Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 255 (2003) 57–68
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Galactosylated low molecular weight chitosan as DNA carrier for hepatocyte-targeting

Shuying Gao, Jiangning Chen, Xuerong Xu, Zhi Ding, Yong-Hua Yang, Zichun Hua, Junfeng Zhang*

State Key Laboratory of Pharmaceutical Biotechnology, Department of Biochemistry, Nanjing University, Nanjing 210093, PR China

Received 18 July 2002; received in revised form 24 December 2002; accepted 13 January 2003

Abstract

Chitosan has the potential for DNA complexation and is useful as a non-viral vector for gene delivery. Highly purified low molecular weight chitosan (LMWC) was prepared. Lactobionic acid (LA) bearing galactose group was coupled with LMWC for liver-specificity. A series of galactosylated-LMWC (gal-LMWC) samples covering a range of galactose group contents were prepared. The chitosan/DNA complexes were obtained using a complex coacervation process. Gal-LMWCs were used to transfer pSV-β-galactosidase reporter gene into human hepatocellular carcinoma cell (HepG2), L-02, SMMC-7721, and human cervix adenocarcinoma cell line (HeLa) cell lines in vitro. Transfection efficiency of gal-LMWCs was evaluated by β-galactosidase assay and compared with those of lipofectin, calcium phosphate (CaP), high molecular weigh chitosan (HMWC) and LMWC. Gal-LMWC/DNA complex shows a very efficient cell selective transfection to hepatocyte. The transfection efficiency of gal-LMWCs increased with the improvement of the galactosylation degree. Cytotoxicity of gal-LMWC was determined by 3-(4,5-dimethylthiazd-2-yl)-2,5-diphenyltentrazolium bromide (MTT) assay and the results show that the modified chitosan has relatively low cytotoxicity, giving the evidence that the modified chitosan vector has the potential to be used as a safe gene-delivery system.

Keywords: Chitosan; Non-viral gene delivery; Galactosylated chitosan; Liver-specificity

1. Introduction

Gene therapy has gained a significant interest due to its ability to treat human diseases by correcting genetic deficiency of key metabolic enzyme. Although gene therapy, as a treatment for disease, holds great promise, progress in developing effective clinical protocols has been quite slow (Hackett and Crystal, 2000). The problem lies in the development of safe and efficient gene-delivery systems. Viral systems, including vectors based on RNA viruses and DNA viruses are potentially very efficient, but two factors suggest that non-viral gene-delivery systems will be the preferred choice in the future: safety, and ease of manufacturing. A totally synthetic gene-delivery system could be engineered to avoid the danger of producing recombinant virus or other toxic and immunogenic effects induced by biologically active viral particles. Furthermore, manufacturing a synthetic product should be less difficult than using tissue

* Corresponding author. Tel.: +86-25-3594220; fax: +86-25-3324605.
E-mail address: jfzhang@nju.edu.cn (J. Zhang).

0378-5173/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0378-5173(03)00082-6

culture cells as bioreactors, and QA/QC procedures should be simplified (Anderson, 1998).

Since the studies with poly-L-lysine in the late 1980s (Wu and Wu, 1987), many cationic polymers have been explored as non-viral vectors. These include polyethylenimine (PEI) (Boussif et al., 1995; Remy et al., 1998), polybrene (Mumper et al., 1996), poly(amidoamine) (PAMAM) dendrimers (Tang et al., 1996). However, the transfection efficiency of these polymers has been relatively poor till now and many of cationic polymers are relatively toxic.

Chitosan is a naturally occurring polysaccharide produced by deacetylation of chitin. Given the numerous studies reporting the low toxicity and biocompatibility of chitosan (Arai et al., 1968; Hirano et al., 1989; Knapczyk et al., 1989; Hirano et al., 1990; Bersch et al., 1995; Weiner, 1991), recent interest has been focused on this natural polycation as a gene therapy vector. As has been shown with several other polycations, the interaction between chitosan and DNA is electrostatic (Mao et al., 2001). This charge interaction appears to be so strong that the chitosan/DNA complex does not dissociate until it has entered the cell (Roy et al., 1999a,b; Ishii et al., 2001). The molecular weight of chitosan is an important factor which influences particle size, chitosan/DNA complex stability and transfection efficiency of the complex (Sato et al., 2001; MacLaughlin et al., 1998). High efficiency of transfection was observed in the case of chitosan/DNA complex containing 40 kDa chitosan (MacLaughlin et al., 1998).

Hepatocyte possesses asialoglycoprotein receptors (ASGR) that binds and internalizes galactose-terminal (asialo) glycoproteins (Wall et al., 1980). Wu et al. reported that poly-L-lysine, coupled with asialoorsomucoid (ASOR) and specifically bound to cell receptors, improved cell specificity of gene drug (Wu and Wu, 1987). Roche et al. reported that lactosylated poly-L-lysine/DNA complexes were used to specifically transfer in vitro a luciferase reporter gene into human hepatocellular carcinoma cell (HepG2) cells (Midoux et al., 1993). Galactose was conjugated with PEI for the purpose of hepatocyte-targeting (Zanta et al., 1997). While the results of the conjugation were successful, it had been reported that the transfection efficiency of galactosylated PEI/DNA complexes might be improved without a loss in cell specificity by manipulating the length of the conjugated galactose chains (Bettinger et al., 1999).

In our study, we report the gene transfection by using modified chitosan vector into mammalian cells. In order to investigate the possibility to form particles with a smaller diameter, high molecular weigh chitosan (HMWC) was depolymerized into low molecular weight chitosan (LMWC), which was further modified with galactose group. Chitosan/DNA complexes were prepared using a complex coacervation process. Several important parameters for the complex synthesis were investigated, including the molecular weight of chitosan, the ratio of the positive amino group of chitosan to the negative phosphate group of DNA (N/P ratio), and the galactosylation degree of galactosylated-LMWCs (gal-LMWCs). Gal-LMWC was used to transfer pSV-β-galactosidase reporter gene into HepG2, L-02, SMMC-7721 and human cervix adenocarcinoma cell line (HeLa) cell lines in vitro. Transfection efficiency of gal-LMWC was evaluated by β-galactosidase assay and compared with those of lipofectin, calcium phosphate (CaP), HMWC and LMWC. The results showed that gal-LMWC/DNA complex allowed a very efficient cell selective transfection to hepatocyte. Cytotoxicity of gal-LMWC was determined by 3-(4,5-dimethylthiazd-2-yl)-2,5-diphenyltentrazolium bromide (MTT) assay and the results show that the modified chitosan vector has potential to be used as a safe gene-delivery system.

2. Materials and methods

2.1. Materials

The pSV-β-galactosidase control vector containing SV40 early promoter, enhancer and *LacZ* gene was supplied by Promega Corporation (Madison, WI). The pSV-β-galactosidase plasmid was amplified in *Escherichia coli*, and then the plasmids were isolated and purified. Chitosan (from crab shells, minimum 85% deacetylated) was obtained from Sigma Chemical Company (St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was obtained from Dojindo (Japan). LA, *O*-nitrophenyl-β-D-galactopyranoside (ONPG), β-galactosidase and MTT were

purchased from Sigma Chemical Company. Xylidine Ponceau 2R was purchased from Aldrich Chemical Company (St. Louis, MO). Lipofectin® and RPMI Medium 1640 were obtained from Gibco, BRL (Grand Island, NY).

HeLa and HepG2 were obtained from American Type Culture Collection (ATCC). Human non-malignant hepatocellular cell line (L-02) and human hepatocellular carcinoma cell line (SMMC-7721) were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Science (CBTCC-CAS).

HeLa, HepG2, L-02, SMWC-7721 were grew in RPMI1640 containing 10% born bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin. Cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere.

2.2. Depolymerization of chitosan

A total of 5 g HMWC was placed in a 250 ml glass flask equipped with a magnetic stirring bar and 4 M HCl (125 ml) was added. The gas phase was thoroughly purged with argon for 30 min and the reaction bottle was closed tightly. The chitosan solution was vigorously stirred and kept at 100 °C for 15 h, and then the reaction mixture was filtrated. After cooling, 85 ml of ethanol was added to the solution and the mixture was kept at 2–4 °C overnight. Precipitated material was centrifuged with 10,000 rpm for 10 min in the cold and the supernatant was discarded. Precipitated material was washed with 50 ml of cold 50% ethanol in Milli Q water (v/v) and the supernatant was discarded. The washing step was repeated three times. Precipitated material was resuspended in 15 ml of Milli Q water, frozen and lyophilized, and a white powder, chitosan fraction was obtained finally.

2.3. Preparation of gal-LMWC

LMWC was coupled with LA via an active ester intermediate using EDC. A total of 100 mg of LMWC was dissolved in 2 ml of 10 mM *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED)/HCl buffer solution (pH 4.7). A total of 180 mg of EDC 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.

2.4. Characterization of LMWC and gal-LMWC

The compositions of HMWC, LMWC, and gal-LMWC were determined by element analysis (EA-240C, Perkin-Elmer). The FT-IR spectra were recorded with a Nicolet 170SX FT-IR spectrometer. 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. (Gummow and Roberts, 1985).

2.5. Preparation of chitosan/DNA complexes

HMWC, LMWC, and gal-LMWC were dissolved in 0.5% acetic acid with a gentle stirring, pH of the solutions was adjusted to 5.6–6.96 with NaOH, respectively. The final concentration was 1 mg/ml. The solutions were sterile filtered through a 0.22 μm filter and diluted to 0.2 mg/ml.

The required volume of 0.2 mg/ml chitosan solution was added to an aqueous suspension containing 10 μg of DNA by gentle pipetting to form complexes of a selected N/P ratio. N/P ratio was defined as the molar ratio of the positive chitosan amino group and the negative DNA phosphate group. The mixture was vortexed rapidly for 3–5 s and left for 1 h in room temperature for the complexes to completely form.

2.6. Characterization of chitosan/DNA complexes

The diameters of the complexes were determined by photon correlation spectroscopy by using 90 Plus Particle Sizer (Brookhaven Instruments, Holtsville, NY). The physical stability of each complex was studied by agarose gel electrophoresis (0.8% agarose in TAE buffer).

2.7. In vitro transfection with chitosan/DNA complexes

In a typical transfection experiment, HepG2 cells were seeded in 6-well plates at the density of 2.0×10^5 cells/well in 3 ml of complete medium (RPMI1640 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 phosphate buffered saline (PBS). The chitosan/DNA complexes (containing 10 μg of DNA) were diluted in 1 ml RPMI1640, and then were used to refilled the wells. After incubated at 37 °C for 16 h, the medium containing complex was removed. The cells were rinsed twice with PBS and the well was refilled with 3 ml of complete medium. Transfection with CaP/DNA complexes or lipofectin/DNA complexes was performed as positive control according to the reported method (Chang, 1994) or the manufacturer's protocol. Transfection of other cell lines, including HeLa, SMMC-7721, and L-02, was carried out according to the same protocol as mentioned before.

At indicated time-points, ranging from 12 to 196 h after transfection, cells were washed with PBS, lysed, harvested, and analyzed for gene expression. A 30 μl of cell extracts was measured for β-galactosidase activity through ONPG assay (Hall et al., 1983) by using UV spectrophotometer (Shimadzu UV-2201).

The gene expression is presented as the amount of β-galactosidase activity per 10^6 transfected cells. All data are expressed as means ± S.D. Statistical differences between mean values were investigated using Student's *t*-test. Difference between group means were considered significant at $P < 0.05$.

2.8. Cytotoxicity assay

Cytotoxicity of different reagents was measured using the MTT dye reduction assay. Cells were seeded in a 96-well plate at a density of 2.0×10^4 cell/well and incubated overnight as described in the transfection session. Then the cells were incubated in 100 μl serum free medium containing selected amount (from 1 to 35 μg) of lipofectin, CaP, HMWC, LMWC, and gal-LMWC. After 16 h, the medium was removed and the cells were rinsed twice with 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. A total of 150 μl of DMSO was added to each well and the plate was incubated for 30 min at room temperature. Absorbance at 490 nm was measured with an ELISA plate reader (Bio-Rad, Microplate Reader 3550).

3. Results and discussion

3.1. Preparation of LMWC and gal-LMWC

In recent years, because of its properties such as biocompatible, lowly immunogenic, and biodegradable and its capability of condensing DNA to form small particles, chitosan has been found suitable for gene delivery (Hirano et al., 1989; Roy et al., 1999b).

To further understand the potential of chitosan as a gene-delivery system, LMWC and gal-LMWC were prepared, and their properties and transfection efficiency were carefully checked and compared. Fig. 1 illustrates the chemical structure of LMWC and the schematic representation of the synthetic process.

Table 1 summarizes the molecular weight results from the HPLC measurements, together with the N content results from the element analysis, and the $–NH_2$ group content results from the metachromatic titration experiment for HMWC, LMWC, and gal-LMWC samples.

The molecular weight of HMWC decreases from 145 to about 21 kDa after acid depolymerization. It was found that the $–NH_2$ content of LMWC was also decreased compared with that of HMWC. It might be explained by the fact that the amino groups of HMWC contacted with HCl to form hexosamine hydrochloride salt or the oxidation of $–NH_2$ groups in the depolymerization process. The element analysis results are consistent well with these of $–NH_2$ group content measurements.

Fig. 2 shows the degree of galactosylation as the function of LA/LMWC reaction ratio. The galactosylation degree increases linearly with the increase of LA/LMWC molar ratio. After the LA/LMWC molar ratio reaches about 0.5, the degree of galactosylation get a constant value even as the ratio increases.

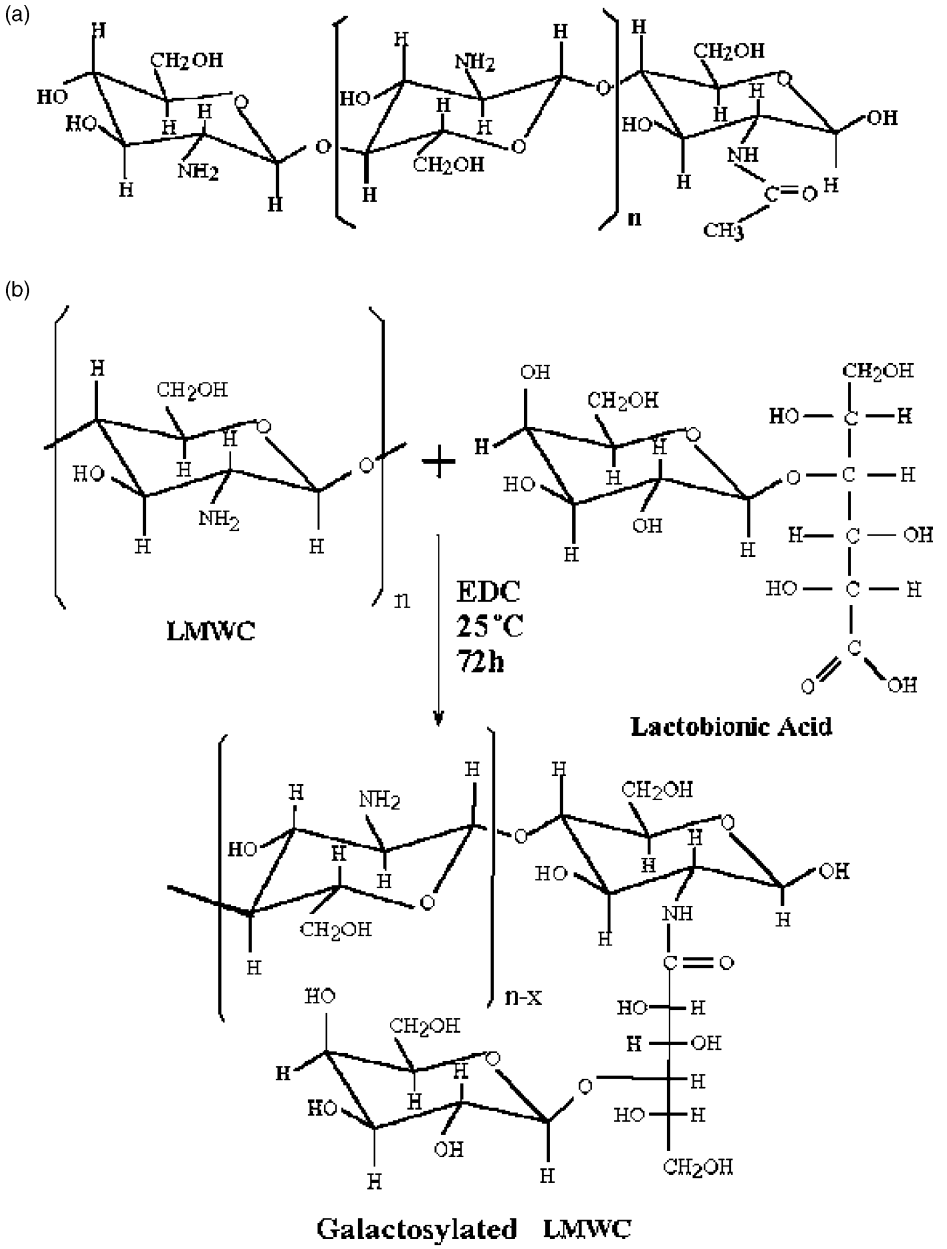

Fig. 1. Chemical structure of LMWC (a) and gal-LMWC (b).

Fig. 3 shows the FT-IR spectra for LA, LMWC, and gal-LMWC. From the IR spectra of gal-LMWC (5.1% galactosylated LMWC if no special annotation) and LA, it can be seen that the carboxyl group of LA at 1749 cm^{-1} in the spectrum of gal-LMWC was not observed due to the coupling reaction between carboxyl group of LA and amino group of LMWC. Since the degree of deacetylation of the LMWC is not 100%, there is not a specific band between gal-LMWC and LMWC. However, the transmittance of amide group at 1613 cm^{-1} in LMWC shifted to 1642 cm^{-1} in gal-LMWC. It may

Table 1
Physicochemical characteristics of HMWC, LMWC, and gal-LMWC (galactosylation degree 5.1%)

	Molecular weight (kDa)	N content (%)	Experimental NH_2 (%)
HMWC	145	8.18	71
LMWC	21	7.01	58
Gal-LMWC	25	6.51	52

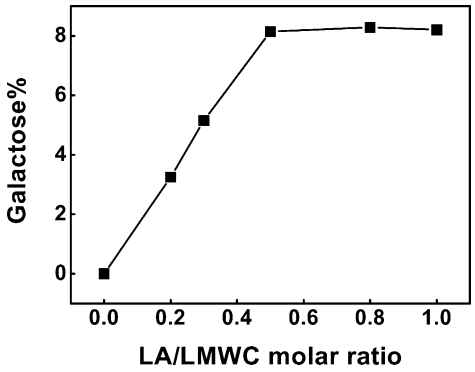

Fig. 2. Galactose percentage of gal-LMWCs as a function of LA/LMWC molar ratio.

be associated with the completion of the occurred reaction.

3.2. Formation of chitosan/DNA complex

Fig. 4 shows the plasmid DNA in complexes migrated in agarose gels. Before the electrophoresis experiment, defined amount of chitosan and DNA was mixed to form the complex. As the proportion of

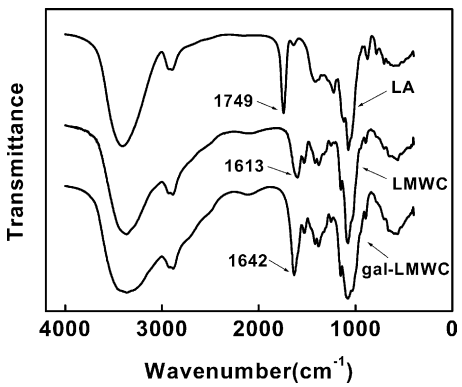

Fig. 3. FT-IR spectra of LA, LMWC, and gal-LMWC.

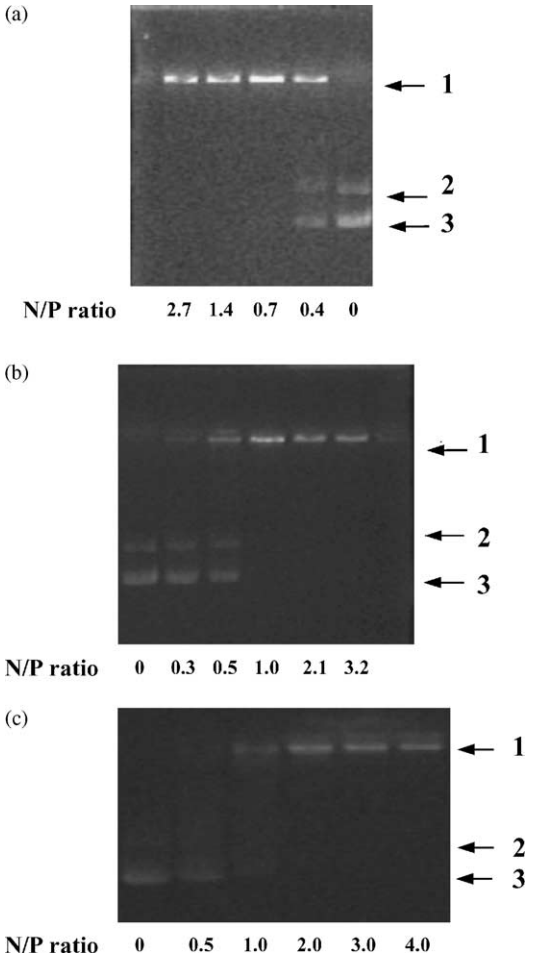

Fig. 4. Gel retardation assay of chitosan/DNA complexes: HMWC/DNA complex (a), LMWC/DNA complex (b), and gal-LMWC/DNA complex (c). Arrows indicate (1) loading position, (2) open circle, and (3) supercoiled form of pDNA.

HMWC, LMWC, and gal-LMWC increased, there was a decrease in the staining intensity of DNA that entered the gel. As N/P ratio reached 0.7 or above, HMWC retained most of the DNA in the gel (Fig. 4a), while the N/P ratio of LMWC and DNA was 1:1 (Fig. 4b) in order to achieve the same retardation effect as HMWC. This fact illustrates that HMWC with higher change density can interact with, and thus, condense DNA more efficiently than LMWC. However, this is outweighed by the fact that HMWC is less soluble, and a great increase in particle diameter or even all aggregation might happen.

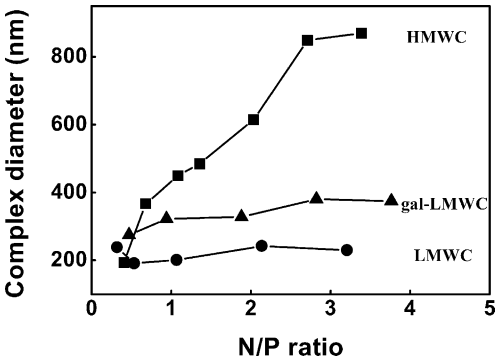

Fig. 5. Particle size of HMWC/DNA, LMWC/DNA, and gal-LMWC/DNA complexes with different N/P ratios.

A complete retardation was achieved at N/P ratio 2:1 in the case of gal-LMWC (Fig. 4c). Although some amino groups are galactosylated, charge density of gal-LMWCs only decreases slightly and gal-LMWCs' ability to condense DNA is retained. This character is important in condensing DNA and transfecting cells.

Effective diameters of chitosan/DNA complexes were determined by Laser Light Scattering (LLS) and Fig. 5 shows the particle diameters of HMWC/DNA, LMWC/DNA, and gal-LMWC/DNA complexes with different N/P ratios. The particle diameter of HMWC/DNA complexes continues to increase while the N/P ratio goes up. However, the particle diameter of complexes prepared with LMWC remains constant of about 220 nm as N/P increases, indicating that LMWC is more suitable to form uniform, stable nanoparticles with DNA.

In the case of gal-LMWC, the changes in particle size follow the same trend as that of LMWC. When the N/P ratio of gal-LMWC and DNA increases from 0.5 to 3.8, the complexes are almost at the homogeneous size 350 nm, slightly greater than those prepared with LMWC. This can be associated with the fact that the LMWC has higher charge density than gal-LMWC and contacts tighter with DNA.

Particle size is an important factor that influences the transfection efficiency. The complexes of great particle diameter have their disadvantages in the process of cell endocytosis, and thus the corresponding transfection efficiency is not good enough (Seymour, 1992). Although the HMWC acts more efficiently than LMWC and gal-LMWC in condensing DNA, because of the large diameter of their particles, the transfection of HMWC/DNA complexes is less efficient than that of gal-LMWC/DNA complexes.

These results also indicate that it is possible to formulate complexes of a specific particle diameter by adjusting condensing conditions, such as chitosan molecular weight, the N/P ratio of the complexes, concentration of DNA and concentration of chitosan.

3.3. Cytotoxicity study of modified chitosans

Fig. 6 shows the cytotoxicity of five kinds of transfection vector in HepG2 cell line and HeLa cell line, respectively. CaP is the most cytotoxic either in HepG2 or in HeLa. Cells almost died when CaP concentration reached 20 μg/ml. The cytotoxicity of lipofectin increased as concentration increased and cells almost died at concentration of 250 μg/ml. However, HMWC,

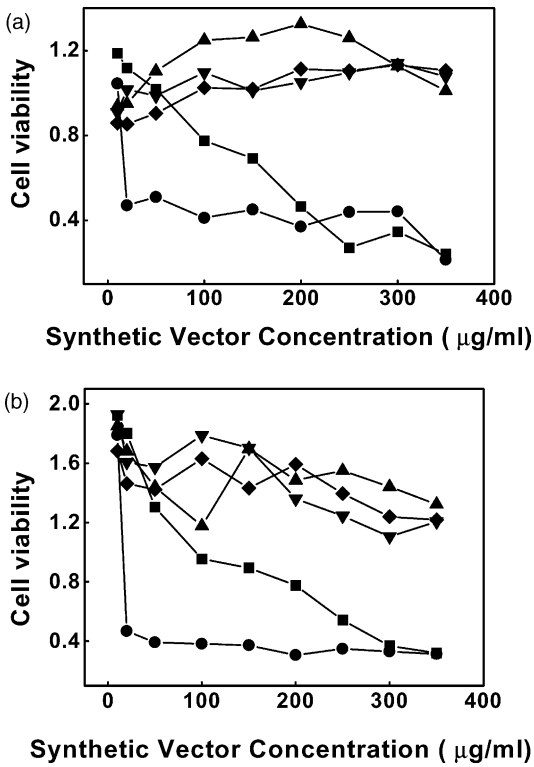

Fig. 6. MTT assay for cytotoxicity of lipofectin (■), CaP (●), HMWC (▲), LMWC (▼), and gal-LMWC (◆) in HepG2 cell line (a) and HeLa cell line (b).

LMWC, and gal-LMWC were found to be less cytotoxic compared with lipofectin and CaP both in HepG2 and HeLa. Cells grew well even at the concentration as high as 350 μg/ml. HMWC, LMWC, and gal-LMWC were found to improve viability of HepG2 cells at some concentration such as 100 and 150 μg/ml. The results further demonstrate that modified chitosan is non-cytotoxic, biocompatible, and safe vector.

3.4. Gene transfection into cells

The pSV-β-galactosidase control vector was used as the reporter gene to monitor the transfection efficiency. To determine the optimal N/P ratio of gal-LMWC/DNA complex for transfection, HepG2 cells were transfected respectively with the complexes prepared with gal-LMWC at different N/P ratios and the results are shown in Fig. 7. It can be found that the β-galactosidase activity increases as the N/P ratio goes up from 0.94:1 to 5.6:1. Maximal gene expression occurs at N/P ratio 5.6:1 and the β-galactosidase activity decreases as N/P ratio is above 5.6:1. Because gal-LMWC/DNA complex is taken up into cell via the receptor-mediated endocytosis pathway, the galactose density of complexes influences the endocytosis. Although a complete retardation was achieved at N/P ratio as low as 2:1 in the case of gal-LMWC, gal-LMWC can condense DNA completely in the reaction system at the N/P ratio 5.6:1. When the galactose density of complex increases with the N/P ratio, it is assumed that the complexes can contact with the receptors on the cell surface more easily, resulting in the increase of transfection efficiency. When the ratio is above 5.6:1 there is undoubtedly increased amount of free gal-LMWC in the reaction system. The free gal-LMWC combines with the receptors on the cell surface competitively and reduces the coupling of gal-LMWC/DNA complexes with the receptors, which inhibits the endocytosis process and results in the transfection efficiency decreasing.

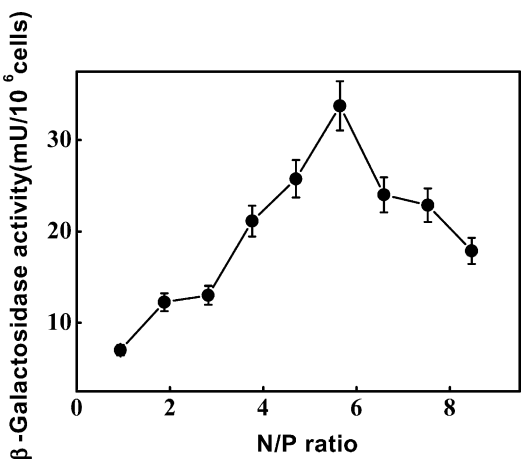

Fig. 7. Charge-dependent β-galactosidase expression in HepG2 cells transfected with gal-LMWC/DNA complexes. Results are expressed as mean values ± S.D. from one representative experiment ($n = 4$) of three performed ($P < 0.05$).

Next, naked DNA, CaP/DNA, lipofectin/DNA, HMWC/DNA, LMWC/DNA, and gal-LMWC/DNA complexes were used to transfect HepG2, L-02, SMMC-7721 cell lines, and HeLa cell line. Fig. 8 shows the efficiency of the six complexes. It can be observed that the transfection efficiency of gal-LMWC/DNA complex is higher than those of naked DNA, CaP/DNA, HMWC/DNA, LMWC/DNA complexes in HepG2 cells and lower than that of lipofectin/DNA complex. This result was also observed in L-02 cells. The gal-LMWC/DNA complex gives the best gene expression in SMMC-7721 cells and even better than lipofectin/DNA complex. It is most probable of the different transfection mechanism with lipofectin/DNA complex and gal-LMWC/DNA complex. Lipofectin/DNA complex appears to be taken up into membrane vesicles, but it is anticipated that the presence of membrane-active oleoyl group in the lipid component endow the complex with a fusogentic, or membrane-disrupting activity, which is important for transfection (Zabner et al., 1995). Gal-LMWC/DNA complex is taken up into cell via the receptor-mediated endocytosis pathway, and the plasmid DNA has to escape from the endosome before it expresses (Mao et al., 2001).

However, in HeLa cells without ASGR on the cell membrane surface, transfection efficiency of gal-LMWC/DNA complex was almost the same as that of of naked DNA, much lower than those of lipofectin/DNA complex and CaP/DNA complex, and even lower than those of HMWC/DNA and LMWC/DNA complexes. These results suggest that the gal-LMWC/DNA complex has the capability to transfect the cell lines with ASGR selectively.

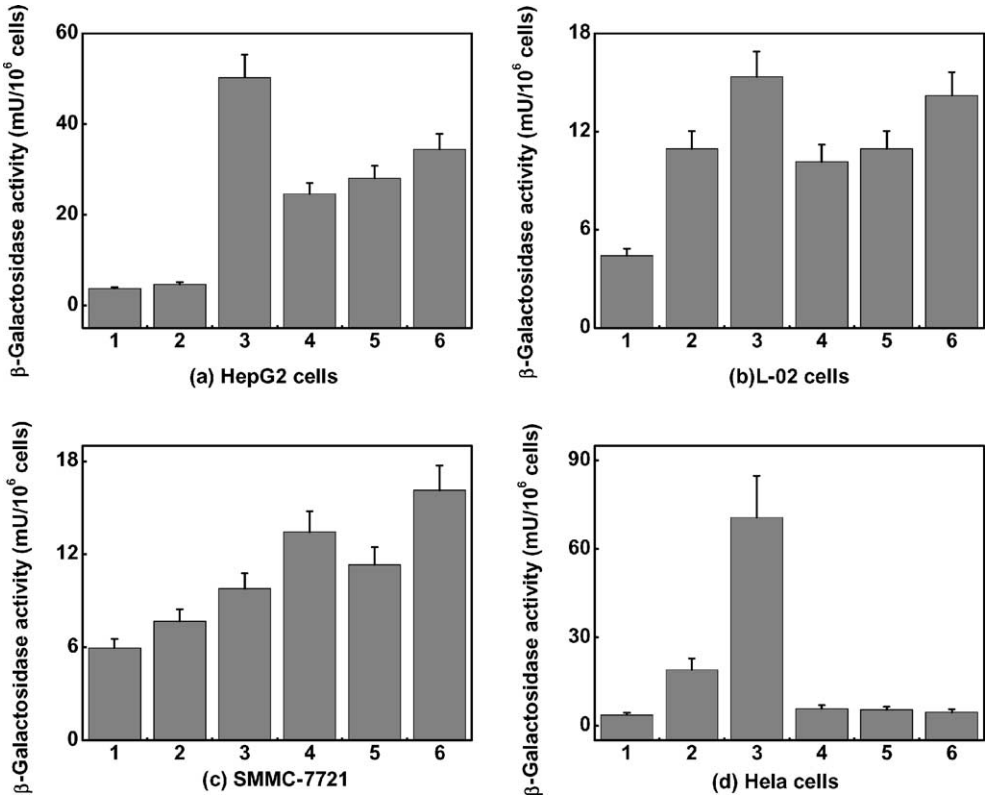

Fig. 8. Comparison of transfection efficiency of the synthetic vectors in HepG2, L-02, SMMC-7721, and HeLa cell lines: (1) naked DNA, (2) CaP/DNA, (3) lipofectin/DNA, (4) HMWC/DNA, (5) LMWC/DNA, and (6) gal-LMWC/DNA. Results are expressed as mean values ± S.D. from one representative experiment ($n = 4$) of three performed ($P < 0.05$).

Based on the results mentioned before, the time-dependent transgene expression in HepG2 cells was studied at a N/P ratio of 5.6:1 and results show that the gal-LMWC/DNA complex can give a long term gene expression (Fig. 9). Gal-LMWC/DNA complex displayed different kinetics of gene expression in HepG2 cells compared with lipofectin/DNA complex. The gene expression obtained with the gal-LMWC/DNA complex increased over time, and even after 177 h, a high transgene expression of about 43.5 mU β-galactosidase activity was obtained. However, lipofectin/DNA complex induced a more rapid onset of gene expression. Incubated only for 24 h after transfection with lipofectin/DNA complex, HepG2 cells obtained the highest β-galactosidase activity and decreased sharply after 50 h while the cell viability decreased at the same time (data not shown). The difference in the kinetics of gene expression can be probably attributed to the different mechanism with which gal-LMWC/DNA complexes and lipofectin/DNA complexes pass the cell membrane, and also the fact that chitosan molecules can protect plasmid DNA from degradation effect of enzymes in cells.

In order to investigate the effect of galactosylation degree, the gal-LMWCs of different galactosylation degree were complexed with DNA at N/P ratio 6:1 and tested for complex stability, particle size, and transfection efficiency both in HepG2 cell line and HeLa cell line. At N/P ratio 6:1, all the four modified chitosans with galactosylation degree of 0, 3.2, 5.1, and 8.3%, respectively, formed stable complexes with DNA in a gel retardation assay as shown in Fig. 10a. Particle size of all the four complexes got a little raise from 314 to 341 nm as the galactosylation degree went up shown in Fig. 10b. The result is consistent with the

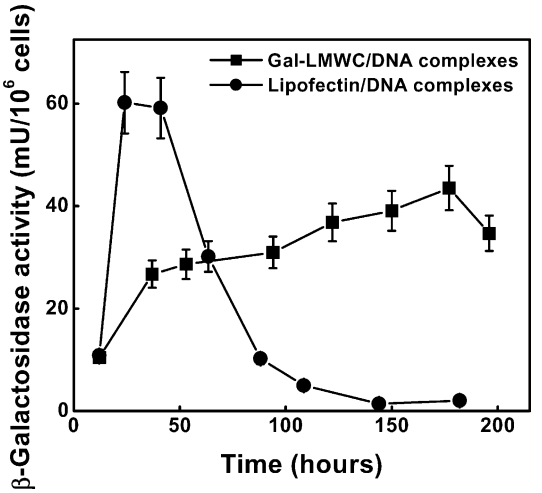

Fig. 9. Time-dependant β-galactosidase expression in HepG2 cells transfected with gal-LMWC/DNA complexes and lipofectin/DNA complexes. Results are expressed as mean values ± S.D. from one representative experiment ($n = 4$) of three performed ($P < 0.05$).

trend shown in Fig. 5. Fig. 10c gives the evidence that the in vitro transfection efficiency of the complexes for HepG2 cells correlates strongly with the galactosylation degree of gal-LMWCs. This result implies that the gal-LMWCs give a very efficient cell selective transfection to HepG2 cells by ASGR-mediated endocytosis process. The selectivity of gal-LMWCs to hepatocyte depended on their galactosylation degree strongly.

This study was designed to provide insight into the in vitro properties of gal-LMWC as a non-viral gene-delivery system. Gal-LMWCs were prepared and characterized for hepatocyte-targeting and complexed with pSV-β-galactosidase control vector. Gal-LMWCs/DNA complexes transfected more effectively in HepG2, L-02, and SMMC-7721 cell lines coming from human hepatocyte than in HeLa cell line coming from human cervix, indicating that gal-LMWC vectors can transfect gene into hepatocyte selectively via the receptor-mediated endocytosis pathway. The galactosylation degree and the N/P ratio were important factors that influence the transfection efficiency.

Both the gene transfection and cell cytotoxicity studies suggest that gal-LMWC is a versatile gene vector with a minimal cytotoxicity. This would pave

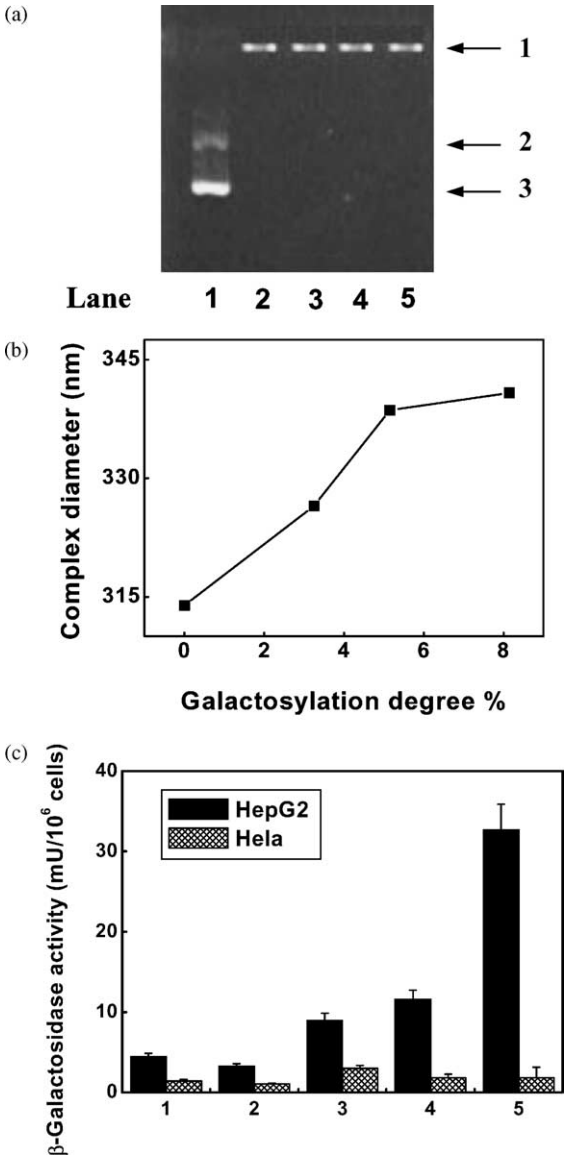

Fig. 10. (a) Gel retardation assay of gal-LMWC/DNA complexes with different galactosylation degrees. Lane 1: Naked DNA, lanes 2–5: galactosylation degree: 0, 3.2, 5.1, and 8.3%, respectively. Arrows indicate (1) loading position, (2) open circle, and (3) supercoiled form of pDNA. (b) Particle size measurements for gal-LMWC/DNA complexes with different galactosylation degrees. (c) In vitro transfection efficiency for gal-LMWC/DNA complexes with different galactosylation degrees. (1) Naked DNA, (2)–(5) galactosylation degree: 0, 3.2, 5.1, and 8.3%, respectively. Results are expressed as mean values ± S.D. from one representative experiment ($n = 4$) of three performed ($P < 0.05$).

the way to realizing the concept of applying gene as a drug.

Acknowledgements

This work was supported by National Natural Science Foundation of China (59703007), Young Investigator Grant of Jiangsu Province (BK2001405) and Chinese High Technology(863) Project (No. 2001AA218061).

References

Anderson, W.F., 1998. Human gene therapy. Nature 392, 25–30.

Arai, K., Kinumaki, T., Fujita, T., 1968. Toxicity of chitosan. Bull. Tokai Feg. Fish Res. Lab. 56, 89–95.

Bersch, P.C., Nies, B., Liebendorfer, A., 1995. Evaluation of the biological properties of different wound dressing materials. J. Mater. Sci. Med. 6, 231–240.

Bettinger, T., Remy, J.S., Erbacher, P., 1999. Size reduction of galactosylated PEI/DNA complexes improves lectin-mediated gene transfer into hepatocytes. Bioconjug. Chem. 10, 558–561.

Boussif, O., Lezoualch, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.-P., 1995. A versatile vector for gene and oligonucleotide delivery into cells in culture and in vivo: polyethylenimine. Proc. Natl. Acad. Sci. U.S.A. 92, 7297–7310.

Chang, P.L., 1994. Calcium phosphate-mediated DNA transfection. In: Wolff, J.A. (Ed.), Gene Therapeutics: Methods and Applications of Direct Gene Transfer. Birkhauser, Boston, pp. 157–159.

Gummow, B.D., Roberts, G.A.F., 1985. 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, 1239–1244.

Hackett, N.R., Crystal, R.G., 2000. Adenovirus vector for gene therapy. In: Templeton, N.S., Lasic, D.D. (Eds.), Gene Therapy: Therapeutic Mechanisms and Strategies. Marcel Dekker, New York, pp. 17–40.

Hall, C.V., Jacob, P.E., Ringold, G.M., Lee, F., 1983. Expression and regulation of *Escherichia coil lacZ* gene fusions in mammalian cell. J. Mol. Appl. Genet. 2, 101–108.

Hirano, S., Seino, H., Akiyama, Y., Nonaka, I., 1989. Biocompatibility of chitosan by oral and intravenous administration. Polym. Eng. Sci. 59, 897–901.

Hirano, S., Seino, H., Akiyama, I., Nonaka, I., 1990. Chitosan: a biocompatible material for oral and intravenous administration. In: Gebelein, C.G., Dunn, R.L. (Eds.), Progress in Biomedical Polymers. Plenum, New York, pp. 283–289.

Ishii, T., Okahata, Y., Sato, T., 2001. Mechanism of cell transfection with plasmid/chitosan complexes. Biochim. Biophys. Acta 1514, 51–64.

Knapczyk, J., Krowczynski, L., Krzck, J., Brzeski, M., Nimberg, E., Schenk, E., Struszcyk, H., 1989. Requirements of chitosan for pharmaceutical and biomedical applications. In: Skak-Braek, G., Anthonsen, T., Sandford, P. (Eds.), Chitin and Chitosan: Sources, Chemistry, Biochemistry, Physical Properties and Applications. Elsevier, London, pp. 657–663.

MacLaughlin, F.C., Mumper, R.J., Wang, J., Tagliaferri, J.M., Gill, I., Hinchcliffe, M., Rolland, A.P., 1998. Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. J. Control. Release 56, 259–272.

Mao, H.Q., Roy, K., Janes, K.A., Truong-Le, V.L., Lin, K.Y., August, J.T., Leong, K.W., 2001. Chitosan–DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficacy. J. Control. Release 70, 399–421.

Midoux, P., Mendes, C., Legrand, A., Raimond, J., Mayer, R., Monsigny, M., Roche, A.C., 1993. Specific gene transfer mediated by lactosylated poly-L-lysine into hepatoma cells. Nucleic Acids Res. 21, 871–878.

Mumper, R.J., Duguid, J.G., Anwer, K., Barron, M.K., Nitta, H., Rolland, A.P., 1996. Polyvinyl derivatives as novel interactive polymers for controlled gene delivery to muscle. Pharm. Res. 13, 701–709.

Remy, J.-S., Abdallah, B., Zanta, M.A., Boussif, O., Behr, J.-P., Demeneix, B., 1998. Gene transfer with lipospermines and polyethylenimines. Adv. Drug Deliv. Rev. 30, 85–95.

Roy, K., Chu, W., McGrath, J., Kuo, S.C., Huang, S.-K., Leong, K.W., 1999a. Nanoparticle mediated oral gene delivery for DNA vaccination. Proc. Int. Symp. Control. Rel. Bioact. Mater. 26, 161–162.

Roy, K., Mao, H.Q., Huang, S.-K., Leong, K.W., 1999b. Oral gene delivery with chitosan–DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. Nat. Med. 5, 387–391.

Sato, T., Ishii, T., Okahata, Y., 2001. In vitro gene delivery mediated by chitosan. Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. Biomaterials 22, 2075–2080.

Seymour, L.W., 1992. Passive tumor targeting of soluble macromolecular and drug conjugates. CRC Crit. Rev. Ther. Drug Carrier Syst. 9, 135–187.

Tang, M.X., Redemann, C.T., Szoka, F.C., 1996. In vitro gene delivery by degraded polyamidoamine dendrimers. Bioconjug. Chem. 7, 703–714.

Wall, D.A., Wilson, G., Hubbard, A.L., 1980. The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes. Cell 21, 79–93.

Weiner, M.L., 1991. An overview of the regulatory status and of the safety of chitin and chitosan as food and pharmaceutical ingredients. In: Brine, C.J., Sandford, P.A., Zikakis, J.P. (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, New Jersey, USA, pp. 663–672.

Wu, G., Wu, C., 1987. Receptor-mediated *in vitro* gene transformation by soluble DNA carrier system. J Biol. Chem. 262, 4429–4432.

Zabner, J., Fasbender, A.J., Moniger, T., Poellinger, K.A., Welsh, M.J., 1995. Cellular and molecular barriers to gene transfer by a cationic lipid. J. Biol. Chem. 270, 18997–19007.

Zanta, M.A., Boussif, O., Adib, A., Behr, J.P., 1997. In vitro gene delivery to hepatocytes with galactosylated polyethylenimine. Bioconjug. Chem. 8, 839–844.