Release of Indomethacin from a Novel Chitosan Microsphere Prepared by a Naturally Occurring Crosslinker: Examination of Crosslinking and Polycation–Anionic Drug Interaction

FWU-LONG MI,[1] HSING-WEN SUNG,[2] SHIN-SHING SHYU[3]

[1] Division of Chemistry, Department of Mathematics, Physics, and Chemistry, Chinese Naval Academy, 669 Jiun Shiaw Road, Kaohsiung 813, Taiwan, Republic of China

[2] Department of Chemical Engineering, National Tsing Hua University, Hsinchu 300, Taiwan, Republic of China

[3] Laboratory of Polymer Materials Research, Department of Chemical Engineering, National Central University, Chung-Li 320, Taiwan, Republic of China

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ABSTRACT: Novel chitosan microspheres with lower cytotoxicity were fabricated in this study and their drug release characteristics were investigated. Genipin, a naturally occurring crosslinking reagent that has been used in herbal medicine and in the production of food dye, was used to prepare crosslinked chitosan microspheres by a water-in-oil dispersion method. The crosslinking mechanism examined by FTIR and ^{13}C–NMR suggests that the crosslinking of chitosan by genipin leads to the formation of secondary amide and heterocyclic amino linkage. The polycation–anionic drug interaction between chitosan and indomethacin was pH dependent and could affect the dissolution property of indomethacin. By examination of the release profiles of the crosslinked chitosan microsphere, it was found that the release of indomethacin from the microsphere was sustainable and influenced by factors such as crosslinking of microsphere and chitosan–indomethacin interaction, thus establishing crosslinked chitosan microsphere as a very promising polymeric carrier for drug release. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 81: 1700–1711, 2001

Key words: chitosan; microspheres; genipin; naturally occurring crosslinker

INTRODUCTION

Recently, a variety of synthetic or natural polymers have been reported to degrade in human.[1] Biodegradable and biocompatible polymers have become increasingly important in the development of implantable biomaterials and drug-delivery devices. These polymers can function as a matrix to control the diffusion of drug, followed by polymer biodegradation and elimination of the degradation products from the body. Biodegradable and biocompatible microspheres have been extensively investigated to use as possible drug carriers for drug targeting and controlled release.[2–6] Chitosan has proved to be exceptional among the enzymatically de-

Correspondence to: S.-S. Shyu.

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gradable polymers developed and evaluated for drug delivery usage.

Chitosan is a copolymer of glucosamine and *N*-acetylglucosamine, derived from the natural polymer chitin, which is commercially available. Chitosan has been reported to be a potentially useful pharmaceutical material because of its good biocompatibility and low toxicity.[7] Microencapsulated drugs such as theophylline, *cis*-platinum(II) diamine dichloride (CDDP), and 5-fluorouracil, among glutaraldehyde-crosslinked chitosan microspheres, were previously investigated.[8–12] The preparation of chitosan microspheres must include the use of a synthetic crosslinker such as diisocyanate or epoxy compound, especially for glutaraldehyde.[13,14] However, these synthetic crosslinking reagents are all cytotoxic, which may impair the biocompatibility of the microspheres. It is thus desirable to provide a crosslinking reagent suitable for use in biomedical applications, one that has low cytotoxicity and could form stable and biocompatible crosslinked products.

Genipin was obtained from its parent compound, geniposide, which may be isolated from the fruits of Gardenia jasminoides Ellis, as described in the literature.[15,16] In vitro evaluation of cytotoxicity of genipin for biological tissue fixation was investigated in a previous study.[17] It was also found that genipin might be about 5000 to 10,000 times less cytotoxic than glutaraldehyde. In the previous study of Sung et al.,[18] it was found that the mechanical strength and resistance against in vitro enzymatic degradation of genipin-fixed tissue was comparable to that of glutaraldehyde-fixed tissue. The result of this study prompted us to evaluate the possibility of using this crosslinking reagent to prepare a biocompatible microsphere for long-acting drug-delivery application.

A novel chitosan microsphere was fabricated in this study by interfacial crosslinking of the water-in-oil dispersed chitosan droplets using genipin. Chitosan microspheres prepared in this way have good sphericity. The acidic drug indomethacin was used as the model drug in this study. The objective of the present study was to examine the crosslinking characteristics of the chitosan microspheres and polycation–anionic drug interaction that dominated the drug-release behavior.

EXPERIMENTAL

Materials

Chitosan (M_w = 70,000) was purchased from Fluka Chemie (Buchs, Switzerland). Indomethacin was purchased from Sigma Chemical Co. (St. Louis, MO). Genipin was obtained from Challenge Bioproducts Co. (Taiwan). All other reagents and solvents used were of reagent grade.

Preparation of Chitosan Microspheres by Water-in-Oil Dispersion

Chitosan powder (3 g) was dispersed in 50 mL of water containing 0.5 wt % acetic acid. The polymer solution was prepared by mechanical stirring at 600 rpm for 3 h to dissolve the powder. The final solution was dispersed into 500 mL of soybean oil, without adding surfactant, in a l-L round-bottom flask at room temperature. The dispersion was stirred at 500 rpm with a mechanical stirrer (RAW 20; IKA) for 30 min to form a water-in-oil (w/o) dispersion, after which aqueous genipin (1 wt %) was slowly added to the medium and stirring continued for several hours. The solidified microspheres were blue color and collected by centrifugation at 3000 *g* for 5 min using a high-speed centrifuge (ZK 365; Hermle). After the upper layer was removed by decantation, the microspheres were rinsed twice with ethyl acetate then dried in air for 24 h. The final products were screened through standard sieves for particle size distribution analysis.

Characterization of Crosslinking Mechanism by FTIR and ^{13}C–NMR

The genipin-crosslinked chitosan microspheres were mixed with KBr. The mixed samples were pressed to disk. IR spectra of the crosslinked chitosan derivative were recorded as KBr pellets on a Perkin–Elmer 983 spectrometer (Perkin–Elmer Cetus Instruments, Norwalk, CT). The solid ^{13}C–NMR spectra of chitosan microspheres were recorded on an MSL-200 Bruker spectrometer (Bruker Instruments, Billerica, MA) equipped with a process controller.

Examination of Chitosan–Indomethacin Complex

Indomethacin and chitosan, in a weight ratio of 1 : 1, were placed in a mortar. The mixtures were kneaded with water (pH 4.0 and 6.5, respectively) for 3 h, then dried in vacuum at room temperature for 24 h. The dried sample was subjected to IR spectrum analysis.

Swelling Studies

The water-sorption capacities of crosslinked chitosan microspheres were determined by swelling

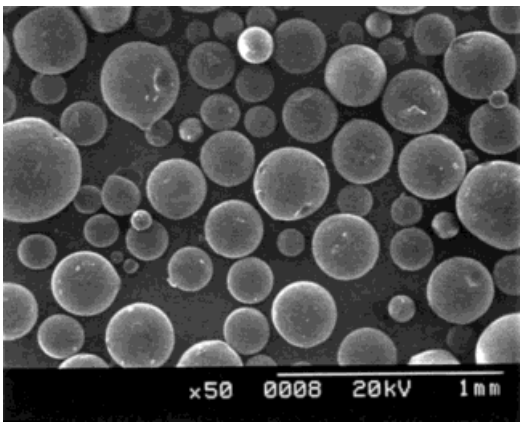


Figure 1 Morphology of genipin-crosslinked chitosan microspheres.

the crosslinked microspheres in distilled water at room temperature. Chitosan microspheres of known weight (200 mg) were placed in the media for the required period of time. The swollen chitosan microspheres were collected by centrifugation using a high-speed centrifuge (ZK 365; Hermle) and the wet weight of the chitosan microspheres was determined first by blotting the crosslinked microspheres with filter paper, to remove adsorbed water on the surface, then by weighing immediately on an electronic balance. The percentage swelling of crosslinked chitosan microspheres in the media was then calculated from the formula

$$E_{sw} = [(W_e - W_o)/W_o] \times 100$$

where E_{sw} is the percentage swelling of crosslinked chitosan microspheres at equilibrium. W_e denotes the weight of the crosslinked microspheres at equilibrium swelling and W_o is the initial weight of the crosslinked microspheres. Each swelling experiment was repeated three times and the average value was taken as the percentage swelling value.

Scanning Electron Microscopy (SEM)

The chitosan microspheres were sprinkled onto double-sided tape and sputter-coated with gold to about 500×10^{-8} cm thickness using a Hitachi coating unit IB-2 coater (Hitachi, Japan) under a high vacuum, 133.3 Pa, high voltage, 1.2 kV, and 50 mA. Coated samples were examined using a Hitachi S-2300 scanning electron microscope.

In Vitro Drug Release

Release of indomethacin from chitosan microspheres was measured by using the dissolution (Dissoette II; Hanson Research) and autosampling (SR6; Hanson Research) systems. The dissolution medium was 500 mL of distilled water. The medium was placed in a 1-L round-bottom flask fitted with a pump for autosampling, to remove the medium, and stirred with a mechanical stirrer at a rate of 100 rpm. The dissolution medium temperature was maintained at 37°C. An equivalent quantity of 100 mg chitosan microspheres was dispersed in the dissolution medium. After a predetermined period, 5 mL of the medium was removed and the amount of indomethacin was analyzed with a UV spectrophotometer at 320 nm. To maintain the original volume, each 5 mL of medium removed was replaced with fresh water.

Table I Particle Size Distributions of Genipin Crosslinked Chitosan Microspheres

Chitosan (wt %)	Stirring Rate (rpm)	Particle Size (μm)			
		<149	149–297	297–420	>420
2.0	500	19.9	48.8	16.5	14.8
	800	24.3	53.6	13.1	9.1
	1000	29.9	54.4	12.8	2.9
1.5	500	22.3	50.4	19.3	8.0
	800	25.7	53.9	14.8	5.6
	1000	28.1	58.7	7.3	5.9
1.0	500	31.5	46.7	11.2	10.6
	800	34.2	51.6	10.1	4.1
	1000	39.3	52.4	7.7	—

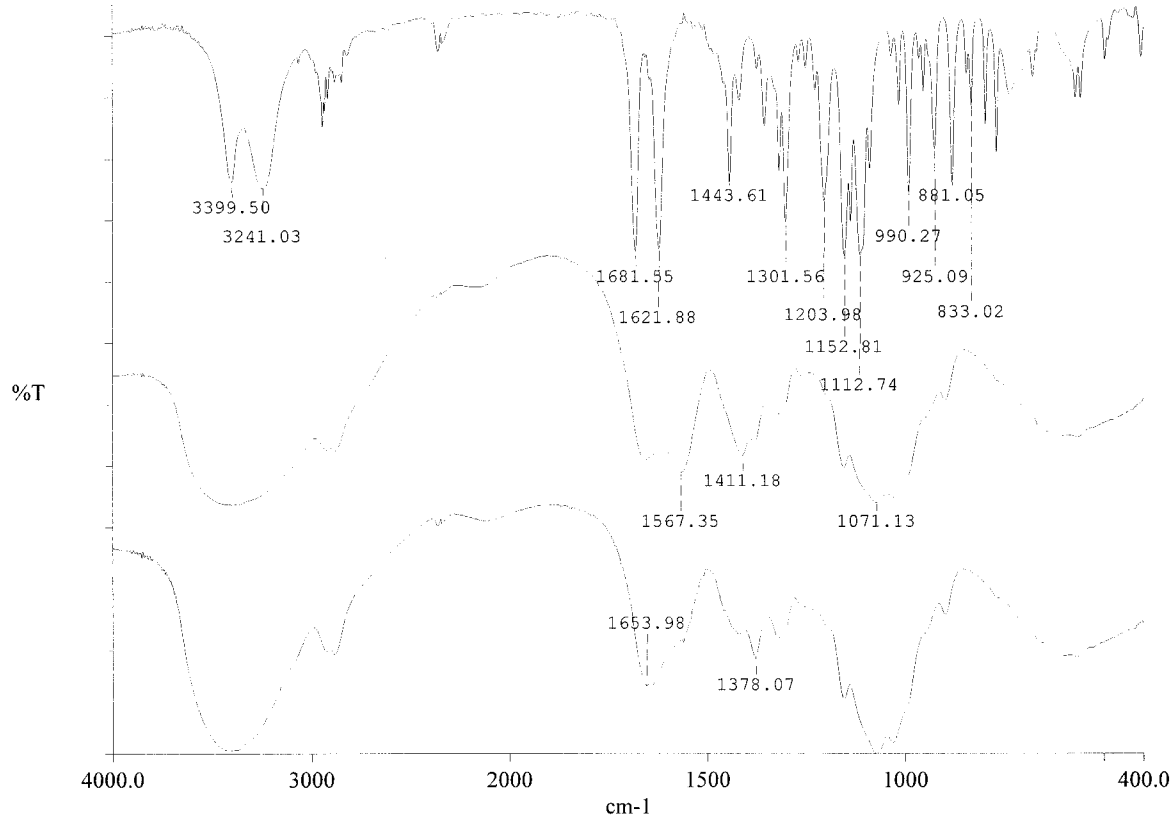


Figure 2 FTIR spectra analysis of genipin, chitosan, and crosslinked chitosan.

RESULTS AND DISCUSSION

Properties of Chitosan Microspheres

Chitosan solution and soybean oil could easily form stable water-in-oil dispersion only by stirring. Crosslinking with genipin is a slow reaction and leads to gradual formation of microspheres from the dispersed chitosan droplets. When 1 wt % of genipin was added to the chitosan/soybean oil dispersion, a substantial amount of genipin may be partitioned into the continuous phase. Therefore, slow and uniform crosslinking of the chitosan droplets occurred, thus fixing the shape and surface morphology of the microspheres, to generate granules of good sphericity (Fig. 1). Morphology of chitosan microspheres prepared by this interfacial crosslinking method showed a good sphericity and dense cross section. The size distribution of the genipin-crosslinked chitosan microspheres is shown in Table I. The particle sizes of the chitosan microspheres range mainly from 100 to 200 μm.

Characterization of Crosslinking Mechanism by FTIR and ^{13}C–NMR

In this study, crosslinking was observed as the color changed from transparent to blue color on treatment with genipin. The IR spectra of chitosan, together with that of genipin-crosslinked chitosan microspheres, are shown in Figure 2. The IR spectrum of chitosan shows peaks of assigned saccharide structure at around 905 and 1153 cm^{-1} and a strong protonated amino characteristic peak at around 1570 cm^{-1}. The stronger absorption band at 1649 cm^{-1} was characteristic of amide absorption. The absorption bands at 1270 and 1110 cm^{-1} could be attributed to the hydroxyl group on chitosan. Comparison of the IR spectrum of chitosan to IR spectra of genipin-crosslinked chitosan microspheres shows a pronounced difference. For genipin-crosslinked chitosan, the significantly increased adsorption at 1643 cm^{-1} and decreased adsorption at 1570 cm^{-1} could be attributed to the formation of secondary amide and the absorption of NH_2 group as

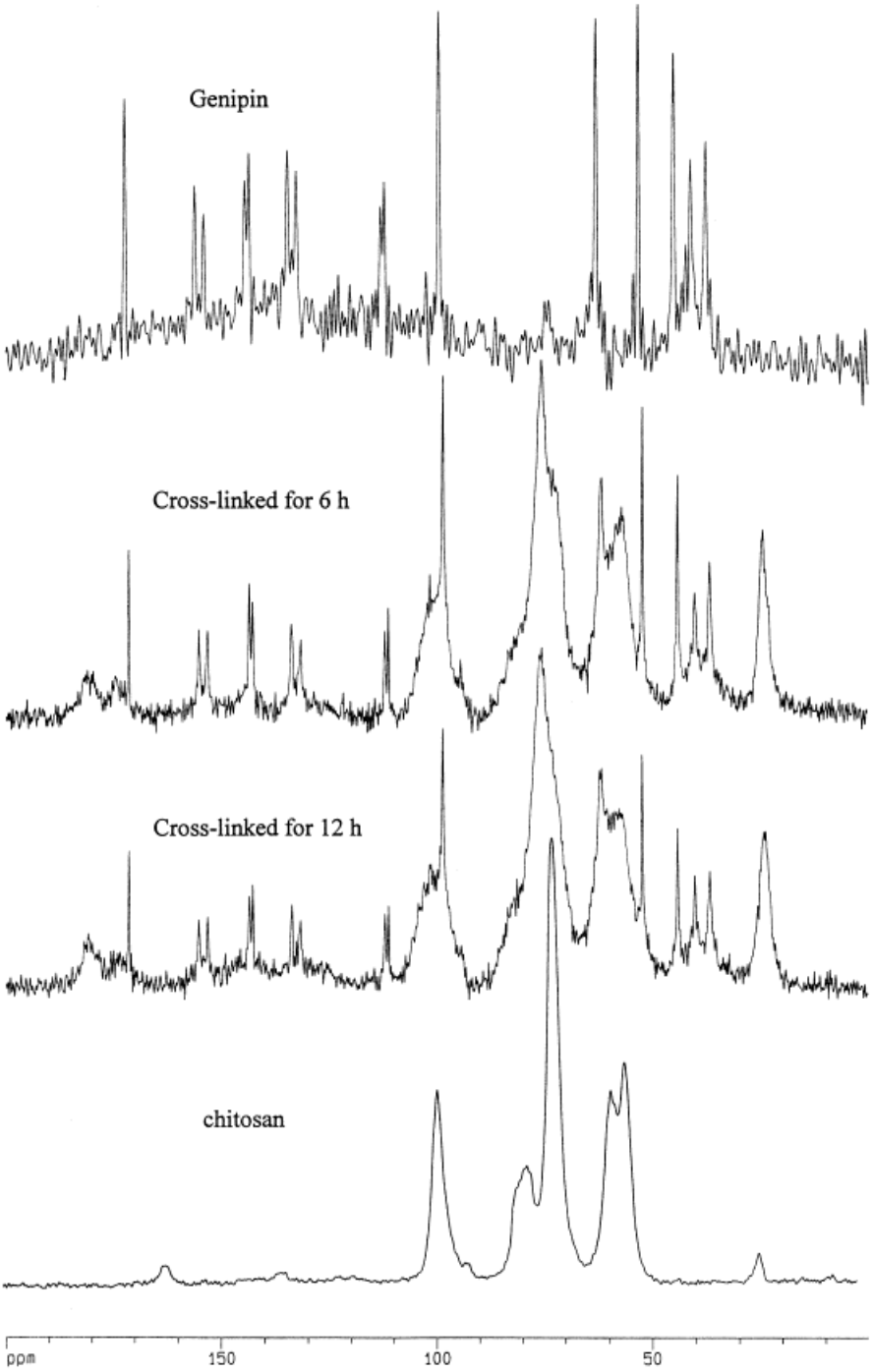


Figure 3 ^{13}C–NMR analysis of genipin, chitosan, and crosslinked chitosan.

a result of the reaction between amino groups on chitosan and ester groups of genipin.

The crosslinking mechanism was further examined by solid ^{13}C–NMR (Fig. 3). In the frequency range 155–160 ppm, the resonance attributed to C-3 of genipin is decreased after the crosslinking of chitosan by genipin, indicating a nucleophilic attack by amino group of chitosan on the olefinic carbon atom at C-3 of genipin, followed by opening of the dihydropyran ring. More-

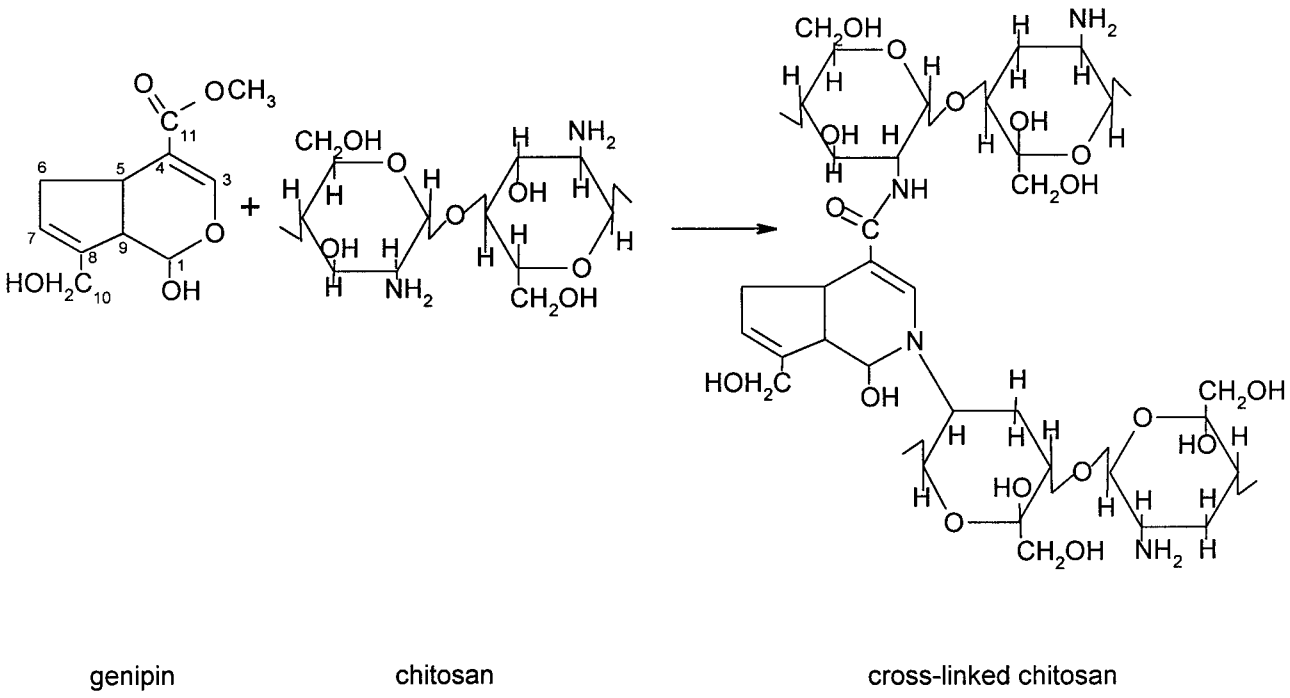


Figure 4 Reaction mechanism for the crosslinking of chitosan using genipin.

over, the decreased resonance attributed to C-6 at 62 ppm carbon atoms of chitosan revealed the transformation of the primary amino group in glucosamine unit into a crosslinked bridge, thus revealing that the reaction of chitosan by genipin leads to the formation of a heterocyclic amine. The resonance at 172 and 53 ppm attributed to C-11 and OCH_3 of genipin is also decreased after the crosslinking of chitosan. The resonance appearing at 180 ppm could be attributed to the

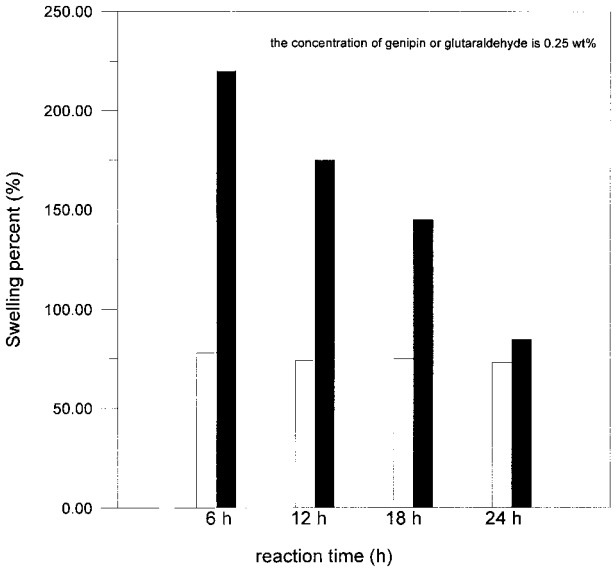


Figure 5 Swelling ability of genipin and glutaraldehyde-crosslinked chitosan microspheres: ●, genipin-crosslinked chitosan microspheres; ○, glutaraldehyde-crosslinked chitosan microspheres.

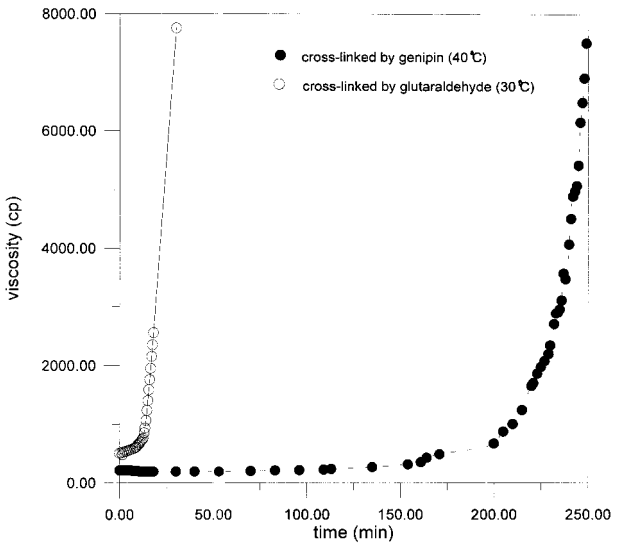
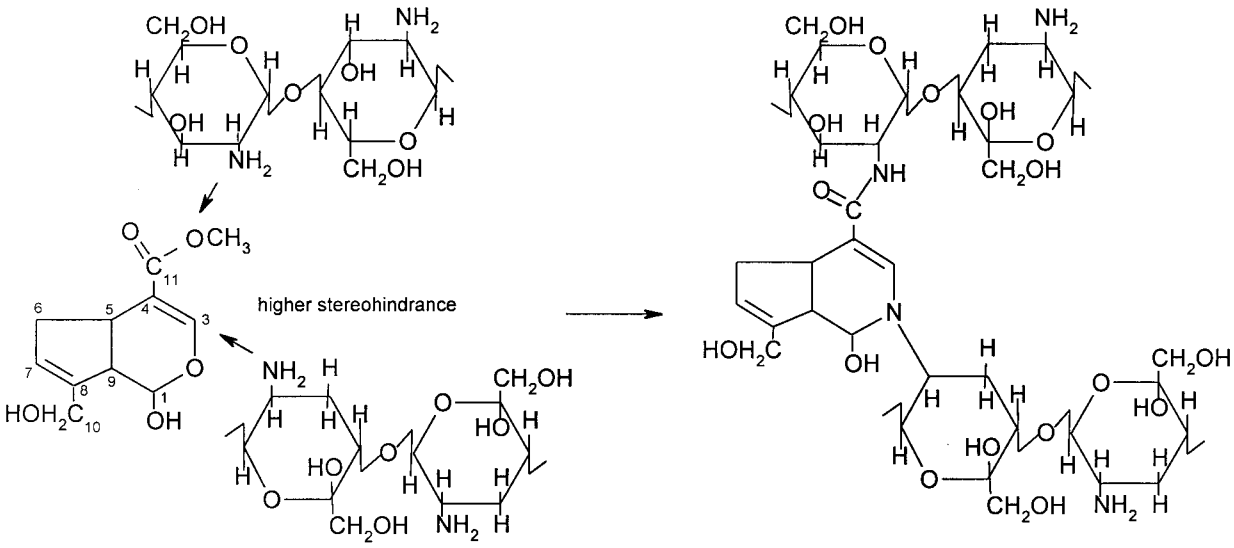
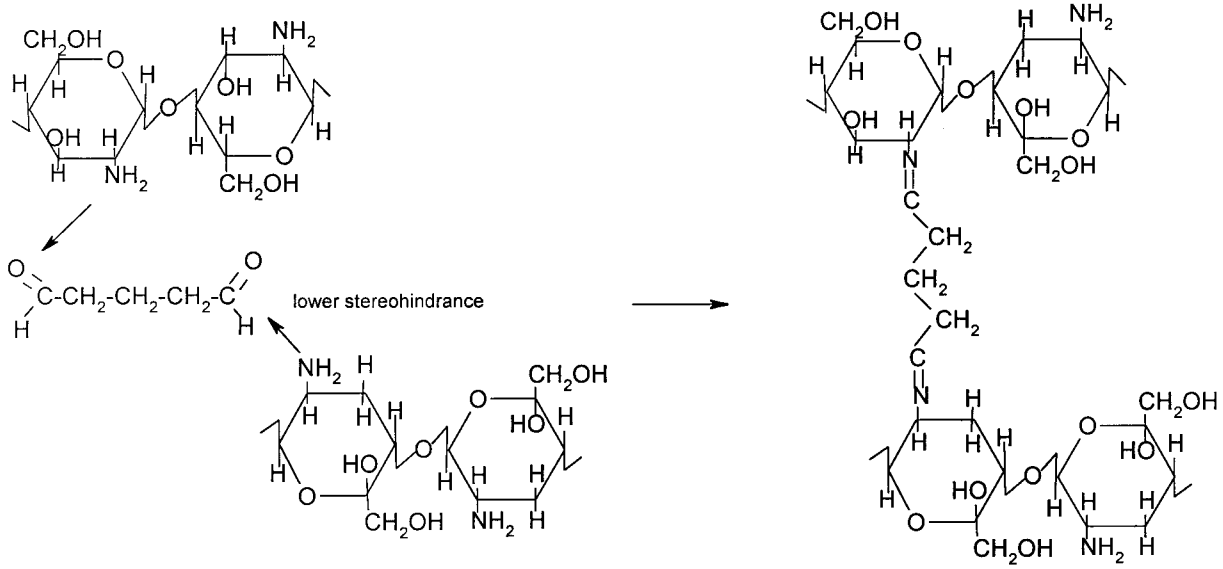


Figure 6 The viscosity changes in the chitosan solution during gelation: ●, crosslinked by genipin; ○, crosslinked by glutaraldehyde.



chitosan cross-linked by genipin



chitosan cross-linked by glutaraldehyde

Figure 7 The stereohindrance for bifunctional glutaraldehyde or genipin to link with chitosan.

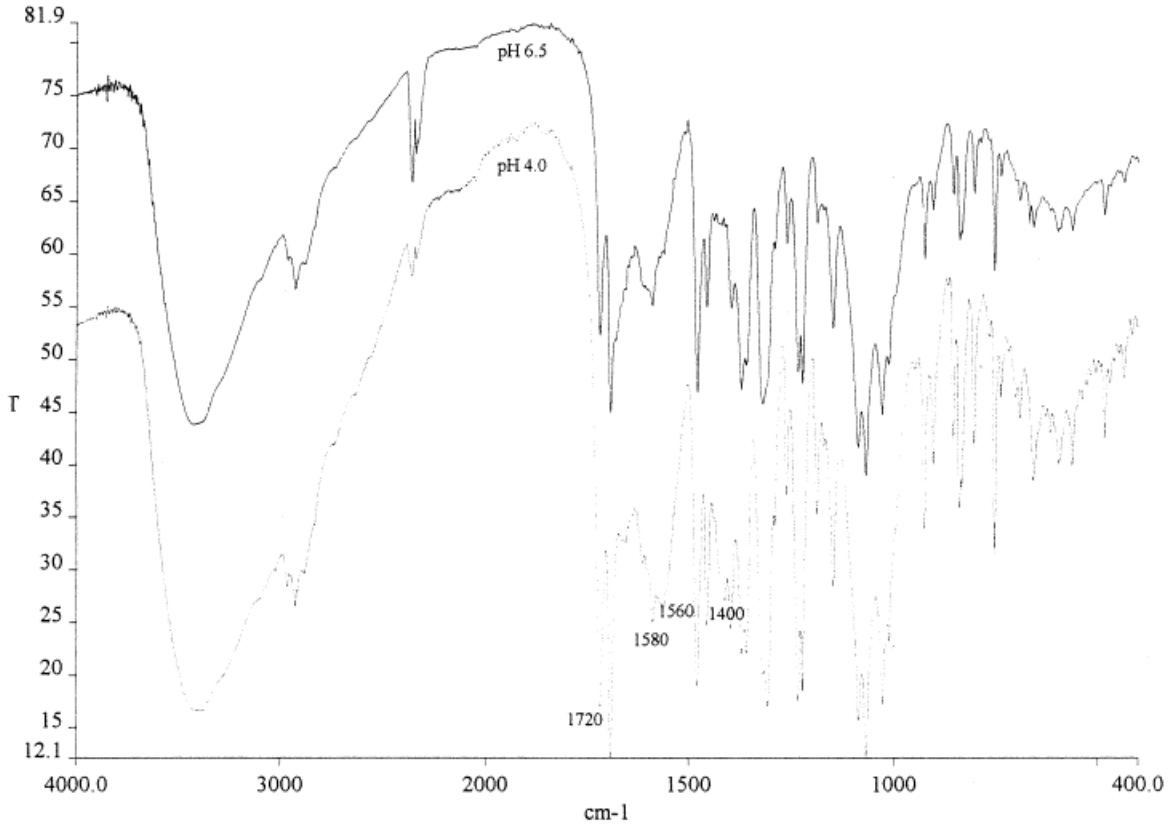


Figure 8 FTIR spectra analysis of chitosan–indomethacin interaction

formation of amide through the reaction of the amino group on chitosan with the ester group of genipin. Splittings of the peak observed in carbon resonance may be ascribed either to differences in the packing of the polymeric chains or to different internal torsion angles. It is well known that chitosan exhibits polymorphism, and the disappearance of the resonance attributed to C-4 at 81 ppm indicated that the conformation was transferred from the linear structure of original chitosan to crosslinked chitosan. This revealed the formation of a secondary amide and heterocyclic amino linkage, leading to the crosslinking of chitosan (Fig. 4).

Swelling Studies

A convenient examination of the hydrophilicity of the chitosan microspheres could be investigated by the swelling of the microspheres in aqueous media. Swelling studies of crosslinked microspheres were carried out in distilled water. Figure 5 shows the equilibrium swelling behavior of the chitosan microspheres, which demonstrates that the swelling ability of the crosslinked chitosan microspheres increased with the decrease of reaction time. The lower swelling ability of chitosan gel is attributed to the increased intermolecular or intramolecular linkage of the $-NH_2$ sites in chitosan, which could be achieved by a more complete crosslinking reaction. By maintaining the same reaction time for crosslinking and comparing the equilibrium swelling ratio of genipin-crosslinked chitosan microsphere to that of the traditional glutaraldehyde-crosslinked one, it was found that the genipin-crosslinked chitosan microsphere has the higher swelling ability. The result is ascribed to the slower gelation rate of genipin-crosslinked chitosan. By measuring the viscosity changes in the chitosan solution during gelation using a Brookfield viscometer, one can find that the viscosity of glutaraldehyde-crosslinked chitosan solution increased significantly faster than that of the genipin-crosslinked one (Fig. 6), thus suggesting a faster gelation rate

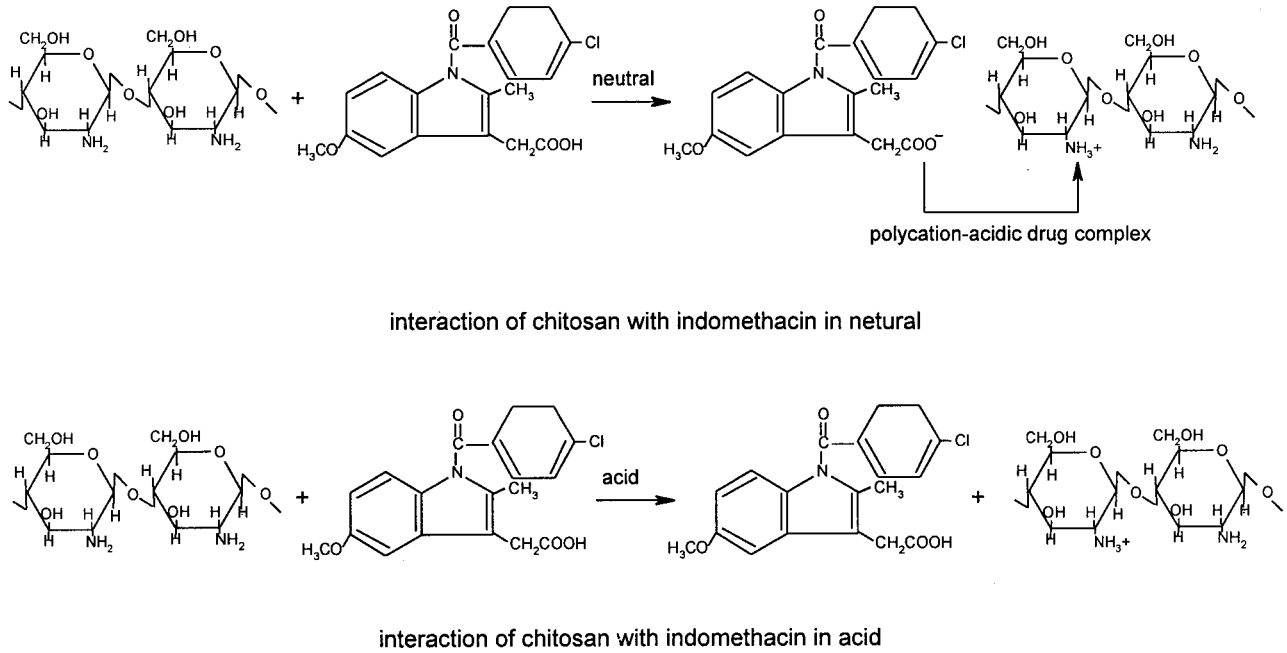


Figure 9 The scheme for the formation and inhibition of chitosan–indomethacin complex.

of the glutaraldehyde-crosslinked chitosan network. Figure 7 shows the interaction of chitosan with bifunctional glutaraldehyde or genipin to form a crosslinked network. The binding sites on genipin responsible for linking two amino groups of chitosan are much more hindered compared to those on the more flexible glutaraldehyde, which could decrease the reactivity of crosslinking because of the stereohindrance effect. Furthermore, the higher swelling in genipin-crosslinked microspheres may be attributed to the hydrogen bonding of water with –OH and $–CH_2OH$ groups on genipin crosslinks.

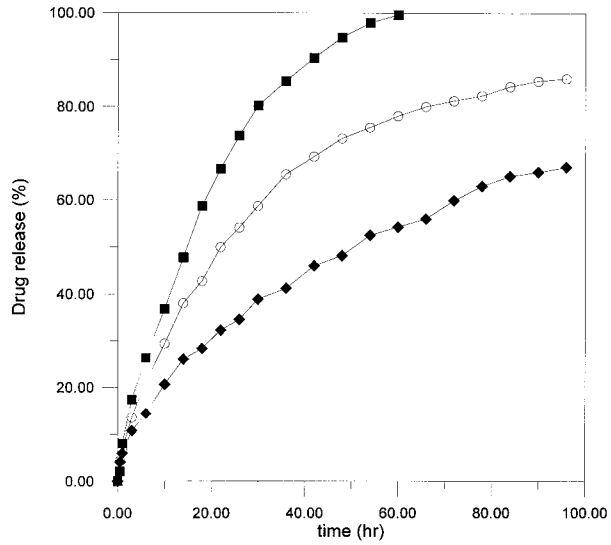


Figure 10 Release profiles of indomethacin from genipin-crosslinked chitosan microspheres. Preparative conditions: ◆, chitosan microspheres crosslinked for 24 h; ○, chitosan microspheres crosslinked for 18 h; ■, chitosan microspheres crosslinked for 12 h.

Examination of Polycation–Anionic Drug Interaction

Chitosan is a polyelectrolyte that is expected to interact with acidic drugs such as indomethacin.[19] The difference in the interaction mode of indomethacin with chitosan depends on the pH of chitosan. Figure 8 shows the IR spectra of chitosan–indomethacin systems. The IR spectrum of indomethacin shows two sharp peaks at 1715 and 1690 cm^{-1}, which are assigned to the carboxy carbonyl stretching and benzoyl carbonyl stretching bands, respectively. The carboxyl groups in indomethacin might be dissociated and associated with amino groups through the complexation with chitosan. As shown in Figure 8, the shift of the carboxy carbonyl stretching band in the complex at pH 6.5 may be explained by the association with the amino

Table II Regression Coefficient and Release Power *n* Value for Indomethacin Releasing from Chitosan Microspheres

Microsphere Type	Crosslinking Time (h)	Power *n* Value[a]	Regression Coefficient[a]
Chitosan microsphere	12	0.72	0.992
Chitosan microsphere	18	0.67	0.989
Chitosan microsphere	24	0.54	0.988

[a] The power n value and regression coefficient R^2 are derived from the release data calculated from Peppas's model: $M_t/M_\infty = kt^n$. M_t is the drug release at time t, and M_∞ is the total drug.

group in chitosan. The significantly increased adsorption at 1560 cm^{-1} and disappeared adsorption at 1720 cm^{-1} could be attributed to the ionization of carboxyl groups in indomethacin, and the protonation of amino group in chitosan attributed to complexation. This suggested that the binding between the amino group in chitosan and the carboxyl group in indomethacin played an important role in the complexation at this pH value. However, it was found that the IR spectra of the chitosan–indomethacin complex at pH 4.0 displayed both peaks at 1560 and 1720 cm^{-1}, indicating the presence of a protonated amino group and intact indomethacin in this pH value. The protonation of the amino group in chitosan and the deionization of the carboxyl groups in indomethacin in acidic medium inhibit the formation of a chitosan–indomethacin complex. The scheme for the formation and inhibition of the chitosan–indomethacin complex is shown in Figure 9.

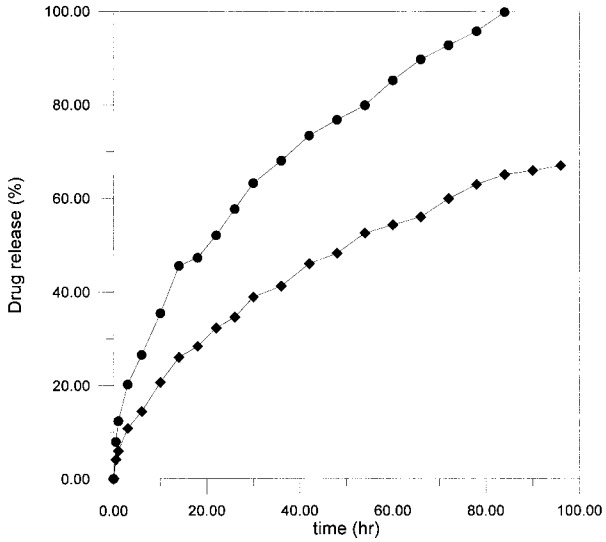


Figure 11 Release profiles of indomethacin from genipin-crosslinked chitosan microspheres. Preparative conditions: ◆, pH 6.5 chitosan–indomethacin suspension; ○, pH 4.0 chitosan–indomethacin suspension.

Effect of Crosslinking and Polycation–Anionic Drug Complex on Drug Release

In distilled water, the release of a drug from the crosslinked chitosan gel matrix is considered to be a swelling-controlled model. Figure 10 shows the release profiles of indomethacin from chitosan microspheres in water. The drug-release kinetics of chitosan microspheres could be expressed by Peppas's model.[20] The time-dependent released percentage of indomethacin from the chitosan microspheres was fitted to Peppas's model and the power n value and regression coefficient R^2 were calculated (Table II). The results show that the power n value derived from the release data of chitosan microspheres decreased from 0.72 to 0.54, indicating an approach of the release mechanism to Fickian diffusion control (a value of $n = 0.43$ is Fickian diffusion controlled) attributed to the increased hydrophobicity of the crosslinked chitosan microspheres.

Figure 11 shows the release profiles of indomethacin from the chitosan microspheres prepared from chitosan–indomethacin suspensions at various pH values. The chitosan microspheres prepared from a chitosan–indomethacin suspension at higher pH exhibited a significantly increased dissolution rate compared to that of chitosan microspheres prepared from a chitosan–indomethacin suspension at lower pH. The faster rate of indomethacin release from chitosan microspheres may be explained by the difference in the ionization ability of indomethacin. Indomethacin in its original form was difficult to be ionized in

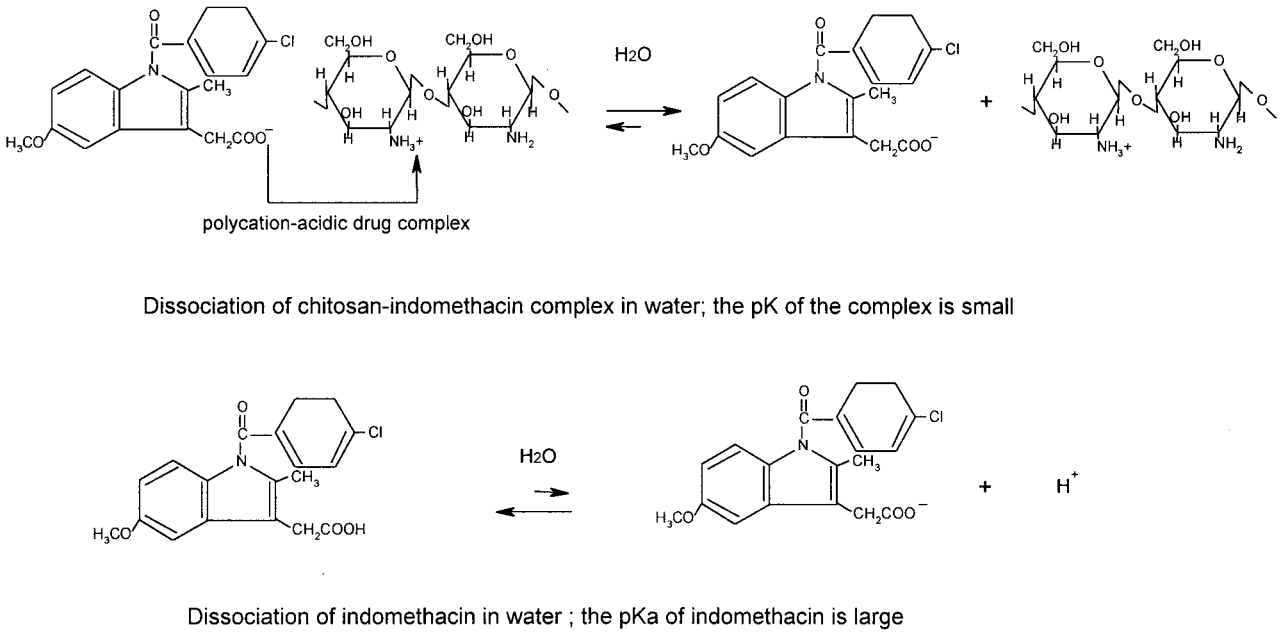


Figure 12 The scheme for the dissolution of indomethacin and chitosan–indomethacin complex.

water. The interaction of chitosan with indomethacin, through complexation at higher pH, enhanced the dissolution of indomethacin because of the improvement in ionization of its carboxyl groups after hydrolysis of the chitosan–indomethacin complex (Fig. 12).

CONCLUSIONS

In this study, we prepared a novel crosslinked chitosan microsphere with lower cytotoxicity. Genipin, an herbal medicine that is about 5000 to 10,000 times less cytotoxic than glutaraldehyde, was used as a crosslinking agent. The release of indomethacin from crosslinked chitosan microsphere in the dissolution medium was slow and sustainable, and therefore appears to be a very promising polymeric carrier for the long-acting release of drugs. The polycation–anionic drug interaction between chitosan and indomethacin was also examined in this study. By examination of the release kinetics of the crosslinked chitosan microsphere using Peppas's model, one can find that the drug-release mechanism of chitosan microsphere approaches that of this model.

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