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ORIGINAL ARTICLE

Microencapsulation of isoniazid in genipin-crosslinked gelatin-A–κ-carrageenan polyelectrolyte complex

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Abstract

Background: Microspheres of gelatin-A and κ-carrageenan were prepared by using genipin, a naturally occurring crosslinker, and sunflower oil as reaction media. *Method*: The variations in the size of the microspheres formed by varying the amount of surfactant (0.33–1.0 g/g of polymer), polymer (1.5–3.0 g), and crosslinker (0.2–0.8 mmol) were studied by scanning electron microscopy. The encapsulation of isoniazid was carried out by absorption. The isoniazid content in the prepared microspheres was determined. The release characteristic of isoniazid was also studied at pH values 1.2 and 7.4 by using UV-spectrophotometer. *Results*: Characterization of the isoniazid-loaded microspheres was carried out by using Fourier transform infrared spectrophotometry, differential scanning calorimetry, and X-ray diffractometery.

Key words: *Controlled release; genipin; isoniazid; microspheres; polyelectrolyte complex*

Introduction

Current short-term chemotherapy for tuberculosis (TB) requires daily administration of one or several antitubercular drugs for a period of at least 6 months, which leads to patient noncompliance and therapeutic failure. Thus, TB continues to be a leading cause of mortality in spite of the availability of an effective chemotherapeutic regimen[1,2]. The efficient treatment of the disease is limited by the toxicity of the drugs, the degradation of drugs before reaching required zones in the body, and low permeability of cell membranes to the drugs[3]. As a rule, liposomes[4,5], polymers[6,7], and microcontainers[8] are used as antitubercular drug carriers.

Although the experience with synthetic polymers is extensive and encouraging[9,10], the recent trend has been to shift toward natural polymers. The major advantage of natural polymers includes their availability and compatibility with the encapsulation of wide range of drugs, with minimal use of organic solvents[11]. Furthermore, bioadhesion, stability, safety, and their approval for human use are additional advantages[12].

Varieties of crosslinking agents like glutaraldehyde, formaldehyde, and epoxy compounds[13–16] are reported to be employed for improving the controlled release behavior. These crosslinking agents can cause physiological toxicity if released into the host because of biodegradation. This leads to an increasing demand for a crosslinking agent capable of forming stable and biocompatible crosslinked products with less cytotoxicity problems. Genipin, a naturally occurring crosslinking agent, is biocompatible and less toxic[17–19]. It can react spontaneously with amino acids or proteins like gelatin[20]. As carrageenan contains some proteins[21], it can react with genipin[22] to form crosslinking.

The use of paraffin oil as a reaction medium has been cited in the literature[14,23]. The toxicity of organic solvent is higher compared to that of vegetable oil. This work aims at to produce microspheres as antitubercular drug carrier by using gelatin-A, κ-carrageenan as polymers, genipin as crosslinker, and sunflower oil as reaction medium. Efforts are also made to characterize and study the drug release behavior of microspheres under different conditions.

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Experimental

Materials

Carrageenan Type I, containing predominantly κ-carrageenan and lesser amount of λ-carrageenan, was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Gelatin type A was purchased from Sigma-Aldrich Inc. Glacial acetic acid (E. Merck, Mumbai, India), Tween 80 (E. Merck, Mumbai, India), and genipin (M_w 226.22; Challenge Bioproducts Co., Ltd., Taichung, Taiwan) were used as such received. Isoniazid was purchased from Sigma-Aldrich Inc. Edible grade refined sunflower oil was purchased from local market. Double-distilled deionized water was used throughout the study. Other reagents used were of analytical grade.

Microencapsulation procedure

In a beaker, known amount of (350 mL) sunflower oil was taken. This oil was allowed to be stirred (magnetic stirrer) for approximately 10 minutes. Under stirring condition, 25–50 mL of κ-carrageenan solution [2%(w/v)] at temperature 60 ± 1°C was added to the beaker containing sunflower oil at the same temperature to form an emulsion. Tween 80 (1–3 g dissolved in 10 mL of water) was added to the beaker to stabilize the emulsion. A known amount of (25–50 mL) gelatin-A solution of 4% (w/v) was added slowly to the beaker. The optimum ratio between carrageenan and gelatin was determined from the measurement of turbidity, coacervate yield (%), and viscosity of the supernant solution. Maximum turbidity, coacervate yield (%), and minimum supernant viscosity would develop when interaction between carrageenan and gelatin would be maximum. The studied range of pH and carrageenan to gelatin ratio were 2.5–5.0 and 0:1–1:0, respectively. The optimum ratio of carrageenan to gelatin and pH at which complete phase separation, that is, maximum interaction, occurred were 1:2 and 3.5, respectively. The pH of the mixture was then brought down to 3.5 by adding 2.5% (v/v) glacial acetic acid solution. The beaker containing the microspheres was left to rest at this temperature (60 ± 1°C) for approximately 15 minutes. The system was then brought to 5–10°C to harden the microspheres. The crosslinking of the microspheres was achieved by slow addition of certain amount of genipin solution. The temperature of the beaker was then raised to 45°C and stirring was continued for another 3–4 hours to complete the crosslinking reaction. The beaker was then cooled to room temperature slowly while stirring. The microspheres were filtered through 300-mesh nylon cloth, washed with acetone rapidly to remove oil, if any, adhered to the surface of microspheres. This was further washed with distilled water and freeze-dried. The dried microspheres were then dipped in isoniazid solution (0.5–10%, w/v) for different time period (20–120 minutes), filtered through 300-mesh nylon cloth, and quickly washed with water to remove isoniazid, if any, adhered to the surface. The isoniazid-encapsulated microspheres were again freeze-dried and stored in a glass ampule in a refrigerator.

Calibration curve of isoniazid

A calibration curve is required for the determination of amount of isoniazid released from the microspheres. To make calibration curve, isoniazid solution (in double-distilled deionized water) of different concentrations (0.001–0.01 g/100 mL) were prepared and scanned in the range of 200–500 nm by using UV-visible spectrophotometer. For isoniazid having concentration in the range 0.001–0.01 g/100 mL, a prominent peak at 261 nm was noticed. The absorbance values at 261 nm obtained with the respective concentrations were recorded and plotted. From this calibration curve, the unknown concentration of isoniazid was obtained by knowing the absorbance value.

Swelling and stability study

The swelling behavior of carrageenan–gelatin microspheres were studied in two systems at pH 1.2 (0.1 N HCl) and pH 7.4 (phosphate buffer). The microspheres were immersed in either 0.1 N HCl at pH 1.2 or phosphate buffer at pH 7.4. The diameters of swollen microspheres were determined after a stipulated time period (0–8 hours).

The swelling behavior was determined by measuring the change of the diameter of the microspheres using a microscope with a micrometer. The swelling ratio for each sample determined at time t was calculated using the following equation[24,25].

$$S_w = \frac{D_t}{D_0},$$

where D_t is the diameter of the microspheres at time (t) and D_0 is the initial diameter of the dried microspheres. The experiments were performed in triplicate and represented as a mean value.

The stability of microspheres was judged by immersing the microspheres in (0.5–10%, w/v) isoniazid solution. This was followed by recording the time till the occurrence of disintegration. The more the time required to disintegrate, the more is the stability.

Percent encapsulation

A known amount of accurately weighed microspheres was grounded in a mortar, transferred with precaution

to a volumetric flask containing 100 mL of water (having pH 7.4 maintained by phosphate buffer), and kept overnight with continuous stirring to dissolve the drug inside the microspheres. The solution was collected and the drug inside the microspheres was determined using UV spectrophotometer. The encapsulation (%) was calculated by using the calibration curve and the following formulae:

$$\text{Encapsulation}\,(\%) = \frac{w_1}{w_2} \times 100,$$

where

w_1 = amount of isoniazid encapsulated in a known amount of microspheres
w_2 = weight of microspheres.

Drug release studies

Isoniazid release studies of encapsulated isoniazid were done by using UV-visible spectrophotometer (UV-2001 Hitachi, Tokyo, Japan). A known quantity of microspheres was placed into known volume of water having different pH (1.2 and 7.4). This pH was maintained by using HCl and phosphate buffer solution. The content was shaken every 5- to 10-min interval and the temperature throughout was maintained at 30°C (room temperature). An aliquot sample of known volume (5 mL) was removed at appropriate time intervals, filtered, and assayed spectrophotometrically at 261 nm for the determination of cumulative amount of drug release up to a time *t*. Each determination was carried out in triplicate. To maintain a constant volume, 5 mL of the solution having same pH was returned to the container.

Scanning electron microscopy study

The samples were deposited on a brass holder and sputtered with platinum. Sizes of the microspheres were studied at room temperature (30°C) using scanning electron microscope (model JSM-6390; JEOL, Singapore) at an accelerated voltage of 5 kV.

Fourier transform infrared study

Fourier transform infrared (FTIR) spectra were recorded using KBr pellet in a Nicholet (model Impact-410) spectrophotometer. Gelatin-A; κ-carrageenan; polyelectrolyte complex of gelatin-A; and κ-carrageenan, isoniazid, and isoniazid containing microspheres were each separately finely grounded with KBr, and FTIR spectra were recorded in the range of 4000–400 cm^{-1}.

Thermal property study

Thermal properties of carrageenan–gelatin microspheres, isoniazid, and isoniazid-loaded microspheres were evaluated by using differential scanning calorimeter (model DSC-60; Shimadzu, Tokyo, Japan). A differential scanning calorimetry (DSC) study was done at a heating rate of 10°C/min upto 450°C. All the studies were done under nitrogen atmosphere.

X-ray diffraction study

X-ray diffractograms of gelatin-A, κ-carrageenan, isoniazid, and microspheres without isoniazid and microspheres containing isoniazid were recorded on an X-ray diffractometer (model MiniFlex; Rigaku Corporation, Tokyo, Japan). The samples were scanned between 2θ = 10° and 50° at the scan rate of 4°/min.

Results and discussion

Effect of variation of surfactant and polymer concentration on size of microsphere

Preliminary studies indicated that surfactant (Tween 80) had important role in preparation and stabilization of carrageenan–gelatin microspheres using sunflower oil as dispersing medium. A matrix-type product was formed in the absence of surfactant. The presence of surfactant of varying amounts was able to form different sizes of microspheres.

Figure 1 represents the scanning electron microscope photographs of microspheres prepared by using different surfactant : polymer (mixture of 1 part carrageenan+2 part gelatin) ratio. With the increase of concentration of surfactant from 0.33 g to 1.0 g/g of polymer (Figure 1a–c), the size of the microspheres was found to decrease. A possible explanation for reduction in size is as follows. At higher concentration of the surfactant, the aqueous polymeric phase became easily dispersed into finer droplets because of the higher activity of the surfactant. This would lead to decrease the interfacial free energy of the system and provide mechanical barrier to coalescence[26]. Interfacial polymerization of polyisocyanates produced smaller microcapsules of polyurea by using higher concentration of emulsifier was reported[27].

Similarly with the decrease in polymer amount from 3.0 to 1.5 g, a decrease in the size of microsphere was observed (Figure 1d). At higher polymer concentration, the amount of surfactant might not be sufficient to cover the surface of all the microspheres properly. This resulted in the coalescence of some microspheres and leads to the formation of larger microspheres. Moreover, the dispersive force of the stirrer became less

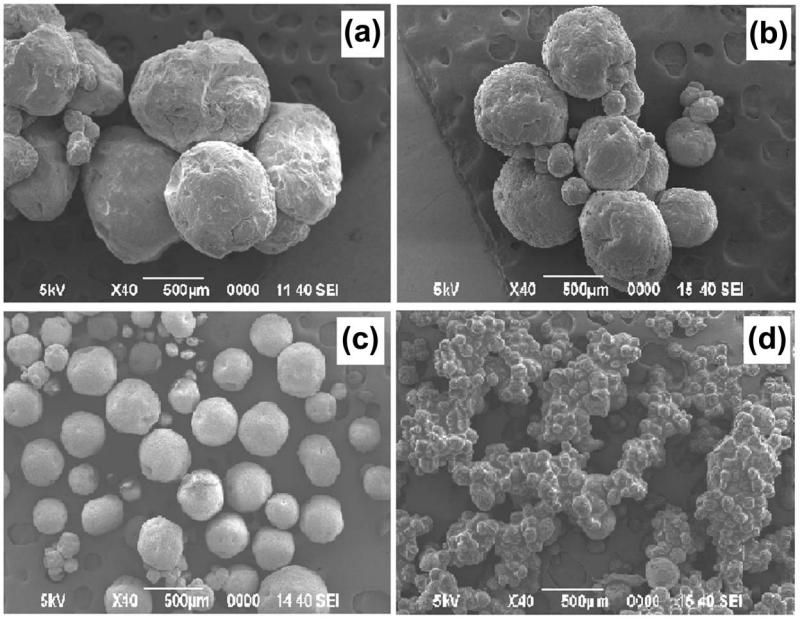

Figure 1. Scanning electron micrographs of microcapsules prepared by using (a) Tween 80: 0.33 g/g of polymer, polymer: 3.0 g; (b) Tween 80: 0.55 g/g of polymer, polymer: 3.0 g; (c) Tween 80: 1.0 g/g of polymer, polymer: 3.0 g; (d) Tween 80: 1.0 g/g of polymer, polymer: 1.5 g.

efficient at high polymer concentration and a larger microsphere might be produced as a result.

Swelling and stability study

The plot of variation of swelling ratio of microsphere against time of immersion is shown in Figure 2. Swelling ratio was determined both at acidic (pH 1.2) and basic (pH 7.4) medium. At lower pH, the microsphere showed a lower swelling ratio compared to those of microspheres at higher pH. This could be attributed to the fact that the microsphere formed by the complexation between gelatin and carrageenan became more stable at lower pH. The observed higher swelling ratio at higher pH might be because of the decomplexation between carrageenan and gelatin. Similar observations were reported by Liu et al.[28] during studying the swelling of gelatin-DNA semi-interpenetrating network at different pH. Further, microsphere with higher crosslinking showed lower swelling ratio. The lower swelling ratio might be because of the formation of more compact wall caused by the formation of crosslinking[29].

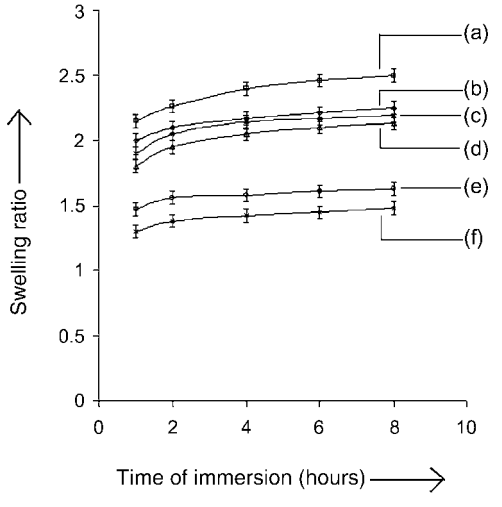

Figure 2. Effect of variation of time of immersion on swelling ratio of microspheres [at pH 7.4, polymer: 3.0 g: (a) crosslinker: 0.2 mmol; (c) crosslinker: 0.4 mmol; (e) crosslinker: 0.8 mmol. At pH 1.2, polymer: 3.0 g: (b) crosslinker: 0.2 mmol; (d) crosslinker: 0.4 mmol; (f) crosslinker: 0.8 mmol].

Effect of variation of drug concentration on stability and percent encapsulation

The effect of variation of isoniazid concentration on stability of microspheres is shown in Table 1. It was observed that crosslinked microspheres were more stable than uncrosslinked microsphere on immersing in isoniazid solution of similar concentration. The increase in stability of crosslinked microspheres might be because of the formation of more compact wall compared to those of uncrosslinked microspheres. Further in the case of uncrosslinked microspheres, it was observed that the higher the concentration of isoniazid, the lower was the stability. Percent encapsulation increased with the increase in the concentration of isoniazid. It was reported that isoniazid was weakly basic in nature[30]. Higher percent encapsulation and basic

Table 1. Effect of variation of isoniazid concentration and crosslinking on stability of microspheres.

Isoniazid concentration (w/v) (%)	Amount of genipin (mmol)	Stability of the microspheres (minutes)
0.5	—	60
1.0	—	50
3.0	—	45
5.0	—	30
7.0	—	25
10.0	—	<25
10.0	0.2	30
10.0	0.4	45
10.0	0.8	>60

nature of isoniazid might help to decompose rapidly the carrageenan–gelatin complex, which in turn would decrease the stability of the microspheres.

The effect of variation of isoniazid concentration on percent encapsulation is shown in Table 2. At a fixed time of immersion, the encapsulation (%) was found to increase with the increase in the concentration of isoniazid. An increase in encapsulation (%) was also observed as time of immersion increased. Again, the higher the crosslinking, the lower was the absorption. The increase in encapsulation (%) was because of more absorption of isoniazid. The decrease in encapsulation (%) might be attributed to the formation of more compact wall in the microspheres because of crosslinking, which led to decrease in the diffusion rate of isoniazid.

Effect of variation of crosslinker concentration on release rate of isoniazid

The effect of variation of crosslinker concentration (0.067–0.267 mmol/g of polymer) on release rate at pH values 1.2 and 7.4 are shown in Figures 3 and 4, respectively. Microspheres having approximately similar loading of isoniazid were considered for the study of the release rate at different pH.

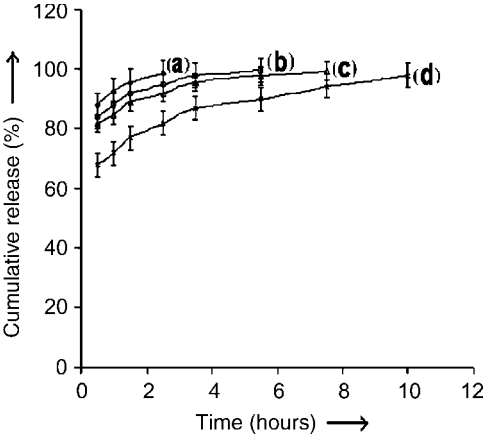

Figure 3. Effect of variation of crosslinker concentration on release profile at pH 1.2. [(a) polymer: 3.0 g, crosslinker: 0 mmol; (b) polymer: 3.0 g, crosslinker: 0.2 mmol; (c) polymer: 3.0 g, crosslinker: 0.4 mmol; (d) polymer: 3.0 g, crosslinker: 0.8 mmol].

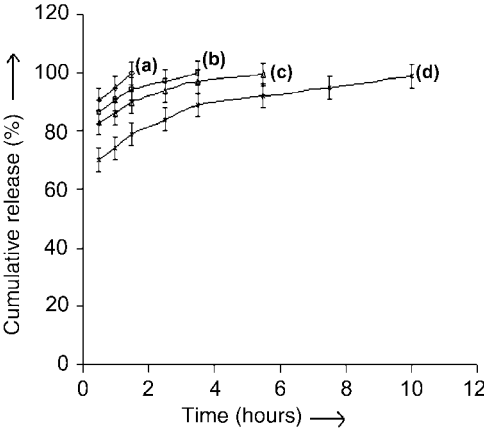

Figure 4. Effect of variation of crosslinker concentration on release profile at pH 7.4. [(a) polymer: 3.0 g, crosslinker: 0 mmol; (b) polymer: 3.0 g, crosslinker: 0.2 mmol; (c) polymer: 3.0 g, crosslinker: 0.4 mmol; (d) polymer: 3.0 g, crosslinker: 0.8 mmol].

The release rate of isoniazid was found to decrease with the increase in the crosslinker in microspheres. In all the cases, a burst release was observed at the beginning, reaching maximum, and then almost leveled off.

Table 2. Effect of variation of isoniazid concentration on percent encapsulation.

Amount of polymer (g)	Concentration of isoniazid (%) (w/v)	Time of adsorption (minutes)	Amount of genipin (mmol)	Encapsulaton (%)
3.0	0.5	20	—	2.0 ± 0.045
3.0	1.0	20	—	7.5 ± 0.12
3.0	3.0	20	—	9.3 ± 0.21
3.0	3.0	30	—	15.5 ± 0.22
3.0	3.0	30	0.2	9.8 ± 0.21
3.0	3.0	30	0.4	9.0 ± 0.18
3.0	3.0	30	0.8	5.4 ± 0.10
3.0	3.0	60	0.8	6.1 ± 0.15
3.0	3.0	120	0.8	9.0 ± 0.19

The compact microsphere wall was responsible for the decrease in release rate as explained earlier.

Further, the amount of isoniazid release at lower pH (1.2) was less compared to that of at higher pH (7.4) throughout the time duration studied for all the crosslinked samples. The lower and higher release in acidic and basic medium, respectively, might be explained by considering the complexation and decomplexation between gelatin and carrageenan as discussed earlier.

Fourier transform infrared study

The spectra of carrageenan (curve-a), gelatin-A (curve-b), carrageenan–gelatin complex (curve-c), isoniazid (curve-d), and isoniazid-loaded crosslinked carrageenan–gelatin microspheres (curve-e) are shown in Figure 5. The spectrum of carrageenan showed absorption bands at 3423, 2910, 1643, 1434, 1379, 1265, and 846 cm^{-1}, which were because of O−H stretching vibration, CH_3 symmetric+CH_2 assymetric vibration, N−H bending, CH_3 + CH_2 bending vibration, sulfonic acid group, C−O stretching band, and glycosidic linkages. The notable absorption bands for gelatin-A appeared at 3421 cm^{-1} (NH stretching), 1630.44 cm^{-1} (amide-I, CO, and CN stretching), 1530 cm^{-1} (amide-II), and 1250 cm^{-1} (amide-III). Among the absorption bands, the amide I band between 1600 and 1700 cm^{-1} is the most important peak for infrared analysis of the secondary structure of protein like gelatin[31]. In the complex of gelatin and carrageenan, a slight shift of the peak of amide I from 1630.44 to 1628.25 cm^{-1} was observed. This indicated that the negatively charged sulfate ester groups might associate with positively charged gelatin. Similar type of observation was reported by Pranoto et al.[32] The probable interaction between carrageenan and gelatin is shown in Figure 6. A shift of the sulfonic acid absorption band to higher wave number because of interaction between carrageenan and gelatin was reported by Li et al. during studying of electrosynthesis of κ carrageenan–gelatin complex[33]. However, this type of shifting was not observed in this case. In the spectrum (shown as curve-d)

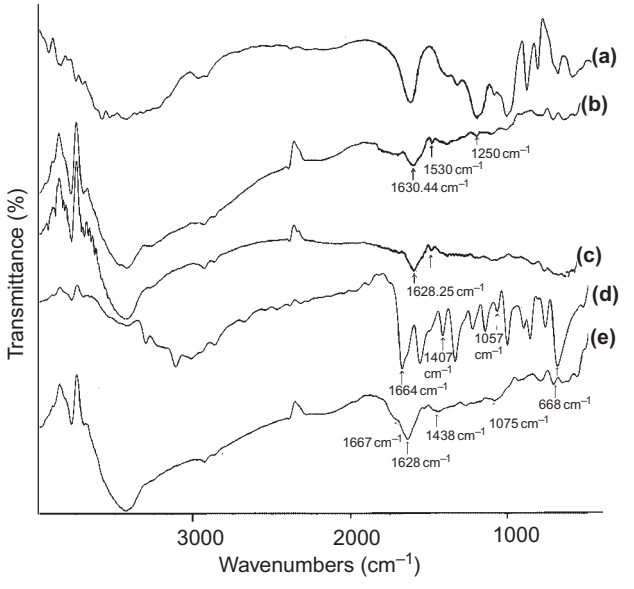

Figure 5. FTIR spectra of (a) carrageenan, (b) gelatin, (c) gelatin–carrageenan complex, (d) isoniazid, and (e) isoniazid-loaded microcapsules.

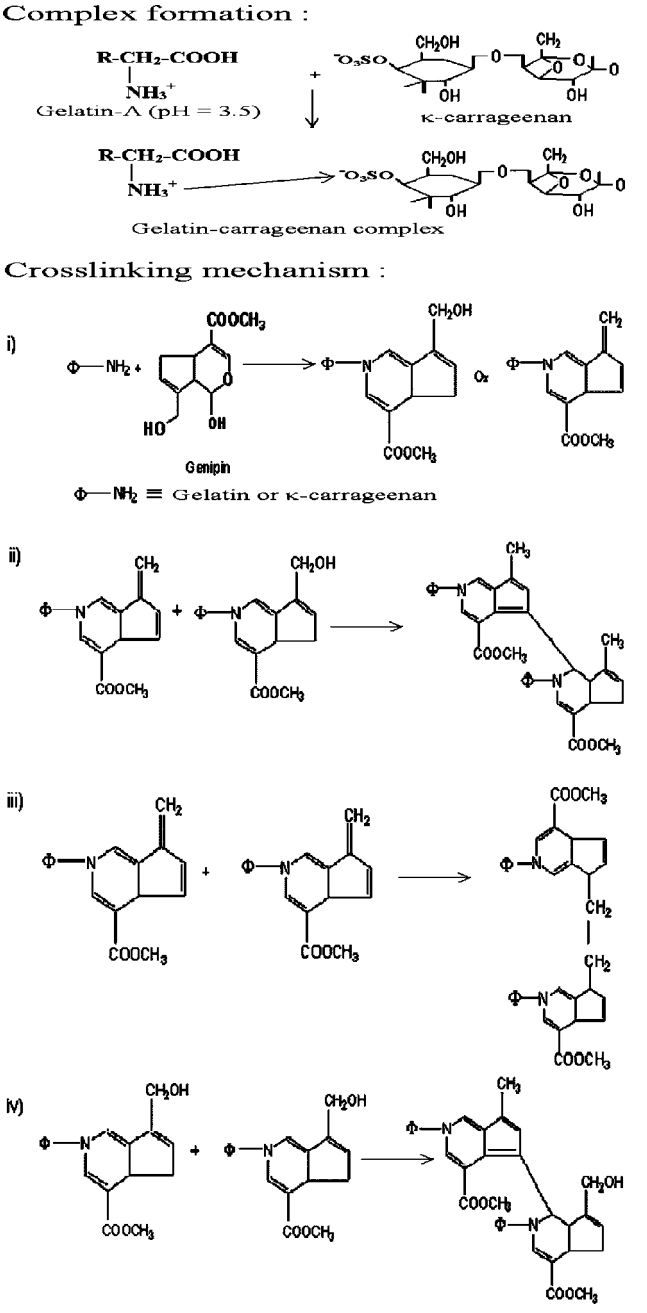

Figure 6. Probable reaction scheme for interaction between carrageenan and gelatin, and genipin with carrageenan–gelatin complex.

of isoniazid, the carbonyl absorption (amide I band) appeared at 1664 cm^{-1}. The amide II band that occurred at 1552.32 cm^{-1} was because of N−H bending of the secondary amide group. Moreover, multiple bands appeared between 1407 and 668.53 cm^{-1} in the spectrum of isoniazid. Some of these characteristic bands of isoniazid appeared in the isoniazid-loaded microspheres (curve-e), suggested the successful loading of isoniazid in the microcapsules. Similar type of infrared spectral pattern for isoniazid and isoniazid containing capsules were reported by Kim et al.[34]

Thermal property study

DSC thermograms of carrageenan–gelatin microspheres (curve-a), isoniazid (curve-b), and isoniazid-loaded microspheres (curve-c) are shown in Figure 7. The endotherm appeared in all the thermograms except isoniazid at around 100°C were because of removal of moisture. The thermograms of isoniazid showed an endothermic peak because of melting at around 190°C. There was no characteristic peak of isoniazid in the thermograms of isoniazid-loaded microspheres. These results indicated that isoniazid was molecularly dispersed in the microspheres. Similar observation was reported by Patil et al. during DSC analysis of carvedilol drug encapsulated within alginate microspheres[35].

X-ray diffraction studies

X-ray diffractograms of carrageenan–gelatin microspheres (curve-a), isoniazid-loaded microspheres (curve-b), and isoniazid (curve-c) are shown in Figure 8. Isoniazid showed multiple sharp peaks at 2θ varying from 12 to 50°, which were because of the crystalline nature of

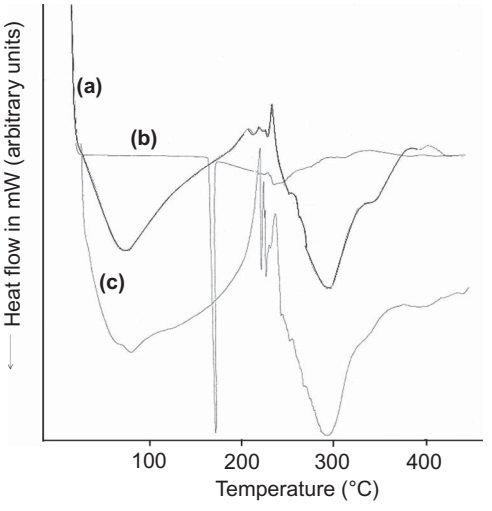

Figure 7. DSC thermograms of (a) carrageenan–gelatin complex, (b) isoniazid, and (c) isoniazid-loaded microcapsules.

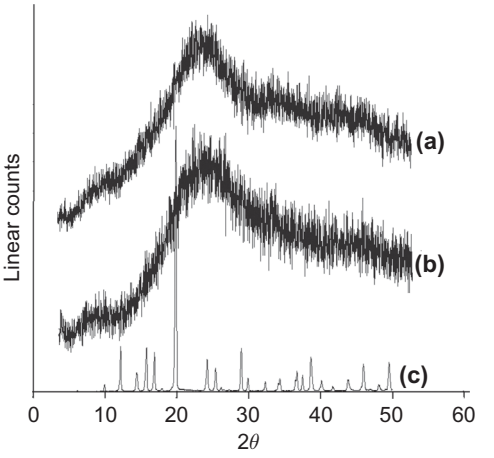

Figure 8. X-ray diffractograms of (a) carrageenan–gelatin microspheres, (b) isoniazid-loaded microspheres, and (c) pure isoniazid.

isoniazid. However, these peaks were not observed in the diffractograms of isoniazid-loaded microspheres indicating the occurrence of a molecular level dispersion of isoniazid in the isoniazid-loaded microspheres. Similar type of findings was reported by Patil et al. in the study of encapsulation of drug[35].

Conclusions

Microspheres of carrageenan–gelatin were prepared successfully using sunflower oil as the dispersing medium. The sizes of the microspheres were controlled by varying concentrations of surfactant and polymer. Higher amount of surfactant and lower polymer concentration favored the formation of smaller microspheres. In the basic medium, the microspheres swelled more compared to that of acidic medium. The absorption of isoniazid into the microspheres was dependent on the concentration of isoniazid solution. The higher the concentration of isoniazid solution, the higher was the percent encapsulation. Stability of microsphere was dependent on crosslinking and the concentration of isoniazid in the solution. The higher the concentration of isoniazid solution, the lower was the stability. Higher pH medium facilitated the release of isoniazid more than lower pH medium. FTIR spectroscopy indicated the loading of isoniazid into the microspheres. DSC and X-ray diffraction results indicated a molecular level dispersion of isoniazid in the microspheres.

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Declaration of interest

The authors report no conflicts of interest.

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