Synthesis, Characterization, and Microsphere Formation of Galactosylated Chitosan

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ABSTRACT: Chitosan derivative with galactose groups, which was recognized specifically by the asialoglycoprotein receptor (ASGR), was synthesized by introduction of the galactose group into the amino group of chitosan. The chemical structure of galactosylated chitosan was characterized by FT-IR, ^{1}H-NMR, ^{13}C-NMR, WAXD, and DSC techniques. The results indicated that although acyl reaction on the part of amino groups of chitosan took place, the degree of galactosylated substitution was 20%, and the crystallization, solubility, stability, and other physical properties were different from chitosan. Microspheres of chitosan and galactosylated chitosan were prepared by the physical precipitation and coacervation method with sodium sulfate, respectively. The characterizations of microspheres were determined by means of scanning electron microscopy (SEM), particle size/ζ potential analysis, and DSC methods. Spherical, positively charged chitosan and galactosylated chitosan microspheres were formed, with an average diameter of 0.54 and 1.05 μm, and average ζ potential of +17 mV and +15 mV, respectively. The novel galactosylated chitosan microspheres may be used as a potential drug delivery system with passive and active hepatic targeting properties. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 91: 659–665, 2004

Key words: polysaccharides; FTIR; NMR; morphology; TEM

INTRODUCTION

Recently, there have been many studies of targeting liver systems using methods such as passive trapping of microparticles by reticuloendothelium[1] or active targeting based on hepatic receptor recognition.[2] It is important that the passive targeting to the liver be related to particle diameter.[3] Alternatively, active targeting systems of receptor recognition can be attained by using the molecules with receptor trapping specific ligands. The galactose moiety is recognized specifically by the asialoglycoprotein receptor (ASGR), which is localized on the liver parenchymal cells, so it has been utilized as a useful site for liver targeting by many researchers.[4–7] The macromolecules with galactose moiety and drug conjugates have been examined as useful liver-specific drug carriers.[8–13] For example, galactose moieties were introduced into serum albumin,[4] *N*-(2-hydroxy-propyl)methacrylamide copolymer,[5,7] asialofetuin,[6] and poly-L-glutamic acid.[8]

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Chitosan is a naturally occurring polysaccharide with excellent biodegradable and biocompatible characteristics. Because of its unique polymeric cationic character and gel-forming properties, chitosan has been extensively examined in the pharmaceutical field for potential in the development of drug delivery systems.[14–19] Taking advantage of chitosan, a few lactosaminated or galactosylated derivatives that chemically conjugated with drugs have been reported. For example, lactosaminated N-succinyl-chitosan was utilized as a liver-specific drug carrier in mice.[20] Galactosylated chitosan-*graft*-poly(ethylene glycol) and galactosylated chitosan-*graft*-dextran as a hepatocyte-targeting DNA carrier had excellent specificity to liver cells.[21,22] There are few studies using galactosylated derivatives of chitosan as targeting microparticle materials. However, the microparticles system composed of galactosylated chitosan may possess both passive and active targeting properties to liver cells.[23–25]

In this article, galactosylated chitosan was synthesized by introduction of the galactose group into part of amino groups of chitosan so the residual amino groups of chitosan would be responsible for the positive charges. The chemical structure of modified chitosan was confirmed by FT-IR, ^{1}H-NMR, ^{13}C-NMR, WAXD, and DSC techniques. On the basis of physical interaction of opposed charges, the physical cross-linking microspheres of chitosan and galactosylated

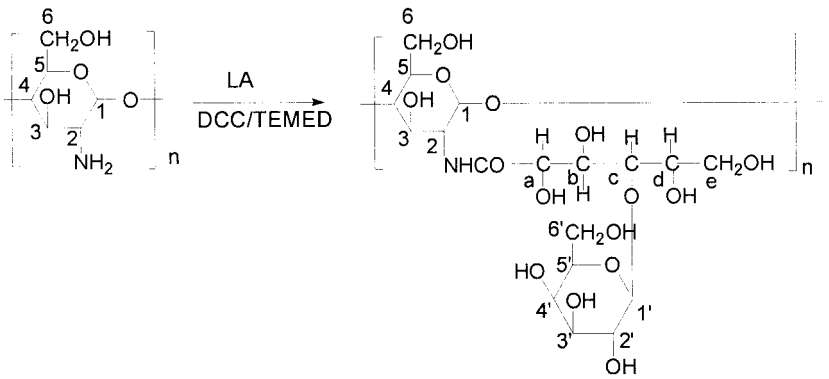

Figure 1 Synthesis route of galactosylated chitosan.

chitosan were prepared by precipitation/coacervation method with sodium sulfate. The microspheres were further characterized by scanning electron microscopy (SEM), particle size/ζ potential analysis, and DSC methods.

EXPERIMENT

Materials

Chitosan was provided by Nantong Suanglin Biochemical Co. Ltd (China) and has a degree of deacetylation of 91.5° and viscosity average molecular weight of 25000D. N, N, N_9, N_9-tetramethylethylenediamine (TEMED) and lactobionic acid (LA) were purchased from Acros (Fairlawn, NJ). N, N′-dicyclohexylcarbodiimide (DCC) was obtained from Shanghai Chemical Regent Company (China). All other chemical solvents and reagents were used without further purification.

Measurements

^{1}H-NMR and ^{13}C-NMR spectra were performed on a Bruker (AVACE) AV-500, AV-300 spectrometer. The sample was dissolved in the mixed solvent D_2O and F_3CCOOD. Presaturation (zgpr) pulse for suppressing water was used in ^{1}H-NMR. Infrared (IR) spectra were recorded on a Fourier-transform infrared spectrometer (Nicolet 2000) in KBr discs. X-ray diffraction spectrometry was obtained by using XD-3A powder diffraction meter with CuKα radiation in the range 5 to 40° (2θ) at 40 kV and 30 mA. The results of DSC were obtained with NETZSCH DSC 204 equipment in a nitrogen atmosphere at a heating rate of 10 °C/min. The shapes and surface characteristics of the microspheres were examined by SEM (SX-40, Japan) at 20 kV. The samples for SEM analysis were mounted on metal grids using double-sided adhesive tape and coated with gold under vacuum before observation.

Particle size analysis of the microspheres was measured by a Zetasizer 2000 instrument (Malvern Instruments, Malvern, UK) with 633 nm He-Ne lasers at 25 °C. The microspheres were dispersed in an aqueous medium and particle size was analyzed after dispersion of the sample.

The ζ potential of both the chitosan and modified chitosan microspheres was measured in distilled water, using a Malvern 3000 HS zetasizer (Malvern Instruments, Malvern, UK).

Galactosylated chitosan synthesis

Galactosylated chitosan was synthesized by the method that Y.K. Park et al. reported.[21,22]

Chitosan (1 g, 6.2 mM) was dissolved in 120 ml of 2% hydrochloric acid aqueous solution. Then the mixed solution of 3.3 g (9.2 mM) of LA in 5 ml water and 1.5 g (7.3 mM) of DCC in 5 ml of TEMED was dropped into the chitosan solution and stirred for 72 h at room temperature. The reaction solution was filtered, and the filtered solution was dialyzed (MWCO 10000) against distilled water for 5 days and then lyophilized to give 0.3 g of galactosylated chitosan.

Galactosylated chitosan microsphere preparation

The microspheres were prepared according to the method reported by Berthold et al.[23] In brief, a 0.25% (w/v) galactosylated chitosan solution was prepared in a mixture of 2% (v/v) acetic acid and 1% (w/v) Tween 80. Then 0.4 ml of 20% (w/v) sodium sulfate was added drop by drop (about 5 min) to 50 ml galactosylated chitosan solution under mechanical stirring and continuous sonication. After complete addition of the sodium sulfate, stirring and sonication were continued for 1 h. The suspension solution formed was subsequently centrifuged for 15 min (3000 rpm). The precipitate was washed twice using distilled water and then freeze-dried.

Chitosan microspheres were prepared under similar reaction conditions.

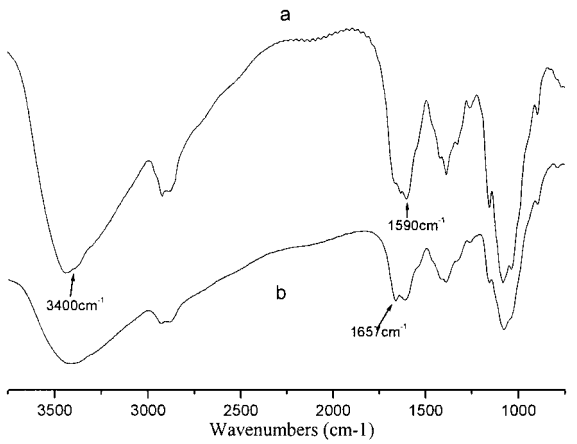

Figure 2 IR spectra of (a) chitosan and (b) galactosylated chitosan.

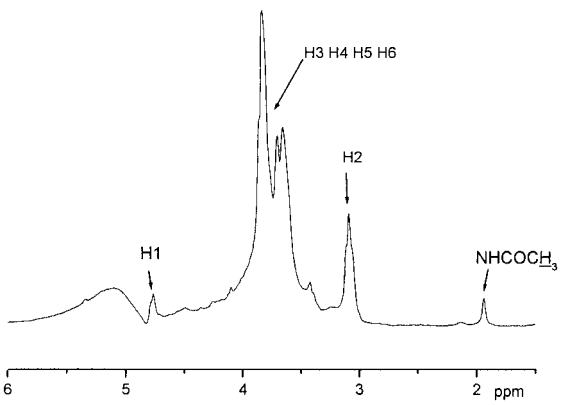

Figure 3 ^{1}H-NMR spectra of chitosan.

Swelling studies of microspheres

Ten milligrams of chitosan and modified chitosan microspheres were placed in 10 ml of different solutions (distilled water and KH_2PO_4/NaOH buffer; pH = 7.4) and allowed to swell at 37 °C. The microspheres were removed, blotted with filter paper, and weighed at various time intervals. The changes in weight were measured as the microspheres swelled and the swelling ratio (SR) was then calculated from the formula:

$$SR = \frac{(W_e - W_0)}{W_0}$$

where W_0 is the initial weight of the dry microparticles and W_e is the weight of the swollen microparticles at time t. Each experiment was repeated three times and the average value ±S.D. was taken as the SR value (Fig.1).

RESULTS AND DISCUSSION

Characterization of galactosylated chitosan

The IR of chitosan and galactosylated chitosan is shown in Fig. 2. From the chitosan spectrum, it can be found that the distinctive absorption bands appear at 1662 cm^{-1} (amide I), 1598 cm^{-1} ($-NH_2$ bending), and 1380 cm^{-1} (amide III). The absorption bands at 1156 cm^{-1} (asymmetric stretching of the C—O—C bridge), 1075 , and 1033 cm^{-1} (skeletal vibration involving the C—O stretching) are characteristic of its saccharine structure.[27] Compared with chitosan, the IR spectrum of galactosylated chitosan showed that the new signals at 1657 cm^{-1} were assigned to the acylamino group, and the peak at 1590 cm^{-1} was attributed to the unreacted amino groups. The intensity of the peak at 3400 cm^{-1} was increased largely because of the increase of the hydroxyl groups. It indicated that the amino group of chitosan was partly substituted by the galactose group.

The ^{1}H-NMR spectra of original chitosan and modified chitosan are given in Figs. 3 and 4, respectively. The ^{1}H-NMR assignment of chitosan was as follows:[28–30] ^{1}H-NMR (D_2O/F_3CCOOD) δ = 4.76 (H1), δ = 3.09 (H2), δ = 3.43 ~ 3.81 (H3, H4, H5, H6), δ = 1.96 ($NHCOC\underline{H}_3$) ppm. Compared with chitosan, the ^{1}H-NMR (D_2O/F_3CCOOD) spectrum of galactosylated chitosan showed that the new signal at δ = 4.45 ppm was assigned to the proton of H1′ and Hc, and the others were assigned to δ = 4.76 (H1), δ = 3.4 ~ 4.4 (H3, H4, H5, H6, H2′, H3′, H4′, H5′, H6′, Ha, Hb, Hd, He), δ = 3.06 (H2), δ = 1.96 ($NHCOC\underline{H}_3$) ppm. The results indicated that the galactose groups were introduced into the structure of chitosan.

Figs. 5 and 6 showed the ^{13}C-NMR (D_2O/F_3CCOOD) spectra of chitosan and galactosylated chitosan. The signals at 97.5 (C1), δ = 76.5 (C4), δ = 75 (C5), δ = 70 (C3), δ = 60 (C6), and δ = 55.6 (C2) ppm of chitosan were detected.[28–30] Compared with chitosan, the chemical shift at δ = 168.2 ppm showed the carbon signal of carbonyl groups. The chemical shifts of the other carbons were attributed as follows: 97.6–97.35 (Ca, C1, C1′), δ = 80.5–68.5 (C4, C4′, C5, C5′, C3,

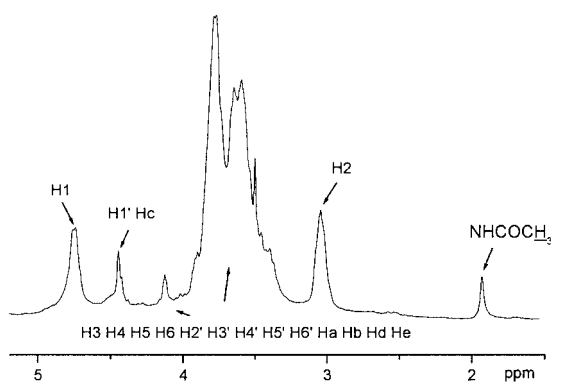

Figure 4 ^{1}H-NMR spectra of galactosylated chitosan.

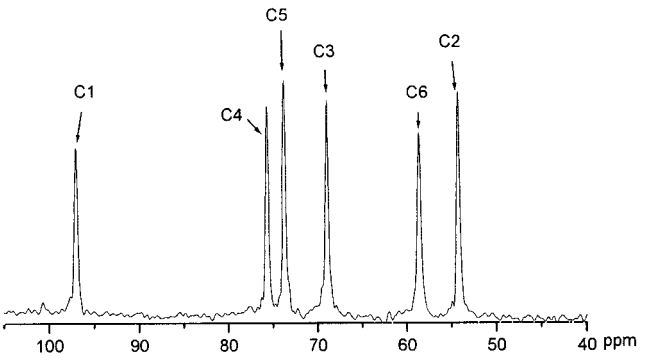

Figure 5 ^{13}C-NMR spectra of chitosan.

C3′, C2′, Cb, Cc, Cd), δ = 61.9–60.1 (C6, C6′, Ce), and δ = 55.7 (C2) ppm. The results evidenced that acyl reaction took place.

It was also observed from the ^{1}H-NMR spectra of chitosan and galactosylated chitosan that the degree of galactosylated substitution was about 20%.[31]

According to FTIR, ^{1}H-NMR, and ^{13}C-NMR, the chemical structure of galactosylated chitosan was established.

X-ray diffraction diagrams of chitosan and galactosylated chitosan (Fig. 7) show that chitosan exhibits three reflection falls at 2θ = 11°, 2θ = 20°, 2θ = 22°. Samuels et al. reported that the reflection fall at 2θ = 11° was assigned to crystal form I, and the strongest reflection appears at 2θ = 20°, which corresponds to crystal form II.[32] The WAXD pattern of galactosylated chitosan shows only one broad peak at around 2θ = 20°. It indicated that its ability to form hydrogen bonds within the ordering structure was decreased after chemical modification and galactosylated chitosan was amorphous.

DSC thermograms of chitosan and galactosylated chitosan are shown in Fig. 8. The spectrum of chitosan show a broad endothermic peak around 103.9 °C and a sharp exothermic peak at 322.6 °C. The former endothermic peak may be caused by the water vapor

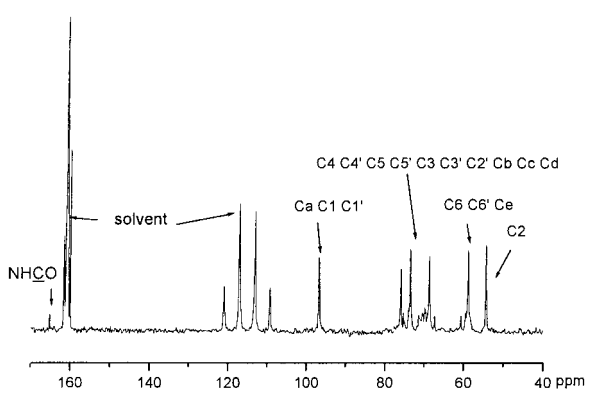

Figure 6 ^{13}C-NMR spectra of galactosylated chitosan.

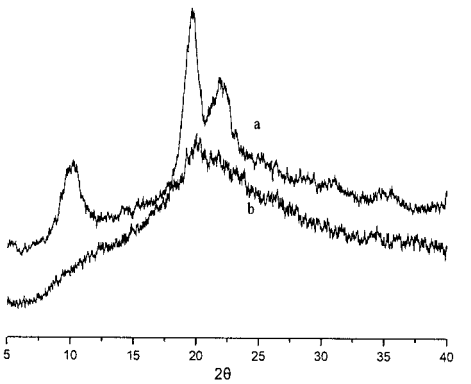

Figure 7 WAXD patterns of (a) chitosan, (b) galactosylated chitosan.

that the chitosan contained and some of the polymer with low molecular weight. The latter may be attributed to the decomposition of chitosan. The endothermic peak of galactosylated chitosan around 72.6 °C and 170.6 °C may be caused by the loss of water and moisture content of the polysaccharide, respectively. The broad exothermic peak at 311.5 °C corresponds to its thermal decomposition. The result indicated that the structure of chitosan chains has been changed by the introduction of the galactosylated group and the decreasing ability of crystalization.

Preparation of microspheres

There are many approaches to preparing chitosan microspheres, such as using the chemical cross-linking agent of glutaraldehyde combined with emulsion technique,[33–35] emulsion solvent evaporation technique,[36] and spray drying.[37] Berthold's method of using complexation between oppositely charged macro-

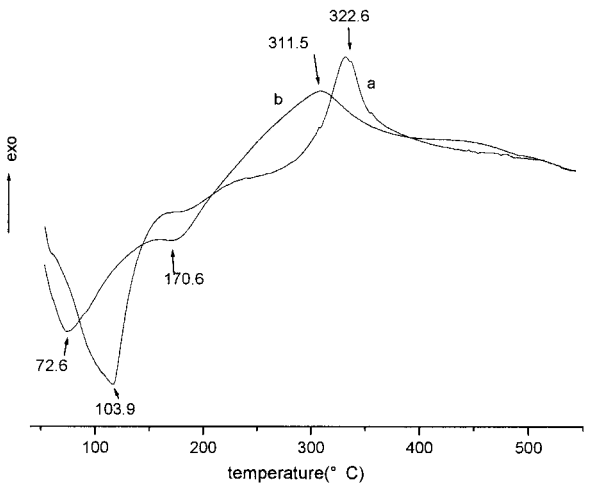

Figure 8 DSC thermograms of (a) chitosan and (b) galactosylated chitosan.

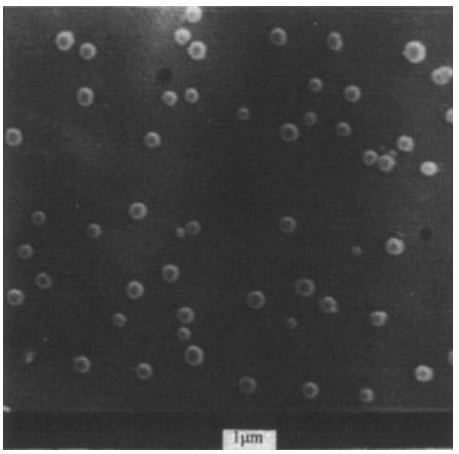

Figure 9 SEM of chitosan microspheres (×10,000).

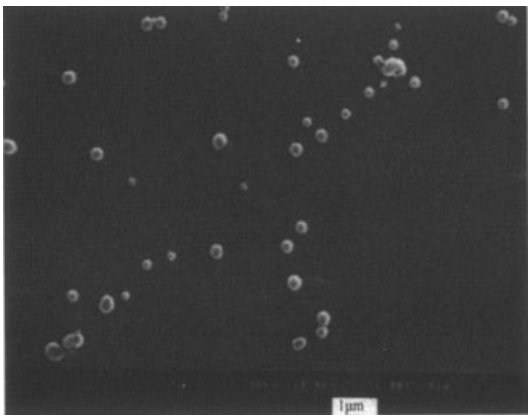

Figure 10 SEM of galactosylated chitosan microspheres (×10,000).

molecules to prepare chitosan microspheres showed good antigen binding and controlled drug release capacities.[26,38] The physical method has attracted much attention because this process is very simple and mild.[39–42] In addition, the reversible physical cross-linking by electrostatic interaction instead of chemical cross-linking avoids the possible toxicity of reagents and other undesirable effects. In the paper, Berthold's method has been successfully applied in chitosan derivative microspheres. The microspheres of galactosylated chitosan were formed by ionic interaction between positively charged amino groups of galactosylated chitosan and negatively charged counter ions of sodium sulfate. Although part of the amino groups of chitosan was substituted by the galactose group, the residual amino groups of galactosylated chitosan would be responsible for the positive charges.

Characterization of microspheres

SEM of microspheres

A typical scanning electron micrograph of chitosan and galactosylated chitosan microspheres is shown in Figs. 9 and 10, respectively. The microspheres are spherical and regular. The surface of the microspheres is smooth.

DSC of microspheres

Fig.11 shows the DSC thermoanalysis of chitosan and galactosylated chitosan microspheres. Both showed three endothermic peaks similarly, but they were significantly different from the starting materials. The endothermic peak of galactosylated chitosan around 95.1 °C is associated with the loss of water, and the peaks around 239.9 °C and 280 °C are ascribed to a complex process including dehydration of the saccharide rings, depolymerization of acetylated and deacetylated units and the galactose group of the polymer. It indicated that the degradation temperature of cross-linking galactosylated chitosan was increased after forming microspheres compared with galactosylated chitosan.

Particle size and ζ potential analysis

The size of the chitosan and galactosylated chitosan microspheres was found to be 0.54 μm and 1.05 μm, respectively. The change of precipitation agent concentration and adding velocity may adjust the size of microspheres but did not influence their shapes.

The average ζ potential of chitosan and galactosylated chitosan microspheres was +17 mV and +15 mV, respectively. Both the chitosan and galactosylated chitosan microspheres were charged positively, and the average ζ potential of chitosan microspheres is higher than that of galactosylated chitosan microspheres. Because part of the amino groups of chitosan was substituted by the galactose group, the amount of the free amino groups of galactosylated chitosan is smaller than that of chitosan. The residual amino

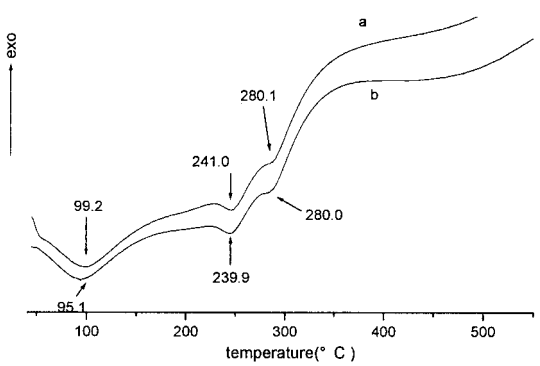

Figure 11 DSC thermograms of (a) chitosan microspheres and (b) galactosylated chitosan microspheres.

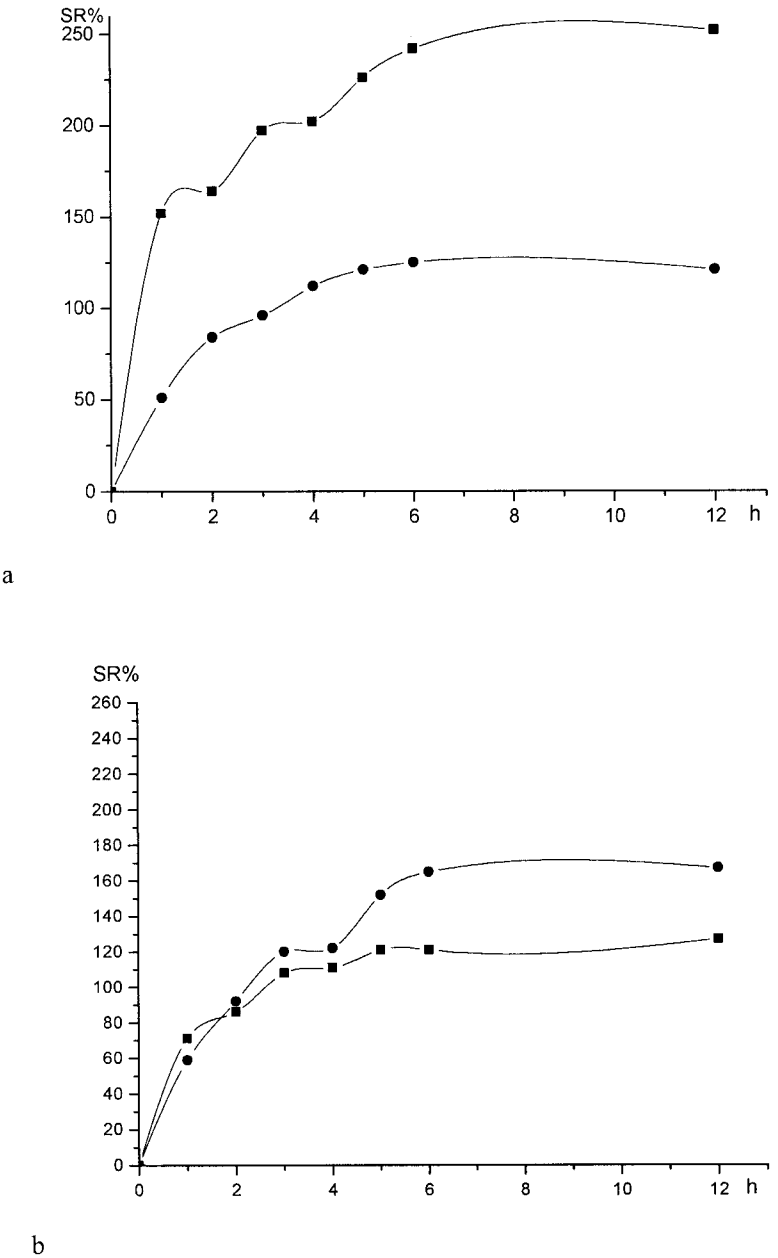

Figure 12 The effect of the swelling media on the swelling ratio of (a) chitosan microspheres and (b)galactosylated chitosan microspheres; ■ = distilled water, ● = PBS (pH = 7.4); data points = mean ± S.D., n = 3.

groups of galactosylated chitosan would be responsible for the positive ζ potential.

Swelling ratio of chitosan and galactosylated chitosan microspheres

Chitosan and galactosylated chitosan microspheres are well swollen in aqueous media over a 6- to 12-h time period (Fig. 12) without an appreciable gain in volume.

CONCLUSIONS

Chitosan derivative containing galactose moiety, which was recognized specifically by the ASGR, was synthesized by introduction of the galactose group into the amino group of chitosan. The structure of galactosylated chitosan was characterized by FT-IR, ^{1}H-NMR, ^{13}C NMR, WAXD, and DSC techniques. Microspheres of chitosan and galactosylated chitosan were prepared by the precipitation/coacervation method based on physical electrostatic interaction. In the paper, Berthold's method was successfully applied in chitosan derivative microspheres, although the galactosylated polymer differs from chitosan in solubility and crystallization. The microspheres were characterized by SEM, particle size/ζ potential analysis, and DSC methods. The average particle size of chitosan and galactosylated chitosan microspheres is

around 0.54 and 1.05 μm, respectively. Both the chitosan and galactosylated chitosan microspheres were charged positively. The novel galactosylated chitosan microspheres may be used as a potential drug carrier with passive and active hepatic targeting.

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