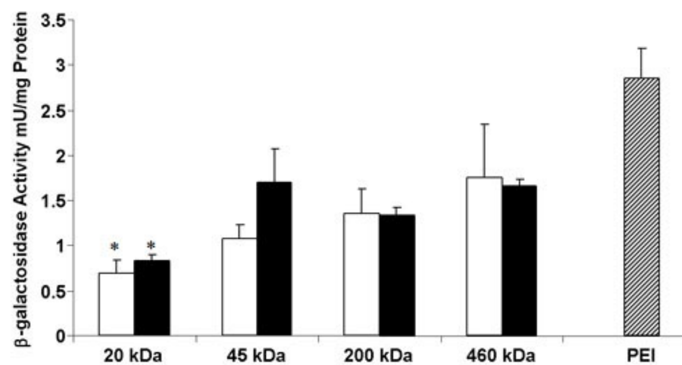

Figure 2. β-galactosidase expression in COS-1 cells transfected with chitosan/plasmid DNA complex prepared at an N/P ratio of 12:1 with 4 molecular weights (20, 45, 200, 460 kDa) of chitosan (□ = chitosan acetate; ■ = chitosan lactate). Each value represents the mean ± SD of tree wells. * $P < .05$. PEI indicates polyethylenimine.

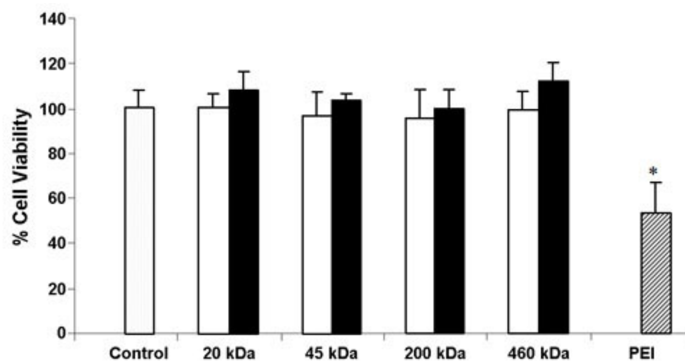

Figure 3. Effect of CA/DNA, CL/DNA, and PEI/DNA complexes on COS-1 cell viability (□ = CA; ■ = CL). Each value represents the mean ± SD of tree wells. * $P < .05$. CA indicates chitosan acetate; CL, chitosan lactate; PEI, polyethylenimine.

complexes. Their average cell viability was over 90%. On the other hand, 5 μg of PEI/DNA complexes showed a drastic decrease in cell viability to ~50%. This result is similar to that previously reported by Kim et al[30] in which the 45% cell viability of 293T cells treated with PEI of MW 25 kDa at 5 μg/mL was obtained. Various chitosans and chitosan derivatives have been reported for gene delivery. However, the toxicity of those chitosans was different depending on the type of cells and derivatives studied.[6-20] Therefore, this study clearly proved that CL is safe.

CONCLUSIONS

CL was successfully prepared by the spray-drying method. The CA/DNA and CL/DNA complexes yielded nanosized particles. The transfection efficiency of CL and CA at various MWs was comparable. Both forms of chitosan proved to be nontoxic to COS-1 cells; however, CL had an advantage over CA in its ease of processing in the polymer/DNA preparation because of its water solubility. This study suggests that CL is a safe and efficient gene carrier.

ACKNOWLEDGMENTS

This work was supported by the Faculty of Pharmacy, Research and Development Institute, Silpakorn University, and the National Nanotechnology Center (Nanotec), Thailand.

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