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ORIGINAL PAPER

Genipin crosslinked microcapsules of gelatin A and κ-carrageenan polyelectrolyte complex for encapsulation of Neem (*Azadirachta Indica A.Juss.*) seed oil

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Abstract Microcapsules containing neem seed oil (NSO) were prepared using complex coacervation technique and employing gelatin A and κ-carrageenan polyelectrolyte complex. The yield of the coacervate was dependent on the ratio of the two polymers and on the pH of the medium. Viscosity and turbidity measurements were carried out in order to support the ratio of the two polymers that produced the highest yield. The encapsulation efficiency and the release rates of NSO were dependent on the amount of crosslinker, oil loading and polymer concentration. Scanning electron micrographs showed the formation of free flowing spherical microcapsules. The size of microcapsules increased with the increase of the concentration of the polymer. Fourier Transform Infrared Spectroscopy and Differential Scanning Calorimetry study showed that there was no significant interaction between NSO and carrageenan–gelatin complex.

Keywords Microcapsule · Gelatin · Carrageenan · Complex coacervation · Crosslinking · Characterisation

Introduction

Considerable efforts are made worldwide, now a days, to promote the use of environmentally friendly and biodegradable natural pesticides. The indiscriminate use of synthetic pesticides has resulted in insurgence of pests and diseases and their resistance to chemicals [1]. The residues of chemicals in the human blood have now become a common phenomenon causing health hazards. To overcome this problem, scientists all over the world now recommend bio-control measures as the alternative for chemical pesticides. The advantage of bio-control measures over chemical

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pesticides is that they are cost effective, have no residues, are target specific, harmless to organisms, promote growth of natural enemies of pests and diseases [1]. *Azadirachta Indica A.Juss*., commonly known as the "neem" tree, produces seeds which can be extracted to get neem seed oil (NSO), that has proven its advantages over many synthetic pesticides [2, 3]. Thus, NSO, a potent botanical pesticide is now attracting worldwide interest [4]. Apart from many advantages, the application of it to the soil is limited due to its liquid nature. Controlled release by microencapsulation seems to be the best way to convert NSO into a solid form and at the same time to protect the oil from environmental damage and thus securing a long shelf-life [5].

Natural polymers, due to their eco-friendly nature, cost effectiveness, free availability and most important—their biodegradability nature, are undoubtedly the best choice for soil applications. Different natural or synthetic biodegradable polymers have been used for controlled release purposes. Starch urea formaldehyde matrix has been used for encapsulation of agrochemicals [6]. The use of starch-g-poly(butyl acrylate) as a material for encapsulating carboxylic containing herbicides for controlled release has been reported by Zhu et al. [7].

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 [8]. They consist of the sulphate esters of galactose and 3,6 anhydrogalactose joined by alternating α-1,3 and β-1,4 glycosidic linkages [9]. The carrageenan mixture has three types of carrageenan, namely, κ-carrageenan, λ-carrageenan and ι-carrageenan [10]. κ-carrageenan has one (produces a weak gel which suffer syneresis), ι-carrageenan has two (produces an elastic gel without syneresis) and λ-carrageenan (no gelling) has three sulphate groups per two galactose residues. Carrageenan has been used for various purposes. Few reports are available regarding its use as a matrix for encapsulation.

Gelatin is the denatured collagen and has been widely used as a fundamental material for microspheres [11], sealants [12], tissue adhesives [13] and carriers for controlled delivery systems [14–16]. Gelatin has also been widely used in combination with other polymers for encapsulation [17, 18].

In order to improve the controlled release behaviour, varieties of crosslinking agents like glutaraldehyde, formaldehyde and epoxy compounds [5, 19–21] are reported to be employed for improving the controlled release behaviour. These crosslinking agents can cause physiological toxicity if released into the host due to biodegradation. This leads to an increasing demand for a crosslinking reagent able to form stable and biocompatible crosslinked products with less cytotoxicity problems. Genipin is a naturally occurring crosslinking agent and is biocompatible and less toxic [22, 23]. It can react spontaneously with amino acids or proteins like gelatine [24]. Since carrageenan contains some proteins [25], it can react with genipin [26] to provide crosslinking.

The present work is aimed to produce gelatin-κ-carrageenan complex microcapsules containing NSO by complex coacervation technique using the natural crosslinker, genipin so that the whole system becomes fully natural and biodegradable. Efforts have also been made to study the release characteristics of oil from microcapsules prepared under different conditions.

Experimental

Materials

Carrageenan Type I containing predominantly κ and lesser amount of λ-carrageenan was purchased from Sigma-Aldrich Inc. (USA). Gelatin type A was purchased from Sigma-Aldrich Inc. (USA). Glacial acetic acid (E. Merck, India), Tween 80 (E. Merck, India) and Genipin (Mol. wt. 226.22) (Challenge Bioproducts Co., Ltd., Taiwan) were used as such received. The core material, cold pressed NSO was obtained from Ozone Biotech., Faridabad, India. Double-distilled deionised (DDI) water was used throughout the study. Other reagents used were of analytical grade.

Microencapsulation procedure

Known amount of (100 mL) 0.5–1.5% (w/v) of carrageenan solution in distilled water was taken in a beaker. This polymer solution was stirred by mechanical stirrer under high agitation (approximately 800 rpm) at 70 ± 1 °C. This temperature was maintained throughout the experiment. To this, NSO (1–4 g) was added under high agitation to form an emulsion. A known amount of (200 mL) gelatin A solution of 1–3% (w/v) was added to the beaker drop wise to attain complete phase separation. However, the weight ratio of carrageenan to gelatin was maintained at 1:2 during all the experiments. At this ratio, interaction between gelatin and carrageenan took place completely in accordance with the coacervate % yield and viscosity measurements. The pH of the mixture was then brought down to 3.5 by adding 2.5% (v/v) glacial acetic acid solution. At this pH, the yield was maximum as judged by % yield and turbidity measurements. The beaker containing the microcapsules was left to rest at the same temperature for approximately 15 min. The system was then brought to 5–10 °C to harden the microcapsules. The cross linking of the polymer capsule was achieved by slow addition of certain amount of genipin solution (0.5225% w/v). 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 slowly while stirring. The microcapsules were filtered through 300-mesh nylon cloth, washed with 0.1% (w/v) Tween 80 surfactant solution to remove oil, if any, adhered to the surface of microcapsules. This was further washed with distilled water, freeze dried and stored inside a refrigerator in a glass ampule.

Measurements

Measurement of turbidity, viscosity and coacervate yield

In order to optimise the ratio of gelatin (type-A) and κ-carrageenan, the measurement of turbidity, viscosity and coacervate yield (%) is essential. The mixing of gelatin and carrageenan in different ratios would produce solutions of different turbidity. The optimal ratio at which complete phase separation occurred between gelatin and carrageenan was the point where the solution would have the

maximum turbidity. The change in absorbance due to turbidity was monitored at a particular wavelength employing UV spectrophotometer. The viscosity of the supernatant solution was measured by using an ubbelohde viscometer at 30 °C. Polymer in the supernatant solution would be either negligible or absent when the interaction between carrageenan and gelatin would be maximum. At this stage, the viscosity of the supernatant would be close or similar to the solvent viscosity.

The coacervate yield (%) obtained by mixing of carrageenan and gelatin in different ratios was measured gravimetrically. The coacervates remained after decantation of supernatants were washed with distilled water and then dried at 40 °C till the attainment of constant weight.

Calibration curve of oil content

A calibration curve is required for the determination of release rate of oil from the microcapsules. It was found that 0.1 g of oil could be easily dissolved in 100 mL of water containing 0.1 g Tween 80. A known concentration of NSO in DDI water containing 0.1% (w/v) Tween 80 was scanned in the range of 200–400 nm by using UV visible spectrophotometer. For NSO having concentration in the range 0.001–0.08 g/100 mL, a prominent peak at 254 nm was noticed. The absorbance values at 254 nm obtained with the respective concentrations were recorded and plotted. From the calibration curve, the unknown concentration of NSO was obtained using the determined absorbance value.

Encapsulation efficiency, oil content and oil load

A known amount of accurately weighed microcapsules was grounded in a mortar, transferred with precaution to a volumetric flask containing a known amount of 0.1% (w/v) aqueous Tween 80 solution and kept for overnight with continuous stirring. The encapsulation efficiency (%), oil content (%) and oil loading (%) were calculated by using the calibration curve and the following formulae [5]:

$$\text{Encapsulation efficiency } (\%) = (w_1/w_2) \times 100$$
$$\text{Oil content } (\%) = (w_1/w) \times 100$$
$$\text{Oil load } (\%) = (w_2/w_3) \times 100,$$

where w is the weight of microcapsules, w_1 is the actual amount of oil encapsulated in a known amount of microcapsules, w_2 is the amount of oil introduced in the same amount of microcapsules and w_3 is the total amount of polymer used including crosslinker.

Oil release studies

Oil release studies of encapsulated oil were done by using UV–visible spectrophotometer (UV-2001 Hitachi). A known quantity of microcapsules was placed into a known volume of 0.1% (w/v) Tween 80 surfactant solution. The microcapsule–Tween 80 mixture 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 254 nm for the determination of cumulative amount of oil release up to a time *t*. Each determination was carried out in triplicate. To maintain a constant volume, 5 mL of 0.1% (w/v) Tween 80 solution was returned to the container.

Scanning electron microscopy study

The samples were deposited on a brass holder and sputtered with gold ions. Surface characteristics of the microcapsules were studied at room temperature using scanning electron microscope (model JEOL, JSM-6390) at an accelerated voltage of 5 KV.

Fourier transform infrared study

FTIR spectra were recorded using KBr pellet in a Nicolet (model Impact-410) spectrophotometer. Gelatin A, carrageenan, complex of gelatin A–carrageenan, NSO and NSO loaded microcapsules were each separately finely grounded with KBr and FTIR spectra were recorded in the range of 4,000–400 cm^{-1}.

Thermal property study

Thermal properties of carrageenan–gelatin complex, NSO, NSO loaded microcapsules and physical mixture of (NSO + carrageenan–gelatin complex) were evaluated by employing differential scanning calorimeter (DSC). DSC study was done in a differential scanning calorimeter (model DSC-60, Shimadzu) at a heating rate of 10 °C/min up to 450 °C. All the study were done under nitrogen atmosphere.

Results and discussion

Solution of gelatin (1% w/v) and carrageenan (1% w/v) were prepared in DDI. Both the solutions were mixed in a definite ratio. The pH of the mixing solution was varied from 2.5 to 5.0 by using 2.5% glacial acetic acid. Maximum turbidity and maximum yield (%) were found to appear at pH 3.5. Therefore, all the microencapsulation reactions were carried out at this pH.

The ratio between the gelatin and carrageenan was optimised by measuring the coacervate yield (%), turbidity and viscosity of the supernatant. Solutions of carrageenan (0.5% w/v) and gelatin A (0.5% w/v) were prepared in acetic acid/sodium acetate buffer (pH 3.5). Both solutions were mixed in different proportions to make 45 mL. The mixtures were incubated at 40 °C for 24 h, and turbidity was measured. The supernatant solution was separated. Coacervate yield (%) of the precipitate and viscosity of the supernatant were measured. Each measurement was done in triplicate and the results reported were the average values.

Turbidity, viscosity and coacervate yield

Turbidity measurements were done in order to optimise the ratio at which maximum coacervation occurred between gelatin A and carrageenan. Solutions of gelatin A (0.5% w/v), carrageenan (0.5% w/v) and the mixture of both at different ratios were scanned in the range 200–600 nm employing UV spectrophotometer. Solutions of both the polymers showed low absorbance at 490 nm while the mixture showed higher absorbance at this wavelength. In all the subsequent experiments, therefore, the wave length of 490 nm was used to scan and study the phase separation behaviour of gelatin–carrageenan mixture.

20 mL of the 0.5% solution of carrageenan was titrated by 0.5% (w/v) solution of gelatin. The plot of absorbance (%) against volume of gelatin (mL) was shown in Fig. 1a. The absorbance increased initially, reaching maximum and then decreased later. The maximum absorbance occurred when 40 mL of the gelatin solution was consumed, i.e. when the % of gelatin in the mixture was 66.66%, i.e. when the carrageenan to gelatin A ratio was 1:2. The turbidity increased due to increase in interaction between gelatin A and carrageenan. The maximum turbidity developed when the interaction between gelatin A and carrageenan was maximum. The % of increased gelatin latter would decrease the turbidity and hence absorbance decreased.

Figure 1b shows the change in supernatant viscosity with variation in percentage of gelatin in gelatin–carrageenan mixture. Viscosity was found to decrease initially, reaching a minimum value, and after that it increased with the increase in the percentage of gelatin. The minimum viscosity observed when the percentage of gelatin in the mixture was 66.66%. At this percentage of gelatin, both the polymers probably reacted maximum to form an insoluble complex. The percentage of polymer at this stage in the supernatant would be minimum, which in turn would develop lowest viscosity. The observed higher viscosity at the latter stage might be due to the presence of unreacted gelatin in the supernatant.

The plot of coacervate yield (%) against % of gelatin is shown in Fig. 1c. The trend was similar to that of turbidity measurement and could be explained as above.

Effect of variation of pH

The effect of variation of pH (2.5–5.0) on turbidity was shown in Fig. 2a, which was plotted as absorbance against pH. The absorbance data were monitored at 490 nm. The turbidity was found to increase up to pH 3.5 beyond that it decreased. This implied that the coacervation between the two polymers was highest at this pH. The explanation for this was similar to that given earlier for the turbidity.

This finding was further confirmed by plotting the coacervate % yield against pH. The plot was shown in Fig. 2b. The % yield was highest at pH 3.5 and the trend was similar to that of absorbance (%) against pH plot.

Scanning electron microscopy study

SEM photographs of neat carrageenan + gelatin complex and NSO loaded microcapsules are shown in Fig. 3. Photographs of neat carrageenan + gelatin

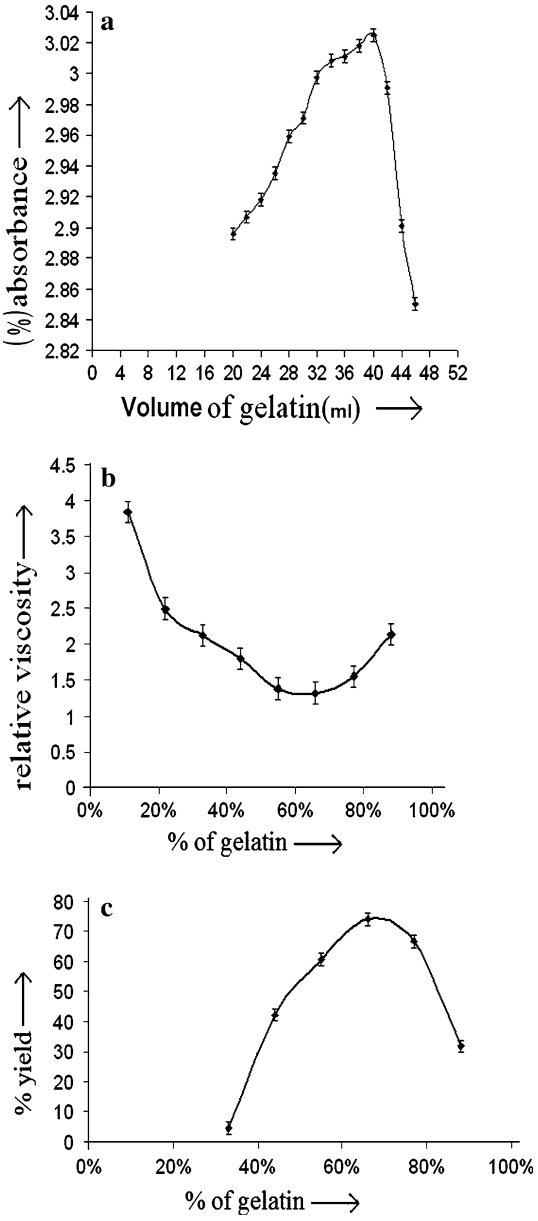

Fig. 1 Effect of variation of gelatin concentration in gelatin–carrageenan mixture on **a** turbidity of the mixture solution and **b** relative viscosity of the supernatant. **c** coacervate yield (%)

complex (Fig. 3a) appeared agglomerated with no definite structure. In contrast, the NSO loaded samples (Fig. 3b–d) were having free flowing spherical shape. With the increase of the amount of polymer concentration (Fig. 3b–d) the size of the microcapsules increased. This might be due to the increase of the thickness of the wall of the microcapsules. Again, the surface of the microcapsules having high NSO loading (Fig. 3e) appeared sticky and agglomerated compared to the microcapsules with low NSO loading (Fig. 3b).

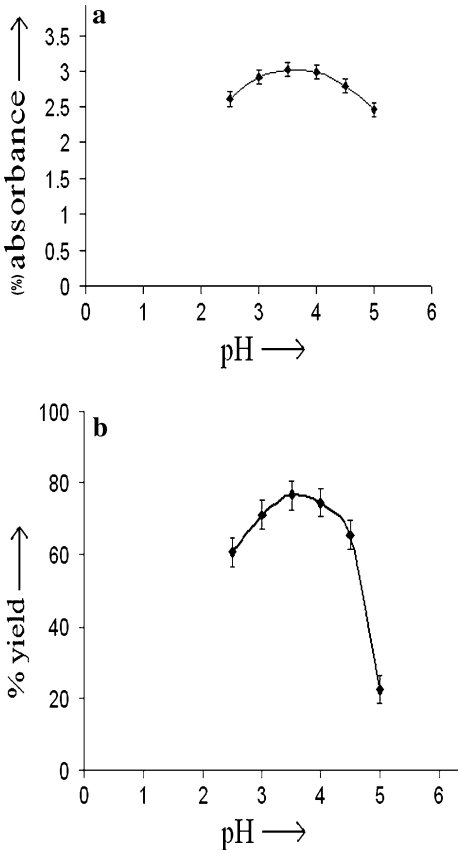

Fig. 2 Effect of variation of pH on **a** turbidity and **b** coacervate yield (%)

Effect of variation of oil loading

The effect of variation of oil loading on oil content, encapsulation efficiency and release rate is shown in the Table 1 and Fig. 4. With the increase in oil loading, the encapsulation efficiency, the release rate and % oil content were found to increase throughout the range of oil concentration studied. At low oil load, the dispersion force of the stirrer was more efficient resulting in the generation of smaller oil droplets. The polymer present in the mixture was enough to encapsulate these droplets. The dispersion force became progressively difficult as the oil load increased. This would develop large oil droplets and as a result encapsulation efficiency would increase. As the amount of polymer was fixed, therefore, the polymers would encapsulate all the large oil droplets at the expense of decrease of thickness of microcapsule wall. The faster release rate might be due to the decrease of thickness of the capsule wall. With the decrease in wall thickness, diffusional path for the oil release became short, which resulted in an increase in release rate. With increase in percent oil load, the oil content (%) increased. At very low oil load, many of the microcapsule probably contained few oil droplets indicating that there was an abundance of the encapsulating polymer for the oil present. With the

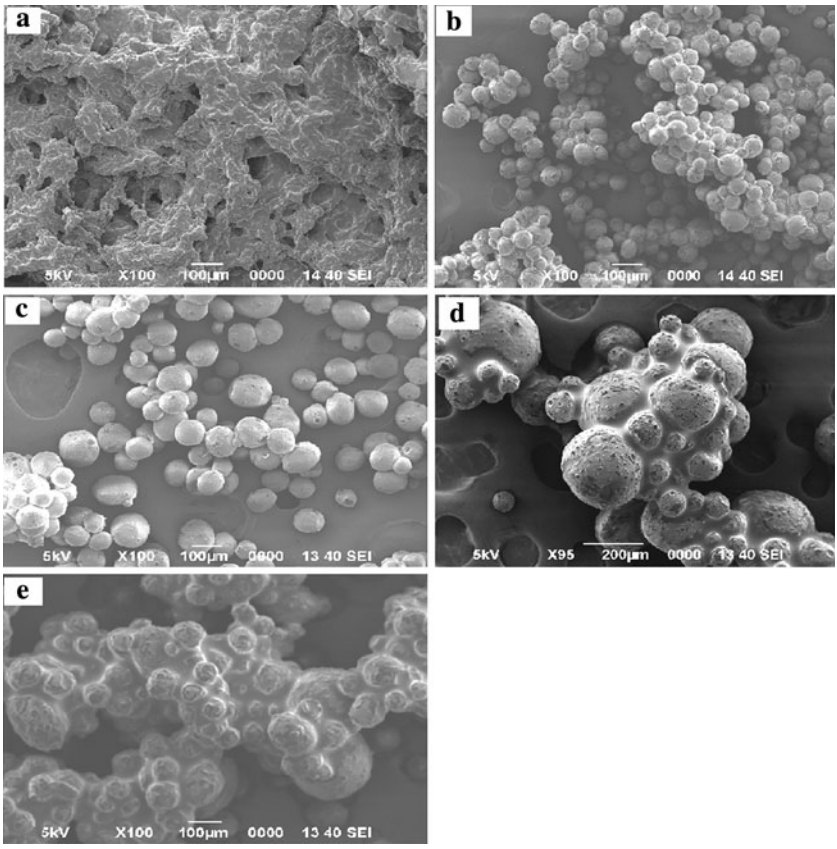

Fig. 3 Scanning electron micrographs **a** neat carrageenan + gelatin complex; microcapsules loaded with **b** NSO = 2 g, polymer = 1.5 g, **c** NSO = 2 g, polymer = 3 g, **d** NSO = 2 g, polymer = 4.5 g, **e** NSO = 4 g, polymer = 1.5 g

increase in oil load (%), the number of oil droplets in the microcapsule increased which resulted in an increase in oil content. The surface characteristics of the microcapsules were found to change as oil content (%) varies as revealed by SEM study.

Effect of variation of cross-linker concentration

The effect of variation of cross-linker concentration on oil loading (%), oil content (%), encapsulation efficiency (%) and release rate is shown in the Table 1 and Fig. 5. The trends of oil loading (%) and oil content (%) shown in the table were as per expectation. With the increase in genipin concentration, oil loading decreased for all but oil content and encapsulation efficiency increased. The increase in encapsulation efficiency (%) could be due to the improvement of oil retention capacity of the microcapsules caused by the formation of crosslinking. The crosslinking reaction took place between genipin and polyelectrolyte complex of

Table 1 Effect of variation of oil loading, polymer and genipin concentration on the behaviour of microcapsules

Sample formulations				Oil load (%)	Oil content (%)	Encapsulation efficiency (%)
Carrageenan (g)	Gelatin A (g)	Genipin (mmol)	NSO (g)			
0.5	1.0	0.1	1.0	65.67	30 ± 0.4	75.68 ± 1.01
0.5	1.0	0.1	2.0	131.35	45 ± 1.0	79.25 ± 1.76
0.5	1.0	0.1	3.0	197.03	60 ± 0.5	90.45 ± 0.75
0.5	1.0	0.1	4.0	262.70	66 ± 0.3	91.12 ± 0.42
0.5	1.0	0.4	2.0	125.80	49 ± 1.0	87.95 ± 1.79
0.5	1.0	0.8	2.0	119.07	51 ± 0.7	93.82 ± 1.29
1.0	2.0	0.4	2.0	64.72	35 ± 0.5	89.08 ± 1.27
1.5	3.0	0.4	2.0	43.57	29 ± 0.8	95.55 ± 2.63

Carrageenan: 0.5–1.5 g; gelatin A: 1.0–3.0 g; water: 200 mL; NSO: 1.0–4.0 g; genipin: 0.1–0.8 mmol; temperature: 70 ± 1 °C

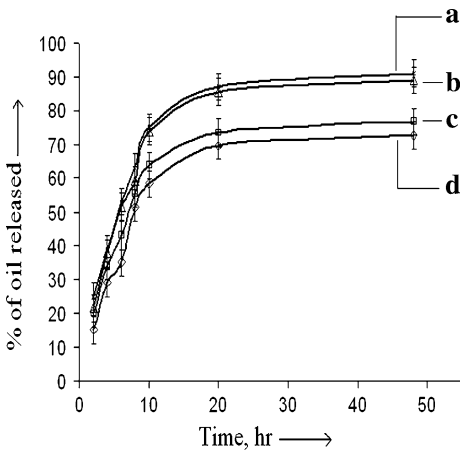

Fig. 4 Effect of variation of oil loading on release profile (**a** polymer 1.5 g; crosslinker 0.1 mmol; NSO 4 g, **b** polymer 1.5 g; crosslinker 0.1 mmol; NSO 3 g, **c** polymer 1.5 g; crosslinker 0.1 mmol; NSO 2 g, **d** polymer 1.5 g; crosslinker 0.1 mmol; NSO 1 g)

carrageenan and gelatin. The possible interaction of carrageenan–gelatin complex with genipin is shown in Fig. 6. The release rate of oil was found to decrease as the % of genipin increased. The microcapsule wall became compact as degree of crosslinking increased. This resulted in the decrease of diffusion rate through the microcapsule wall. Similar findings were cited in the literature [5].

Effect of variation of polymer concentration

Table 1 and Fig. 7 shows the results of the effect of variation of total polymer concentration on oil loading, oil content and encapsulation efficiency. Both oil loading (%) and oil content (%) decreased with the increase in total polymer content but encapsulation efficiency increased. With the increase in polymer content, more

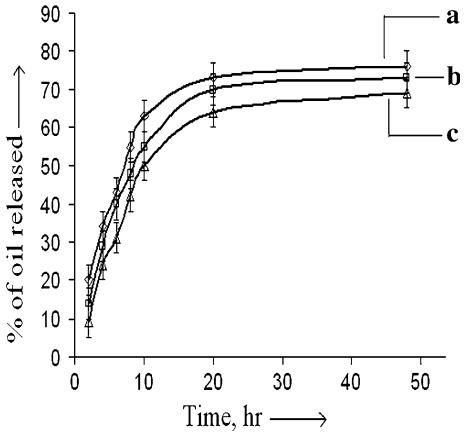

Fig. 5 Effect of variation of crosslinker concentration on release profile (**a** polymer 1.5 g; crosslinker 0.1 mmol; NSO 2.0 g, **b** polymer 1.5 g; crosslinker 0.4 mmol; NSO 2.0 g, **c** polymer 0.68 g; crosslinker 0.8 mmol; NSO 2.0 g)

and more polymer would be available to encapsulate the oil vesicles and thereby efficiency increased. The excess polymer after complete encapsulation would enhance the thickness of the microcapsule, which was also clear from SEM photographs. The release profile is shown in Fig. 7. The release rate was found to decrease with the increase in polymer concentration. The increase in wall thickness of the microcapsules might be responsible for this type of behaviour [5].

Fourier transform infrared study

The spectra of carrageenan (curve a), gelatin-A (curve b), carrageenan–gelatin complex (curve c), NSO (curve d), NSO loaded crosslinked carrageenan–gelatin microcapsules (curve e) are shown in Fig. 8. The spectrum of carrageenan showed absorption bands at 3,423, 2,910, 1,434, 1,379, 1,265 and 846 cm^{-1}, which were due to O–H stretching vibration, CH_3 symmetric + CH_2 asymmetric vibration, CH_3 + CH_2 bending vibration, sulphonic acid group, C–O stretching band and glycosidic linkages. The notable absorption bands for gelatin-A appeared at 3,421 cm^{-1} (NH-stretching), 1630.44 cm^{-1} (amide I, CO and CN stretching), 1,530 cm^{-1} (amide II) and 1,250 cm^{-1} (amide III). Among the absorption bands, the amide I band between 1,600 and 1,700 cm^{-1} is the most important peak for IR analysis of the secondary structure of protein like gelatin [27]. 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 sulphate ester groups might associate with positively charged gelatin. Similar type of observation was reported by Pranoto et al. [28]. The probable interaction between carrageenan and gelatin is shown in Fig. 6. A shifting of the sulphonic acid absorption band to higher wave number due to interaction between carrageenan and gelatin was reported by Li et al. during studying of electrosynthesis of κ carrageenan–gelatin complex [29]. However, this type of shifting was not observed in this case. The absorption band appeared in the spectrum of NSO at 1,743, 1,456 and 1,163 cm^{-1} were due to the carbonyl stretching, CH_2 asymmetric

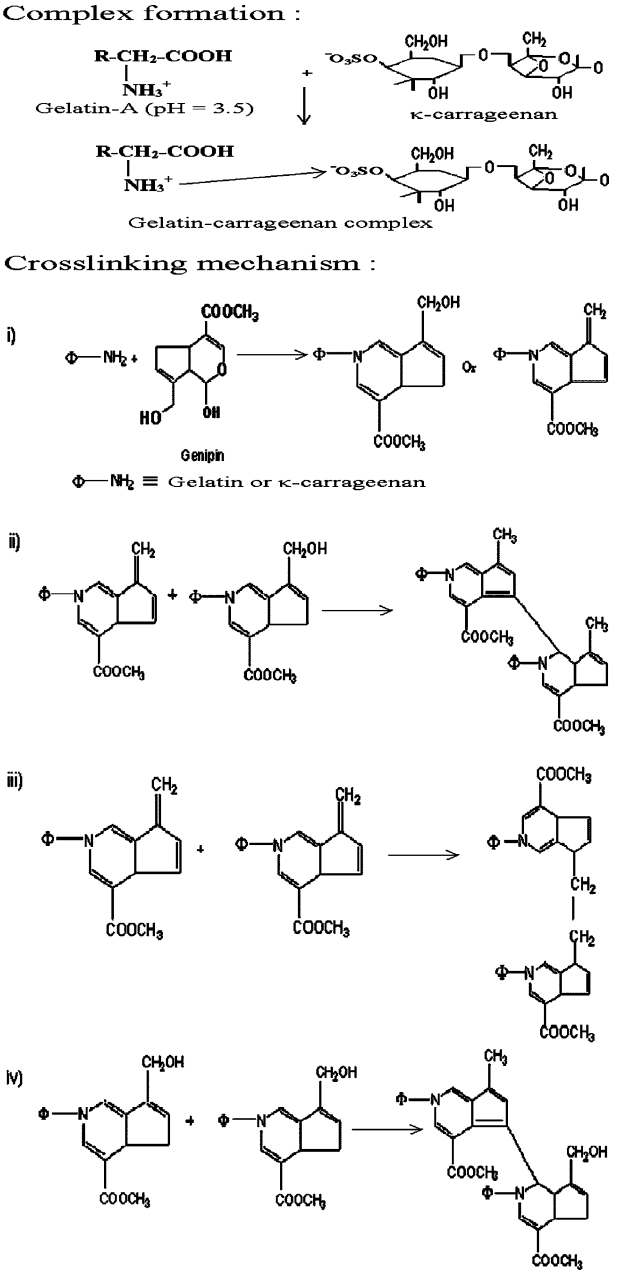

Fig. 6 Probable reaction scheme for interaction between carrageenan and gelatin and genipin with carrageenan–gelatin complex

deformation and C–C stretching vibration. The position of these bands remained almost unchanged in NSO loaded microcapsules. These indicated the absence of any significant interaction between NSO and carrageenan–gelatin complex.

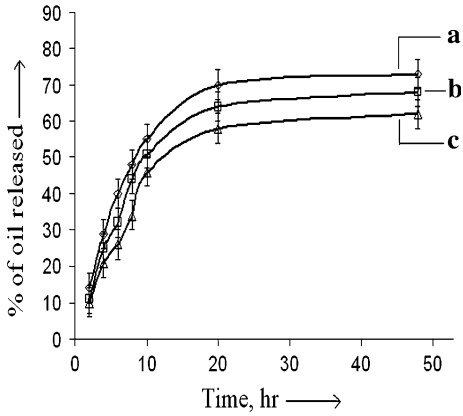

Fig. 7 Effect of variation of polymer concentration on release profile (**a** polymer 1.5 g; crosslinker 0.4 mmol; NSO 2.0 g, **b** polymer 3.0 g; crosslinker 0.4 mmol; NSO 2.0 g, **c** polymer 4.5 g; crosslinker 0.4 mmol; NSO 2.0 g)

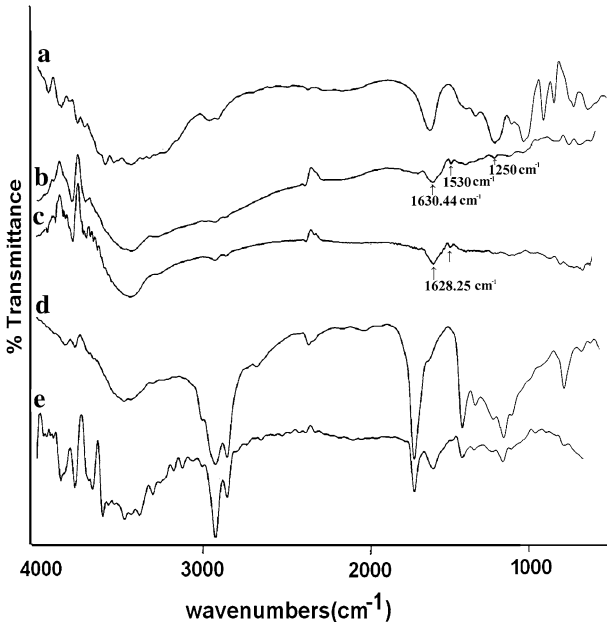

Fig. 8 FTIR spectra of **a** carrageenan, **b** gelatin, **c** gelatin–carrageenan complex, **d** NSO and **e** NSO loaded microcapsules

Thermal property study

DSC thermograms of carrageenan–gelatin complex (curve a), NSO(curve b), NSO loaded microcapsules (curve c) and physical mixture of (NSO + carrageenan–gelatin complex) (curve d) are shown in Fig. 9. The ratio of carrageenan–gelatin to NSO was kept similar to that of microcapsules loaded with NSO. The endotherm appeared in all the thermograms (except NSO) at around 100 °C were due to removal of moisture. The thermograms of NSO showed an endothermic peak at

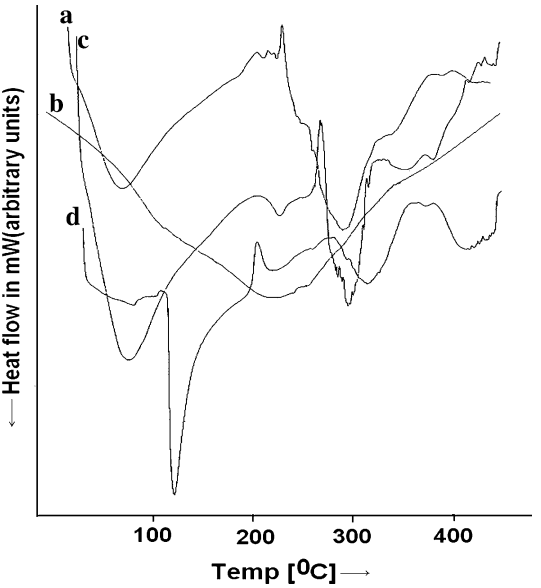

Fig. 9 DSC thermograms of **a** carrageenan–gelatin complex, **b** NSO, **c** NSO loaded microcapsules and **d** physical mixture of (NSO + carrageenan–gelatin complex)

around 220 °C. Both NSO loaded microcapsules and physical mixture showed two endothermic peaks corresponding to NSO and carrageenan–gelatin complex in the range of 218–225 °C and 305–315 °C, respectively. There was no significant change in the position of the endothermic peak of NSO in both the thermograms. These results indicated that there was no remarkable interaction between NSO and carrageenan–gelatin complex.

Conclusion

NSO could be encapsulated efficiently using κ-carrageenan–gelatin A and genipin as complex and crosslinker, respectively. Maximum coacervation occurred at 2:1 gelatin to carrageenan ratio and pH value of 3.5. The encapsulation efficiency was found to increase with the increase in the concentration of NSO, genipin and carrageenan–gelatin complex. SEM study revealed that the size of the microcapsules was increased as the polymer concentration increased. FTIR and DSC study did not provide evidence of any remarkable interaction between NSO and carrageenan–gelatin complex.

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