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Research paper

Targeting delivery of oligonucleotide and plasmid DNA to hepatocyte via galactosylated chitosan vector

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Abstract

Delivery of oligonucleotide to specific cells and maintenance of its biological function are important for nucleic acid therapy. The objective of this paper is to demonstrate that galactosylated low molecular weight chitosan (gal-LMWC) is a safe and effective vector of antisense oligonucleotide (ASO) and plasmid DNA for the hepatocyte targeting delivery. Gal-LMWC has been successfully prepared and MTT cytotoxic assay shows that cytotoxicity of gal-LMWC is lower than that of high molecular weight chitosan (HMWC) and low molecular weight chitosan (LMWC) in HepG2 cells. Using a complex coacervation process, gal-LMWC can form stable nano-complexes with plasmid DNA or with ASO by the electrostatic interaction. The morphometrics, particle size, and the zeta potential of gal-LMWC/ASO complexes and gal-LMWC/plasmid DNA complexes are very similar. The transfection efficiency by using gal-LMWC vector is significantly higher than that of naked DNA or naked ASO in HepG2 cells. Transfection efficiency of gal-LMWC/ASO complexes and gal-LMWC/plasmid DNA complexes depends on the molar ratio of the positive chitosan amino group and the negative DNA phosphate group (N/P ratio) strongly. Inhibition experiments confirm that the enhanced transfection efficiency is due to the ASGR mediated endocytosis of the gal-LMWC/ASO complexes or gal-LMWC/DNA complexes. These results suggest that gal-LMWC can be used in gene therapy to improve the transfection efficiency in vitro and in vivo.

Keywords: Galactosylated low molecular weight chitosan; Plasmid DNA; Antisense oligonucleotide; Hepatocyte targeting; Transfection efficiency; Non-viral vector

1. Introduction

Antisense oligonucleotides are a promising therapeutic strategy that has been successfully applied in oncology, inflammatory and viral infective diseases. The possible mechanisms by which antisense molecules result in decreased protein expression include the modulation of protein translation by disrupting ribosome assembly, RNase H mediated cleavage of targeted mRNA, and pre-modification of splicing [1]. However, because of the poor stability in biological medium and the weak intracellular penetration of antisense oligonucleotides, the major problem of antisense therapy lies on the development of safe and efficient systems for the delivery of oligonucleotides. The two main types of vectors that are used in oligonucleotide delivery are based on viral or non-viral gene delivery systems. The viral gene delivery system shows a high transfection yield but it has many disadvantages, such as oncogenic effects and immunogenicity [2,3]. Considering the properties of safety and ease of manufacturing, non-viral gene transfer vectors based on nucleic acid complexes with polycations have recently been used for gene delivery applications [4–8].

Among the large number of cationic polymers described, chitosan is shown to be an effective vector that is able to condense and deliver DNA in vitro and in vivo [9]. Chitosan is a naturally occurring linear polysaccharide consisting of β-(1,4) linked monomers of D-glucosamine and *N*-acetyl-D-glucosamine. It is a positively charged biodegradable and biocompatible polymer. There are numerous reports highlighting the low toxicity and biocompatibility of chitosan

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[10–14]. Chitosan has been extensively examined in the pharmaceutical industry for its potential in the development of controlled release drug delivery system due to its unique polymeric cationic character and its gel and film forming properties [15,16].

Chitosan is a good candidate for gene delivery system because it is able to spontaneously form interpolyelectrolyte complexes with DNA as a result of cooperative electrostatic interactions between the positive amino groups of chitosan and the negative phosphate groups of DNA [17,18]. Chitosan can effectively bind DNA and protect it from nuclease degradation [19,20]. High efficiency of transfection has been observed in the case of chitosan/DNA complexes both in vitro and in vivo and chitosan has been one of the candidates for practical gene vector. The molecular weight of chitosan and the amino content of chitosan, i.e. the charge of the polymer, are two important factors that influence particle size, stability and transfection efficiency of chitosan/DNA complexes [8,9,21,22].

Hepatocyte possesses asialoglycoprotein receptors (ASGR) that binds and internalizes galactose-terminal (asialo) glycoproteins [23]. Recently, Kim et al. described galactosylated chitosan/DNA nanoparticles as a gene carrier which gave a higher transfection efficiency into ASGR beared cells HepG2 than that into Hela without ASGR [24]. We also coupled lactobionic acid (LA) bearing galactose group to LMWC to improve hepatocytes specificity of plasmid DNA in our previous works [25].

Though chitosan has been evaluated as a carrier for plasmid DNA into various cell types and experimental animals, there was little report on its use for the delivery of ASO. ASO are typically 10–25 nucleotides long and the difference of length and other properties between plasmid DNA and ASO make their quite different requirements on the vectors in transfection. Unfortunately, most important cationic polymers being studied now are too large, and their surface charge densities are too high for ASO delivery. Their complexation with ASO is a difficult problem because of the large difference between the sizes of the two macromolecules. Due to their high surface charge densities, the complexes of cationic polymers with ASO are so stable that they are taken up by the cells without release the ASO, which therefore are not able to show antisense activity [26]. However, gal-LMWC has low molecular weight and low surface charge densities and it is probably feasible for the specific delivery of ASO in vitro and in vivo.

In this study, we report that gal-LMWC can be used not only as the vector of plasmid DNA, but also as the vector of ASO. Gal-LMWC can form stable nano-complexes with plasmid DNA or with antisense oligonucleotide by the electrostatic interaction. The morphometrics, the particle size, and the zeta potential of gal-LMWC/ASO complexes and gal-LMWC/plasmid DNA complexes are very similar. When gal-LMWC is used as vector transfection efficiency is significantly higher than that of naked plasmid DNA or naked ASO in HepG2 cells. Transfection efficiency of gal-LMWC/ASO complexes or gal-LMWC/plasmid DNA complexes largely depends on the N/P ratio. The results of inhibition experiments further confirmed that the enhanced transfection efficiency was due to the ASGR mediated endocytosis of the gal-LMWC/ASO complexes or gal-LMWC/plasmid DNA complexes. The study suggests that gal-LMWC is an effective and safe vector of ASO and plasmid DNA for hepatocyte targeting.

2. Materials and methods

2.1. Materials

Chitosan (minimum 85% deacetylated), 3-(4,5-dimethylthiazd-2-yl)-2,5-diphenyltentrazolium bromide (MTT), *O*-nitrophenyl-β-D-galactopyranoside (ONPG), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), lactobionic acid (LA) and β-galactosidase were obtained from Sigma Chemical Company (St Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was obtained from Dojindo (Japan). The pSV-β-galactosidase control vector containing SV40 early promoter, enhancer and *LacZ* gene is supplied by Promega Corporation (Madison, WI).

The phosphorothioate-modified ASO used in this study, TJU-2755, was synthesized by Genset (La Jolla, CA). For cell treatment, the ASO was sterilized by filtration through 0.22 μm filter and stored at −70 °C prior to use. The sequence of TJU-2755 is: 5′-TGATCCACTCCCCCCTC CACT-3′. It was designed to target the 3′-UTR of the primary RNA transcript of TNF-α and was found to strongly inhibit the TNF-α production [27]. The sequence of the sense strain is 5′-AGTGGAGGGGGGAGTGGATCA-3′. The sense strain was 5′ end labeled with [^{32}P]-γ-ATP using T4 polynucleotide kinase (Promega).

Human hepatocellular carcinoma cell line (HepG2) was obtained from American Type Culture Collection (ATCC) and was grown in RPMI Medium1640 containing 10% born bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. Preparation and characterization of gal-LMWC

HMWC (minimum 85% deacetylated) was chemically treated with sodium nitrite in 0.1 mol/l acetic acid at 25 °C for 10 h to produce LMWC according to a method described by Peniston et al. [28]. LMWC was coupled with LA via an active ester intermediate using EDC. LMWC of about 100 mg was dissolved in 2 ml of 10 mM *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED)/HCl buffer solution (pH 4.7). EDC of about 180 mg was added to this solution and stirred at 25 °C for 24 h. Then, different amounts of LA were added into tubes, respectively, and stirred for another 72 h at 25 °C. The resulting gal-LMWCs were dialyzed for 4 days against Milli Q water and lyophilized.

The molecular weights of HMWC, LMWC and gal-LMWC were determined by HPLC (TL-9800, Shimadzu). The mobile phase was Milli Q water with flow-rate of 0.5 ml/min. PEGs of different molecular weight were used as molecular weight standards. Amino group content of each sample was determined by metachromatic titration using Xylidine Tonceau 2R (C.I. Acid Red 26) according to the modified method of Roberts et al. [29].

2.3. Cytotoxicity assay of gal-LMWC

Cytotoxicity of gal-LMWC was measured using the MTT dye reduction assay and compared with other regents in HepG2 cells. Cells were seeded in a 96-well plate at a density of 2.0×10^4 cells/well and incubated overnight. Then the cells were incubated in 100 μl serum free medium containing selected amount of Lipofectin, calcium phosphate (CaP), HMWC, LMWC and gal-LMWC. After 16 h, the medium was removed and the cells were rinsed twice with 1× PBS. The wells were refilled with complete medium and cells were cultured for another 24 h. Next, 10 μl of MTT (5 mg/ml) solution was added into each well and was allowed to react for 4 h at 37 °C. The solution was removed and 150 μl of dimethylsulfoxide (DMSO) was added to each well. Then the plate was incubated for 30 min at room temperature. Absorption at 490 nm was measured with an ELISA plate reader (Bio-RAD, Microplate Reader 3550).

2.4. Preparation and characterization of gal-LMWC/DNA complexes

Gal-LMWC was dissolved in 0.5% acetic acid with a gentle stirring; pH of the solution was adjusted to 7.0 with 0.1 mol/l NaOH solution. The final concentration was 1 mg/ml. The solution was sterile filtered through a 0.22 μm filter and diluted to 0.2 mg/ml by RPMI 1640.

The required volume of 0.2 mg/ml gal-LMWC solution was added to 50 μl of 0.2 mg/ml ASO solution (diluted from 1 mg/ml by RPMI Medium1640) to form complexes of a selected N/P ratio by gentle pipetting. The mixture was vortexed rapidly for 3–5 s and left for 1 h in room temperature for the gal-LMWC/ASO complexes to completely form. For the atomic force microscope (AFM) measurements, 20 μl of the complexes was deposited on to silicon, and remained in a place for 90 s to make the silicon surface dry. The images were observed by tapping mode in air using SPI3800 (Seiko Instruments, Inc., Japan) AFM microscope. The diameters and the zeta potential of the complexes were determined by light scattering by using 90Plus Particle Sizer (Brookhaven Instruments, Holtsville, NY). The preparation and characterization of gal-LMWC/plasmid DNA complexes are similar to those of gal-LMWC/ASO complexes.

2.5. Transfection with complexes in vitro

In a typical transfection experiment, HepG2 cells were seeded at the density of 5.0×10^5 cells/dish in 60 mm culture dishes with 5 ml of complete medium (RPMI 1640 containing 10% serum) and incubated for 24 h prior to transfection. Transfections were performed on cells that were approximately 70% confluence. Before transfection, the complete medium was removed and cells were rinsed once with 1× PBS. The naked ASO and gal-LMWC/ASO complexes (containing 20 μg of ASO) were diluted in 2 ml RPMI 1640 medium, and then were used to refill the dishes. After incubated at 37 °C for 6 h, added serum and RPMI 1640 medium to the dishes so that the final volume of medium was 5 ml and containing 10% serum. After another 18 h, the medium containing complexes was removed. The cells were rinsed twice with 1× PBS, harvested, and resuspended in 1× PBS. The transfection of naked plasmid DNA and gal-LMWC/plasmid DNA complexes were carried out according to the same protocol as mentioned above.

In order to investigate the inhibition of cell uptake of ASO, different amount of potential competitor (LA or LMWC) in 2 ml RPMI 1640 medium were pre-incubated with HepG2 cells for 60 min at 37 °C. After pretreatment, gal-LMWC/ASO complexes (containing 20 μg of ASO, N/P = 3) were added into the dishes. The latter procedures were same as the transfection experiment. The inhibition of gal-LMWC to the transfection efficiency of gal-LMWC/plasmid DNA complexes was measured by the same method.

2.6. Determination of transfection efficiency

To determine the transfection efficiency, the β-galactosidase activity was measured through ONPG assay with UV spectrophotometer (Shimadzu UV-2201) [30].

For histochemical staining [31], cells were washed three times with ice-cold Dulbecco's phosphate buffered saline (D-PBS) and fixed for 5 min in fixation solution (2% formaldehyde, 0.05% glutaraldehyde in D-PBS) at 4 °C. After removal of fixation solution, cells were washed three times with D-PBS and then stained with X-gal solution (5 mM $K_3Fe_3(CN)_6$, 5 mM $K_4Fe_2(CN)_6$, 2 mM $MgCl_2$ and 1 mg/ml X-gal in 1×PBS (pH 8.0)) at 37 °C overnight. Then the cells were washed three times with D-PBS. In order to prevent expression of endogenous mammalian β-galactosidase, histochemical staining was carried out at pH 8.0. Under these conditions, only bacterial β-galactosidase was shown to be active. Blue cells were counted per each well by light microscopy.

2.7. Quantification of the ASO in cells

The ASO was extracted from transfected cells and the extracted ASO was separated by electrophoresis in 20%

polyacrylamide denaturing gel containing 7 M urea. The separated ASO in gel were transferred to Hybond nylon membrane (Amersham Pharmacia Biotech) and then cross-linked by UV irradiation. The membrane was prehybridized in tube for 2 h at 40 °C in prehybridization buffer and hybridized for 4 h at 40 °C in fresh hybridization buffer containing ^{32}P-labeled sense oligonucleotides. Following hybridization, the membrane was sequentially washed in 5× standard saline citrate (SSC) buffer, and 5× SSC/0.05% SDS buffer for 20 min at 40 °C, respectively. The membrane was exposed to phosphor screen overnight at room temperature. The autradiogram were scanned and the densities associated with bands were compared using the Packard Cyclone™ Storage Phosphor System (Parkard Instrument Company, Inc., USA). In each hybridization another membrane containing different amount of the standard ASO was also included. The amount of the unknown was extrapolated from the standard curve.

3. Results and discussion

3.1. Characterization of chitosan

Table 1 summarizes the molecular weight results from the HPLC measurements, and the $-NH_2$ group content results from the metachromatic titration experiment for HMWC, LMWC and gal-LMWC samples. The molecular weight of gal-HMWC is 21 kDa, the $-NH_2$ group content is 52% and the galactosylation degree is 8.1%.

3.2. Cytotoxicity study

MTT assay was performed to determine the cytotoxicity of the pure transfection reagents, including: CaP, lipofectin, HMWC, LMWC, and gal-LMWC in HepG2 cells and the results are shown in Fig. 1. CaP is the most cytotoxic and cells almost die when CaP concentration reaches 20 μg/ml. The cytotoxicity of lipofectin increases with a concentration dependent manner and cells almost die at concentration of 250 μg/ml. However, HMWC, LMWC and gal-LMWC are found to be less cytotoxic. Cells grow well even at the concentration as high as 400 μg/ml. Cells almost die when the concentration of HMWC reaches 600 μg/ml. Both LMWC and gal-LMWC are found to improve viability of HepG2 cells at low concentrations. More then 60% cells die when the concentration of LMWC reaches 1400 μg/ml.

Table 1
Physicochemical characteristics of HMWC, LMWC and gal-LMWC

Types	Molecular weight (kDa)	Experimental NH_2 (%)
HMWC	145	71
LMWC	21	58
Gal-LMWC	25	50

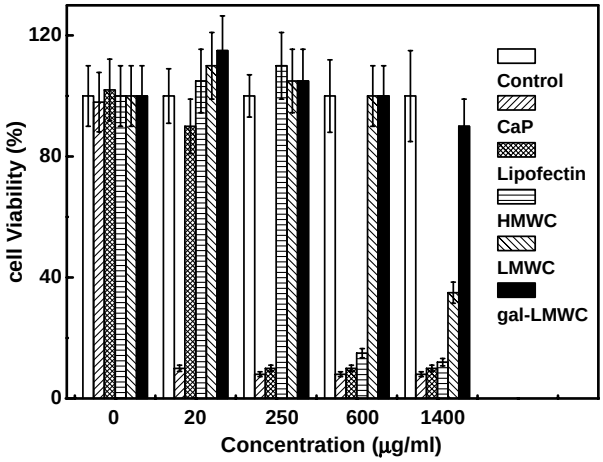

Fig. 1. MTT assay for cytotoxicity of CaP, Lipofectin, HMWC, LMWC, and gal-LMWC in HepG2 cell line.

On the contrary, gal-LMWC does not show any cytotoxicity at the same concentration. These results indicate that modification might alleviate the cytotoxicity of HMWC, probably because of the decrease of the positive charge density. The results demonstrate clearly that gal-LMWC is non-cytotoxic, biocompatible, and safe gene vector.

3.3. Characterization of complexes

Preliminary studies were performed to identify the experimental conditions for the formation of gal-LMWC/plasmid DNA complexes or gal-LMWC/ASO complexes. Results show that gal-LMWC can condense both ASO and plasmid DNA effectively and form stable complexes.

The particle images of the gal-LMWC/ASO complexes (N/P = 3) and the gal-LMWC/plasmid DNA complexes (N/P = 6) were examined by AFM as shown in Fig. 2. From the AFM observation, the gal-LMWC/ASO complexes (N/P = 3) are found to be near spherical of 80 nm in diameter with homogeneous structure and smooth surface (Fig. 2b). The morphometrics of gal-LMWC/ plasmid DNA complexes (N/P = 6) measured by AFM are very similar to that of gal-LMWC/ASO complexes (Fig. 2a).

Effective diameters of gal-LMWC/plasmid DNA complexes and gal-LMWC/ASO complexes were determined by laser light scattering (LLS). When the N/P ratio of gal-LMWC and plasmid DNA increases from 1 to 6, the effective diameter of complexes decreases from 384.2 to 288.2 nm, and the zeta potential increases from −15.2 to 12.2 mV. In the case of gal-LMWC/ASO complexes, the changes in particle size and zeta potential follow the same trend as that of gal-LMWC/plasmid DNA complexes. When the N/P ratio of gal-LMWC and ASO increases from 1 to 6, the zeta potential increases from −7.6 to 13.7 mV, and the effective diameter of complexes decreases from 325.8 to 253.8 nm, slightly smaller than that of gal-LMWC/plasmid DNA complexes. This can be associated with the fact that

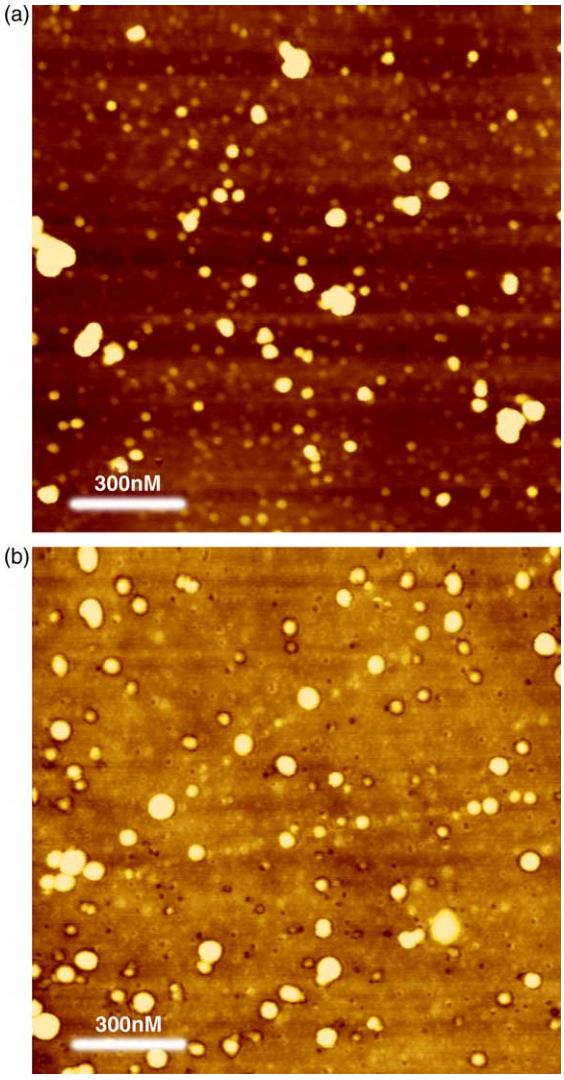

Fig. 2. Atomic force microscopic images of gal-LMWC/plasmid DNA complexes (N/P=6) (a) and gal-LMWC/ASO complexes (N/P=3) (b). The panels shown are typical of the results of multiple evaluations.

the interaction between gal-LMWC and DNA is electrostatic. ASO and plasmid DNA have the same charge density, but segment length of ASO is great shorter than that of plasmid DNA, so it is able to contact with gal-LMWC more easily and form complexes more compactly.

The particle size obtained from the AFM is smaller than that measured by laser light scattering, and this might be due to the fact that AFM measurements were performed in a dried state, while the light scattering measurements were conducted in aqueous solution and the particles were hydrated.

3.4. Transfection with complexes in vitro

To verify the ability of gal-LMWC to increase transfection efficiency, HepG2 cells were transfected with the naked plasmid DNA and gal-LMWC/plasmid DNA complexes at different N/P ratios, respectively. Cells were stained with X-gal solution for presence of active β-galactosidase expression 48 h after transfection. When cells are transfected with naked plasmid DNA, a complete absence of β-galactosidase enzymatic activity is observed (Fig. 3b). However, the staining cells are observed in HepG2 cells transfected with gal-LMWC/plasmid DNA complexes, and the number of blue cells increases with the N/P ratio of the complexes. The frequency of active β-galactosidase expression is over 20% in the total cells transfected with gal-LMWC/plasmid DNA complexes at N/P=6 as shown in Fig. 3a.

To determine the optimal N/P ratio for transfection, HepG2 cells were transfected, respectively, with the complexes at different N/P ratios and the results are shown in Fig. 4. Using the gal-LMWC/plasmid DNA complexes, it can be found that the β-galactosidase activity increases as the N/P ratio goes up from 1 to 6. The maximal activity of gene expression occurs at N/P ratio of 6. When the N/P ratio extends to 7, a slight decrease is observed (Fig. 4a). Similar to gal-LMWC/plasmid DNA complexes, the transfection efficiency of gal-LMWC/ASO complexes is dependent on the N/P ratio. The most effective transfection is obtained at the N/P ratio of 3 (Fig. 4b). The different optimum N/P ratios in transfection of the two kinds of complexes may also be associated with the fact that the segment length of ASO is much smaller than that of plasmid DNA, and thus it can contact with gal-LMWC completely and form complexes compactly at a lower N/P ratio.

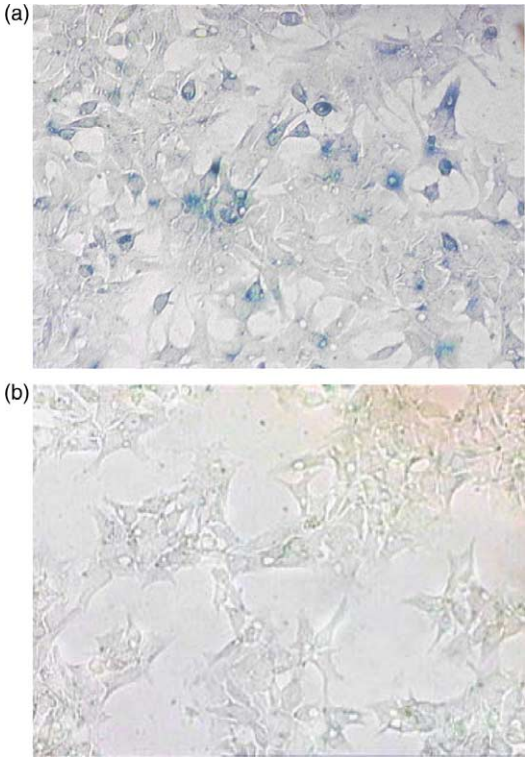

Fig. 3. The histochemical staining of transfection by gal-LMWC/plasmid DNA complexes (N/P=6) (a) and naked plasmid DNA (b) in HepG2 cell line.

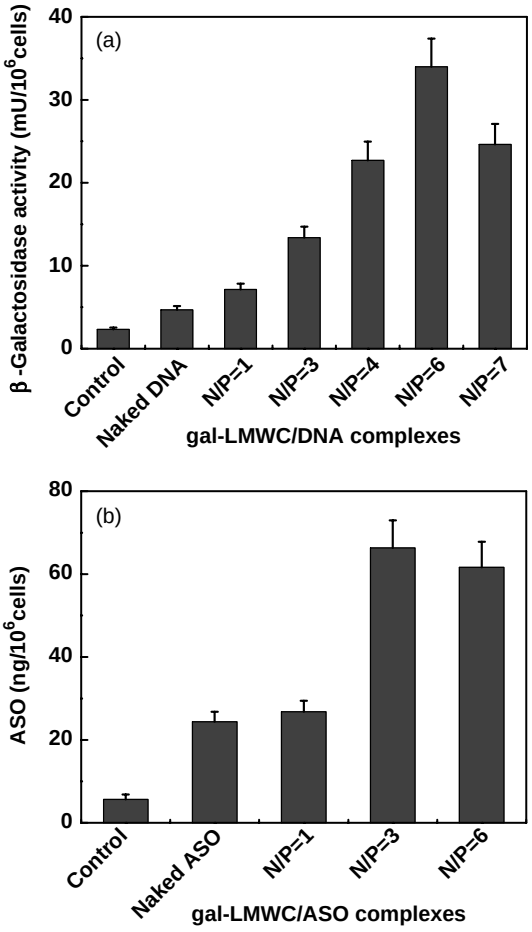

Fig. 4. N/P ratio-dependent transfection efficiency of gal-LMWC/plasmid DNA complexes (a) and gal-LMWC/ASO complexes (b) in HepG2 cell line. Results are expressed as mean values ± SD ($n = 4$) from one representative experiment of three performed.

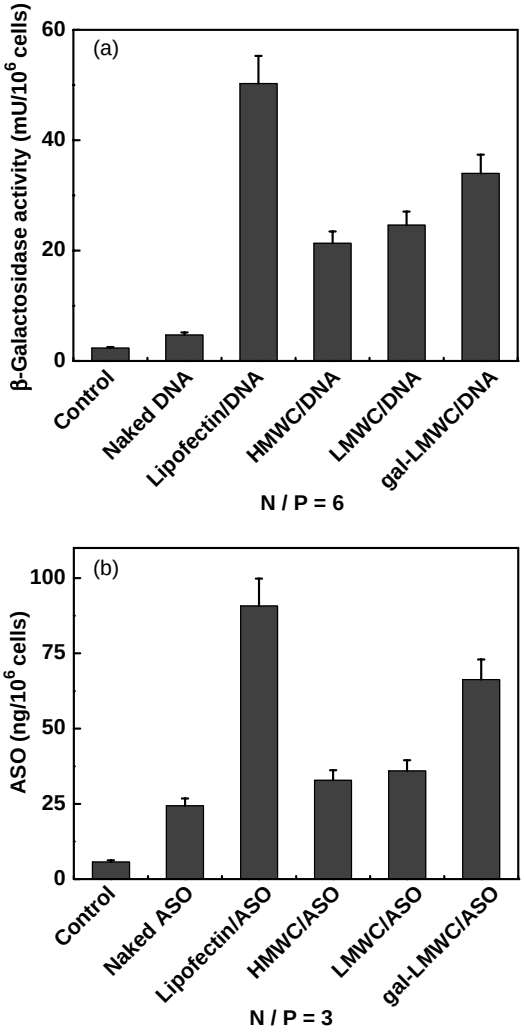

Fig. 5. Comparison of transfection efficiency of the synthetic vectors with DNA (a) and ASO (b) in HepG2 cell line. Results are expressed as mean values ± SD ($n = 4$) from one representative experiment of three performed.

Next, naked DNA, lipofectin/DNA complexes, HMWC/DNA complexes (N/P = 6), LMWC/DNA complexes (N/P = 6) and gal-LMWC/DNA complexes (N/P = 6) were used to transfect HepG2 cells. Fig. 5a shows the transfection efficiency of the five complexes. It can be observed that active β-galactosidase expression of gal-LMWC/DNA complexes is higher than those of naked DNA, HMWC/DNA, and LMWC/DNA complexes and only lower than that of lipofectin/DNA complexes. The similar results are observed in the experiments of ASO. The transfection efficiency of gal-LMWC/ASO complexes with N/P = 3 is a little lower than that of lipofectin/ASO complexes, and greatly higher than those of naked ASO, HMWC/ASO complexes, and LMWC/ASO complexes in HepG2 cells (Fig. 5b). These results suggest that the gal-LMWC have the capability to increase the transfection efficiency of plasmid DNA and ASO in the HepG2 cells that have ASGR on their membrane.

We did not find any cytotoxicity of chitosan/DNA and chitosan/ASO complexes at any ratio in the transfection experiments mentioned above. These results give another evidence to verify that gal-LMWC is non-cytotoxic, biocompatible, and safe gene vector. The viability of the cells, which were transfected by lipofectin/DNA complexes, decreased a little in the transfection experiments and data are not shown here.

3.5. Inhibition to transfection in vitro

To confirm that the enhanced transfection efficiency of the gal-LMWC/plasmid DNA complexes is due to the ASGR mediated endocytosis process; we use gal-LMWC with different concentration as competitors in transfection experiments. Fig. 6 shows the relationship between the transfection efficiency of gal-LMWC/plasmid DNA complexes (N/P = 6) and the gal-LMWC inhibition concentration. The transfection efficiency decreases when the concentration of gal-LMWC increases. β-Galactosidase activity is inhibited 50% at gal-LMWC 10-fold (molar

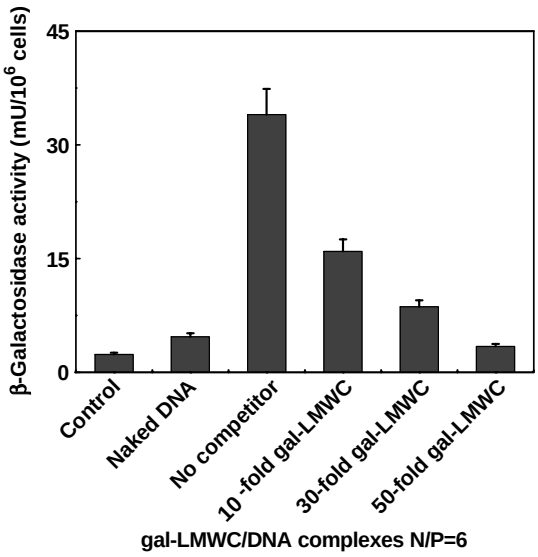

Fig. 6. Inhibition of transfection efficiency of gal-LMWC/plasmid DNA complexes by gal-LMWC in HepG2 cell line. Results are expressed as mean values ± SD ($n = 4$) from one representative experiment of three performed.

ratio) excess, and the activity is lower than that of naked plasmid DNA transfection at gal-LMWC 50-fold excess.

We also carried out control experiments using LMWC as potential competitor to determine whether specific binding sites were involved in the uptake of gal-LMWC/ASO complexes. As shown in Fig. 7, uptake of gal-LMWC/ASO complexes (N/P = 3) is inhibited by LMWC in a dose-dependent manner and LMWC can completely inhibit the uptake of gal-LMWC/ASO complex at 50-fold molar excess. These results suggest that the uptake of gal-LMWC/ASO complexes involve specific cell surface binding sites, which are subjected to competition. The specific cell surface binding site may be ASGR. ASGR can bind and internalizes galactose-terminal (asialo) glycoproteins in high assinity. However, the ASGR can also recognize, bind and internalize glycoproteins having glucose residues or glucose derivatives, on their oligosaccharide chains with low affinity [32]. Skalko et al. used a *N*-acetyl-D-glucosamine derivative of BSA conjugated to a pH-sensitive liposome with FITC-dextran as a mimic of ASO. The complexes were efficiently taken up by an avian hepatoma cell line expressing ASGR when compared to liposomes with no targeting ligand [33]. LMWC is composed of two subunits: D-glucosamine and *N*-acetyl-D-glucosamine, which can be recognized and bound by ASGR. These facts give us some clues that why excess LMWC can inhibit the cell uptake of gal-LMWC/ASO complexes.

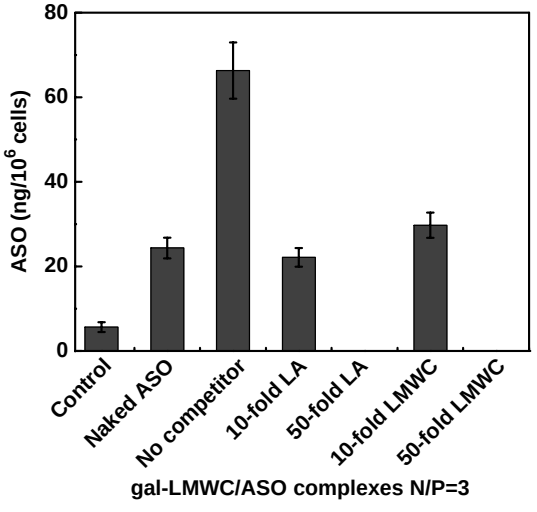

Fig. 7. Inhibition of transfection efficiency of gal-LMWC/ASO complexes by LA and LMWC in HepG2 cell line. Results are expressed as mean values ± SD ($n = 4$) from one representative experiment of three performed.

We also investigated the inhibition effect of excess LA on cellular uptake of gal-LMWC/ASO complexes and the results are also shown in Fig. 7. It can be observed clearly that uptake of gal-LMWC/ASO complexes (N/P = 3) is also inhibited by LA in a dose-dependent manner. LA can completely inhibit the uptake of gal-LMWC/ASO complexes at 50-fold excess. However, LA inhibits about 70% hepatic uptake at 10-fold excess, whereas LMWC can only inhibit 50% uptake at the same excess amount. The results demonstrate that the capability of hepatic targeting of gal-LMWC/ASO complexes is coming from the galactosylation of chitosan by coupling with LA. Gal-LMWC vectors transfect ASO into hepatocyte selectively via the ASGR mediated endocytosis pathway.

4. Conclusion

This work reports that gal-LMWC can be used as vector of plasmid DNA and ASO for hepatocyte targeting in gene therapy. MTT assay shows that gal-LMWC is low cytotoxicity. Gal-LMWC can form stable nano-complexes with ASO or plasmid DNA by a complex coacervation process. Both of the two complexes are nearly spherical of 200–300 nm in diameter with homogeneous structure and smooth surfaces. As a vector, gal-LMWC shows significantly higher transfection efficiency than that of naked DNA or naked ASO in HepG2 cells. Transfection efficiency depends on the N/P ratio strongly. The results of inhibition experiments confirm that the enhanced transfection efficiency is due to the ASGR mediated endocytosis of gal-LMWC/ASO complexes or gal-LMWC/plasmid DNA complexes. Our studies suggest that gal-LMWC is an effective and safe vector of ASO and plasmid DNA for the hepatocyte targeting delivery in vitro and can be used in gene therapy to improve the transfection efficiency. The in vivo delivery investigating will be carried out in further studies.

Acknowledgements

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References

[1] I. Tamm, B. Dorken, G. Hartmann, Antisense therapy in oncology: new hope for an old idea? Lancet 358 (2001) 489–497.
[2] N. Somia, I.M. Verma, Gene therapy: trials and tribulations, Nat. Rev. Genet. 1 (2000) 91–99.
[3] D. Ferber, Gene therapy: safer and virus-free? Science 294 (2001) 1638–1642.
[4] K. Lappalainen, A. Urtti, E. Söderling, I. Jääskeläinen, K. Syrjänen, S. Syrjänen, Cationic liposomes improve stability and intracellular delivery of ASOs into CaSki cells, Biochim. Biophys. Acta 1196 (1994) 201–208.
[5] O. Zelphati, F.C. Szoka Jr., Intracellular distribution and mechanism of delivery of oligonucleotides mediated by cationic lipids, Pharm. Res. 13 (1996) 1367–1372.
[6] M.G. Ferreiro, L.G. Tillman, G. Hardee, R. Bodmeier, Alginate/poly-L-lysine microparticles for the intestinal delivery of ASOs, Pharm. Res. 19 (2002) 755–764.
[7] Y. Nakada, E. Fattal, M. Foulquier, P. Couvreur, Pharmacokinetics and biodistribution of oligonucleotide adsorbed onto poly (isobutylcyanoacrylate) nanoparticles after intravenous administration in mice, Pharm. Res. 13 (1996) 38–43.
[8] P. Erbacher, S. Zou, T. Bettinger, A.-M. Steffan, J.-S. Remy, Chitosan-based vector/DNA complexes for gene delivery: biophysical characteristics and transfection ability, Pharm. Res. 15 (1998) 1332–1339.
[9] M. Köping-Höggård, I. Tubulekas, H. Guan, K. Edwards, M. Nilsson, K.M. Vårum, P. Artursson, Chitosan as a nonviral gene delivery system. Structure–property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo, Gene Ther. 8 (2002) 1108–1121.
[10] S. Hirano, H. Seino, Y. Akiyama, I. Nonaka, Biocompatibility of chitosan by oral and intravenous administration, Polym. Eng. Sci. 59 (1989) 897–901.
[11] J. Knapczyk, L. Krowczynski, J. Krzck, M. Brzeski, E. Nimberg, E. Schenk, H. Struszcyk, Requirements of chitosan for pharmaceutical and biomedical applications, in: G. Skak-Braek, T. Anthonsen, P. Sandford (Eds.), Chitin and Chitosan: Sources, Chemistry, Biochemistry, Physical Properties and Applications, Elsevier, London, 1989, pp. 657–663.
[12] S. Hirano, H. Seino, I. 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, 1990, pp. 283–289.
[13] P.C. Bersch, B. Nies, A. Liebendorfer, Evaluation of the biological properties of different wound dressing materials, J. Mater. Sci. Med. 6 (1995) 231–240.
[14] M.L. Weiner, An overview of the regulatory status and of the safety of chitin and chitosan as food and pharmaceutical ingredients, in: C.J. Brine, P.A. Sandford, J.P. Zikakis (Eds.), Advances in Chitin and Chitosan, Se Teata de un Libro de Proceedings from the Fifth International Conference on Chitin and Chitosan Held in Princeton (1991), pp. 663–672.
[15] L. Illum, Chitosan and its use as a pharmaceutical excipient, Pharm. Res. 15 (1998) 1326–1331.
[16] V. Dodane, V.D. Vilivalam, Pharmaceutical applications of chitosan, Pharm. Sci. Technol. Today 1 (1998) 246–253.
[17] F.C. MacLaughlin, R.J. Mumper, J. Wang, J.M. Tagliaferri, I. Gill, M. Hinchcliffe, A.P. Rolland, Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery, J. Controlled Release 56 (1998) 259–272.
[18] S.C.W. Richardson, H.V.J. Kolbe, R. Duncan, Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA, Int. J. Pharm. 178 (1999) 231–243.
[19] Z. Cui, R.J. Mumper, Chitosan-based nanoparticles for topical genetic immunization, J. Controlled Release 75 (2001) 409–419.
[20] L. Illum, I. Jabbal-Gill, M. Hinchcliffe, A.N. Fisher, S.S. Davis, Chitosan as a novel nasal delivery system for vaccines, Adv. Drug Deliv. Rev. 51 (2001) 81–96.
[21] H.Q. Mao, K. Roy, K.A. Janes, V.L. Truong-Le, K.Y. Lin, J.T. August, K.W. Leong, Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficacy, J. Controlled Release 70 (2001) 399–421.
[22] K. Roy, H.Q. Mao, S.-K. Huang, K.W. Leong, Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy, Nat. Med. 5 (1999) 387–391.
[23] D.A. Wall, G. Wilson, A.L. Hubbard, The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes, Cell 21 (1980) 79–93.
[24] S.Y. Gao, J.N. Chen, X.R. Xu, Z. Ding, Y.-H. Yang, Z.C. Hua, J.F. Zhang, Galactosylated low molecular weight chitosan as DNA carrier for hepatocyte-tarrgeting, Int. J. Pharm. 255 (2003) 57–68.
[25] T.H. Kim, I.K. Park, J.W. Nah, Y.J. Choi, C.S. Cho, Galactosylated chitosan/DNA nanoparticles prepared using water-soluble chitosan as a gene carrier, Biomaterials 25 (2004) 3783–3792.
[26] T.V. Chirila, P.E. Rakoczy, K.L. Garrett, X. Lou, I.J. Constable, The use of synthetic polymers for delivery of therapeutic antisense oligodeoxynucleotides, Biomaterials 23 (2002) 321–342.
[27] G.-C. Tu, Q.-N. Cao, F. Zhou, Y. Israel, Tetranucleotide GGGA motif in primary RNA transcripts, novel target site for antisense design, J. Biol. Chem. 273 (9) (1997) 25125–25131.
[28] Q.P. Peniston, E.L. Johnson, Process for depolymerization of chitosan, U.S. Patent 3,922,260 (1975).
[29] B.D. Gummow, G.A.F. Roberts, Studies on chitosan-induced metachromasy. 1. Metachromatic behavior of sodium 2′-hydroxy-1, 1′-azonaphthalene-4-sulfonate in the presence of chitosan, Makromol. Chem. 186 (1985) 1239–1244.
[30] C.V. Hall, P.E. Jacob, G. Ringold, F. Lee, Expression and regulation of *Escherichia coil lacZ* gene fusions in mammalian cell, J. Mol. Appl. Genet. 2 (1983) 101–108.
[31] T. Sato, N. Shirakawa, H. Nishi, Y. Okahata, Formation of a DNA/polygalactosamine complex and its interaction with cells, Chem. Lett. 297 (1996) 725–726.
[32] A.L. Hubbard, G. Wilson, G. Ashwell, H. Stukenbrok, An electron microscope autoradiographic study of the carbohydrate recognition system in rat liver. 1. Distribution of ^{125}I-ligands among the liver cell types, J. Cell Biol. 83 (1979) 47–64.
[33] N. Skalko, R. Peschka, U. Altenschmidt, A. Lung, R. Schubert, pH-sensitive liposomes for receptor-mediated delivery to chicken hepatoma (LMH) cells, FEBS Lett. 434 (3) (1998) 351–356.