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Effect of Crosslinking Agent on Neem (*Azadirachta Indica A. Juss.*) Seed Oil (NSO) Encapsulated Microcapsules of κ-Carrageenan and Chitosan Polyelectrolyte Complex

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Microencapsulation of neem (*Azadirachta Indica A. Juss.*) seed oil (NSO) was carried out by polyelectrolyte complexation of κ-carrageenan and chitosan. The microcapsules were crosslinked by using three different crosslinking agents - glutaraldehyde, genipin and tannic acid. The lowest and highest water uptake capacities were exhibited by glutaraldehyde and tannic acid crosslinked matrices, respectively. The release behavior of NSO from encapsulated crosslinked microcapsules followed the order: tannic acid > genipin > glutaraldehyde. Polyelectrolyte complex formation and its interaction with crosslinker was studied. Crosslinking improved thermal stability without affecting crystallinity. Roughness appeared on microcapsule's surface indicated interaction between microcapsules and crosslinker.

Keywords: Chitosan, κ-carrageenan, microcapsules, neem seed oil, crosslinking agent

1 Introduction

Synthetic pesticides that are used to control plant diseases are doing irreparable harm and damage to our fragile environment. The increasing awareness and concern about the impact of agricultural practices on the environment and in food and fiber production is promoting the concept of sustainable agriculture, thus, raising the thrust for biopesticides over synthetic pesticides. Neem has been in high regard due to its potent insecticidal properties. But, due to its liquid nature, application of it to the soil is limited. This limitation can be overcome by the encapsulation of NSO to give rise to a solid formulation.

NSO, though a potent natural pesticide, shows toxicity to fish like tilapia and carp (1). NSO produces toxic effect in human and in several isolated cases (1–2). Microencapsulation and controlled delivery technology seems to be the most useful technique to minimize toxicity and make efficient use of this natural resource NSO.

Different natural or synthetic biodegradable polymers have been used for controlled release purposes. Natural polymers are better compared to synthetic polymers in terms of availability and effect on environment. The membrane produced by crosslinked starch and copolymers of acrylic acid and acrylamide has been used to encapsulate urea fertilizer (3). Starch urea formaldehyde matrix has been used for encapsulation of agrochemicals (4). The use of starch-g-poly(butyl acrylate) as a material for encapsulating carboxylic containing herbicides for controlled release was studied by Zhu et al. (5).

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 (6). Chitosan have been broadly evaluated by the industries due to its biocompatibility, antibacterial, biodegradability, antifungal and antimicrobial properties (7). It also acts as a water binding agent and inhibits various enzymes.

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 sulfate esters of galactose and 3,6 anhydrogalactose joined by alternating α-1,3 and β-1,4 glycosidic linkages (9). The carrageenan mixture has, namely, three types of carrageenan (10) κ-carrageenan, λ-carrageenan, and *ι*-carrageenan. κ-carrageenan has one sulfate group per two galactose residues (produces a weak gel which suffer syneresis), *ι*-carrageenan has two sulfate groups per two galactose residues (produces an elastic gel without syneresis), and λ-carrageenan has three sulfate

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groups per two galactose residues (no gelling). Carrageenan has been used for various purposes. Few reports are available regarding its use as a matrix for encapsulation.

When two oppositely charged polyelectrolytes are mixed in an aqueous solution, a polyelectrolyte complex is formed by the electrostatic attraction between the polyelectrolytes. Complexes between oppositely charged polyelectrolytes such as chitosan-sodium alginate (11), chitosan-polyacrylic acid (12) and chitosan-gelatin (13) have been used for controlled release formulations. Tomida et al. (14) has suggested that κ-carrageenan-chitosan membrane spherical capsules can release theophylline as a model drug from the capsules. Tapia et al. (15) has 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. Although chitosan and carrageenan are little bit costly but considering their multiple advantages, they can be exploited for controlled release of agrochemicals. Some reports regarding the use of different polyelectrolyte complexes for drug delivery are available. However, far less is known regarding the use of this polyelectrolyte complex in delivery of agrochemicals.

In order to improve the controlled release behaviour, varieties of crosslinking agents are employed. Glutaraldehyde, a crosslinker of synthetic origin, has been used as a successful crosslinking agent in many studies to cross link chitosan, and the chitosan-carrageenan polyelectrolyte complex (16, 17). However, glutaraldehyde, like many other crosslinking agents are synthesized chemically and not free from problem caused by physiological toxicity (18). Genipin, a naturally occurring crosslinker, can react spontaneously with amino acids or proteins (13). Its toxicity is much less than glutaraldehyde (19). In our present work, attempts have been made to compare the effect of various cross linking agents namely, glutaraldehyde, genipin and tannic acid on the release profile of NSO encapsulated in the polyelectrolyte complex microcapsules of κ-carrageenan and chitosan.

2 Experimental

2.1 Materials

Carrageenan Type I, containing predominantly *k*-and lesser amount of λ-carrageenan was purchased from Sigma-Aldrich Inc. (USA). Chitosan, medium molecular weight with brookfield viscosity ~200cps was purchased from Sigma-Aldrich Inc. (USA). Glacial acetic acid (E. Merck, India), Tween 80 (E. Merck, India), glutaraldehyde 25% w/v (E. Merck, Germany), tannic acid (E. Merck, Worli, Mumbai), 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. DDI (double-distilled deionised) water was used throughout the study. Other reagents used were of analytical grade.

2.2 Microencapsulation Procedure

In a beaker, a known amount of (100 ml) 0.5% (w/v) of carrageenan solution was taken. This polymer solution was stirred by mechanical stirrer under high agitation at 70 ± 1°C. This temperature was maintained throughout the experiment. To this, neem seed oil (2.04 g) was added under high agitation to form an emulsion. Chitosan solution of 0.5% was added dropwise to attain complete phase separation. The beaker, containing the microcapsules was left to rest at this temperature for 15 min. The system was then brought to 5–10°C to harden the microcapsules. The crosslinking of the polymer capsule was achieved by slow addition of certain amount of crosslinkers. The temperature of the beaker was then raised to 45°C and stirring was continued for about 3–4 h in order to complete the crosslinking reaction. The beaker was then cooled to room temperature. The microcapsules were filtered, washed with 0.1% Tween 80 surfactant solution, to remove any oil adhered to the surface of microcapsules, dried and stored inside a refrigerator in a glass ampoule.

2.3 Calibration Curve of Oil

A calibration curve is required for the study of release rate of oil from the microcapsules. It was observed that 0.1 g of NSO 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 wt% 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 to 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 by knowing the absorbance value.

2.4 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 wt% 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 (20).

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

$$\text{Oil content (\%)} = w_1/w \times 100$$

$$\text{Oil load (\%)} = w_2/w_3 \times 100$$

Where w = weight of microcapsules
w_1 = actual amount of oil encapsulated in a known amount of microcapsules
w_2 = amount of oil introduced in the same amount of microcapsules
w_3 = total amount of polymer used including crosslinker

2.5 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 immersed into a known volume of 0.1 wt% 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% Tween 80 solution was returned to the container.

2.6 Water Uptake Studies

A known amount (w_1) of the NSO free crosslinked products, prepared with similar amounts of different crosslinkers—glutaraldehyde, genipin and tannic acid, were allowed to swell in DDI water at room temperature (30°C) for a certain time period. The wet samples were taken out after stipulated time period and wiped dry with filter paper to remove excess water, and weighed (w_2). The water uptake (%) by the crosslinked products was calculated according to the formula:

$$\text{Water uptake (\%)} = [(w_2 - w_1) / w_1] \times 100$$

Where w_1 is initial weight of microcapsules before swelling, and w_2 is the final weight of microcapsules after swelling for time "t".

2.7 Fourier Transform Infrared (FTIR) Study

FTIR spectra were recorded using KBr pellet in a Nicholet (model Impact-410) spectrophotometer. Chitosan, carrageenan, polyelectrolyte complex of chitosan–carrageenan, NSO, NSO loaded uncrosslinked and crosslinked microcapsules, were each separately finely grounded with KBr and FTIR spectra were recorded in the range of 4000–400 cm^{-1}.

2.8 Thermal Property Study

Thermal properties of NSO, NSO loaded microcapsules crosslinked with different crosslinker and NSO loaded microcapsules without crosslinker were evaluated using a thermogravimetric analyzer (Model TGA-50, Shimadzu) instrument at a heating rate of 5°C/min up to 500°C under nitrogen atmosphere.

2.9 Scanning Electron Microscopy Study

The samples were deposited on a brass holder and sputtered with gold. Surface characteristics of the microcapsules were studied at room temperature using scanning electron microscope (Model JEOL, JSM-6360) at an accelerated voltage of 10 kv.

2.10 X-ray Diffraction Study

X-ray diffractograms of microcapsules crosslinked with different crosslinkers and without crosslinker were recorded on a X-ray diffractometer (Model MiniFlex, Rigaku corporation, Japan). The samples were scanned between 2θ =10° to 50° at the scan rate of 4°/min.

3 Results and Discussion

3.1 Effect of Variation of Type and Concentration of Crosslinker

The effect of variation of crosslinker type and concentration on oil loading (%), oil content (%), encapsulation efficiency (%) and release rate are shown in Table 1 and Figures 1–3. As per expectation, glutaraldehyde produced highest oil loading (%) followed by genipin and tannic acid. Oil content (%) and encapsulation efficiency (%) were highest and lowest for glutaraldehyde and genipin crosslinked samples.

Further, in all the cases, as the amount of crosslinker increased, oil load (%) decreased while oil content (%) and encapsulation efficiency (%) increased. The increase in encapsulation efficiency (%) could be due to the improvement in oil retention capacity of the microcapsules caused by the formation of crosslinking. In the chitosan-carrageenan complex, the amino group of chitosan interacted with the sulphate group of carrageenan as revealed by FTIR study.

Moreover, carrageenan contained some proteins (21). Tannic acid possessed large number of free phenolic hydroxyl groups, which could form strong hydrogen bonds with proteins and carbohydrates (22). Tannic acid might also form complex with proteins (23). The interaction with proteins and carbohydrates were not so strong like those of produced by glutaraldehyde. But still this weak complex might capable of retaining large proportion of oil. On the other hand, glutaraldehyde could form strong covalent bonding with the hydroxyl groups present in the chitosan-carrageenan microcapsules. It could also interact with the proteins present in carrageenan. The crosslinking intensity would be more due to formation of covalent bonding and availability of more crosslinkable sites. Genipin could react with protein part of carrageenan (24). The crosslinking intensity would be less due to the

Table 1. Effect of variation of type and concentration of crosslinker on the behavior of microcapsules. (chitosan: 0.1 g; carrageenan: 0.5 g; water: 136 ml; NSO: 2.04 g; crosslinker: 0.2–0.8 mmol; temperature: 70 ± 1°C)

Sample particulars					
Name of crosslinker	*Amount of crosslinker (mmol)*	*NSO (g)*	*Oil load (%)*	*Oil Content (%)*	*Encapsulation efficiency (%)*
Glutaraldehyde	0.2	2.04	291.40	50 ± 2.0	67.15 ± 2.68
Glutaraldehyde	0.4	2.04	283.33	55 ± 1.0	74.42 ± 1.35
Glutaraldehyde	0.8	2.04	268.42	62 ± 2.0	85.16 ± 2.75
Genipin	0.2	2.04	281.30	27 ± 1.0	36.60 ± 1.35
Genipin	0.4	2.04	265.00	28 ± 1.0	38.50 ± 1.44
Genipin	0.8	2.04	236.98	30 ± 1.0	42.60 ± 1.48
Tannic acid	0.2	2.04	200.00	44 ± 1.0	65.0 ± 1.50
Tannic acid	0.4	2.04	150.00	46 ± 2.0	71.60 ± 2.72
Tannic acid[a]	0.8	2.04	100.00	—	—

[a]Appearance of turbidity makes difficulty in assessing.

presence of lower amount of protein in carrageenan. Therefore the crosslinking would be highest and lowest for glutaraldehyde and genipin crosslinked samples respectively. The high crosslinking intensity might be responsible for showing high encapsulation efficiency. In the similar way, the high encapsulation efficiency observed in crosslinked samples prepared by varying the crosslinker amount could be explained as before.

Oil content (%) and encapsulation efficiency (%) of the microcapsules prepared by adding higher concentration of tannic acid could not be performed due to the appearance of turbidity in the release medium. This creates difficulty in assessing the amount of oil entrapped by spectroscopically.

The release rate of oil was found to be lowest and highest for glutaraldehyde and tannic acid crosslinked samples. The order of release rate of oil was as follows: tannic acid > genipin > glutaraldehyde. In the case of glutaraldehyde and genipin, a strong covalent bonding took place between chitosan-carrageennan microcapsules and crosslinker. But tannic acid formed weak bonding with the microcapsules. The available sites in the microcapsules for crosslinking with genipin was less compared to those of glutaraldehyde. Therefore, glutaraldehyde would produce highest crosslinking in the microcapsule wall followed by genipin and tannic acid. Further, it was observed that release rate decreased with the increase in the concentration

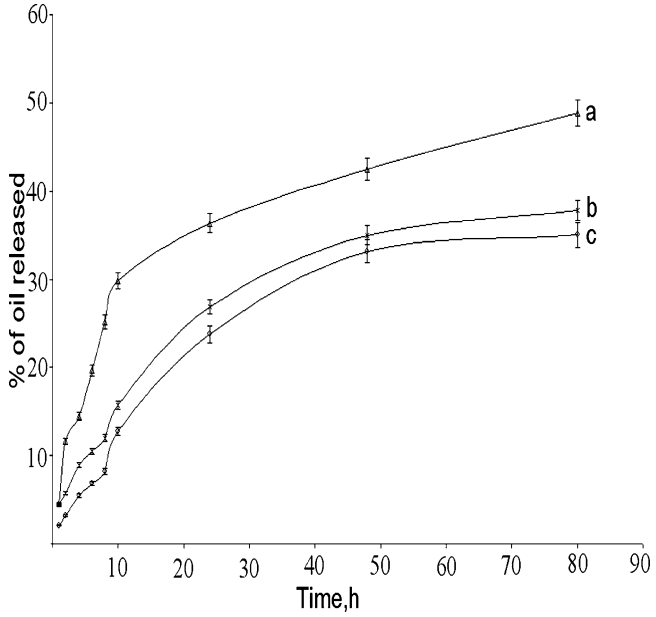

Fig. 1. Oil release profiles of microcapsules crosslinked with 0.2 mmol a) tannic acid, b) genipin, c) glutaraldehyde.

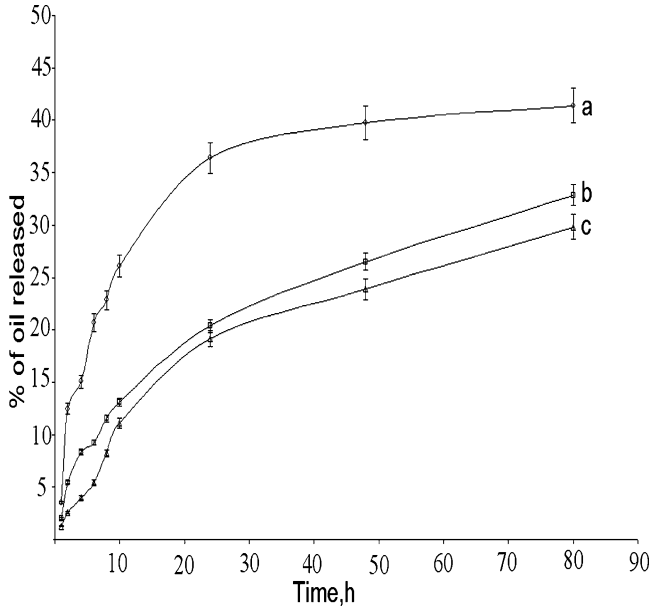

Fig. 2. Oil release profiles of microcapsules crosslinked with 0.4 mmol a) tannic acid, b) genipin, c) glutaraldehyde.

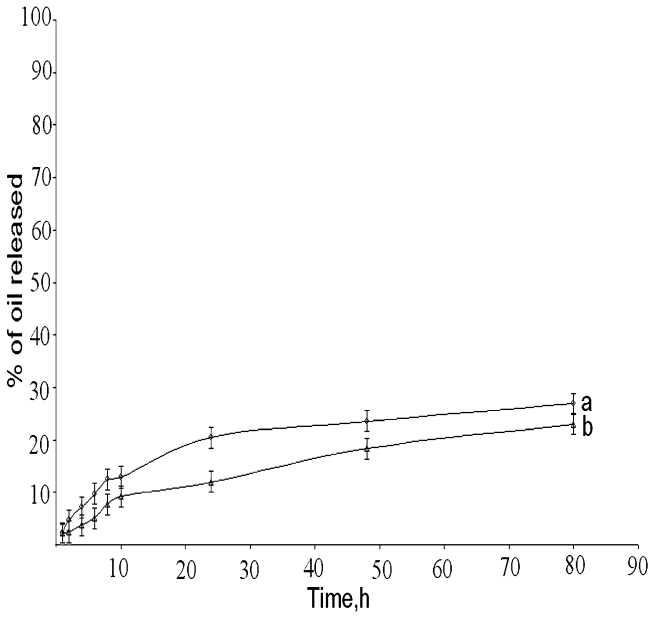

Fig. 3. Oil release profiles of microcapsules crosslinked with 0.8 mmol a) genipin, b) glutaraldehyde.

of crosslinker. The microcapsule wall became more compact as degree of crosslinking increased. This resulted in the decrease of diffusion rate of oil through the microcapsule wall. Similar observations were reported in the literature (20).

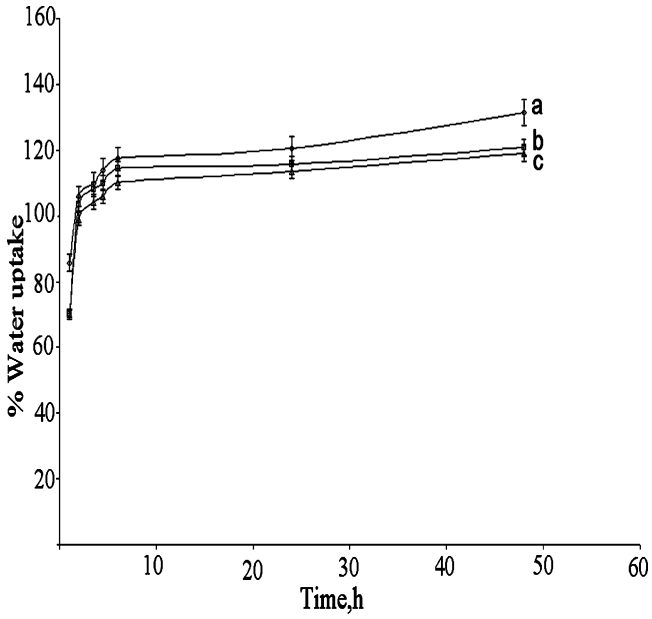

Fig. 4. Percentage water uptake of matrices crosslinked with a) tannic acid, b) genipin, c) glutaraldehyde.

3.2 Water Uptake Studies

The results of swelling experiments of different crosslinked products with different crosslinkers are shown in Figure 4. The crosslinked products were allowed to swell in water at room temperature for 40 h. The percent water uptake for crosslinked products followed the order: tannic acid > genipin > glutaraldehyde. This behaviour could be explained on the basis of the restriction in mobility of water molecules through the polymer network. Both glutaraldehyde and genipin formed strong covalent bonding with the polyelectrolyte complex. The crosslinking intensity, as explained earlier, was higher in the case of glutaraldehyde. Tannic acid formed weak complex with the polyelectrolyte through hydrogen bonding and hydrophobic association resulting in producing higher water absorption.

3.3 Fourier Transform Infrared (FTIR) Study

FTIR spectra of Chitosan (curve-a), Carrageenan (curve-b), chitosan-carrageenan polyelectrolyte complex (curve-c), NSO (curve-d), NSO loaded uncrosslinked chitosan-carrageenan microcapsules (curve-e) and NSO loaded tannic acid, genipin, glutaraldehyde crosslinked microcapsules (curve-f, g, h) are shown in Figure 5. The spectrum of chitosan showed a strong absorption band at 1635.33 cm^{-1}

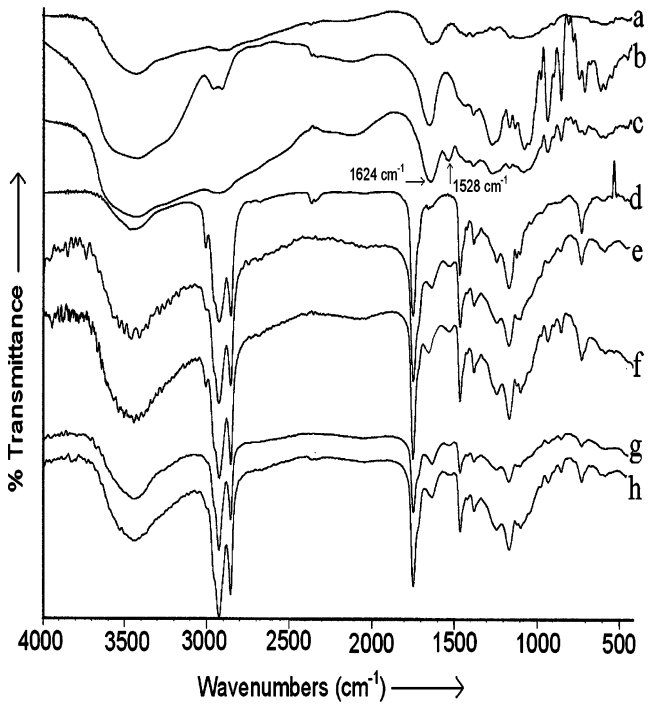

Fig. 5. FTIR spectra of a) chitosan; b) carrageenan; c) polyelectrolyte complex of chitosan and carrageenan; d) NSO; e) NSO loaded uncrosslinked microcapsules; f) NSO loaded tannic acid crosslinked microcapsules; g) NSO loaded genipin crosslinked microcapsules; h) NSO loaded glutaraldehyde crosslinked microcapsules.

Table 2. Temperature of decomposition at different weight loss (%) of carrageenan-chitosan complex and oil containing crosslinked microcapsules

Sample particulars	*Cross linker (mmol)*	*Temperature of decomposition (T_D) (°C) at different weight loss (%)*							*Residue (%) at 500 (°C)*
		20	*30*	*40*	*50*	*60*	*70*	*80*	
NSO loaded microcapsules without crosslinker	—	195	205	210	220	250	375	—	23
NSO	—	315	340	358	375	395	415	430	7
NSO loaded microcapsules crosslinked with-									
-Tannic acid	0.2	195	217	255	320	370	400	—	20
	0.4	210	242	300	342	365	390	467	15
	0.8	235	285	350	378	397	405	421	13
-Glutaraldehyde	0.2	215	254	317	350	370	395	450	16
	0.4	216	262	328	365	384	400	—	16
	0.8	235	288	342	368	382	398	412	20
-Genipin	0.2	190	219	256	320	368	400	—	17
	0.4	212	253	314	349	370	390	460	21
	0.8	200	230	280	340	369	418	495	19

assigned to NH bending. The absorption bands appeared in the spectrum of carrageenan at 1379.23, 1265.70 and 846.33 cm^{-1} were due to sulphonic acid group, C–O stretching band and glycosydic linkages. Further the appearance of a new band at 1528 cm^{-1} due to NH_3^+ groups and reduction of intensity of the absorption band of sulphonic acid groups in the spectrum of chitosan-carrageenan complex indicated the formation of strong polyelectrolyte complex (15). The absorption bands appeared in the spectrum of NSO (curve-d) 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 intensity of the peaks at 1528 cm^{-1} and 1624 cm^{-1} observed in the uncrosslinked polyelectrolyte microcapsules were found to change with the introduction of crosslinker. The intensity of the peak corresponding to NH_3^+ groups decreased while that corresponds to NH bending increased. The change noticed was highest and lowest in the case of glutaraldehyde and tannic acid crosslinked microcapsules. The trend observed in genipin crosslinked samples was in between to those of glutaraldehyde and tannic acid crosslinked samples. These results indicated that the interaction of crosslinker with polyelectrolyte complex was highest and lowest in the case of glutaraldehyde and tannic acid, respectively.

3.4 Thermal Property Study

Temperature of decomposition (T_D) values and residual weight (%) of NSO loaded microcapsules without crosslinker, NSO, and NSO loaded microcapsules crosslinked with glutaraldehyde, genipin and tannic acid at different weight loss (%) are presented in Table 2. T_D values for crosslinked microcapsules were found to be higher than those of microcapsules without crosslinker. In all the cases, T_D values increase with the increase in the amount of crosslinker. Among the various crosslinkers studied, T_D values for crosslinked microcapsules were as follows: glutaraldehyde > genipin > tannic acid. The increasing trend of the T_D values might be due to the decreasing chance of elimination of small molecules like CO_2, CO etc. with the formation of crosslinking, which acted as an infusible support and provided thermal resistance to the microcapsules. The reason for higher and lower thermal stability, shown by glutaraldehyde and genipin crosslinked samples respectively, could be as explained as earlier. Water uptake study and oil release studies also supported the above observation.

3.5 Scanning Electron Microscopy Study

Figure 6 shows the scanning electron micrographs of chitosan-carrageenan complex (Fig. 6a) NSO loaded crosslinked microcapsules (Figs. 6b, c, d) and uncrosslinked microcapsules (Fig. 6e), respectively. The photograph of chitosan-carrageenan complex appeared powdery. Similarly, photograph of NSO loaded uncrosslinked (Fig. 6e) appeared spherical, smooth and agglomerated. Partly spherical and partly bean like structure were observed in glutaraldehyde crosslinked microcapsules (Fig. 6b). In the case of genipin crosslinked microcapsules (Fig. 6c), the particles were agglomerated and spherical. In both the cases, a roughness was observed on the surface of the particles. The roughness seems to be more in glutaraldehyde crosslinked samples. Tannic acid crosslinked microcapsules (Fig. 6d) were spherical and smooth. The roughness

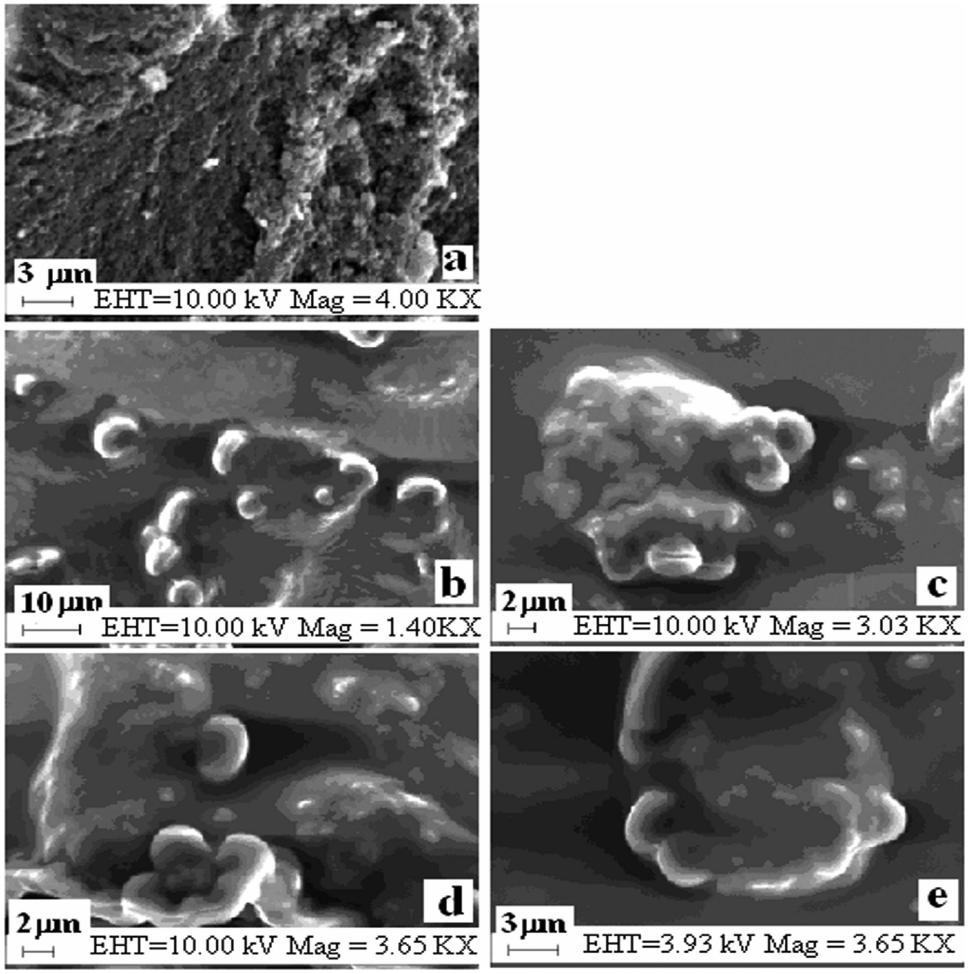

Fig. 6. Scanning electron micrographs of a) carrageenan-chitosan complex; b) glutaraldehyde crosslinked microcapsules; c) genipin crosslinked microcapsules; d) tannic acid crosslinked microcapsules; e) uncrosslinked microcapsules.

appeared on the surface of the microcapsules might be due to the interaction between chitosan-carageenan microcapsules and crosslinker. Piyakulawat and colleagues (17) observed and reported the appearance of irregular and rough surfaces while studying the SEM micrographs of glutaric acid crosslinked chitosan-carrageenan beads.

3.6 X-Ray Diffraction Studies

The X-ray diffractogram of chitosan, carrageenan, and oil loaded chitosan-carrageenan microcapsules uncrosslinked and crosslinked with glutaraldehyde, genipin and tannic acid are presented in Figure 7. Chitosan showed its characteristic peak at $2\theta = 20^\circ$. This was similar to that reported in the literature (25). Similarly, carrageenan showed sharp peaks at $2\theta = 29^\circ$ and 41°, respectively. Bhise and coworkers (26) observed the appearance of multiple peaks in the diffractogram of κ-carrageenan between 2θ values of 15° to 30°. Meena and colleagues (27) reported the formation of broad peak at around $2\theta = 20^\circ$. In the uncrosslinked microcapsule, a broad peak appeared at 22.4°. This showed that the uncrosslinked microcapsule was amorphous in nature compared to either chitosan or carrageenan. Furthermore, the nature of the difractograms of oil loaded microcapsules crosslinked with different crosslinking agents appeared similar to that of diffractogram of uncrosslinked microcapsule. The peaks of crosslinked microcapsules were found to shift to lower values of 2θ. The shifting was more in the case of glutaraldehyde crosslinked microcapsules. This was followed by microcapsules crosslinked with genipin and tannic acid respectively. Heat treatment of chitosan film produced a shifting of the peak at lower value of 2θ was reported in the literature (28). These results indicated that both the uncrosslinked and crosslinked microcapsules were amorphous in nature. Both neat carrageenan and genipin crosslinked carrageenan hydrogel produced amorphous compounds, as revealed by X-ray diffraction study, were reported by Meena and colleagues (27).

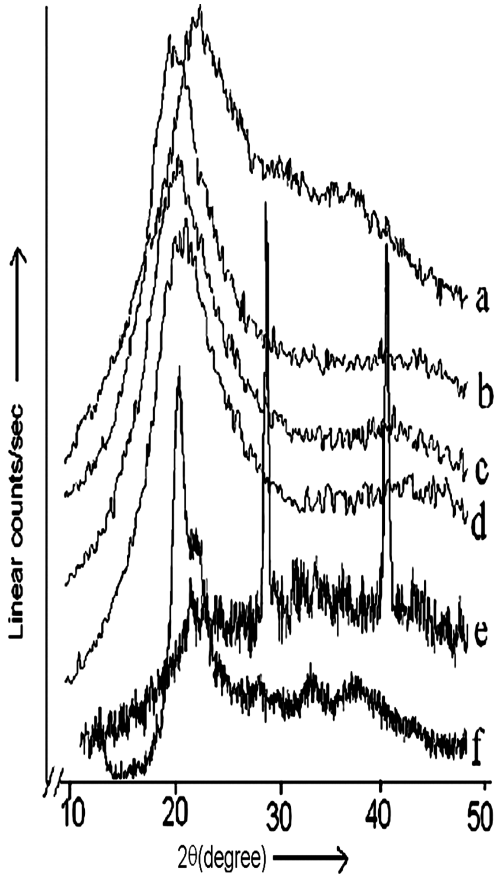

Fig. 7. X-ray diffractograms of NSO loaded chitosan-carrageenan microcapsules a) uncrosslinked; b) glutaraldehyde crosslinked; c) genipin crosslinked; d) tannic acid crosslinked; e) neat carrageenan; f) neat chitosan.

4 Conclusions

Chitosan-carrageenan polyelectrolyte complex could be used as an efficient matrix for encapsulation of NSO. The release rate was found to be dependent on nature and concentration of crosslinkers used in the system. Water uptake capacity, release behaviour of NSO, thermal properties and surface morphology of microcapsules crosslinked with various crosslinkers were compared. Glutaraldehyde was found to be most effective crosslinker followed by genipin and tannic acid. Various properties like decrease in water uptake capacity, release rate and improve in thermal stability were found maximum in the case of glutaraldehyde crosslinked microcapsules. These findings might provide some valuable information in developing a suitable system for controlled release purpose.

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