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Research Article

Folate-Targeted Liposome Encapsulating Chitosan/Oligonucleotide Polyplexes for Tumor Targeting

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Abstract. We previously reported that a liposome encapsulating polyethylenimine/oligonucleotides is suitable for *in vivo* delivery of nucleic acid therapeutics. However, toxicity of polyethylenimine is an obstacle in clinical application. To develop a liposome encapsulating polyplexes applicable to clinical use, we proposed to replace polyethylenimine with chitosan and thus constructed the liposome encapsulating low-molecular weight chitosan (LMWC)/oligonucleotide (ODN) polyplexes [LS(CO)]. ODN was completely complexed to LMWC at pH 5.5 and an N/P ratio 10 with a positive zeta potential of 19.81±1.11. The positively charged polyplexes were encapsulated into anionic liposome by membrane extrusion. Folate-targeted liposome encapsulating LMWC/ODN complex [FLS(CO)] was prepared by adding folate-conjugated phospholipid. The resulting LS(CO) and FLS(CO) were characterized with respect to size distribution, zeta potential, and colloidal stability. The LS(CO) and FLS(CO) were also evaluated for *in vitro* cellular uptake and cytotoxicity. The LS(CO) and FLS(CO) showed a narrow size distribution with a mean diameter of about 130 nm and neutral zeta potentials and remained stable for 7 days in 0.15-M NaCl at room temperature. FLS(CO) showed higher cellular uptake than LS(CO) in B16F10 murine melanoma cells. Furthermore, LS(CO) showed less toxicity as compared to liposome encapsulating polyethylenimine/oligonucleotides, representing a biocompatible nanocarrier of oligonucleotide therapeutics.

KEY WORDS: chitosan; folate targeting; liposomes; oligonucleotide; tumor targeting.

INTRODUCTION

The PEG-stabilized liposomes have been successfully applied for *in vivo* delivery of various nucleic acid-based therapeutics such as plasmid DNA (1,2) or oligonucleotides (3). However, it has been a challenge to encapsulate nucleic acid-based therapeutics, as with other macromolecular therapeutics, within liposomal systems. Condensation of nucleic acid-based therapeutics using a polycationic polymer followed by encapsulation into liposomes has also been reported (4–6).

Among polycationic polymers, polyethylenimine (PEI) has been widely explored for nucleic acid delivery due to its high transfection efficiency (7–10). Nevertheless, polyplexes between PEI and nucleic acids have not shown significant therapeutic efficacy for *in vivo* application due to their rapid plasma clearance and accumulation by reticuloendothelial system (RES) sites. In an effort to improve the poor *in vivo* stability and pharmacokinetic behavior of the polyplex systems, we previously reported a procedure to prepare liposomes encapsulating polyethylenimine/oligonucleotide polyplexes within PEG-stabilized liposomes by rehydrating anionic lipid film in an aqueous buffer containing preformed polyethylenimine/oligonucleotide polyplexes (11) or reverse-phase evaporation technique (12). Our previously established procedure offers a simple method for combining polyplex systems with liposomal gene carrier systems, with a very high loading efficiency. The procedure is based on the sequential charge interaction between oppositely charged macromolecules (*i.e.,* cationic polymers and ODNs) and between polyplexes and phospholipid membranes and can easily be adapted to other cationic polymers. Although previous studies showed that applying low molecular PEI i proven to lead to tolerable toxicity, the repeated use of PEI, however, should be limited due to its toxicity in systemic application (13). Toxicity of PEI has been known not to effect as apoptotic signaling in cells, but it could cause cellular plasma membrane disruption (14).

In this study, we proposed to use low molecular chitosan, as an alternative polycationic polymer, to form polyplexes with nucleic acids. Chitosan and its derivatives have attracted attention as favorable non-viral vectors in the area of *in vivo* delivery of nucleic acid therapeutics owing to their non-toxicity, high biocompatibility, and biodegradability (15,16). The liposomes encapsulating chitosan/oligonucleotide were then targeted to tumor tissues by endowing folic acid as a tumor-specific ligand. Folate receptors were found to be overexpressed in malignant cells, including ovarian cancer and other malignancies. Distribution of folate receptor is largely restricted to malignant tissues, and folate receptors are now considered to be selective drug targets for the treatment of cancer and inflammatory diseases (17,18). The preparation procedure was optimized, and the resulting folate-targeted liposome encapsulating chitosan/oligonucleotide

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polyplexes was characterized with regard to size, zeta potential, colloidal stability, and encapsulation efficiency. *In vitro* cellular uptake and cytotoxicity were also evaluated.

MATERIALS AND METHODS

Materials

Low molecular weight chitosan (LMWC; MW 3–5 kDa) was purchased from Kittolife (Kyounggi, South Korea). LMWC was dissolved in SBG buffer (10-mM sodium borate, 5% glucose, pH 4.5) at a final concentration of 5.0 μg/μL. The sequence of the double-stranded oligodeoxynucleotide (ODN) (5′-CCTTGAAGGGATTTCCCTCC-3′ and 3′-GGAACTTCCCTAAAGGGAGG-5′) contained the NF-κB *cis* element (19). Single-stranded 20-mer oligonucleotides and corresponding 5′-Alexa488-conjugated oligonucleotides were purchased from Genotech (Daejeon, South Korea). Double-stranded ODNs were prepared by annealing the single stranded oligonucleotides with equal molar amounts of complementary single-stranded oligonucleotides at a final ODN concentration of 1 μg/μL. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-91-glycerol] (POPG), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (PEG-PE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-2000] (amine-PEG-PE), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Other reagents were purchased from Sigma-Aldrich (St Louis, MO) and Tokyo Chemical Industry (Tokyo, Japan).

Preparation of Folate-PEG-PE

Folic acid was conjugated to amine-PEG-PE *via* a dicyclohexyl carbodiimide/*N*-hydroxysuccinimide (DCC/NHS) coupling reaction according to a previously published method (20). In brief, 6.3 mg (0.014 mmol) of folic acid in 0.3 mL of dry dimethyl sulfoxide was dissolved before adding 25 mg (0.009 mmol) of amine-PEG-PE, 110 μL of pyridine, and 7.8 mg (0.038 mmol) of dicyclohexyl carbodiimide (DCC) (1:1.6 molar ratio of amine-PEG-PE/folate). The reaction was carried out for 4 h at room temperature, and reaction progress was monitored by TLC (C18) using chloroform/methanol/water (75:36:6) (Rf for product 0.57). After removing pyridine by evaporation, the residue was rehydrated with 6 mL of deionized water. Insoluble by-products were then removed by centrifugation at 4000*g* for 7 min. The supernatant was dialyzed (MWCO 10000) against 1 L of 50 mM of sodium chloride and 1 L of distilled water then lyophilized and stored at −80°C. The conjugation was confirmed by MALDI-TOF. The product (FA-PEG-PE) was obtained in a yield of 87.85%.

Preparation of LMWC/ODN Polyplex

A constant amount of ODN (100 μg) and varying amounts of LMWC were separately diluted in SBG (10 mM Sodium borate, 5% glucose, pH 5.5) to a final volume of 250 μL. After incubating for 10 min at room temperature, the solutions were rapidly mixed and vortexed immediately, yielding 500 μL of LMWC/ODN complexes at +/−charge ratios varying from 1 to 24. The amount of LMWC was calculated to obtain the desired charge ratio by assuming that that 160 g/mol corresponds to each repeating unit of LMWC containing one amine and 330 g/mol corresponds to each repeating unit of ODN containing one phosphate. Each mixture was analyzed by 2% agarose gel electrophoresis (100 V, 30 min) to determine the optimum charge ratio for complete complexation.

Liposome Encapsulation of LMWC/ODN Polyplex

The following amounts of lipids were dissolved in chloroform: POPC (3.7 μmol), POPG (3.0 μmol), cholesterol (3.0 μmol), and either PEG-PE (0.3 μmol) or folate-polyethyleneglycol-phosphatidylethanolamine (FA-PEG-PE) (0.3 μmol) for non-targeted liposome encapsulating LMWC/ODN complex (LS(CO)) and folate-targeted liposome encapsulating LMWC/ODN complex (FLS(CO)), respectively. The amount of folate ligand should be sufficient to exert a targeting effect (21). Chloroform was removed by vacuum evaporation using a rotary evaporator (500 mmHg, 4 h). The positively charged LMWC/ODN complex, preformed at N/P ratio 10 was then added to the dried anionic lipid film and incubated at room temperature for 4 h with intermittent mixing. The resulting suspension was extruded 11 times through a stack of two polycarbonate membranes of a 100-nm pore size by employing a hand-held extruder (Avestin, Ottawa, Canada). Encapsulation efficiency of Alexa488-labeled ODN into liposome was determined by size-exclusion chromatography (Zenix-SEC100, 4.6×150 mm, Waters Corp.) using PBS (pH 7.4) as an eluent at a flow rate of 1.0 mL/min.

Measurement of Size Distribution and Zeta Potential

Each sample was diluted with HBS (10-mM HEPES, 150-mM NaCl, pH 7.4) to obtain an optimal scattering intensity. Hydrodynamic diameter and zeta potential were measured by dynamic light scattering (DLS) and electrophoretic light scattering (laser doppler) using a zeta potential and particle size analyzer (ELSZ-1000, Otsuka Electronics Co, Osaka, Japan). Scattered light was detected at 23°C at an angle of 90°. A viscosity value of 0.933 mPa and a refractive index of 1.333 were used for the data analysis. The instrument was routinely calibrated using a latex microsphere suspension.

Colloidal Stability

Colloidal stability of the nanoparticles against the salt-induced aggregation was determined by monitoring the hydrodynamic diameter of the particles in 150-mM NaCl. Concentrated NaCl solution (5 M) was added to the LS(CO) or FLS(CO) or LMWC/ODN complex in SBG to a final concentration of 0.15 M while measuring the size distribution described above.

In Vitro Cellular Uptake by Confocal Microscopy and Flow Cytometery

The murine melanoma B16F10 cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM, Gibco, NY) supplemented with 10% (*v*/*v*) fetal bovine serum (FBS, Gibco, NY) on cover slips (12 mm, Fisher Scientific) at 37°C and 5% CO_2. The cells were treated with LS(CO) and FLS(CO) labeled with Rhodamine-labeled PE (Rh-PE) and

Alexa488-labeled ODN (A488-ODN) at a final concentration of 2-μM ODN in serum-free DMEM. After a 30-min incubation at 37°C, the cells were washed four times with ice-cold PBS and fixed with 4% (*w*/*v*) paraformaldehyde in PBS. The coverslips with fixed cells were mounted with a mounting medium (Fluoromount, Sigma-Aldrich) and observed with a laser scanning confocal microscope (LSCM, A1Plus, Nikon). Alternatively, the cells, treated as above for 4 h, were harvested with 0.25% trypsin-EDTA and analyzed by a flow cytometer (FACS Caliber, BD).

In Vitro Cytotoxicity

The murine melanoma B16F10 cells (4×10^3) were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) in 96-well plates overnight. The cells were treated by replacing the medium with serum-free medium (100 μL) containing a serial dilution of LS(CO) up to 200 μg/mL of LMWC. The liposome encapsulating PEI/ODN was separately prepared and tested for comparison. After a 4-h incubation, the cells were washed twice with PBS and returned to complete medium (100 μL). After a 24-h incubation, a 110-μL medium containing 10 μL of water soluble tetrazolium (WST) solution (Ez-Cytox Cell Viability Assay Kit, DoGen, South Korea) was added to each well, and the plates were re-incubated for 30 min. The absorbance at 460 nm was measured for each well using a microplate spectrophotometer (Epoch, BioTek Instruments, VT). Relative cell viability was calculated with the cells treated only with the medium as a control.

RESULTS

Preparation and Characterization

Folate-targeted or non-targeted liposome encapsulating LMWC/ODN complex, FLS(CO), or LS(CO) was prepared by a previously established procedure of complexation-membrane extrusion after optimization (Fig. 1). Folate-PEG-PE was synthesized using DCC/NHS reaction according to the previously reported method. First, LMWC was complexed with ODN in an aqueous buffer at varying nitrogen/phosphate (N/P) ratios. The optimal N/P ratio was determined to be 10 where all ODN was completely condensed by LMWC (Fig. 2a). Then, the positively charged LMWC/ODN at N/P 10 was encapsulated into liposomal bilayers leading to folate-targeted or non-targeted liposomal polyplex nanoparticles.

The mean hydrodynamic diameters were found to be 167.92±4.39 nm for LMWC/ODN, 125.38±0.49 nm for LS(CO), and 130.06±0.19 nm for FLS(CO), respectively. Surface charges were also determined to be 19.81±1.11 mV for LMWC/ODN, 1.66±0.38 mV for LS(CO), and −1.0±1.56 mV for FLS(CO), respectively (Table I). The narrow and uniform size distribution of LS(CO) and FLS(CO) with polydispersity index of 0.09±0.005 and 0.1±0.003, respectively (Table I), were obtained after membrane extrusion. The positive surface charge of LMWC/ODN was converted to almost neutral surface charges of LS(CO) or FLS(CO), demonstrating that the positively charged LMWC/ODN polyplexes were encapsulated into liposomal bilayer, thus shielding the positive charge.

Colloidal stability of LS(CO) and FLS(CO) was also measured by monitoring the hydrodynamic mean diameters (Fig. 2b). The naked LMWC/ODN as well as LS(CO) and FLS(CO) remained stable in salt-free condition of 10-mM HBG. Upon salt challenge, the average diameter of the naked LMWC/ODN rapidly increased up to 1000 nm, indicating rapid aggregation of the particles. However, the LS(CO) and FLS(CO) maintained their average diameters and uniform distribution, implicating their colloidal stability in high salt condition.

Encapsulation efficiency of ODN into the liposomal LS(CO) was determined by size exclusion chromatography (SEC). The LS(CO) containing Alexa488-labeled ODN as a tracer was prepared as above. The free Alexa488-labeled ODN was then separated from the encapsulated Alexa488-labeled ODN by a size exclusion column. Encapsulation efficiency of ODN into the liposomal carriers was found to be more than 90%, as calculated from the peak areas of encapsulated ODN and free ODN, of which retention times were 1.97 and 3.78–4.2 min, respectively (Fig. 2c).

In Vitro Cellular Behavior

To demonstrate the feasibility of folate-targeted FLS(CO) for tumor targeting, *in vitro* cellular behavior of non-targeted LS(CO) and folate-targeted FLS(CO) was analyzed in B16F10 cells overexpressing folate receptor by laser scanning confocal microscopy (LSCM) and flow cytometry (FACS). Treatment of B16F10 cells with FLS(CO) led to distinct particulate yellow signals, which are from a merge of green signal of Alexa488-labeled ODN and red signal of Rhodamine-labeled PE throughout the cytosol. The non-targeted LS(CO) also showed particulate yellow signals comparable to FLS(CO) during the incubation time period. These indicated that both LS(CO) and FLS(CO) were able to bind and deliver the cargo LMWC/ODN into the cytosol of the tumor cells during the incubation time of 30 min (Fig. 3a). The cellular uptake was quantitatively analyzed by FACS, revealing that FLS(CO) was taken up greater than LS(CO) by B16F10 cells (Fig. 3b). The mean fluorescence intensity of Alexa488-labeled ODN from FLS(CO) treatment was significantly higher than that from LS(CO) treatment (Fig. 3c) although no significant difference was observed in the mean fluorescence intensity of Rhodamine-PE. The higher cellular uptake of the cargo ODN from FLS(CO) treatment indicates that folate receptor targeting of the liposomal nanocarriers by folic acid could enhance their cellular uptake into B16F10 cells at a given time point. These results also suggest that folate targeting, upon *in vivo* application, could reduce the nanocarrier's non-specific interaction with normal cells and allow active targeting to cancer cells.

Cytotoxicity was also evaluated in murine melanoma (B16F10) cells by MTT assay over an exposure period of 24 h. The liposomal nanocarrier encapsulating chitosan/oligonucleotide polyplexes LS(CO) showed no toxicity with 100% cell viability up to 200 μg/mL of LMWC concentration, while the corresponding liposomal nanocarrier encapsulating polyethylenimine/oligonucleotide polyplexes led to only 60% cell viability at 200 μg/mL of PEI concentration against the same cells (Fig. 4). This cytotoxicity results clearly demonstrate that the liposomal nanocarrier encapsulating chitosan/oligonucleotide polyplexes LS(CO) should be a promising nucleic acid delivery system safer than liposome encapsulating polyethylenimine/oligonucleotide polyplexes.

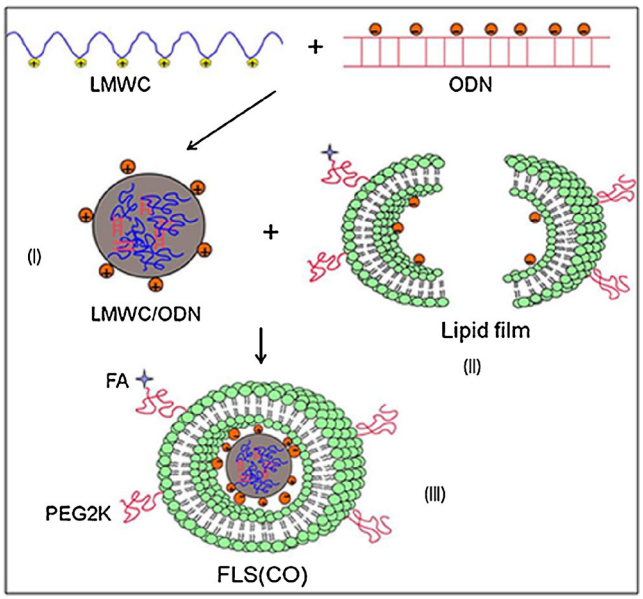

Fig. 1. Schematic diagram for preparation of the folate-targeted liposome encapsulating LMWC/ODN polyplex [FLS(CO)]; (*I*) formation of the LMWC/ODN polyplex in aqueous buffer, (*II*) formation of a dry lipid film, and (*III*) hydration of lipid film in the presence of LMWC/ODN polyplex and size reduction by membrane extrusion

DISCUSSION

Liposomal encapsulation of nucleic acid-based therapeutics such as plasmid DNA, ribozyme, antisense ODN, siRNA molecules into both conventional, and PEG-stabilized liposomes has been studied in several different ways in order to improve the stability of nucleic acid therapeutics and delivery safely into targeted area (2,3,22–25). As an alternative approach, liposomal encapsulation of polyplexes between polymers and nucleic acid has been previously proposed. It was reported that polyplexes between DNA and cationic polymer, such as poly-L-lysine, can be entrapped into folate-targeted anionic liposomes for tumor-specific oligonucleotide delivery (4,26). It was also shown that polyplexes between oligonucleotides and cationic polypeptides such as protamine sulfate can be entrapped into preformed DOTAP cationic liposomes, resulting ternary oligonucleotide delivery systems, so called cationic lipid-protamine-DNA (LPD) particles with enhanced *in vivo* oligonucleotide transfer activity (27). Moreover, polyplexes between PEI and DNA were shown to be entrapped in preformed anionic liposomes, resulting in nano-sized particles, so called artificial virus-like particles with high *in vitro* oligonucleotide transfer activity (5,28).

With a specific aim to develop a non-toxic nucleic acid delivery system with clinical potential, we carried out, in this study, the preparation and characterization of folate-targeted liposome encapsulating low molecular weight chitosan/oligonucleotide polyplexes [FLS(CO)] through the optimization of the previous established procedure. This liposomal nanocarrier was characteristically very similar to the pegylated liposomes encapsulating polyethylenimine/oligonucleotide, which were previously reported (11,12). However, it differs from the previous one in the ways that low molecular weight chitosan (LMWC) is used instead of PEI and folate ligands are introduced into the surface, which would provide the nanocarrier with some properties critical for *in vivo* application, *i.e.*, low toxicity and enhanced tumor targeting.

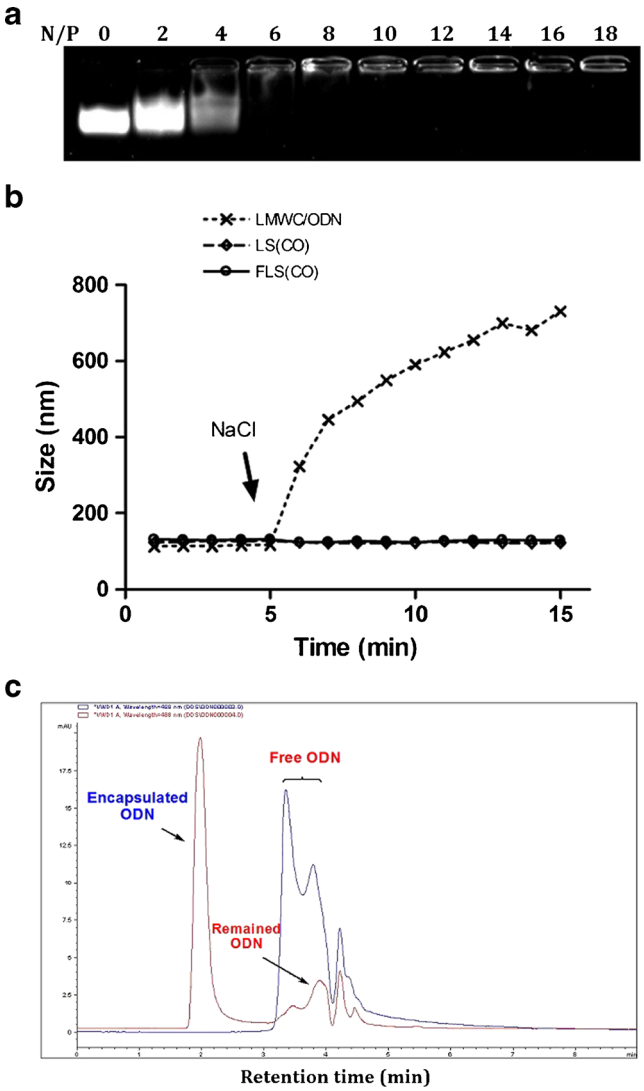

Fig. 2. Formation of the liposome encapsulating LMWC/ODN (FLS(CO). **a** Agarose gel electrophoresis of complexation between LMWC and ODN at varying of N/P ratios. No migration of ODN represents complexation. ODN was complexed completely with LMWC at more than N/P ratio 6. **b** Colloidal stability of FLS(CO) before and after adding 150-mM NaCl. Diameter of LMWC/ODN complex increases dramatically after adding salt, whereas those of LS (CO) and FLS (CO) are continuously stable. **c** Encapsulation efficiency by size exclusion chromatography (SEC). Encapsulated ODN was separated from free ODN with more than 90% encapsulated

Overall, FLS(CO) was confirmed to have the capability of delivering the cargo polycation/nucleic acid polyplexes into cytoplasm of tumor cells where folate receptors are expressed and low toxicity as compared to the polyethylenimine counterpart.

Here, we used LMWC, which is a natural cationic polymer with biodegradability and biocompatibility. Although chitosans and their derivatives have been extensively explored for nucleic acid delivery, transfection efficiency is too low as compared to PEI (29). It was known that chitosans have nucleic acid condensing capacity comparable to PEI at an acidic pH lower than 5.5 (16). At a neutral pH, chitosan shows insufficient nucleic acid condensing capacity due to a low degree of protonation. The nucleic acid condensing capacity of chitosan, however, increases as pH decreases because more

Table I. Physicochemical Characteristics of the Liposome Encapsulated LMWC/ODN in Comparison to the Naked LMWC/ODN. (Mean ±S.E.M, $n=5$)

	LMWC/ODN	LS(CO)	FLS(CO)
Diameter (nm)	167.92±4.39	125.38±0.49	130.06±0.19
Zeta potential (mV)	19.81±1.11	1.66±0.38	−1.00±1.56
Polydispersity index	0.11±0.005	0.09±0.005	0.11±0.003
Encapsulation efficiency (%)	–	–	≥90

LMWC low molecular weight chitosan, *ODN* oligonucleotide, *LS(CO)* liposome encapsulating LMWC/ODN complex, *FLS(CO)* folate-targeted liposome encapsulating LMWC/ODN complex

amino groups are protonated. Thus, stable polyplex at NP 10 was not formed by LMWC at pH 7.4 where stable polyplexes were readily formed by PEI. It was possible, however, to obtain stable polyplexes of NP 10 at pH 4.5, which was maintained during the following encapsulation step to avoid the dissociation of the polyplexes. Upon completion of encapsulation step, the polyplexes were no longer subject to dissociation even at pH 7.4, leading to colloidally stable nanocarrier in physiological pH. Our liposome formulations have high stability because pegylation produces steric hindrance to the liposome molecule (30).

The liposomal nanocarrier encapsulating LMWC/ODN polyplexes are expected to show *in vivo* behavior comparable to pegylated liposomes, *i.e.*, prolonged blood circulation and suppressed RES accumulation. Those behaviors could allow passive tumor targeting by enhanced permeability and retention (EPR) effect. Upon accumulation in tumor tissues by EPR, the nanocarrier should enter the tumor cells across the plasma membrane. The intracellular uptake could be triggered by specific binding between membrane receptors and ligands on the nanocarriers. Folate receptors expressed on tumor cells, including ovarian, brain, lung, and breast (31), have been used for intracellular uptake of various nanocarriers decorated with folic acids (17,31). The feasibility of active targeting of the liposome encapsulating LMWC/ODN into tumor cells was shown in melanoma cells by the an enhanced cellular uptake of folate-targeted FLS(CO) as compared to non-targeted LS(CO). It should be noted that the non-targeted LS(CO) also showed significant cellular uptake probably by non-specific interaction between plasma membrane and the liposomal nanocarriers, implying that the benefit of active tumor targeting by folate ligand should be justified with careful evaluation.

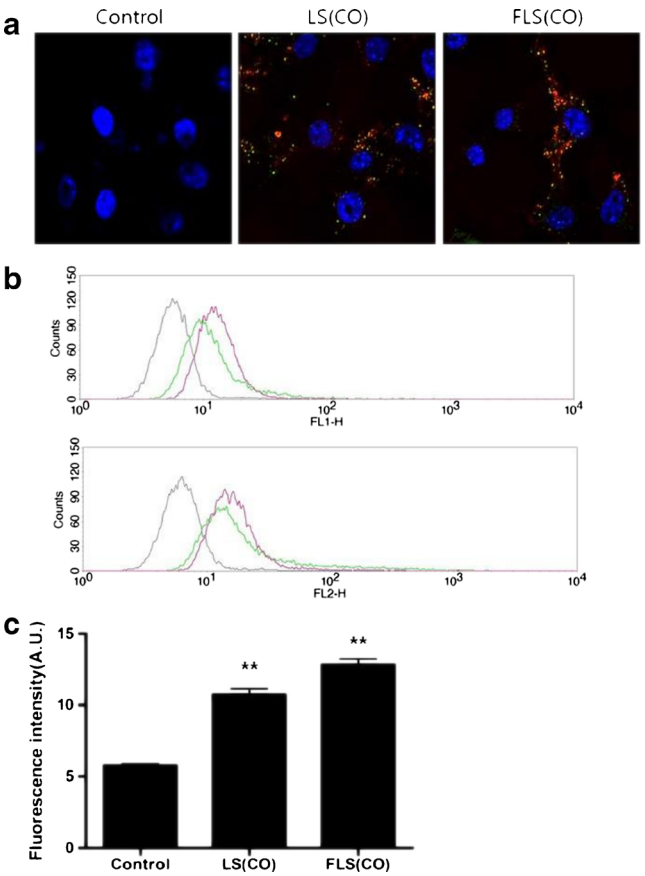

Fig. 3. Cellular uptake of the liposome encapsulating LMWC/ODN in B16F10 cells. **a** Laser scanning confocal microscopy (LSCM) images after a 30-min incubation with LS (CO) or FLS (CO) (*red*, Rhodamine-labeled liposome; *green*, Alexa488-labeled ODN; *blue*, Hoechst). **b** Flow cytometric (FACS) analysis after incubation with LS(CO) or FLS(CO) (*black*, control; *green*, LS(CO); *red*, FLS(CO); *FL1-H* Alexa488-labeled ODN channel; *FL2-H* Rhodamine-labeled liposome). **c** Mean fluorescence intensity of Alexa488 ODN in B16F10 cells (mean±S.E.M, $n=5$; $^{**}p<0.05$)

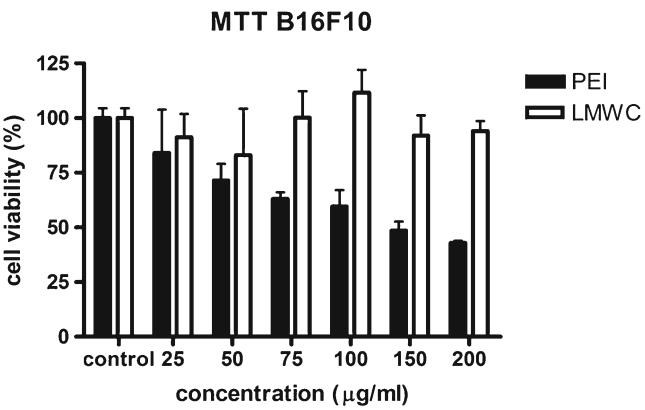

Fig. 4. Comparison of cytotoxicity between PEI and LMWC in B16F10 cells. The melanoma B16F10 cells were treated with free PEI and LMWC at varying concentrations, and cell viability was measured using MTT assay. Relative cell viability was expressed as a percentage of non-treated control cells (mean±S.E.M, $n=5$)

CONCLUSION

The folate-targeted liposome encapsulating low molecular weight chitosan/oligonucleotide polyplex could be a promising delivery system for nucleic acid therapeutics, which is non-toxic and applicable for multiple administrations. Furthermore, the system should be applicable for the various nucleic acid-based therapeutics including siRNA and asODN.

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REFERENCES

1. Shi N, Pardridge WM. Noninvasive gene targeting to the brain. Proc Natl Acad Sci U S A. 2000;97(13):7567–72.
2. Wheeler JJ, Palmer L, Ossanlou M, MacLachlan I, Graham RW, Zhang YP, *et al*. Stabilized plasmid-lipid particles: construction and characterization. Gene Ther. 1999;6(2):271–81.
3. Stuart DD, Kao GY, Allen TM. A novel, long-circulating, and functional liposomal formulation of antisense oligodeoxynucleotides targeted against MDR1. Cancer Gene Ther. 2000;7(3):466–75.
4. Lee RJ, Huang L. Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. J Biol Chem. 1996;271(14):8481–7.
5. Bandyopadhyay P, Ma X, Stieers CL, Kren BT, Steer CJ. Nucleotide exchange in genomic DNA of rat hepatocytes using RNA/DNA oligonucleotides. J Biol Chem. 1999;274(15):10163–72.
6. Heyes J, Palmer L, Chan K, Giesbrecht C, Jeffs L, MacLachlan I. Lipid encapsulation enables the effective systemic delivery of polyplex plasmid DNA. Mol Ther. 2007;15(4):713–20.
7. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, *et al*. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci U S A. 1995;92(16):7297–301.
8. Akinc A, Thomas M, Klibanov AM, Langer R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. J Genet Med. 2005;7(5):657–63.
9. Kircheis R, Wightman L, Wagner E. Design and gene delivery activity of modified polyethylenimines. Adv Drug Deliv Rev. 2001;53(3):341–58.
10. Vinogradov SV, Batrakova EV, Kabanov AV. Nanogels for oligonucleotide delivery to the brain. Bioconjug Chem. 2004;15(1):50–60.
11. Ko YT, Bhattacharya R, Bickel U. Liposome encapsulated polyethylenimine/ODN polyplexes for brain targeting. J Control Release. 2009;133(3):230–7.
12. Ko YT, Bickel U. Liposome-encapsulated polyethylenimine/oligonucleotide polyplexes prepared by reverse-phase evaporation technique. AAPS PharmSciTech. 2012;13(2):373–8.
13. Jones CH, Chen CK, Ravikrishnan A, Rane S, Pfeifer BA. Overcoming nonviral gene delivery barriers: perspective and future. Mol Pharm. 2013;10(11):4082–98.
14. Florea BI, Meaney C, Junginger HE, Borchard G. Transfection efficiency and toxicity of polyethylenimine in differentiated Calu-3 and nondifferentiated COS-1 cell cultures. AAPS PharmSci. 2002;4(3):E12.
15. Fernandes JC, Qiu X, Winnik FM, Benderdour M, Zhang X, Dai K, *et al*. Low molecular weight chitosan conjugated with folate for siRNA delivery in vitro: optimization studies. Int J Nanomedicine. 2012;7:5833–45.
16. Mao S, Sun W, Kissel T. Chitosan-based formulations for delivery of DNA and siRNA. Adv Drug Deliv Rev. 2010;62(1):12–27.
17. Low PS, Antony AC. Folate receptor-targeted drugs for cancer and inflammatory diseases. Adv Drug Deliv Rev. 2004;56(8):1055–8.
18. Stephenson SM, Low PS, Lee RJ. Folate receptor-mediated targeting of liposomal drugs to cancer cells. Methods Enzymol. 2004;387:33–50.
19. Morishita R, Sugimoto T, Aoki M, Kida I, Tomita N, Moriguchi A, *et al*. In vivo transfection of cis element "decoy" against nuclear factor-kappaB binding site prevents myocardial infarction. Nat Med. 1997;3(8):894–9.
20. Saul JM, Annapragada A, Natarajan JV, Bellamkonda RV. Controlled targeting of liposomal doxorubicin via the folate receptor in vitro. J Control Release. 2003;92(1–2):49–67.
21. Pan XQ, Lee RJ. In vivo antitumor activity of folate receptor-targeted liposomal daunorubicin in a murine leukemia model. Anticancer Res. 2005;25(1A):343–6.
22. MacLachlan I, Cullis P, Graham RW. Progress towards a synthetic virus for systemic gene therapy. Curr Opin Mol Ther. 1999;1(2):252–9.
23. Fenske DB, MacLachlan I, Cullis PR. Long-circulating vectors for the systemic delivery of genes. Curr Opin Mol Ther. 2001;3(2):153–8.
24. Wang W, Li W, Ma N, Steinhoff G. Non-viral gene delivery methods. Curr Pharm Biotechnol. 2013;14(1):46–60.
25. Allen TM, Cullis PR. Liposomal drug delivery systems: from concept to clinical applications. Adv Drug Deliv Rev. 2013;65(1):36–48.
26. Huang SL. Liposomes in ultrasonic drug and gene delivery. Adv Drug Deliv Rev. 2008;60(10):1167–76.
27. Li S, Huang L. In vivo gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. Gene Ther. 1997;4(9):891–900.
28. Muller K, Nahde T, Fahr A, Muller R, Brusselbach S. Highly efficient transduction of endothelial cells by targeted artificial virus-like particles. Cancer Gene Ther. 2001;8(2):107–17.
29. Buschmann MD, Merzouki A, Lavertu M, Thibault M, Jean M, Darras V. Chitosans for delivery of nucleic acids. Adv Drug Deliv Rev. 2013;65(9):1234–70.
30. Milla P, Dosio F, Cattel L. PEGylation of proteins and liposomes: a powerful and flexible strategy to improve the drug delivery. Curr Drug Metab. 2012;13(1):105–19.
31. Zwicke GL, Mansoori GA, Jeffery CJ. Utilizing the folate receptor for active targeting of cancer nanotherapeutics. Nano Rev. 2012;3:18496.