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Drug release from chitosan–alginate complex beads reinforced by a naturally occurring cross-linking agent

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Abstract

Cross-linking reinforced chitosan–alginate complex beads were prepared by dropping chitosan droplets into alginate–cross-linker co-existing gelling solution. A novel, naturally occurring material — genipin was used as the cross-linking agent. Genipin and its related iridoid compounds, extracted from the Gardenia fruits, have been used in the traditional Chinese medicine for the treatments of jaundice and various inflammatory and hepatic diseases. The gelation of chitosan droplets in alginate–genipin mixed solution depends on both controlling factors: chitosan–alginate polyelectrolyte complex and chitosan cross-linked by genipin. The reaction of chitosan–alginate complex dominates the formation of skin layer on the surface of beads, but cross-linking of chitosan by genipin dominates the curing of inner core of beads. The swelling ratio of the cross-linking reinforced chitosan–alginate complex bead decreases as the pH or concentration of alginate in the gelling solution was decreased. The protonation of amine group of chitosan by hydrogen ions from acid or the shield of charge of ammonium group of chitosan by Cl^- ions result in the decrease of cross-linking density due to the inhibition of nucleophilic attack on the dihydropyran ring of genipin. Contrary to the swelling properties, the rate of indomethacin releasing out of the chitosan–alginate complex beads increases with the decrease in pH or concentration of alginate in the gelling solution due to the decreased cross-linking density of the beads. The cytotoxic examination suggested that the chitosan–alginate beads cross-linked by genipin had a good cellular compatibility.

Keywords: Chitosan; Alginate; Genipin; Polyelectrolyte complex; Reinforce

1. Introduction

Chitosan is a biopolymer, which could be used for the preparation of various polyelectrolyte complex products with natural polyanions as carboxymethylcellulose (Fukuda, 1980), heparin (Kikuchi & Noda, 1976), xanthan (Dumitriu & Chornet, 1998), alginate (Gåserød, Smidsrød & Skjåk-Bræk, 1998; Gåserød, Sannes & Skjåk-Bræk, 1999; Hari, Candy & Sharma, 1996; Knorr & Daly, 1988; Lee, Ha, Park & Lee, 1997), carrageenan (Hugerth, Caram-Lelham & Sundelöf, 1997 and hyaluronate (Denuziere, Ferrier & Domard, 1996; Denuziere, Ferrier, Damour & Domard, 1998. In the previous studies, we also examined the preparation of chitosan–tripolyphosphate complex (Mi, Shyu, Lee & Wong, 1999; Mi, Shyu, Kuan, Lee, Lu & Jang, 1999; Mi, Shyu, Wong, Jang, Lee & Lu, 1999). Chitosan–polyanion complexes have been widely investigated for applications like drug and protein delivery, cell transplantation, enzyme immobilization (Huguet, Groboillot, Neufeldt, Poncelet & Dellacherie, 1994; Mattew, Salley, Peterson & Klein, 1993; Overgaard, Scharer, Moo-Young & Bols, 1991). Among those chitosan–polyanion complexes, chitosan–alginate complex may be the most important drug delivery system (Douxian, Yan, Anlie, Goosen & Sun, 1991; Hari, Chandy & Sharma, 1996; Takahashi, Takayama, Machida & Nagai, 1996; Murata, Maeda, Miyamoto & Kawashima, 1993; Polk, Amsden, Yao, Peng & Goosen, 1994; Thu, Bruheim, Espevik, Smidsrød, SoonShiong & Skjåk-Bræk, 1996; Thu, Bruheim, Espevik, Smidsrød, SoonShiong & Skjåk-Bræk, 1996). The strong electrostatic interaction of amine groups of chitosan with the carboxyl groups of alginate lead to the formation of chitosan–alginate complex. The chitosan–alginate gel beads with a chitosan core and a chitosan–alginate skin are prepared by dropping the solution of chitosan into the alginate solution. In contrast, chitosan–alginate gel beads with an alginate core and a chitosan–alginate skin are prepared by dropping a

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solution of alginate into chitosan solution. Due to the protonation of amino group on chitosan and the ionization of carboxylic acid group on alginate, the stability of chitosan–alginate complex may be influenced by the environmental parameters, such as pH and ionic strength.

It has been found that the macromolecular chitosan rapidly bind onto the surface of alginate droplet, but were limited to diffuse into the inner core (Gåserød et al., 1998. In order to increase the stability of chitosan–alginate complex, chitosan solution, consisting of $CaCl_2$, was used for the gelation of alginate (Gåserød et al., 1998, 1999; Lee et al., 1997. The presence of calcium ions in the chitosan solution during the incubation had a great effect on the ability of a gel bead to bind chitosan. Chitosan was bound faster and to a higher extent with increasing concentrations of calcium chloride. The presence of calcium salt leading to the competition of gelling reaction and polyelectrolyte complex results in the formation of a more porous gel, allowing the diffusion of chitosan. Alginate–chitosan beads prepared by this method help in increasing the stability and decreasing the permeability of beads (Gåserød, Smidsrød & Skjåk-Bræk, 1998). The stability and permeability of alginate–chitosan beads could also be improved by dropping alginate solution into $CaCl_2$ to form calcium alginate beads. The beads were then transferred directly to chitosan solution for the formation of chitosan–alginate complex membrane (Gåserød et al., 1998).

This work focused on the preparation of a novel chitosan–alginate bead with inner cross-linked chitosan core and outer chitosan–alginate complex membrane. The one-stage procedure for the preparation of cross-linking reinforced chitosan–alginate beads is examined by dropping chitosan solution into alginate solution containing a naturally occurring cross-linking agent — genipin in the solution. Genipin is an aglucone of geniposide, which is a component of the Chinese medicine — gardeniae fructus. It has been reported that genipin can spontaneously react with amino acids or proteins to form dark blue pigments (Fujikawa, Yokota, Koga & Kumada, 1987a; Fujikawa, Fukui & Koga, 1987b; Fujikawa, Nakkamura & Koga, 1988). It has also been examined that genipin might be about 5000–10 000 times less cytotoxic than glutaraldehyde (Sung, Huang, Huang, Tsai & Chiu, 1998; Sung, Huang, & Tsai, 1999. In this study, polyanion–polycation complex membrane is formed instantaneously on the surface of the alginate–chitosan beads and the cross-linked inner core is formed afterward due to the resistance for the diffusion of genipin into the core of the beads.

2. Experimental

2.1. Materials

Chitosan (M_w: 70puncsp;000) were purchased from Fluka Chemical Co. (Switzerland). Indomethacin was purchased from Sigma Chemical Co. (USA). Sodium alginate was a commerical product from Sigma Chemical Co. (USA). Genipin was obtained from Challenge Bioproducts Co., Taiwan. All other reagents and solvents used were of reagent grade.

2.2. Preparation of cross-linking reinforced chitosan–alginate beads

Chitosan solution (1.5 wt%) was prepared by dissolving chitosan in deionized water containing 0.5 wt% of acetic acid. Alginate solution (0.25, 0.5, 0.75 and 1.0 wt%) was prepared by dissolving sodium alginate in deionized water. The chitosan solution was pumped through a syringe connected to a coaxial airflow and dropped into the gelling bath. The gelling bath contained an aqueous of alginate with 0.05 wt% of genipin, which gives gel beads with inner cross-linked core. The gel beads were allowed to harden in the gelling bath for 12 h. The pH of alginate, concentration of alginate in gelling bath are adjusted to examine the influence on the stability of the cross-linking reinforced chitosan–alginate complex beads. The beads were filtered, rinsed with deionized water and dried in air overnight for further analysis.

2.3. Cytotoxicity of chitosan–alginate beads cross-linked by genipin

The chitosan droplets were gelled in alginate solution without genipin. The chitosan–alginate beads formed were placed in the center of each well in a 24-well plate (the diameter of each well is about 16 mm). The beads collapsed into a thin disk, and adhered closely to the bottom of the well after drying. The samples were then placed into an 0.05 wt% genipin solution and cross-linked for 12 h. The test samples were sterilized in a graded series of ethanol solutions with a gradual increase in the concentration from 20 to 75% over a period of 4 h. Subsequently, the test samples were rinsed thoroughly in sterilized phosphate buffered saline (PBS) and prepared for the study of cytotoxicity. Subsequently, 3T3 fibroblasts at 5×10^4 cells/well were seeded evenly in each well of the 24-well plate in 1 ml Dulbecco's modified eagle medium (Gibco 430-2800EG, Grand Island, NY, USA) with 10% fetal calf serum (Hyclone Laboratories. Logan, UT, USA). The cell culture was maintained in a humidified incubator at 37°C with 10% CO_2 in air. During the period of culture, the cells in the well were photographed using an inverted light microscope.

2.4. Drug incorporation

Indomethacin was dispersed into chitosan solution by stirring to prepare chitosan–indomethacin mixed solution. The chitosan–indomethacin mixed solution was pumped through a syringe and dropped into the alginate–genipin mixed gelling medium according to the process described above. The cross-linking reinforced chitosan–alginate

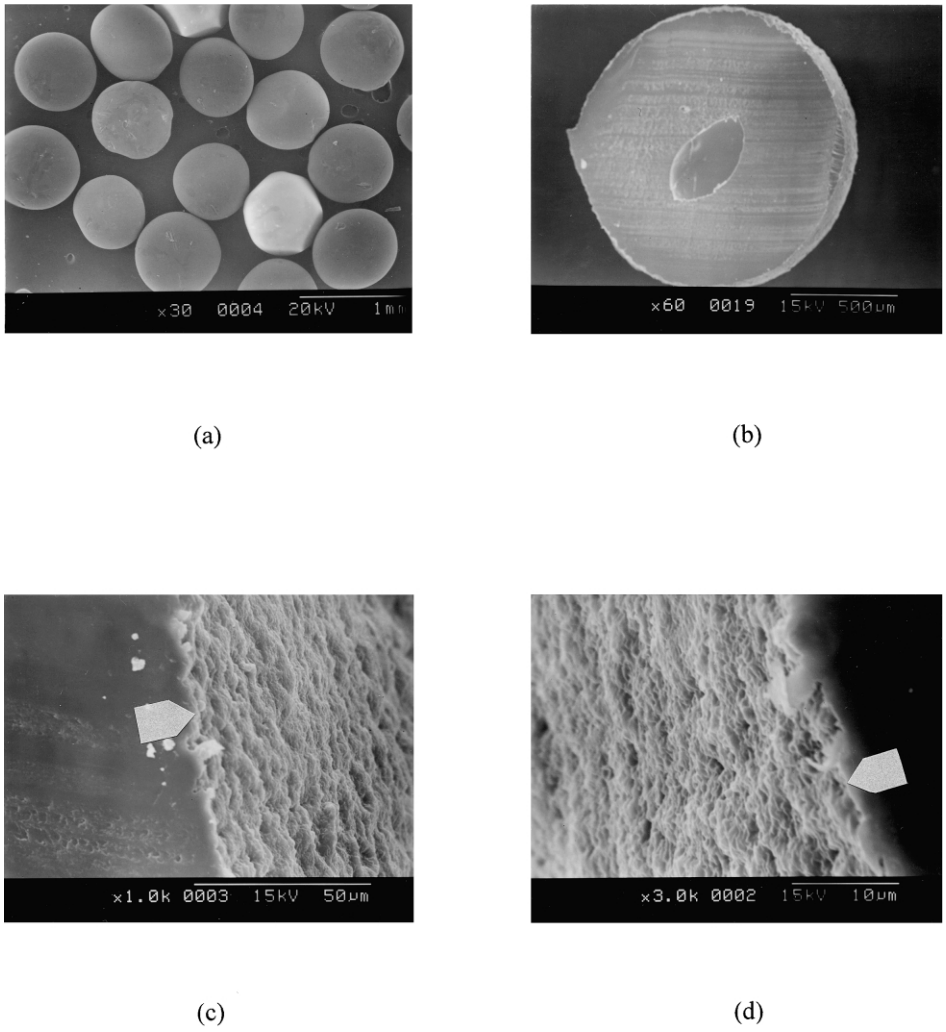


Fig. 1. SEM photographs of cross-linking reinforced chitosan–alginate bead: (a) whole picture (× 30), (b) cross-section morphology (× 60), (c) magnified view of surface and cross-section (× 1000, the arrow indicates the interface of surface and cross-section), and (d) magnified view of surface and cross-section (× 3000, the arrow indicates the interface of surface and cross-section).

beads containing drug were rinsed and dried in air overnight and then stored in a desiccator for future analysis.

2.5. IR spectra analysis

All cross-linked reinforced chitosan–alginate beads prepared from different processes were analyzed by Hitach model 6500 spectrophotometer. The peak variation of adsorption between 400 and 4000 cm^{-1} were detected to monitor the reaction for the preparation of cross-linking reinforced chitosan–alginate beads.

2.6. Swelling studies

The water sorption capacity of the cross-linking reinforced chitosan–alginate beads were determined by swelling the beads in media of pH 1–7 at room temperature. A known weight (200 mg) of the chitosan–alginate beads were placed in the media for the required period of time. The swollen chitosan–alginate beads were collected, and the net weight of the chitosan–alginate beads were determined by first blotting the chitosan–alginate beads with filter paper to remove adsorbed water on the surface, then weighing immediately on an electronic balance. The percentage swelling of the chitosan–alginate beads in the media were then calculated from the formula:

$$E_{sw} = [(W_e - W_0)/W_0] \times 100,$$

where E_{sw} is the percent swelling of chitosan–alginate beads at equilibrium. W_e denotes the weight of the chitosan–alginate beads at equilibrium swelling and W_0 is the initial weight of the chitosan–alginate beads. Each swelling experiment was repeated three times, and the average value was taken as the percentage swelling value.

2.7. Assay of the drug content

Triplicate samples of 0.1 g cross-linking reinforced chitosan–alginate beads were placed in a mortar and triturated

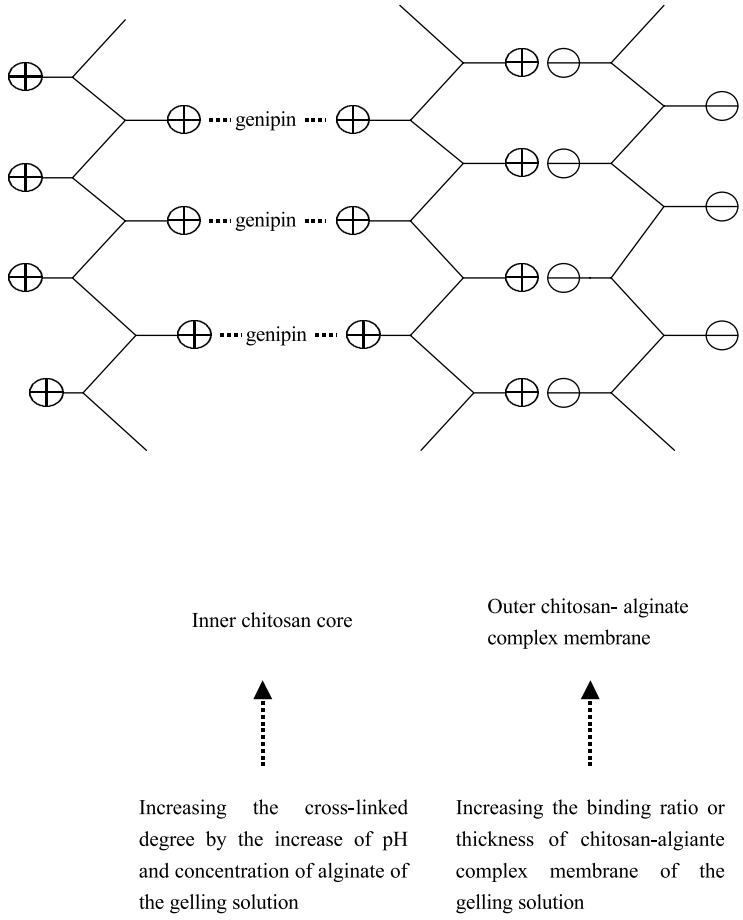


Fig. 2. Schematic sketch of the asymmetric reinforced chitosan–alginate beads: outer chitosan–alginate complex skin layer and inner chitosan core.

thoroughly. Indomethacin was extracted into 500 ml of PBS. After rinsing all equipments thoroughly, the whole mixture was filtered through a sintered glass suction funnel and made up to volume in a 1000 ml volumetric flask. The drug was assayed by a double-beam UV spectrophotometer (Milton roy spectronic 3000) at 320 nm.

2.8. Scanning electron microscopy

The cross-linking reinforced chitosan–alginate beads were adhesived onto double-sided tape, sputter-coated with gold to about 500×10^{-8} cm thickness using a Hitachi coating unit IB-2 coater under a high vaccum, 0.1 Torr, high voltage, 1.2 kV and 50 mA. Coated samples were examined using Hitachi S-2300 scanning electron microscopy (SEM).

2.9. In vitro release

The releasing of indomethacin from cross-linking reinforced chitosan–alginate bead was measured by using the dissolution (Hanson research, Dissoette II) and autosampling (Hanson research, SR6) systems. The dissolution medium was 500 ml of PBS solution. The medium placed in a 1 l round flask fitted with a pump for autosampler to remove medium and stirred with a mechanical stirred at a rate of 100 r.p.m. The dissolution medium temperature was maintained at 37°C. An equivalent quantity of 100 mg chitosan–algiante beads were dispersed in the dissolution medium. After a predetermined period, 5 ml of the medium was removed and the amount of indomethacin was analyzed spectrophotomerically at 320 nm. In order to maintain the original volume, each time 5 ml of the medium was replaced with fresh water.

3. Results and discussion

3.1. Preparation of chitosan–alginate beads

Cross-linking reinforced chitosan–alginate beads formed instantaneously after dropping chitosan droplets into the alginate–genipin mixed solution and the beads gradually turned from transparent into blue color within 12 h. The beads produced in this work had diameters of 800 μm with a narrow size distribution. The color of cross-linked chitosan–alginate beads are colored in different shades of blue; this depends on their cross-linking degree. Fig. 1 shows the SEM micrographs of the cross-linking reinforced chitosan–alginate beads. The dark-blue colored chitosan–alginate

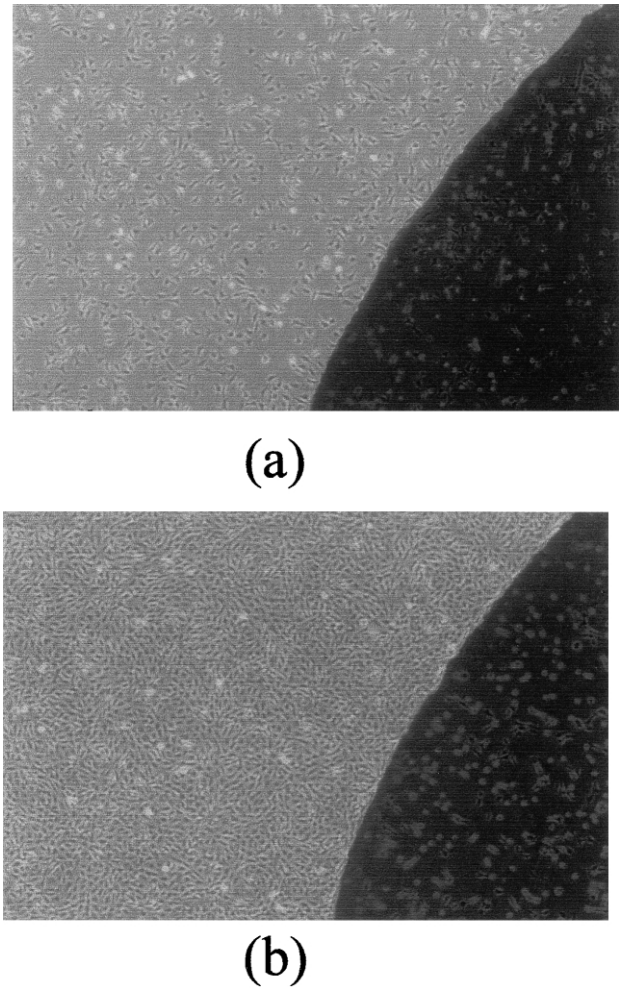


Fig. 3. Photomicrographs of the 3T3 fibroblasts cultured in a 24-mm well containing genipin cross-linked chitosan–alginate complex gel: (a) cultured for 8 h and (b) cultured for 32 h.

beads with higher cross-linking density were spherical in shape and had a coarse surface and relatively smooth cross-section, suggesting that the beads consist of obvious inner core and outer skin layer (Fig. 2). The formation of the interphasic membrane can be attributed to the interaction of anionic alginate with the cationic chitosan, giving a very thin skin layer. Contrary to the skin layer, the inner core was formed due to the cross-linking of chitosan by genipin. The light-blue colored chitosan–alginate beads with lower cross-linking density were different from the dark-blue colored chitosan–alginate beads. The collapsed appearances were observed form those chitosan–alginate beads with lower cross-linked density or without cross-linking. The gelation of chitosan droplets in alginate–genipin mixed solution depends on both controlling factors: chitosan–alginate polyelectrolyte complex and chitosan cross-linked by genipin. In consideration of the kinetic aspect, the reaction rate of chitosan–alginate complex is very quick, but that of cross-linking is very slow. While in consideration of the diffusion aspect, the macromolecular alginate is restricted to penetrate through outer skin layer into the inner core, but genipin is a small molecule that can penetrate chitosan droplets more readily than the macromolecular alginate. Due to this reason, the chitosan–alginate complex dominates the formation of skin layer on the surface of asymmetric chitosan–alginate beads, but cross-linking of chitosan by genipin dominates the curing of inner core of the beads. Both the formations of outer chitosan–alginate complex membrane and cured inner chitosan core lead to the fixing of the sphericity of beads.

3.2. Cytotoxicity of genipin cross-linked chitosan–alginate beads

The potential cytotoxic sources of a chemically modified biomaterial may be from the modified material itself and the residues, such as the remaining cross-linking reagent. Fig. 3a and b gives the photomicrographs of the 3T3 fibroblasts cultured in the well, containing the genipin cross-linked chitosan–alginate disk after a 8-h and 32-h culture, respectively. As shown in Fig. 3b, it was filled with 3T3 fibroblasts in the vicinity of the chitosan gel after 32 h of culture. This indicated that the residues (genipin, algiante or chitosan) released from the genipin cross-linked chitosan–alginate disk had no toxic effect on the seeded cells. This fact implied that the chitosan–alginate bead itself cross-linked with genipin and showed a good cellular compatibility.

3.3. IR spectra analysis

The IR spectrum of chitosan droplet gelled in alginate solution without genipin shows around 905 and 1153 cm^{-1} peaks of assigned saccharide structure and a strong protonated amino characteristic peak at around 1570 cm^{-1}. The stronger absorption bands at 1649 cm^{-1} was characteristic of the amide absorption. As compared to the characteristic adsorption peak of alginate and chitosan, one can easily find that chemical structures of the beads are similar to chitosan (Fig. 4). The result indicated that the binding of alginate to chitosan droplets was limited to a very low binding ratio. The bound alginate was accumulated only on the surface due to the hindrance of macromolecular alginate form further diffusing into the inner core (Gåserød et al., 1998. This resulted in the formation of asymmetric beads with outer chitosan–alginate complex skin and inner chitosan core. The chitosan–alginate beads collapsed after drying due to lack of support of the inner core. Due to this, the bead should be cross-linked for fixing its sphericity. Fig. 4 also shows the IR spectra of chitosan droplets gelled in alginate solution dissolved with the cross-linking agent — genipin. The significantly increased adsorption at 1643 cm^{-1} and decreased adsorption at 1570 cm^{-1} could be attributed to the formation of cross-linked inner chitosan core by genipin. Genipin is a bicyclic compound and have one oxygen atom involved in one of these rings. The ring opening of genipin is performed by a nucleophilic attacked by the amino group of chitosan on the olefinic carbon atom

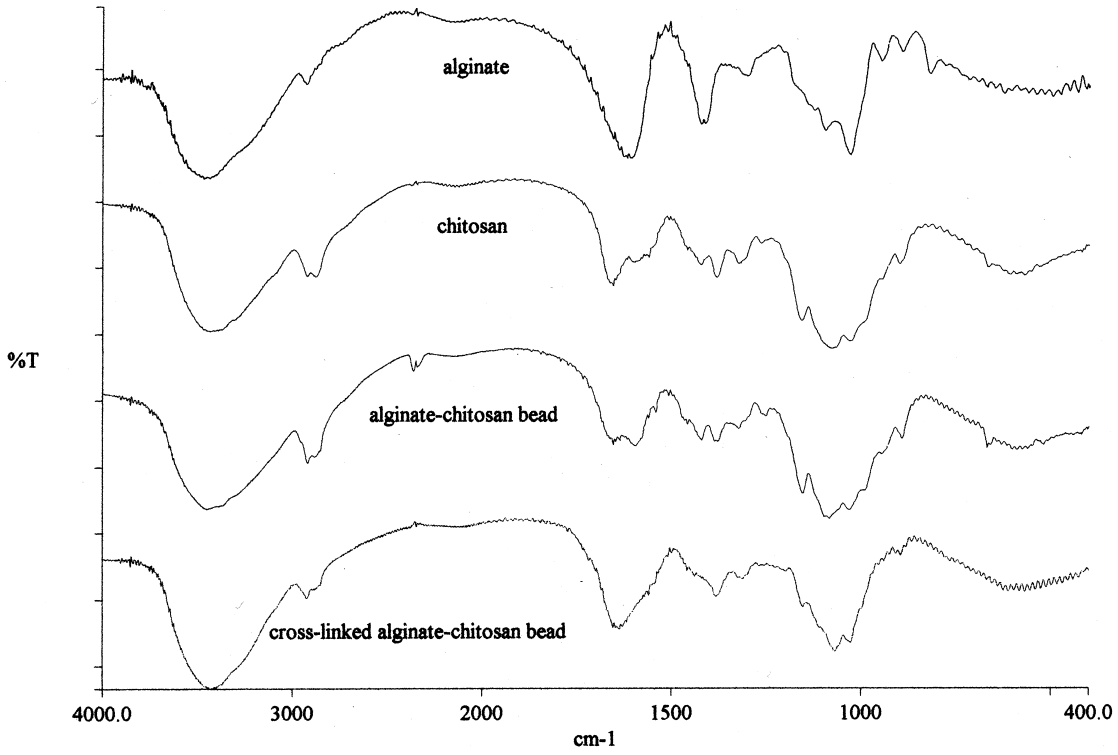


Fig. 4. IR spectra of chitosan, alginate and cross-linked reinforced chitosan–alginate beads.

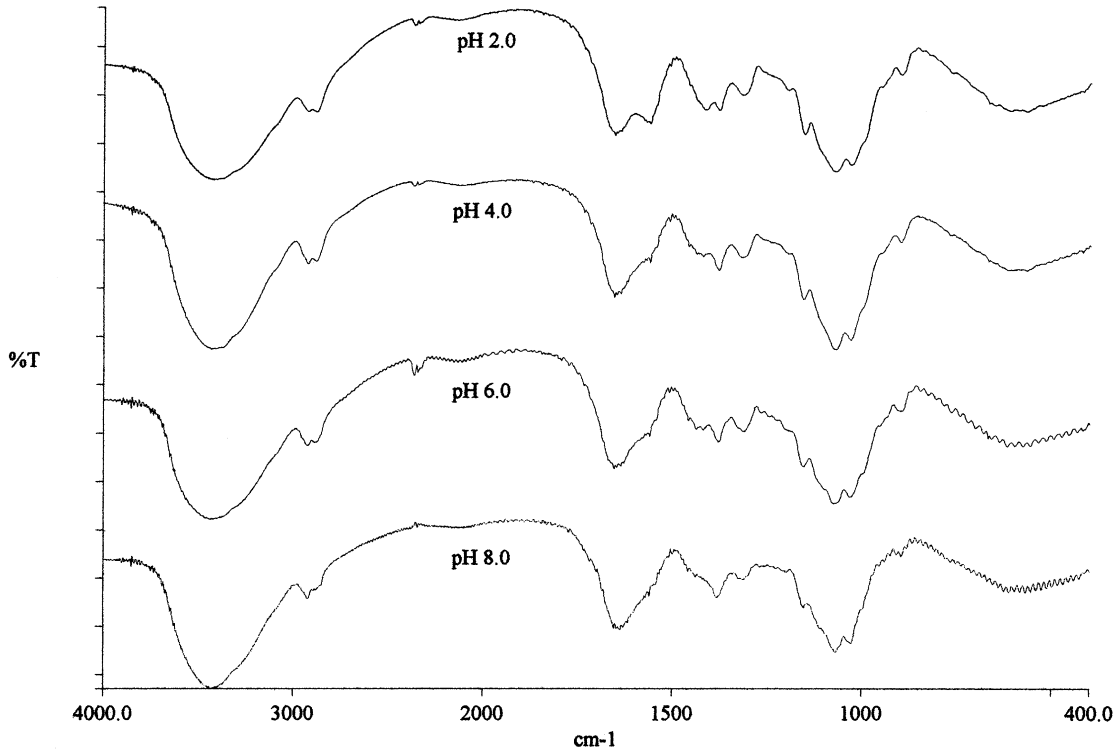


Fig. 5. IR spectra of chitosan–alginate beads using different pH of alginate–genipin mixed solution.

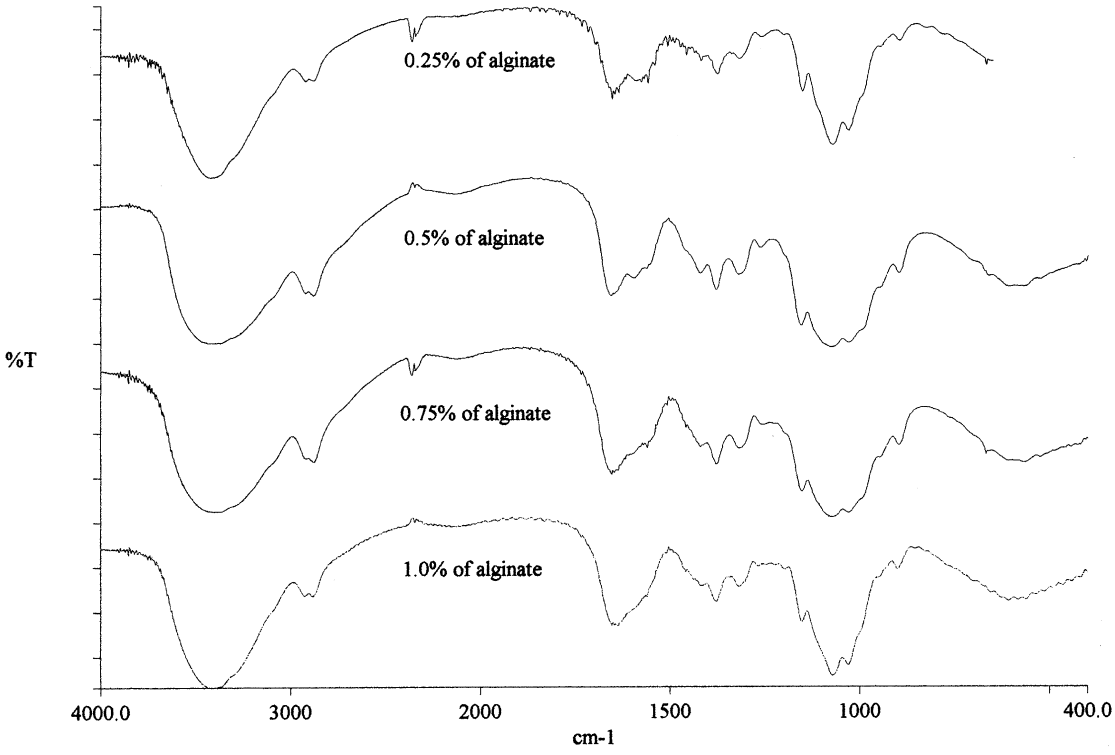


Fig. 6. IR spectra of chitosan–alginate beads using different concentrations of alginate of alginate–genipin mixed solution.

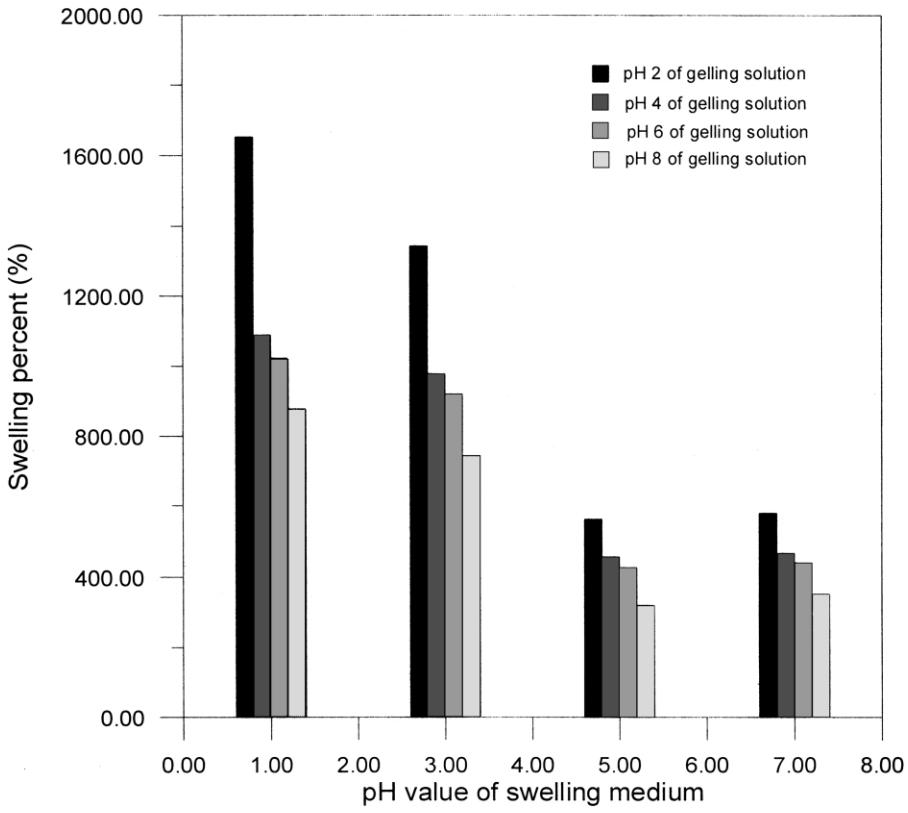


Fig. 7. Swelling degree of chitosan–alginate beads prepared in different pH values of alginate–genipin mixed solution.

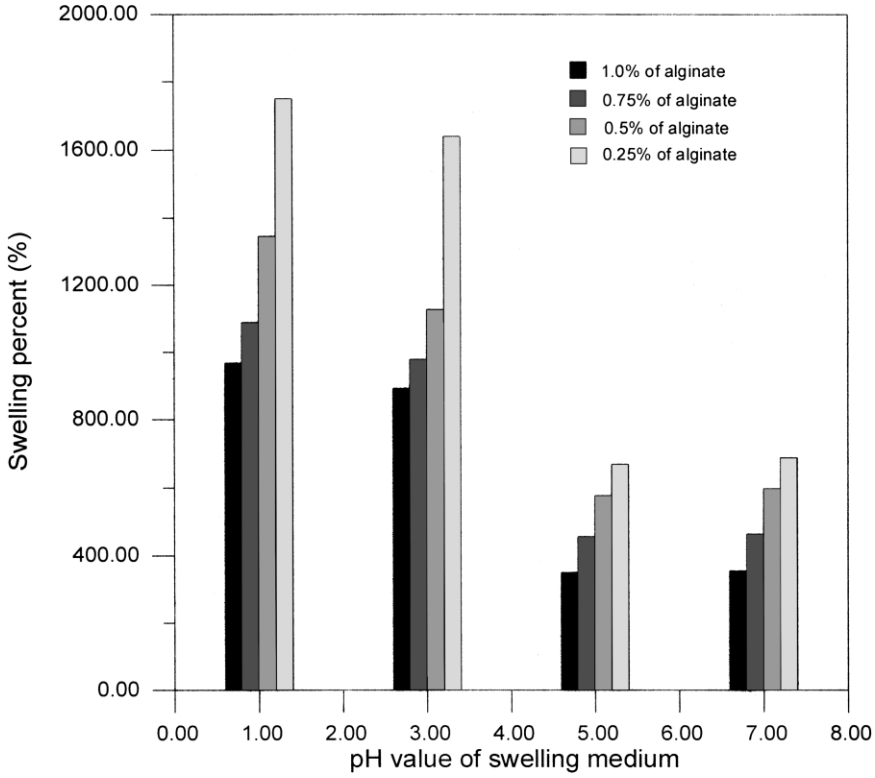
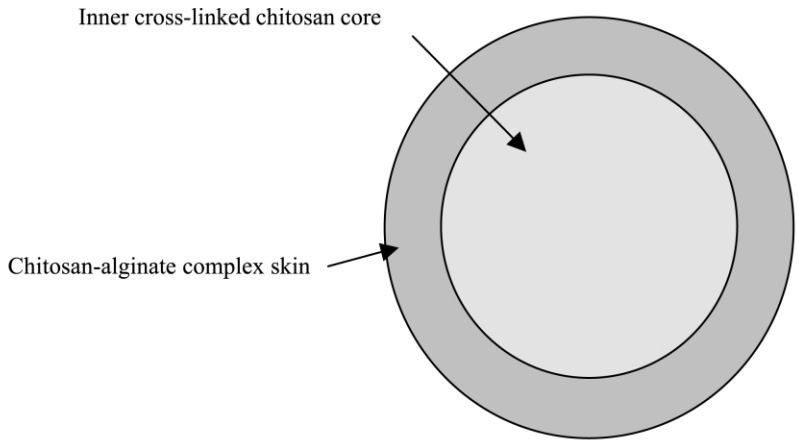


Fig. 8. Swelling degree of chitosan–alginate beads prepared in different concentrations of alginate of alginate–genipin mixed solution.

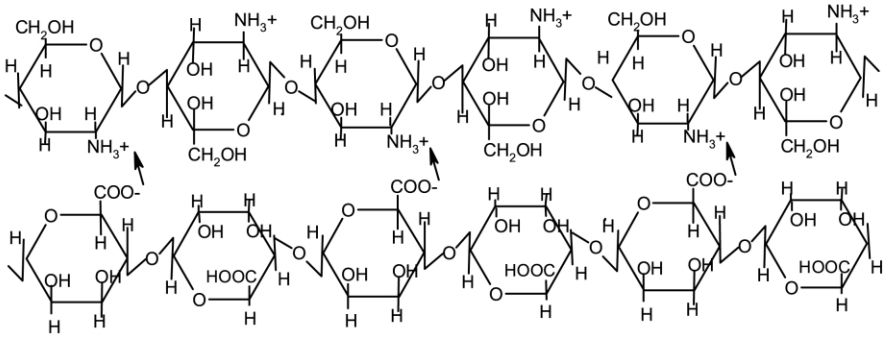
at C-3 of deoxyloganin aglycone, followed by the opening of the dihydropyran ring and attack by the secondary amino group on the resulting intermediate aldehyde group. Furthermore, the carboxymethyl group of genipin could react with amino group of chitosan and lead to the formation of secondary amide. As can be seen from the IR spectra in Fig. 5, the increased adsorption at 1643 cm^{-1} and decreased adsorption at 1570 cm^{-1} could be observed from beads prepared in higher pH of alginate–genipin mixed gelling solution. Fig. 6 shows the effect of the concentration of alginate in the alginate–genipin mixing solution on the cross-linking degree of the beads. It could also be found that the adsorption at 1643 cm^{-1} was increased and the adsorption at 1570 cm^{-1} was decreased for beads prepared in higher concentrations of alginate. These results suggest that cross-linking properties of the chitosan–alginate beads could be improved by the variation of pH value or concentrations of alginate in the gelling solution.

3.4. Swelling ability

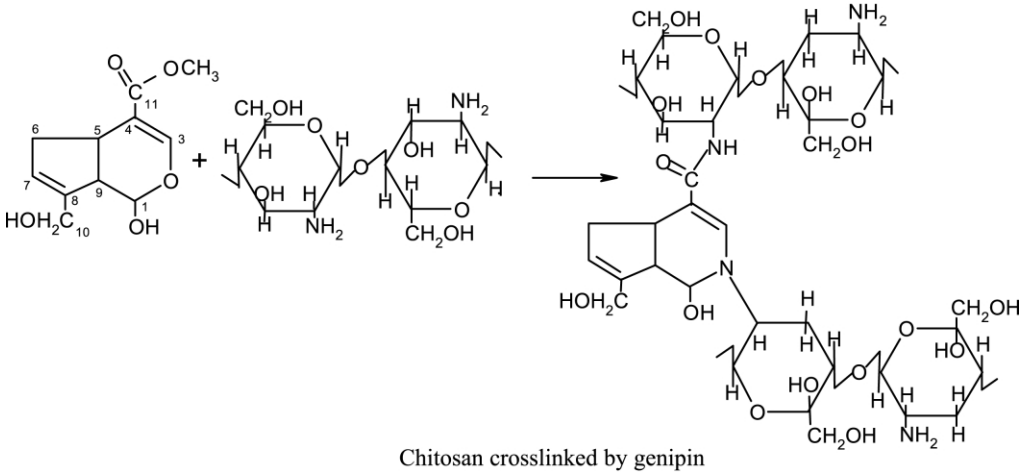
The swelling ability of the cross-linking reinforced chitosan–alginate beads are dependent on the pH value of the swelling medium. The cross-linked chitosan–alginate beads showed the obviously higher swelling degree at pH lower than 2 and slightly higher swelling degree at pH higher than 6. The increased swelling degree at pH lower than 2 is attributed to the protonation of inner chitosan core while the slightly increased swelling degree at pH higher than 5 is attributed to the ionization of carboxyl group of alginate in the outer chitosan–alginate complex layer. The swelling degrees of the chitosan–alginate complex beads also depend on the process for the preparation of beads, such as pH or concentration of alginate of the alginate–genipin gelling solution. The swelling degrees of the prepared beads decrease with the increase in pH value of the alginate–genipin mixed gelling solution (Fig. 7). It leads to the following results: the amine group of chitosan was protonated by hydrogen ions from acid, and the excess of Cl^- ions shield the charge of ammonium group of protonated chitosan, which result in the inhibition of nucleophilic attack on the dihydropyran ring of genipin. The concentration of alginate in the alginate–genipin mixed gelling solution also significantly affects the swelling degree of the prepared chitosan–alginate beads. The swelling degrees of the beads decrease with increasing concentrations of alginate in the gelling solution (Fig. 8). This result may be ascribed to the fact that hydrogen ions could diffuse out of chitosan–alginate beads and be adsorbed by alginate around the beads. The deprotonation of inner chitosan core increases the nucleophilicity of amino group, which in turn leads to the increase in the cross-linking degree due to nucleophilic attack of chitosan on the dihydropyran ring of genipin. The proposed gelation mechanism combining chitosan–alginate complex skin layer and genipin cross-linked inner chitosan core are shown in Fig. 9.



Crosslinking reinforced chitosan-alginate complex bead



Chitosan-alginate polyelectrolyte complex



Chitosan crosslinked by genipin

Fig. 9. A proposed mechanism for chitosan gelled in alginate–genipin mixed solution.

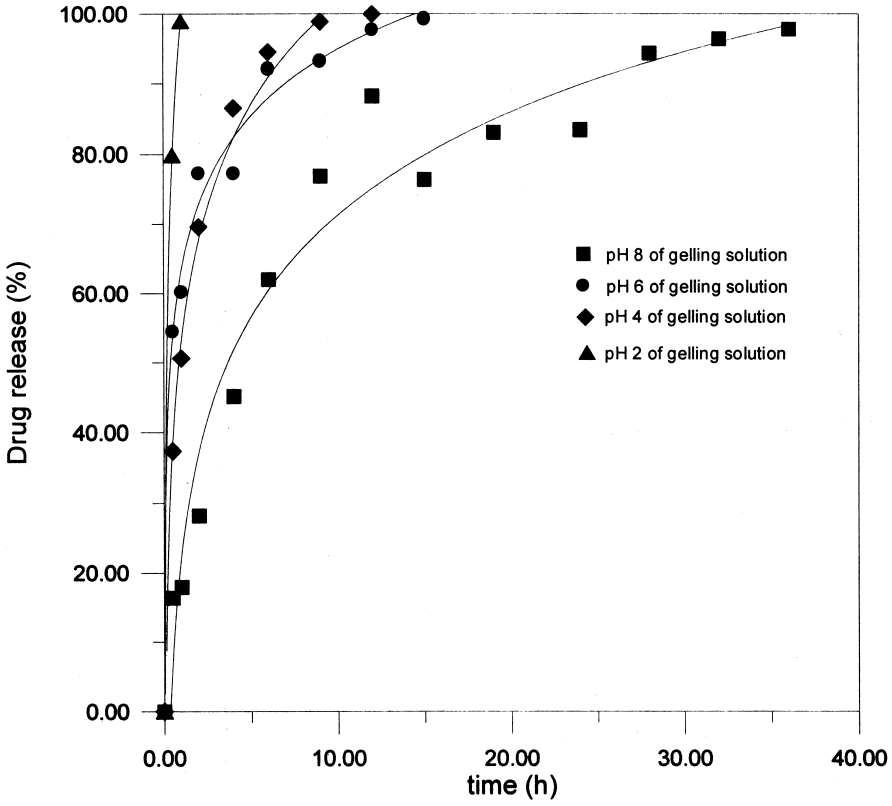


Fig. 10. Indomethacin releasing from chitosan–alginate beads prepared in different pH values of alginate–genipin mixed solution.

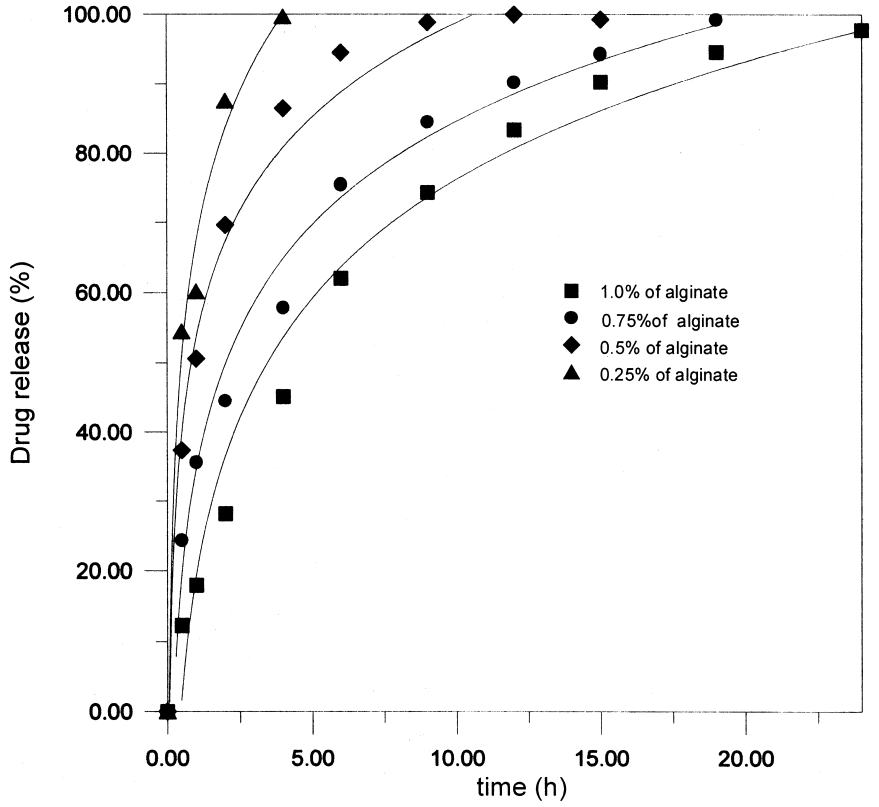


Fig. 11. Indomethacin releasing from chitosan–alginate prepared in different concentrations of alginate of alginate–genipin mixed solution.

3.5. Drug release

The permeability of indomethacin through cross-linking reinforced chitosan–alginate beads was examined by dissolution test. The release rate of indomethacin into dissolution medium could be limited by increasing the cross-linking degree of inner core and the binding ratio of chitosan–alginate complex membrane. Fig. 10 shows the rate of indomethacin release from the cross-linked chitosan–alginate beads prepared in different pH of alginate–genipin gelling solution. The release rate was quicker for beads prepared in low pH but slower for beads prepared in neutral pH of alginate–genipin gelling solution. Due to the fact that the pK_a values of chitosan and alginate are 6.3 and 3.5, respectively, the binding of alginate to form complex layer on the outer layer of chitosan droplet will be higher at pH values between 4.0 and 5.0, whereas, the cross-linking degree of inner chitosan core by genipin was increased by the increase in pH value as described above in the swelling studies. Both the formations of higher binding ratios of chitosan–alginate complex membrane in the outer layer and higher cross-linking degrees of inner chitosan core lead to the decrease in drug release rate. Fig. 11 shows the rate of indomethacin releasing from the cross-linked chitosan–alginate beads prepared in different concentrations of alginate in alginate–genipin mixed gelling solution. The release rate was quicker for beads prepared in low concentrations of alginate but slower for beads prepared in high concentration of alginate. The formation of thicker chitosan–alginate complex membrane in high concentrations of alginate due to the forced diffusion induced from concentration gradient. Besides, the increased concentration of alginate could increase the nucleophilicity of chitosan, which, in turn, will lead to the increase in the cross-linking degree of inner chitosan core. The effects of both the formation of thicker chitosan–alginate complex skin layer and higher degrees of cross-linked inner core lead to the decrease in drug release rate.

4. Conclusion

In this study, we prepare a novel cross-linking reinforced chitosan–alginate beads using a naturally occurring cross-linker genipin for drug delivery. The asymmetric chitosan–alginate beads consist of a chitosan–alginate skin layer and inner cross-linked chitosan core. The release rate of the drug coming out of the asymmetric chitosan–alginate beads could easily be modified by regulating the production process, such as pH or concentration of alginate in the gelling solution. The asymmetric chitosan–alginate bead had a good cellular compatibility according to the examination in the study of cytotoxicity.

Acknowledgements

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