Synthesis of a Novel Glycoconjugated Chitosan and Preparation of Its Derived Nanoparticles for Targeting HepG2 Cells

Fwu-Long Mi,*,†,|| Yong-Yi Wu,‡ Ya-Lin Chiu,‡ Mei-Chin Chen,‡ Hsing-Wen Sung,*,‡ Shu-Huei Yu,§ Shin-Shing Shyu,|| and Mei-Feng Huang⊥

Department of Biotechnology, Vanung University, Chung-Li, Taiwan, Republic of China, Department of Chemical Engineering, National Tsing Hua University, Hsinchu, Taiwan, Republic of China, Department of Polymer Materials, Vanung University, Chung-Li, Taiwan, Republic of China, Nano Materials R&D Center, Vanung University, Chung-Li, Taiwan, Republic of China, and Department of Chemical and Material Engineering, National Central University, Chung-Li, Taiwan, Republic of China

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In the study, a novel chitosan (CS) derivative conjugated with multiple galactose residues in an antennary fashion (Gal-m-CS) was synthesized. A galactosylated CS (Gal-CS) was also prepared by directly coupling lactobionic acid on CS. Using an iontropic gelation method, CS and the synthesized Gal-CS and Gal-m-CS were used to prepare nanoparticles (CS, Gal-CS, and Gal-m-CS NPs) for targeting hepatoma cells. TEM examinations showed that the morphology of all three types of NPs was spherical in shape. No aggregation or precipitation of NPs in an aqueous environment was observed during storage for all studied groups, as a result of the electrostatic repulsion between the positively charged NPs. Little fluorescence was observed in HepG2 cells after incubation with the FITC-labeled CS NPs. The intensity of fluorescence observed in HepG2 cells incubated with the Gal-m-CS NPs was stronger than that incubated with the Gal-CS NPs. These results indicated that the prepared Gal-m-CS NPs had the highest specific interaction with HepG2 cells among all studied groups, via the ligand−receptor-mediated recognition.

1. Introduction

Nanoparticles (NPs) made of biodegradable macromolecules have recently attracted extensive attention because of their potential applications in the biomedical fields as a drug/gene delivery carrier. These NPs may be delivered to the tumor sites via a size-dependent passive targeting or the EPR (enhanced permeability and retention) effect.[1] Additionally, to aim for a specific site of the human body and to enhance uptake of the NP carrier into the targeted cells, active targeting has been attempted by many investigators.[2,3] To achieve this purpose, target-specific ligands such as carbohydrates are commonly conjugated to the surface of the macromolecular carriers.[4,5]

It was reported that asialoglycoprotein (ASGP) receptors are abundantly expressed on the surface of hepatoma cells.[6,7] Therefore, for the treatment of liver cancers, targeting may be achieved by designing the drug delivery carriers conjugated with a ligand that can bind to the ASGP receptors.[8,9] Galactosylated macromolecules, as a drug/gene carrier, have been reported to enhance drug delivery or gene transfection into HepG2 cells (a liver cancer cell line).[10−15] This targeting is mediated by the specific interaction between the galactose residues conjugated on macromolecules and the ASGP receptors present on hepatoma cells.[8]

Chitosan (CS) has been used as a raw material for constructing NPs because its free amino groups can be easily modified and conjugated with target-specific ligands and it readily forms complexes with the negatively charged drug/gene.[16−19] CS is a polysaccharide composed primarily of repeating units of β-(1−4)2-amino-D-glucose (glucosamine) and β-(1−4)2-acetamido-D-glucose (*N*-acetylglucosamine). Being a nontoxic, biocompatible, and biodegradable polymer, CS has been applied for pharmaceutical and biomedical applications.[20,21]

The aim of the study was to develop a CS-based NP carrier for targeting hepatoma cells. To provide the NP carrier with the ability to recognize hepatoma cells, CS was conjugated with multiple galactose residues in an antennary fashion (Gal-m-CS). The Gal-m-CS was prepared by first introducing branched lysine spacers to CS, followed by covalent coupling of lactobionic acids (LA) on the branched lysine spacers. Thus, each conjugated lysine spacer may have four galactose residues in maximum. The Gal-m-CS NPs were prepared by an iontropic gelation method. The particle size, zeta potential, and morphology of the prepared NPs were investigated. The targeting efficacy of the prepared NPs against HepG2 cells was investigated in vitro.

2. Experimental Section

2.1. Materials. CS (MW ~2.5×10^5) with a degree of deacetylation of approximately 85% was acquired from Fluka Chemical Co. (Switzerland). LA was purchased from TCI (Tokoy, Japan). l-Lysine, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), and *N*-hydroxylsuccinimide (NHS) were obtained from Sigma Chemical Co. (U.S.). All other reagents and solvents used were of reagent grade.

* The contributions by the two collaborating parties are equal. To whom correspondence should be addressed. Fax: 886-3-4333063 (F.-L.M.); 886-3-5726832 (H.-W.S.). E-mail: flmi530326@vnu.edu.tw (F.-L.M.); hwsung@che.nthu.edu.tw (H.-W.S.).

† Department of Biotechnology, Vanung University.

‡ National Tsing Hua University.

§ Department of Polymer Materials, Vanung University.

|| Nano Materials R&D Center, Vanung University.

⊥ National Central University.

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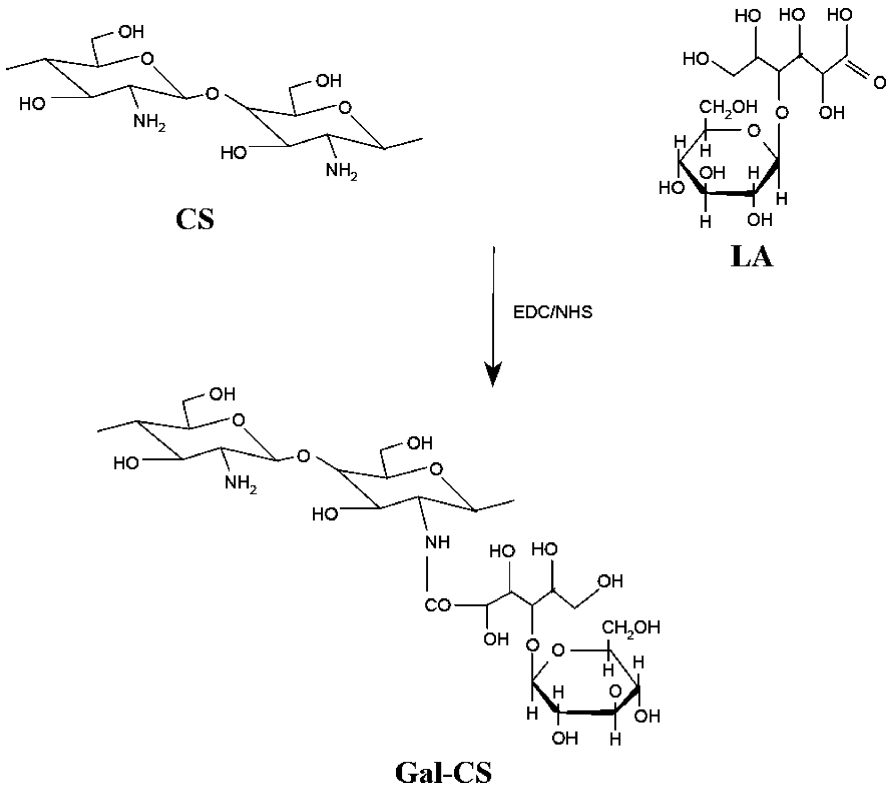

Figure 1. Schematic illustration of the procedures for the synthesis of Gal-CS. CS, chitosan; LA, lactobionic acid; Gal-CS, galactosylated chitosan; EDC, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide; NHS, *N*-hydroxylsuccinimide.

2.2. Syntheses of the Galactosylated CS (Gal-CS) and the Antennary Type of Galactosylated CS (Gal-m-CS). The procedures used in the synthesis of the galactosylated CS (Gal-CS) and the antennary type of galactosylated CS (Gal-m-CS) were schematically illustrated in Figures 1 and 2, respectively. The galactosylated CS without the branched lysine spacer conjugated (Gal-CS) was prepared by directly coupling LA with CS according to a method reported by Cho et al.[22]

For the synthesis of Gal-m-CS, a stock solution of CS in aqueous acetic acid (1.5% by w/v) was prepared by dissolving 1.5 g of CS in 100 mL of aqueous acetic acid (0.5% by v/v) and stirring for 12 h at room temperature. The dissolved CS solution was sonicated to remove the trapped air bubbles. Synthesis of the branched lysine spacer was performed before introducing the galactose residues to CS. L-Lysine with the protected amino groups (Lys_p) was dissolved in deionized water and reacted with an equal molar ratio of EDC/NHS for 24 h to activate the carboxylic acid groups on L-lysine. In the study, the protected lysine was prepared similarly to that reported in the literature.[23,24] t-Boc (tert-butyloxycarbonyl) was used for protecting the amino groups on L-lysine. Afterward, a half molar ratio of L-lysine with the reactive amino groups (Lys_a) was added to the aforementioned Lys_p/EDC/NHS solution for covalent coupling of the amino groups on Lys_a with the carboxylic acid groups on Lys_p. After 24 h of reaction, the branched lysine spacer with the protected amino groups (B-Lys_p) was synthesized through the formation of amide bonds.

B-Lys_p was then reacted with an equal molar ratio of EDC/NHS for 24 h to activate its carboxylic acid groups. Afterward, an equal molar ratio of glucosamine unit of CS was added to the B-Lys_p/EDC/NHS solution for covalent coupling of the amino groups on CS with the activated carboxylic acid groups on B-Lys_p. After 24 h of reaction, the CS derivative conjugated with the branched lysine spacer (B-Lys_p-CS) was synthesized through the formation of amide bonds. The introduction of galactose residues to the branched lysine spacers on CS was performed as follows. The protected amino groups on the lysine spacers of B-Lys_p-CS were deprotected by acid treatment to obtain the CS derivative with the reactive amino groups on its branched lysine spacers (B-Lys_a-CS). LA was dissolved in deionized water and reacted with an equal molar ratio of EDC/NHS for 24 h to activate its carboxylic acid groups. Subsequently, LA was added into the B-Lys_a-CS/EDC/NHS solution at an equal molar ratio for covalently coupling of the carboxylic acid groups on LA with the amino groups on B-Lys_a-CS. The reaction was performed for 24 h at room temperature to obtain a galactosylated CS with multiple galactose residues on every conjugated lysine spacer (Gal-m-CS).

The resulting products (Gal-CS and Gal-m-CS) were purified using dialysis (MWCO 6000−8000) against deionized water for 1 week with water changes several times, followed by lyophilization. The synthesized Gal-CS and Gal-m-CS were identified by analyzing the compounds using ^{1}H NMR (Varian Unityionva 500 NMR spectrometer, MO).

2.3. Preparation of CS and Galactosylated CS NPs. NPs were prepared as per an iontropic gelation method reported by Kumacheva et al.[25] CS (0.2 wt %) was dissolved in an aqueous acetic acid, while galactosylated CS (Gal-CS or Gal-m-CS) was directly dissolved in deionized water in a concentration of 0.2 wt %. Subsequently, 1.0 mL of aqueous tripolyphosphate sodium (TPP) was added dropwise into 5.0 mL of aqueous CS or galactosylated CS solutions under magnetic stirring. Formation of CS NPs and galactosylated CS NPs (Gal-CS NPs and Gal-m-CS NPs) started spontaneously via the TPP initiated ionic gelation mechanism. The NPs were prepared at a weight ratio of CS, Gal-CS, or Gal-m-CS to TPP of 4:1. The NPs were subjected to repeated cycles of washing by deionized water and centrifugation (20 000*g* for 10 min) to remove the unreacted polymers (CS, Gal-CS, or Gal-m-CS) and TPP. The NP suspensions were gently stirred for 60 min at room temperature, without adjusting the pH conditions, before being subjected to further analyses and applications.

2.4. Characterization of the Prepared NPs. Transmission electron microscopy (TEM) was used to observe the morphology of the prepared NPs. TEM samples were prepared by placing a drop of the NP solution onto a 400 mesh copper grid coated with carbon. About 2 min after deposition, the grid was tapped with a filter paper to remove surface water and positively stained by an alkaline bismuth solution. The size distribution and zeta potential of the prepared NPs were measured using a Zetasizer (3000HS, Malvern Instruments Ltd., Worcestershire, U.K.).

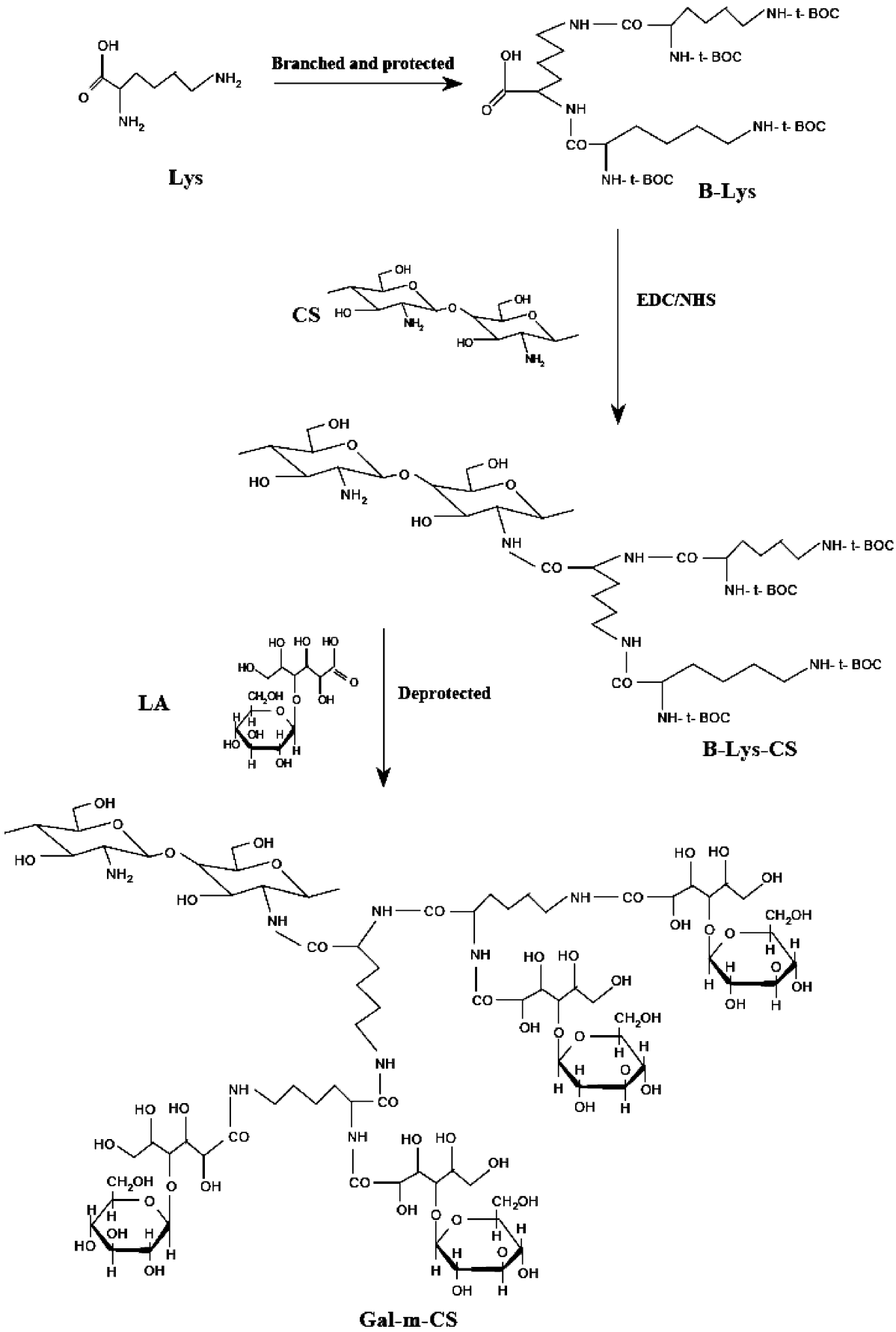

Figure 2. Schematic illustration of the procedures for the synthesis of Gal-m-CS. Lys, L-lysine; B-Lys, branched lysine; B-Lys-CS, chitosan conjugated with branched lysine spacer; LA, lactobionic acid; Gal-m-CS, galactosylated chitosan with multiple galactose residues.

During storage, the precipitation of NPs may occur and thus causes them to lose their structural integrity or form precipitation of NPs. Therefore, the stability of NPs during storage must be evaluated. In the stability study, the particle size and zeta potential of the prepared

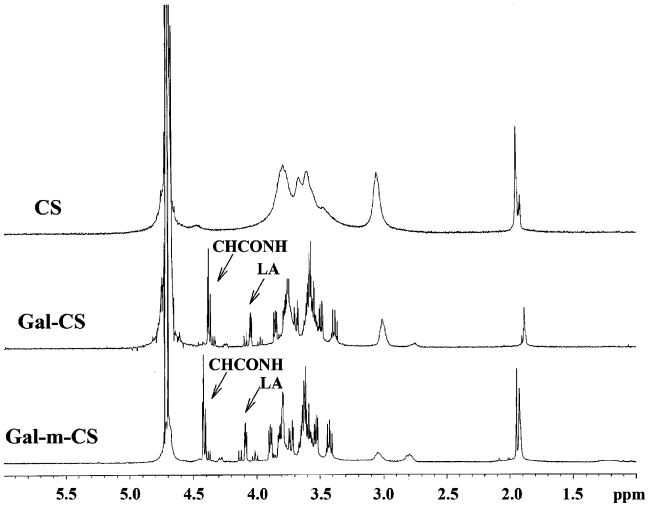

Figure 3. ^{1}H NMR spectra of CS, Gal-CS, and Gal-m-CS. CS, chitosan; Gal-CS, galactosylated chitosan; Gal-m-CS, galactosylated chitosan with multiple galactose residues.

Table 1. Mean Particle Sizes, Polydispersity Indices, and Zeta Potential Values of the Prepared CS, Gal-CS, and Gal-m-CS NPs ($n = 5$)

	CS NPs[a]	Gal-CS NPs[b]	Gal-m-CS NPs[c]
mean particle size (nm)	N/A[d]	159.4 ± 3.4	163.2 ± 5.3
polydispersity index	N/A[d]	0.23 ± 0.17	0.29 ± 0.01
zeta potential (mV)	29.6 ± 1.1	26.7 ± 0.9	35.8 ± 0.8

[a] CS NPs: the nanoparticles prepared by the iontropic gelation of chitosan with TPP. [b] Gal-CS NPs: the nanoparticles prepared by the iontropic gelation of galactosylated chitosan with TPP. [c] Gal-m-CS NPs: the nanoparticles prepared by the iontropic gelation of galactosylated chitosan with multiple galactose residues with TPP. [d] No data were available for the CS NPs because their size distribution was not monodispersed as mentioned in the text.

CS, Gal-CS, or Gal-m-CS NPs were monitored during storage for up to 1 month using the Zetasizer.

2.5. Preparation of the FITC-Labeled CS, Gal-CS, and Gal-m-CS NPs. Fluorescence (FITC)-labeled CS, Gal-CS, and Gal-m-CS NPs were prepared for the confocal laser scanning microscopy study. Synthesis of the FITC-labeled CS was based on the reaction between the isothiocyanate group of FITC and the primary amino groups of CS as reported in the literature.[26] Briefly, 100 mg of FITC in 150 mL of dehydrated methanol was added to 100 mL of 1% CS in 0.1 M acetic acid. After 3 h of reaction in the dark at ambient conditions, the FITC-labeled CS was precipitated by raising the pH to 8−9 with 0.5 M NaOH. To remove the unconjugated FITC, the precipitate was subjected to repeated cycles of washing and centrifugation (40 000*g* for 10 min) until no fluorescence was detected in the supernatant. The FITC-labeled CS dissolved in 80 mL of 0.1 M acetic acid was then dialyzed for 3 days in the dark against 5 L of distilled water, with water replaced on a daily basis. The resultant FITC-labeled CS was lyophilized in a freeze-dryer and used for preparation of the FITC-labeled CS, Gal-CS, and Gal-m-CS NPs as described in sections 2.2 and 2.3.

2.6. Cell Uptake Study. The FITC-labeled CS, Gal-CS, and Gal-m-CS NP suspensions were filtered through a 0.45-μm membrane for sterilization. Subsequently, 100 μL of NPs (1 mg/mL) was added to HepG2 cells, which were pre-cultured on a 35-mm glass dish for 24 h. The experimental temperature was maintained at 37 °C by a temperature control system (DH-35 Culture Dish Heater, Warner Instruments Inc., Hamden, CT). After incubation for specific time intervals, the fluorescence images were obtained using an inverted confocal laser scanning microscope (TCS SL, Leica, Germany). The fluorescence images were observed using an argon laser (excitation at 488 nm, emission collected at a range of 510−540 nm).

2.7. Statistical Analysis. The comparison in the measured properties among the different groups of NPs was accomplished using one-way analysis of variance and determination of confidence intervals, performed with a computer statistical program (Statistical Analysis System, Version 6.08, SAS Institute Inc., Cary, NC). All data are presented as a mean value with its standard deviation indicated (mean ± SD). Differences were considered to be statistically significant when the p values were less than 0.05.

3. Results and Discussion

Biodegradable macromolecular carriers conjugated with glycotargeting ligands have attracted considerable interest as an effective method for delivery of drug/gene to the target site. Glycotargeting takes advantage of a highly specific interaction between the carbohydrate ligands conjugated on macromolecules and the endogenous lectins present on the targeted cells.[5] Because of their high density on the surface of hepatoma cells in the liver cancers, the ASGP receptors are a particularly attractive site for glycotargeting.[8] It was recently reported that macromolecules conjugated with multiple glycotargeting ligands significantly increased their binding efficiency to hepatoma cells (the cluster effect).[14,27] The cluster effect depends on the spatial distances between the glycotargeting ligands, the flexibility of the arm connecting to the glycotargeting ligands, and the structure of their branch points. Among the glycoconjugated macromolecules, galactosylated CS was found to be a suitable material for liver-targeting drug/gene delivery or liver tissue engineering.[28−31]

In this study, to achieve an effective targeting of the NP carrier to hepatoma cells via the ligand−receptor-mediated recognition, a novel antennary type of CS-galactose conjugate was developed. Figures 1 and 2 schematically illustrated the procedures for the synthesis of a galactosylated CS (Gal-CS) and an antennary type of galactosylated CS with multiple galactose residues (Gal-m-CS), respectively. The Gal-CS was prepared by directly coupling LA on CS. To prepare Gal-m-CS, the branched lysine spacers were first synthesized and then conjugated onto CS. Subsequently, LA was introduced to the branched lysine spacers.

The synthesized galactosylated CS (Gal-CS) and an antennary type of galactosylated CS with multiple galactose residues (Gal-m-CS) were characterized by ^{1}H NMR spectra (Figure 3). The signal observed at 2.0 ppm corresponded to the hydrogens of the methyl moieties of the acetamide groups on CS, while the signal at 3.1 ppm was the hydrogen bonded to the C2 of the glucosamine ring. The signals between 3.3 and 3.9 ppm were the hydrogens on the carbon atoms C3, C4, C5, and C6 of CS. The new peaks that appeared in the range $3.9 < \delta < 4.4$ ppm were due to the hydrogens bonded to the introduced galactose residues (3.9−4.2 ppm), and the chemical shift of CHCONH (4.4 ppm) was due to condensation of the amine group on CS and the carboxylic acid on lysine or LA. The degrees of substitution of LA coupled with Gal-CS and Gal-m-CS were calculated by comparing the characteristic peaks of LA groups (∼4.1 ppm) with the 2.0 ppm peak attributed to the original acetamide group of CS.[22,30] The degrees of substitution of LA coupled with CS in Gal-CS and Gal-m-CS were 9.4 and 8.8 mol %, respectively. When compared to the intensity of Gal-CS (0.44), Gal-m-CS (5.65) demonstrated a high intensity of CHCONH signal at 4.4 ppm, suggesting that the branched galactose residues were successfully introduced to the CS backbone.

The size distributions and zeta potential values of the NPs formed by iontropic gelation of CS (CS NPs) or galactosylated

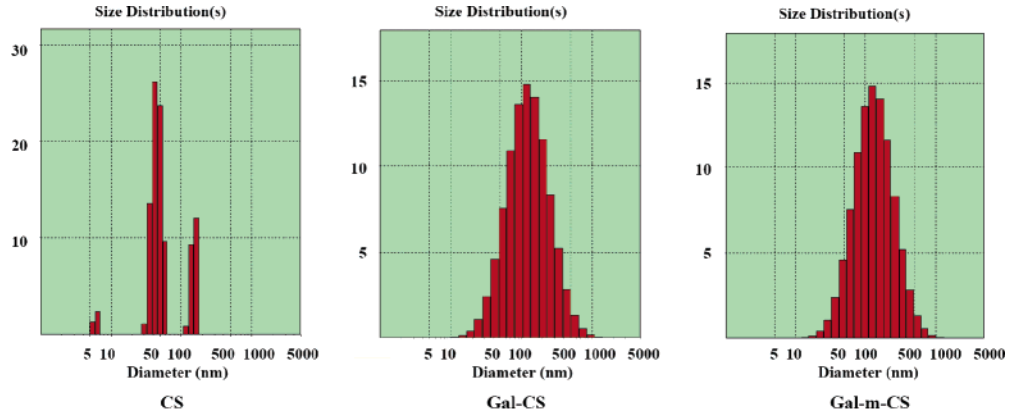

Figure 4. Representative particle size distributions of the CS, Gal-CS, and Gal-m-CS nanoparticles. CS NPs, the nanoparticles prepared by the iontropic gelation of chitosan with TPP; Gal-CS NPs, the nanoparticles prepared by the iontropic gelation of galactosylated chitosan with TPP; Gal-m-CS NPs, the nanoparticles prepared by the iontropic gelation of galactosylated chitosan with multiple galactose residues with TPP.

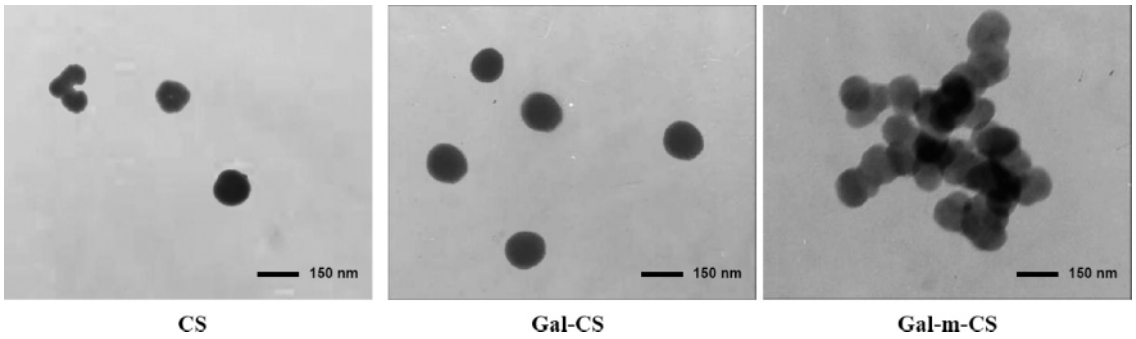

Figure 5. TEM micrographs of the CS, Gal-CS, and Gal-m-CS nanoparticles. CS NPs, the nanoparticles prepared by the iontropic gelation of chitosan with TPP; Gal-CS NPs, the nanoparticles prepared by the iontropic gelation of galactosylated chitosan with TPP; Gal-m-CS NPs, the nanoparticles prepared by the iontropic gelation of galactosylated chitosan with multiple galactose residues with TPP.

CS (Gal-CS and Gal-m-CS NPs) and TPP were investigated by dynamic light scattering. Figure 4 shows representative size distributions of the prepared NPs, while Table 1 presents their mean particle sizes, polydispersity indices, and zeta potential values. As shown in Figure 4, there were three peaks (~8.0, 60.0, and 150.0 nm) observed in the size distribution of the CS NPs. A similar phenomenon was also observed in the studies of Kumacheva et al.[25] and Janes and Alonso.[32] It is speculated that the smaller NPs were obtained from the iontropic gelation of lower molecular-weight CS with TPP; that is, shorter polymer chains readily entangled with TPP to produce smaller particles.

In contrast, the size distributions of the Gal-CS and Gal-m-CS NPs were monomodal, possibly because Gal-CS and Gal-m-CS were thoroughly dialyzed after their synthesis processes, which removed the lower molecular-weight CS derivatives. The mean particle sizes of the Gal-CS and Gal-m-CS NPs were significantly larger than that of the CS NPs. The increases in mean particle size for the Gal-CS and Gal-m-CS NPs may be due to the pendent galactose residues conjugated on CS that may affect the nucleation and growth of NPs with TPP.

The results obtained in the TEM examination showed that the morphology of the prepared NPs was spherical in shape (Figure 5). The diameter of NPs observed by TEM was generally smaller than that obtained by dynamic light scattering because NPs swelled in aqueous solution, while that observed by TEM was the diameter of dried NPs. Aggregation of the Gal-m-CS NPs was due to the removal of surface water from the TEM samples.

It was found that the CS NPs had a positive surface charge with a zeta potential value of 30 mV (Table 1) because of their protonated amino groups (NH_3^+) on CS. This may be an advantage for cellular uptake of the prepared NPs due to the electrostatic interaction between the positively charged NPs and the rather negatively charged surface of cells. However, some studies suggested that the positively charged carriers may inhibit the selective delivery of drug/gene to the liver's parenchymal cells, because their cationic nature may lead to a nonspecific binding to various cells after systemic administration.[33]

The zeta potential value of the Gal-CS NPs was significantly lower than that of the CS NPs, due to the consumption of some of the free amino groups on CS conjugated by the galactose residues. It was found that the Gal-m-CS NPs had the highest value in zeta potential among all studied groups. This is because some of the positively charged amino groups on the branched lysine spacers introduced on CS were still left unconjugated and thus increased the zeta potential value of the prepared NPs.

It is known that size distribution and zeta potential may play important roles in determining the fate of NPs after administration.[34] Hashida et al. reported that the majority of the fenestrate of the liver sinusoid is usually smaller than 200 nm in diameter.[33] Thus, large particles hardly reach the liver's parenchymal cells. Additionally, drug carriers with a diameter larger than 200 nm are readily scavenged nonspecifically by monocytes and the reticuloendothelial system.[35] It was reported that smaller particles tended to accumulate at the tumor sites due to the EPR effect, and a greater internalization was also observed.[36]

During storage, aggregation of NPs may occur and thus leads to precipitation of NPs.[37] In the study, no aggregation or precipitation of NPs in an aqueous environment was observed during storage for up to 1 month for all three studied groups, as a result of the electrostatic repulsion between the positively charged NPs. Figure 6 presents the particle sizes and zeta potential values of the Gal-CS and Gal-m-CS NPs during storage. No data were available for the CS NPs because their

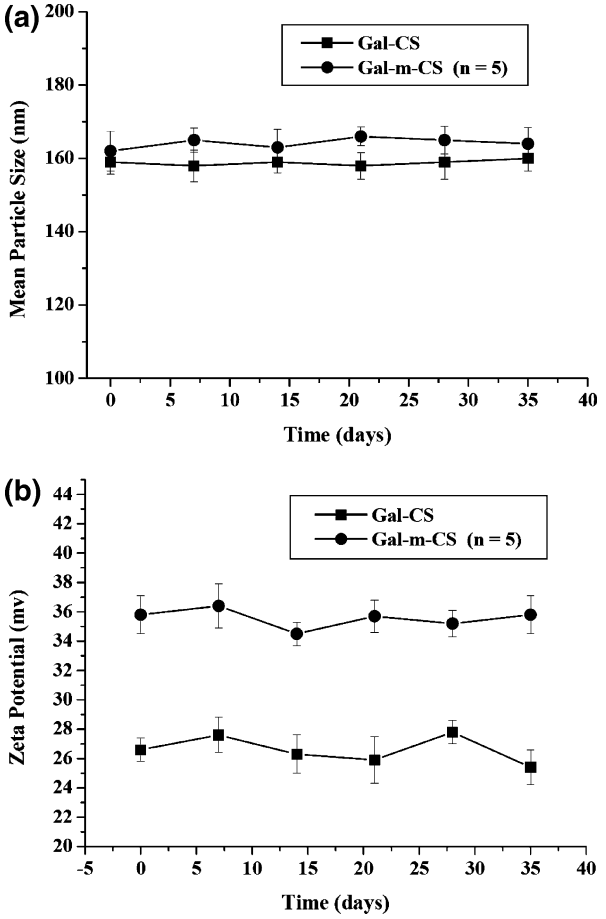

Figure 6. Changes in particle size and zeta potential of the Gal-CS and Gal-m-CS nanoparticles during storage. Gal-CS NPs, the nanoparticles prepared by the iontropic gelation of galactosylated chitosan with TPP; Gal-m-CS NPs, the nanoparticles prepared by the iontropic gelation of galactosylated chitosan with multiple galactose residues with TPP.

size distribution was not monomodal as mentioned earlier. As shown, changes in particle size and zeta potential of the Gal-CS and Gal-m-CS NPs throughout the entire course of the study were minimal. These results further demonstrated that the prepared NPs suspended in an aqueous environment were rather stable during storage.

Results of the differential interference contrast (DIC) and fluorescence images of HepG2 cells after incubation with the FITC-labeled CS, Gal-CS, or Gal-m-CS NPs at 37 °C for distinct durations are shown in Figure 7. As shown, little fluorescence was observed in HepG2 cells for the images taken at 30 min after incubation with the CS NPs. In contrast, with antennary galactose residues conjugated on the NPs (the Gal-m-CS NPs), the intensity of fluorescence observed in HepG2 cells increased significantly. These results indicated that the NPs possessing galactose residues on their surfaces had a specific interaction with HepG2 cells via the ligand−receptor (ASGP)-mediated recognition, leading to a high affinity to HepG2 cells.

Hepatocytes are known to recognize galactose- and *N*-acetylgalactosamine-terminated glycoproteins via the ASGP receptors located on their surfaces.[38] Once a ligand binds to the ASGP receptor, the ligand−receptor complex is rapidly internalized by hepatocytes, and the receptor recycles back to the surface of hepatocytes.[6] It was reported that the ASGP receptors are also abundantly expressed on the surfaces of various hepatoma cell lines.[6]

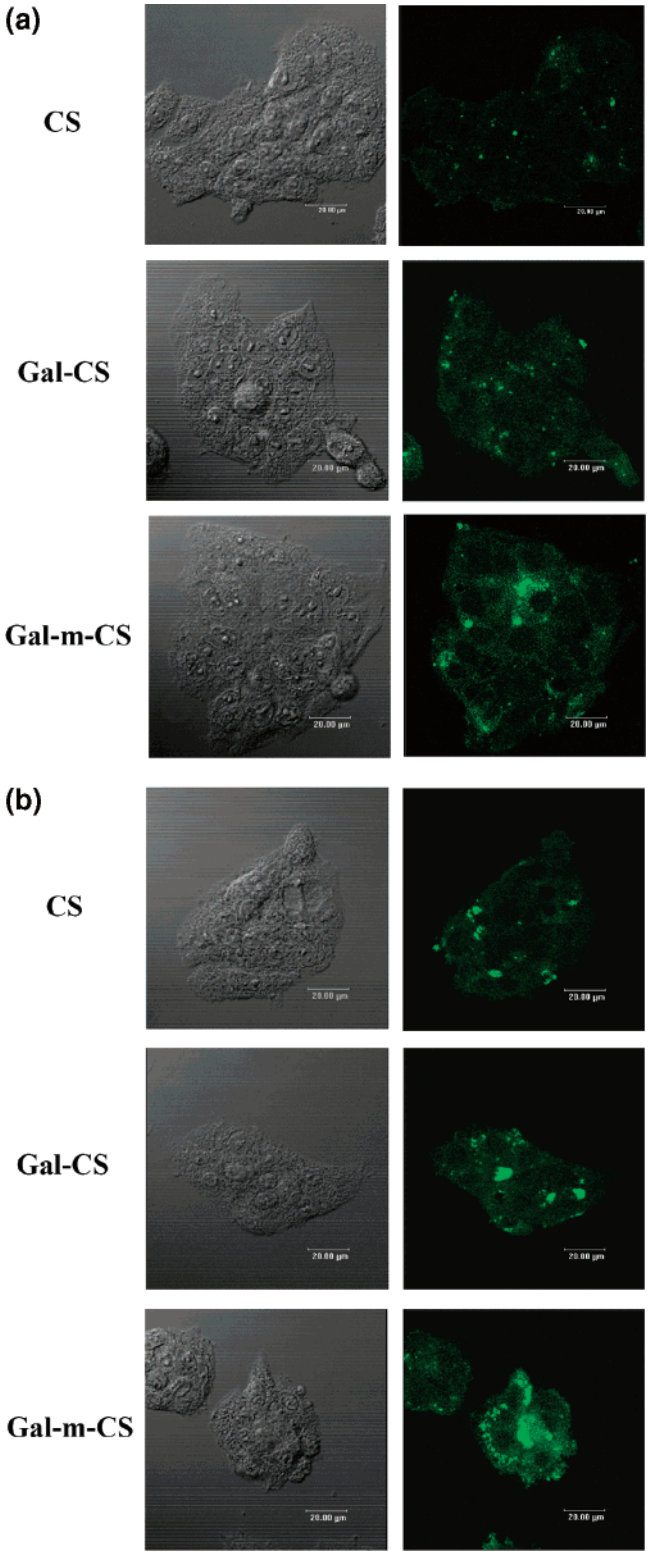

Figure 7. Differential interference contrast (DIC) and fluorescence images of HepG2 cells after incubation with the CS, Gal-CS, or Gal-m-CS NPs for (a) 30 min and (b) 120 min. CS NPs, the nanoparticles prepared by the iontropic gelation of chitosan with TPP; Gal-CS NPs, the nanoparticles prepared by the iontropic gelation of galactosylated chitosan with TPP; Gal-m-CS NPs, the nanoparticles prepared by the iontropic gelation of galactosylated chitosan with multiple galactose residues with TPP.

The intensity of fluorescence observed in HepG2 cells incubated with the Gal-m-CS NPs appeared to be stronger than that incubated with the CS NPs. This indicated that the Gal-m-CS NPs with multiple galactose residues in an antennary

fashion had a higher affinity to the ASGP receptors on HepG2 cells than the CS NPs, possibly due the "cluster effect" of multiple galactose residues present on their surfaces.[14,27] With increasing incubation time (120 min), the fluorescence intensity observed in HepG2 cells increased significantly for all three studied groups.

4. Conclusions

The aforementioned results indicated that the branched galactose residues were successfully introduced to the CS backbone. The NPs conjugated with multiple galactose residues in an antennary fashion (the Gal-m-CS NPs) suspended in an aqueous environment were rather stable during storage. It was demonstrated that the Gal-m-CS NPs had a high affinity to HepG2 cells. Therefore, the prepared Gal-m-CS NPs may be a novel galactosylated carrier for specific liver-targeting drug/gene delivery.

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