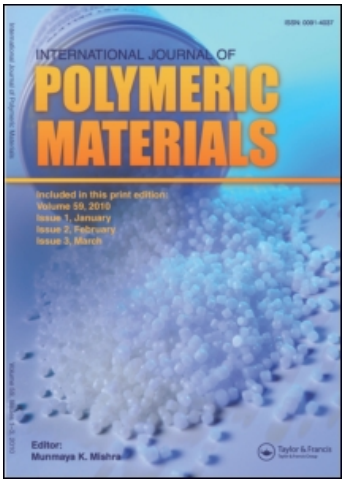

International Journal of Polymeric Materials
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713647664

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Online publication date: 09 August 2010

To cite this Article Devi, Nirmala and Maji, Tarun Kumar(2010) 'Genipin Crosslinked Chitosan-κ-carrageenan Polyelectrolyte Nanocapsules for the Controlled Delivery of Isoniazid', International Journal of Polymeric Materials, 59: 10, 828 — 841
To link to this Article: DOI: 10.1080/00914037.2010.484792
URL: http://dx.doi.org/10.1080/00914037.2010.484792

International Journal of Polymeric Materials, 59:828–841, 2010

ISSN: 0091-4037 print/1563-535X online
DOI: 10.1080/00914037.2010.484792

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Genipin Crosslinked Chitosan-κ-carrageenan Polyelectrolyte Nanocapsules for the Controlled Delivery of Isoniazid

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Polyelectrolyte nanocapsules of κ-carrageenan and chitosan polyelectrolyte complex were prepared by encapsulating neem seed oil (NSO). Nanocapsules were freed from NSO and then loaded with isoniazid. Sonication and surfactant concentration controlled the particle size of the nanocapsules as indicated by the particle size analyzer. TEM study supported the observation obtained from the particle size analyzer. The loading of the drug into the nanocapsules was time-dependent. The release rates of isoniazid were higher at acidic media compared to basic media. FTIR study showed that there was no remarkable interaction between the isoniazid and polyelectrolyte complex. Surface characteristics of the nanocapsules were studied by SEM. X-ray diffraction study showed that the dispersion of isoniazid had not occurred at the molecular level.

Keywords encapsulation, isoniazid, nanocapsules, neem seed oil, polyelectrolyte

INTRODUCTION

The development of suitable carriers for drug delivery still remains an urgent need. Delivering drugs at a controlled rate, slow rate, and targeted area by

Received 6 January 2010; accepted 12 March 2010.
Financial assistance in the form of a fellowship to one of the authors (ND) by Tezpur University is gratefully acknowledged.
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various methods, e.g., matrix, microencapsulation and nanoencapsulation are very attractive possibilities that are being pursued enthusiastically. Polyelectrolyte capsules have recently been introduced as new microscopic vesicles which could have high potential in the biomedical field [1]. These novel types of vesicles were introduced recently [2,3]. They are fabricated using the layer-by-layer (LBL) technique, i.e., the self-assembly of charged species onto an oppositely charged sacrificial colloidal substrate followed by the dissolution of this substrate [1]. Among various LBL self-assembled structures, hollow polyelectrolyte capsules are of special interests as a platform device for applications in drug delivery, bioreactor design and biosensing. These polyelectrolyte capsules have several unique characteristics over conventional capsules (e.g., liposomes) [4].

Polymeric drug carriers are drawing special attention as suitable drug carriers. Although experience with synthetic polymers is extensive and encouraging [5–7], the trend has been to shift towards natural polymers [8]. The major advantage of natural polymers includes their low cost and compatibility with the encapsulation of a wide range of drugs, with minimal use of organic solvents. Furthermore, bio-adhesion, stability, safety and their approval for human use by the US FDA are additional advantages [8–10].

Chitosan is a hydrophilic cationic polyelectrolyte obtained by alkaline *N*-deacetylation of chitin. Chitin is the most abundant natural polymer next to cellulose and is obtained from crab and shrimp shells [11]. Chitosan has been broadly evaluated by industry due to its biocompatibility and its potential use in controlled-release systems as membranes, tablets and microspheres. Carrageenans are naturally occurring high molecular weight polysaccharides extracted from seaweeds and are made up of the repeating units of galactose and 3,6 anhydrogalactose [12]. They consist of the sulfate esters of galactose and 3,6 anhydrogalactose joined by alternating α-1,3 and β-1,4 glycosidic linkages [13].

When two oppositely charged polyelectrolytes are mixed in an aqueous solution, a polyelectrolyte complex is formed by the electrostatic attraction between the polyelectrolytes. Chitosan-carrageenan polyelectrolyte complex has been developed for the controlled release of drugs. Tomida et al. [14] suggested that κ-carrageenan-chitosan membrane spherical capsules could release theophylline as a model drug from the capsules. Tapia et al. [15] evaluated the possibility of using mixtures of chitosan and/or polyelectrolyte complexes of κ-carrageenan and chitosan in a tablet form as a prolonged-release system, using diltiazem hydrochloride as a model drug.

Isoniazid is widely used in the chemotherapy of tuberculosis. The encapsulation of isoniazid tends to be limited due to its hydrophilic characteristics [16]. The procedure of polyelectrolyte complex formation of carrageenan and chitosan also involves unsuitable conditions, i.e., high temperature. Therefore, hollow nanocapsules were formed initially by encapsulating NSO within a

carrageenan-chitosan complex and finally by the removal of NSO completely. This was followed by loading isoniazid in the hollow nanocapsules.

In order to control the release behavior of the polyelectrolyte complex, a variety of crosslinkers can be used. Natural crosslinkers are less toxic compared to synthetic crosslinkers. Genipin, a natural crosslinker, is reported to be much less toxic than glutaraldehyde, a crosslinker of synthetic origin [17]. The present work aims to produce hollow genipin crosslinked nanocapsules of κ-carrageenan and chitosan and to encapsulate isoniazid inside nanocapsules. Efforts have also been made to study the release behavior of drugs from nanocapsules prepared under different conditions.

MATERIALS AND METHODS

Materials

Carrageenan Type I, (predominantly k-and lesser amount of λ-carrageenan) and chitosan (medium molecular weight with Brookfield viscosity ~ 200 cps) were purchased from Sigma-Aldrich Inc. (USA). Glacial acetic acid (E. Merck, India), Tween 80 (E. Merck, India), Genipin (Challenge Bioproducts Co., Ltd., Taiwan), were used without further purification. The core material, cold-pressed neem seed oil, was a gift sample of Ozone Biotech., Faridabad, India. Isoniazid was purchased from Sigma-Aldrich Inc. (USA). DDI (double-distilled deionized) water was used throughout the study. Other reagents used were of analytical grade.

Encapsulation Procedure

In a beaker, a known amount of (100 ml) 0.35% (w/v) carrageenan solution was taken. This polymer solution was stirred by mechanical stirrer under high agitation at $70 \pm 1^{\circ}$C. In a separate beaker Tween 80 (0.1 g–0.3 g) was dissolved in a minimum amount of distilled water by heating. It was then mixed with a stipulated amount of neem seed oil by stirring. This Tween 80 added NSO was mixed with carrageenan solution at 60°C under continuous stirring. This mixture was then sonicated for 90 min. After completion of sonication, this was stirred by mechanical stirrer under high agitation at $70 \pm 1^{\circ}$C. This temperature was maintained throughout the experiment. Chitosan solution of 0.35% (w/v) was added to this dropwise by a syringe to attain complete phase separation. The beaker containing the nanocapsules was left to rest at this temperature for 15 min. The system was then brought to 5–10°C to harden the microcapsules. The pH of the system was then brought to 7.0 by the addition of sodium hydroxide solution, as this pH favored maximum crosslinking with genipin. Now, the crosslinking of the polymer nanocapsules was

achieved by the slow addition of certain amount of genipin. The temperature of the beaker was then raised to 45°C and stirring was continued for another 3–4 h to complete the crosslinking reaction. The beaker was then cooled to room temperature. The nanocapsules were centrifuged and washed. This cycle was repeated until it was freed from alkali.

Wet crosslinked nanocapsules were directly transferred to a beaker containing a sufficient amount of acetone and left for a stipulated time period till complete removal of NSO. This was judged by the absence of the characteristic peak of NSO in the FTIR spectrum of the nanocapsules. These are now filtered several times with fresh acetone through a filter paper and allowed to dry at room temperature. These hollow capsules were immersed in saturated solution of isoniazid for different time periods. The isoniazid-filled nanocapsules were further filtered, and washed with water to remove any isoniazid adhered to its surface. Finally, it was dried in a vacuum oven at room temperature (30 ± 1°C).

Particle Size Analysis

Particle size measurements were carried out using a DLS550V particle size analyzer by dynamic light scattering method. The dispersed NSO-encapsulated nanocapsules, after centrifugation and washing with water, were diluted again with DDI for the measurement of particle size. The particle size analyzer measured and reported the mean size of the nanocapsules.

Transmission Electron Microscopy Study

The transmission electron microscopy was carried out in a transmission electron microscope (model JEOL JEM 100CXII). The dispersed nanocapsules in distilled water was used for transmission electron microscopy study. A small drop of the sample was poured through a micropipette on a copper grid. The copper grid was allowed to dry in an oven. It was now ready for study by transmission electron microscope.

Calibration Curve of Isoniazid

A calibration curve is required for the determination of release rate of isoniazid from the nanocapsules. A known concentration of isoniazid in DDI water was scanned in the range of 200–500 nm by using a UV-visible spectrophotometer. For isoniazid with concentration in the range 0.001 to 0.01 gm/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 the calibration curve, the unknown concentration of isoniazid was obtained by knowing the absorbance value.

Encapsulation Efficiency

A known amount of accurately weighed nanocapsules was ground in a mortar, transferred with precaution to a volumetric flask containing 100 ml of water (with pH = 1.2 maintained by HCl) and kept for overnight with continuous stirring to dissolve the drug inside the capsules. The solution was collected and the drug inside the capsules was determined using UV spectrophotometry. The encapsulation efficiency (%), was calculated by using the calibration curve and the following formula

$$\text{Encapsulation efficiency } (\%) = w_1/w_2 \times 100$$

where

w_1 = amount of isoniazid encapsulated in a known amount of nanocapsules

w_2 = weight of nanocapsules.

Drug Release Studies

Isoniazid release studies of encapsulated isoniazid were done by using UV–visible spectrophotometer (UV-2001 Hitachi). A known quantity of nanocapsules was placed into a known volume of water with a different pH (pH = 1.2 and 7.5). This pH was maintained by using HCl and phosphate buffer solution. The content was shaken from time to time 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 a 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 with the same pH was returned to the container.

Fourier Transform Infrared (FTIR) Study

FTIR spectra were recorded using KBr pellet in a Nicholet (model Impact-410) spectrophotometer. NSO, NSO-loaded nanocapsules, hollow nanocapsules after complete removal of NSO, isoniazid and isoniazid-containing nanocapsules were each separately finely grounded with KBr and FTIR spectra were recorded in the range of 4000–400 cm^{-1}.

Scanning Electron Microscopy Study

Scanning electron micrographs of samples prepared by varying the amount of Tween 80, samples after removal of NSO, and samples after encapsulation of NSO were each separately taken. The samples were deposited on a brass holder and sputtered with platinum. Surface characteristics of the microcapsules were studied at room temperature using a scanning electron microscope (model JEOL, JSM-6390) at an accelerated voltage of 5–10 kV.

X-ray Diffraction Study

X-ray diffractograms of isoniazid, nanocapsules with oil encapsulation and isoniazid-encapsulated samples were recorded on a X-ray diffractometer (Model MiniFlex, Rigaku corporation, Japan). The samples were scanned between $2\theta = 10^\circ$ to 50° at the scan rate of 4°/min.

RESULTS AND DISCUSSION

Particle Size Analysis

Effects of variation of surfactant amount on the average particle size of the capsules are shown in Table 1. It was observed that the size of the capsules decreased as the amount of surfactant (Tween 80) increased. At a fixed oil concentration, the emulsifying capacity of a surfactant increased with the increase in the concentration of surfactant. This resulted in the generation of the smaller oil vesicles, which in turn would lead to the formation of smaller capsules. Sonication also assisted to further decrease the particle size. Landfester reported that the size of the droplets in oil in water miniemulsions decreased [18] with the increase in the concentration of surfactant and sonication time.

Table 1: Effect of variation of amount of surfactant on particle size.

Sample particulars	Tween 80 (g)	Sonication time (mins)	Average diameter (mean) (μm)
Carrageenan/ Chitosan/NSO	–	–	3.2620 ± 0.5275
	0.1	–	2.2525 ± 0.2092
	0.1	90	1.9984 ± 0.1852
	0.2	90	1.5414 ± 0.2911
	0.3	90	0.2344 ± 0.0047

(Carrageenan: 0.35 g; Chitosan: 0.13 g; NSO: 1 g; Sonication: 90 minutes; Temperature: $60 \pm 1^\circ$C, Total volume: 136 ml).

Transmission Electron Microscopy Study

Transmission electron micrographs of NSO encapsulated samples are shown in Figure 1. Micrograph (a) represented the capsules without the addition of surfactant. Micrographs Figure 1(c–e) were taken for samples prepared by employing sonication and varying surfactant concentration. The concentration of surfactant was varied from 0.1–0.3 g/100 ml of polymer solution. The micrograph for the sample prepared by using surfactant and without any sonication was shown in Figure 1(b). The micrograph shown in Figure 1(e) was magnified further in order to get some clear idea about the size of the capsule. This was shown in Figure 1(f). It was observed that average particle size decreased with the increase in the concentration of the surfactant. Sonication also played a role in decreasing the particle size (Figure 1(c–e)). The calculated diameter of a randomly chosen nanocapsule was 230 nm, which was close to that of the average particle size (234 nm)

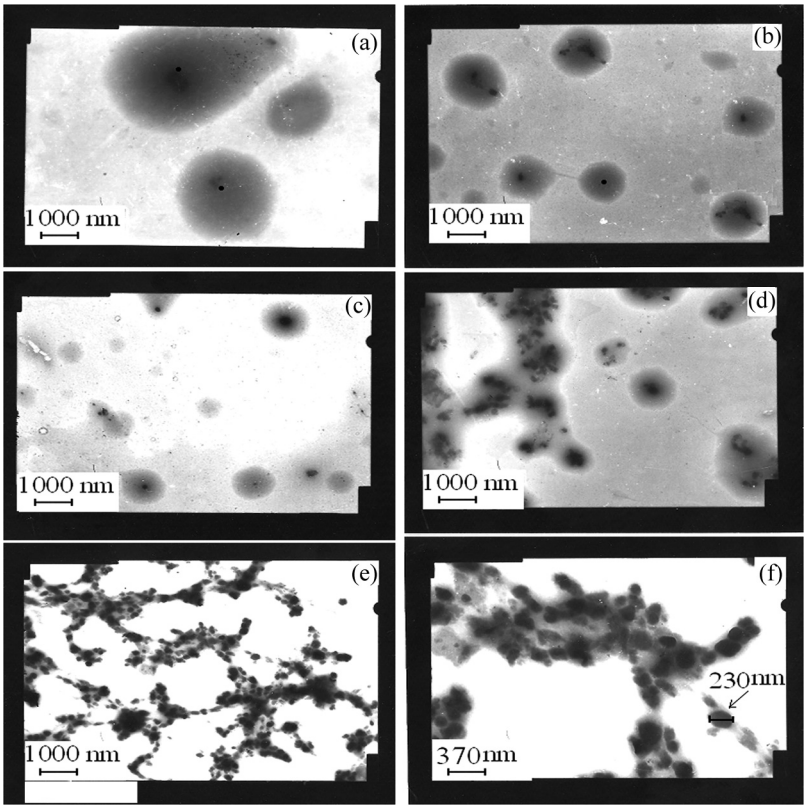

Figure 1: Transmission electron micrographs of NSO-loaded nanocapsules prepared (a) without surfactant (b) 0.1 g Tween 80 (c) 0.1 g Tween 80 and sonication (d) 0.2 g Tween 80 and sonication (e) 0.3 g Tween 80 and sonication (f) 0.3 g Tween 80 and sonication (higher magnification).

measured by particle size analyzer. Moreover, a clear and distinct layer of two types of materials was observed in the nanocapsules. The core material NSO appeared dark and the surrounding polymeric material was a little brighter. A similar observation was reported by Nabi et al. [19] during TEM study of nanoemulsion. On decreasing the size of the capsules, the layer separation was not distinct probably due to the agglomeration of nanocapsules. The results of TEM study were in agreement with the results obtained from the particle size analyzer.

Effect of Variation of Immersion Time in Drug

The effect of variation of immersion time of nanocapsules in the saturated solution of isoniazid is shown in Table 2. With the increase in the time of immersion, the encapsulation efficiency (%) increased initially at a faster rate and later at a slower rate. This could be attributed to the difference in concentration of the drug solution between the outside and inside of the nanocapsules. The higher the concentration gradient, the higher the rate of diffusion. The space availability inside the pore was more initially. The available space gradually decreased with the increase of the immersion time. As a result, a variation in the rate of diffusion occurred. This variation in the rate of diffusion from the beginning of the immersion until attainment of equilibrium governed the encapsulation efficiency.

Effect of Variation of Crosslinker Concentration

The effect of variation of crosslinker concentration on encapsulation efficiency (%) and release rate is shown in Table 2 and Figure 2. The encapsulation efficiency increased as the concentration of genipin was varied from 0.1 to 0.4 mmol. The increase in encapsulation efficiency (%) could be due to the

Table 2: Effect of variation of immersion time in isoniazid solution and concentration of crosslinker on percent encapsulation.

Sample particulars	Genipin (mmol)	Time of immersion in isoniazid solution (hr)	Encapsulation (%)
Chitosan/Carrageenan/ NSO/Genipin	0.2	2	30 ± 1.5
	0.2	6	42.5 ± 2.0
	0.2	12	48.7 ± 1.4
	0.2	24	50 ± 2.7
	0.4	24	52 ± 3.5
	0.1	24	24 ± 1.2

(Carrageenan: 0.35 g; Chitosan: 0.13 g; NSO: 1 g; Genipin: 0.1–0.4 mmol; Total volume: 136 ml; Temperature: $30 \pm 1^{\circ}C$).

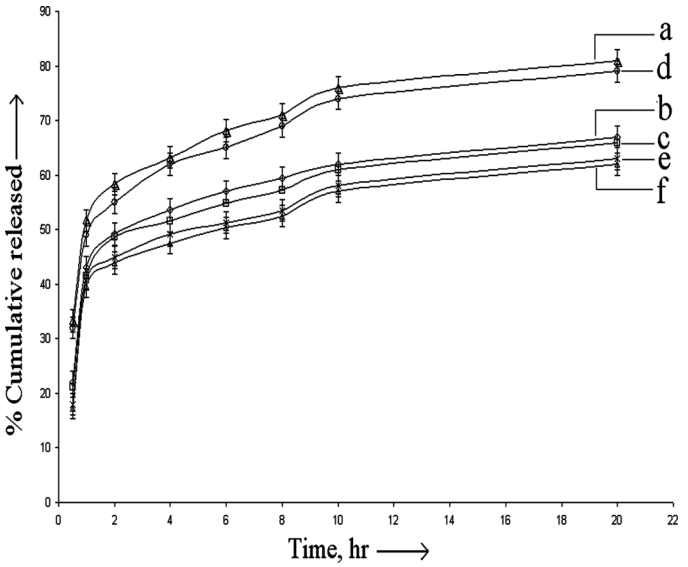

Figure 2: Effect of variation of crosslinker concentration and pH of the medium on the release rate of isoniazid (a) 0.1 mmol genipin, pH = 1.2 (b) 0.2 mmol genipin, pH = 1.2 (c) 0.4 mmol genipin, pH = 1.2 (d) 0.1 mmol genipin, pH = 7.4 (e) 0.2 mmol genipin, pH = 7.4 (f) 0.4 mmol genipin, pH = 7.4.

improvement of drug retention capacity of the nanocapsules caused by the formation of crosslinking. The crosslinking reaction took place between the genipin and polyelectrolyte complex of carrageenan and chitosan. Further, the encapsulation efficiency increased initially at a rapid rate and later at a slow rate. As the concentration of the crosslinker increased, the crosslinking reaction approached saturation due to which encapsulation decreased at a later stage. The release rate of the drug was found to decrease as the % of genipin increased. In all the cases, a burst release was observed at the beginning, reaching maximum within 6 hours, and then almost leveled off. The nanocapsule wall became compact as the degree of crosslinking increased. This resulted in the decrease of diffusion rate through the nanocapsule wall [20]. The swelling capacity of the capsules decreased as crosslinker increased. Agnigotri et al. reported a similar type of phenomenon of swelling of chitosan microparticles [21]. The decrease in release rate could be explained by considering the combined effect of swelling and diffusion.

Effect of Variation of pH

Figure 2 shows the results of the effect of variation of pH on the release rate of drug. Cumulative release rate profiles were studied in acidic (pH = 1.2) and basic (phosphate buffer pH = 7.5) media. The amount of

isoniazid released in acidic medium was higher than that of in basic medium throughout the time duration studied. In both acidic and basic media the release rates were fast initially, up to 6 hours, and after that it remained almost constant during the remaining time period.

At a fixed isoniazid concentration, the release profiles of the isoniazid in both acidic and basic media were affected by two factors-the swelling nature of the polymer and the solubility of the drug. For chitosan-carrageenan polyelectrolyte complex, the swelling did not significantly change on altering the pH of the system as reported by Piyakulawat et al. [22]. The swelling of chitosan and carrageenan gel did not occur below pH 9.0 [23]. Thus the difference in release profile in both the acidic and basic solution observed for this system was not governed by the swelling of the polymer, but by the solubility of isoniazid. The solubility of isoniazid increased at acidic pH due to its weak basic nature as reported in the literature [24]. Therefore, the higher release rate of isoniazid at acidic pH may be attributed to its higher solubility at lower pH.

Fourier Transform Infrared (FTIR) Study

FTIR spectra of the neat polyelectrolyte complex of chitosan and carrageenan (curve a), NSO (curve b), NSO-loaded chitosan-carrageenan nanocapsules

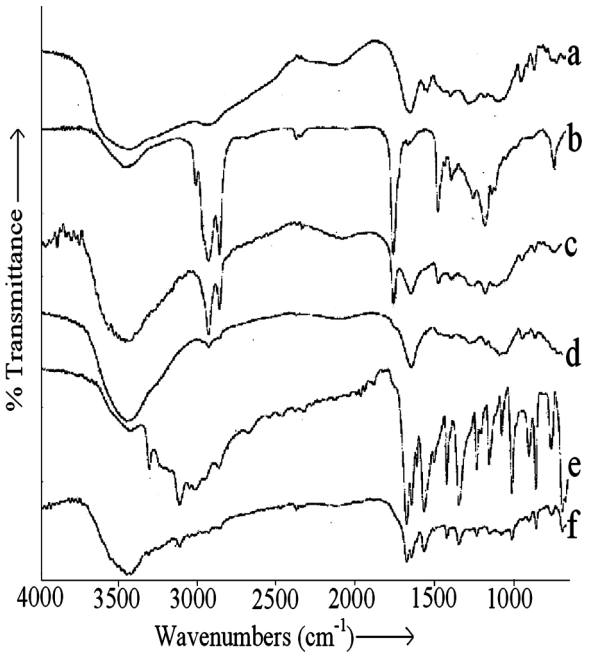

Figure 3: FTIR spectra of (a) polyelectrolyte complex of chitosan and carrageenan (b) NSO c) NSO-loaded crosslinked nanocapsules (d) nanocapsules after removal of NSO (e) isoniazid (f) isoniazid loaded nanocapsules.

(curve c), NSO-free nanocapsules (curve d), isoniazid (curve e) and isoniazid-loaded chitosan-carrageenan nanocapsules (curve f), are shown in Figure 3. In curve (a), the band appeared at 1528 cm^{-1} due to NH_3^+ groups and confirmed the formation of a strong polyelectrolyte complex between chitosan and carrageenan. The absorption bands appearing in the spectrum of NSO (curve-b) at 1745.90 cm^{-1}, 1463.04 cm^{-1} and 1163.85 cm^{-1} were due to carbonyl stretching, CH_2 asymmetric deformation and C-C stretching vibration. The peaks observed in the spectrum of NSO (curve b) also appeared in the NSO-loaded nanocapsules (curve c). This confirmed the successful encapsulation of NSO in the nanocapsules without any interaction between NSO and the polyelectrolyte complex. In the spectrum after removal of NSO

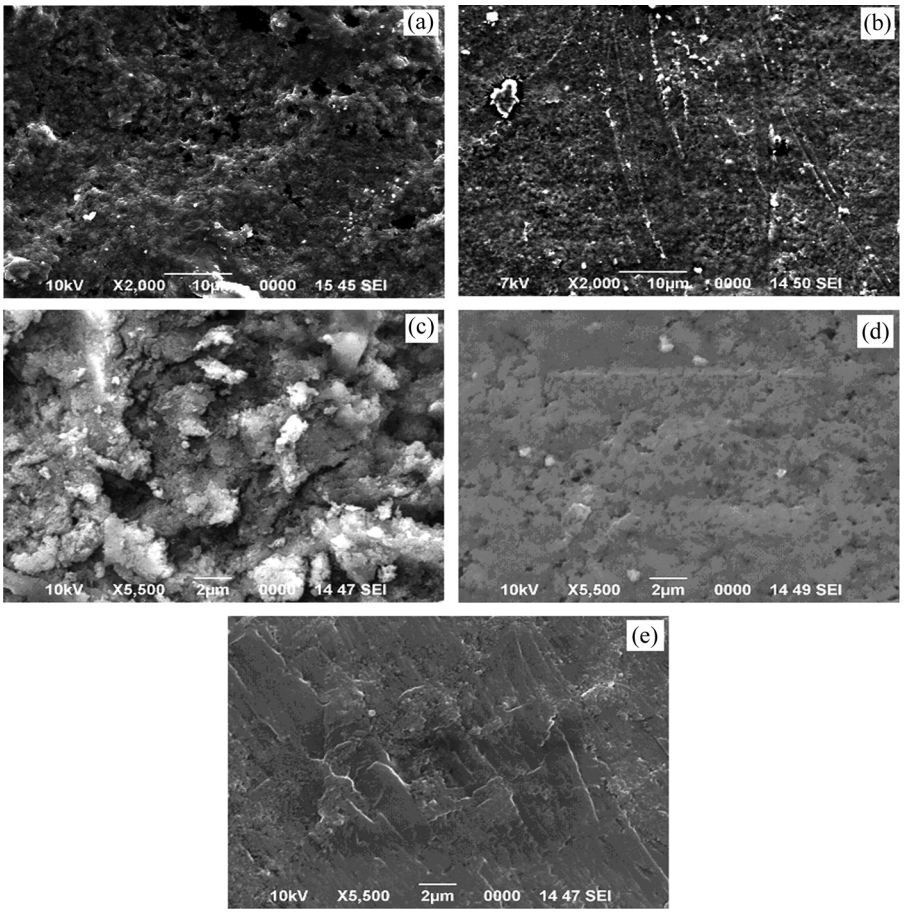

Figure 4: Scanning electron micrographs of NSO-loaded nanocapsules prepared by using (a) 0.1 g Tween 80 and sonication (b) 0.2 g Tween 80 and sonication (c) 0.3 g Tween 80 and sonication (d) nanocapsules after removal of NSO (e) isoniazid-loaded nanocapsules.

(curve d), the absence of any characteristic peak of NSO confirmed the complete removal of NSO from the nanocapsules.

In the spectrum (shown as curve e) of isoniazid, the carbonyl absorption (amide I band) appeared at 1667.00 cm^{-1}. The amide II band that occurred at 1555.90 cm^{-1} was due to N-H bending of the secondary amide group. The band due to stretching of C = N appeared at 1633.51 cm^{-1}. Moreover, bands also appeared at 1335.67 cm^{-1} and 995.54 cm^{-1} in the spectrum of isoniazid. All the characteristic bands of isoniazid appeared in the isoniazid-loaded nanocapsules (curve f), suggesting the successful loading of isoniazid in the hollow nanocapsules. A similar type of IR spectral pattern for isoniazid and isoniazid-containing capsules were reported by Kim et al. [25].

Scanning Electron Microscopy Study

The scanning electron micrographs of the samples prepared by using varying amounts of surfactants (Tween 80, 0.1 g–0.3 g) are shown in Figure 4(a–c). Figure 4(d) represents capsules after removal of NSO. Figure 4(e) shows the micrograph of isoniazid-loaded capsules. The size of the capsules was found to decrease as the amount of surfactant increased. TEM study also confirmed these findings. Micrograph 4(c) shows the capsules in agglomerated form. Figure 4(d) represents the micrograph of the sample after complete removal

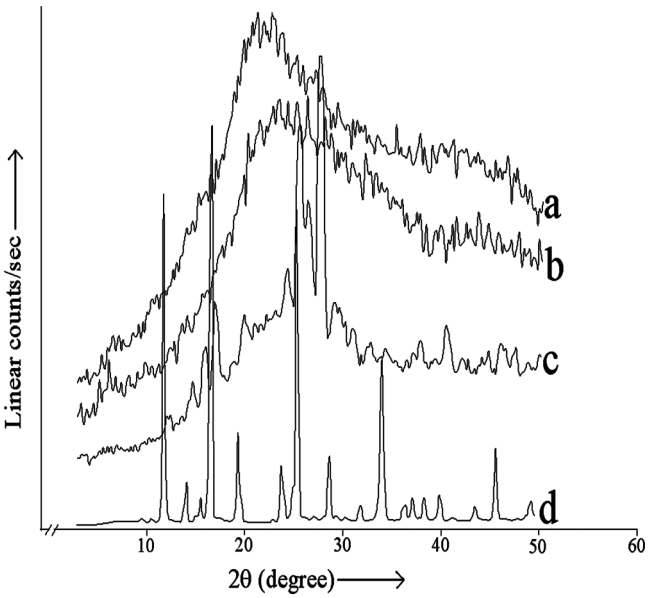

Figure 5: X-ray diffractograms of (a) NSO-loaded nanocapsules (b) NSO-free nanocapsules (c) isoniazid-encapsulated nanocapsules (d) isoniazid.

of NSO. In this case the agglomeration was lost but the capsules seemed to be a little collapsed and deformed after the removal of NSO. Figure 4(e) was the micrograph of the sample loaded with isoniazid. The surface of isoniazid-loaded nanocapsules appeared to be collapsed. A similar type of observation was reported in the literature [26].

X-ray Diffraction Study

The X-ray diffractograms of NSO-loaded samples (curve a), nanocapsules after complete removal of NSO (curve b), isoniazid-loaded nanocapsules (curve c) and isoniazid (curve d) are shown in Figure 5. The X-ray diffraction patterns of NSO-loaded samples (curve a) and nanocapsules after complete removal of NSO (curve b) showed broad peaks due to their amorphous nature. Isoniazid had sharp peaks at $2\theta = 11.8$, 16.8, 25.4 and 34.2. Fukuoka et al. also reported the X-ray diffractogram of isoniazid with the peaks in this region [27]. Some of these peaks also appeared in the isoniazid-loaded nanocapsules. This indicated that the dispersion of isoniazid in the nanocapsules was not very good.

CONCLUSIONS

Hollow nanocapsules prepared by using carrageenan-chitosan polyelectrolyte complex could be used for loading isoniazid successfully. Sonication and concentration of surfactant played a role in decreasing the particle size of the nanocapsules as confirmed by particle size analyzer, TEM and SEM study. The loading of drug took place initially at a rapid rate and finally at a slower rate. The release rate of isoniazid was dependent on the pH of the medium. SEM study also indicated that the capsules were deformed after loading with isoniazid. X-ray diffractogram indicated that the dispersion of isoniazid had not occurred uniformly at the molecular level in the nanocapsules.

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