DEVELOPMENT OF NATURAL BIODEGRADABLE POLYMERIC SYSTEM FOR CONTROLLED DELIVERY APPLICATIONS

A thesis submitted
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

By

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Dedicated to all

who are working altruism for the

cultivation of science...

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CERTIFICATE

This is to certify that the thesis entitled "**DEVELOPMENT OF NATURAL BIODEGRADABLE POLYMERIC SYSTEM FOR CONTROLLED DELIVERY APPLICATIONS**" submitted by Ms. Nirmala Devi, Research Scholar of Department of Chemical Sciences, Tezpur University, Assam for the award of degree of Doctor of Philosophy in Science, is a record of bonafide research work done under my supervision and guidance at Department of Chemical Sciences, Tezpur University, Assam-784028. She has successfully completed the work. A few scientific papers related to the subject have been published/ accepted for publication in international and national journals of repute. A few are also under communication.

She has fulfilled all the requirements for submitting the thesis for award of the degree of Doctor of Philosophy in Science.

The results embodied in the thesis have not been submitted to any other University or Institution for award of any degree or diploma.

Tarun Kanti Maji 14/12/09
(Prof. T. K. Maji)

DECLARATION

I hereby declare that the thesis entitled "DEVELOPMENT OF NATURAL BIODEGRADABLE POLYMERIC SYSTEM FOR CONTROLLED DELIVERY APPLICATIONS" is an authentic work carried out by me under the supervision of Prof. T. K. Maji, Department of Chemical Sciences, Tezpur University, Napaam. No part of this work had been presented for any other degree or diploma earlier.

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ABSTRACT

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Scientists today, more than ever before are being challenged to provide environmentally benign, more economical, and more efficient products for the health and well being of mankind. "Controlled delivery" technologies have emerged as an approach with promise not only to utilize resources in the maximum efficient way but also for the prevention of pollution. Moreover, if the resource is natural or renewable polymer, then it will draw attention as more new, more economical and more eco-friendly source for use of mankind.

Controlled delivery occurs when a polymer, natural or synthetic, is judiciously combined with an active agent (drug or pesticide etc.) in such a way that the active agent is released from the material in a predesigned manner. The purpose behind controlling the delivery of the active agent is to achieve more effective therapies while eliminating the potential for both under- and overdosing. During the last decade, controlled release technology has received increasing attention in the face of a growing awareness that substances ranging from drugs to agricultural chemicals are frequently excessively toxic and sometimes ineffective when administered or applied by conventional means. Thus, conventionally administered drugs in the form of pills, capsules, injectables, and ointments are introduced into the body as pulses that usually produce large fluctuations of drug concentrations in the blood-stream and tissues and consequently, unfavourable patterns of efficacy and toxicity. Conventional application of agricultural chemicals similarly provides an initial concentration far in excess of that required for immediate results in order to assure the presence of sufficient chemical for a practical time period. Such overdosing wastes much of the chemical's potential and all too often causes toxicity problems for nontarget organisms.

Thus the need of controlled delivery device can be understand by their advantages over conventional formulations. Though the advantages of controlled-release technology are impressive, the merits of each application have to be examined individually, and the

positive and negative effects weighed carefully before large expenditures for development work are committed. Exploration of all the advantages and disadvantages (though limited) of controlled release technology for both pharmaceutical and agrochemical applications concludes that natural and biodegradable polymers are the materials of best choice. Therefore, although experience with synthetic polymers is extensive and encouraging, the trend has been to shift towards natural polymers. The major advantage of natural polymers includes their low cost and compatibility with the encapsulation of a wide range of drugs and agrochemicals, with minimal use of organic solvents. Furthermore, bio-adhesion, stability, safety and their approval for human use are the additional advantages. Similarly, 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.

In the present research work, polymeric systems for the controlled delivery of two active agents – (a) Isoniazid, an antitubercular drug (ATD), and (b) Neem seed oil (NSO), a botanical pesticide extracted from the Indian neem plant *Azadirachta Indica A Juss.* were developed and characterized. In order to make the system biodegradable, the polymeric materials used were naturally occurring polymers such as chitosan, carrageenan, gelatin and sodium carboxymethyl cellulose. Microencapsulation technique was used for the preparation of controlled release microspheres. Microencapsulation is a technique that reproducibly applies a uniformly thin polymeric coating around small solid particles, liquid droplets or solid dispersions. The polymeric wall is designed to permit controlled release of the encapsulated material under desired conditions. The release of the active agents can be controlled by crosslinking of the polymeric wall. Both synthetic and naturally occurring crosslinking agents were used in our present work. Synthetic crosslinking agent used was glutaraldehyde and naturally occurring crosslinking agents were genipin and tannic acid.

Chapter I - This chapter includes introduction part. This covers concept of controlled delivery, need of this type of delivery, components of controlled delivery systems, advantages and limitations of controlled delivery, natural and biodegradable polymers

used for controlled delivery, fabrication techniques, crosslinkers, their properties and crosslinking of polymers. This chapter also includes general literature relating to properties of chitosan, carrageenan, gelatin, sodium carboxymethyl cellulose, NSO and isoniazid.

Chapter II - This chapter covers, in general, the available literatures involving on controlled release agrochemicals and drugs. The literatures relating to NSO and isoniazid have been incorporated. Besides this, literatures regarding analysis of controlled release systems in terms of loading efficiency, release characteristics, swelling, Fourier transform infrared (FTIR) spectroscopy, thermal properties, surface properties etc. are covered. The main research objectives and plan of work have also been included in this chapter.

Chapter III - This chapter covers the materials and methods, which includes the raw materials used, techniques for sample preparation, release studies and characterization of the products.

Chapter IV - This chapter includes results and discussion part. This chapter has been divided into the following two parts.

***Part I* - Microencapsulation of Neem Seed Oil (NSO), a potent natural pesticide, in three different polyelectrolyte complex systems-namely (i) chitosan- κ-carrageenan (ii) κ-carrageenan-gelatin A (iii) gelatin A-sodium carboxy methyl cellulose.**

(i) In **chitosan-κ-carrageenan** system, the pH and optimum ratio between carrageenan and chitosan to form a stable polyelectrolyte complex were 4.5 and 1: 0.36 respectively at temperature 40 ^{0}C. This ratio of polymer and pH were maintained for encapsulation of NSO.

The encapsulation efficiency, oil content and release pattern from the microcapsules were determined by using UV spectrophotometer and calibration curve of oil. The release rates of NSO were studied by varying the percentage of oil loading, concentration of crosslinking agent (glutaraldehyde) and polymer concentration.

With the increase in oil loading (%) (73.12 to 219.35), the encapsulation efficiency (%) (73.39± 2.37 to 85.90±0.728), the cumulative release rate and oil content (%) (31±1.0 to 59±0.5) were found to increase throughout the range of oil concentration studied. The effects of variation of cross-linker concentration on oil loading (%), oil content (%), encapsulation efficiency (%) and release rate were studied. It was observed that with the increase in crosslinker i.e. glutaraldehyde concentration (1.5-7.5mmol), oil loading (%) decreased (163.85 to 70.4) but oil content (%) (41±2.0 to 48±0.74) and encapsulation efficiency (%) (66.02±3.22 to 98.47±1.51) increased. A decrease in trend in oil content (%) (40±1.0) was observed in the case of 12.5 mmol concentration of glutaraldehyde. The release rate of oil was found to decrease as the % of glutaraldehyde increased. Both oil loading (%) (342.56-126.04) and oil content (%) (61-46) decreased with the increase in total polymer content (0.408-1.156g). Encapsulation efficiency (%) (78.80±1.17 to 82.49±4.23) increased initially and then leveled off. The release rate was found to decrease with the increase in polymer concentration.

Scanning electron microscopy (SEM) study demonstrated that the surfaces of microcapsules loaded with low percentage of NSO were smooth indicating the formation of continuous film on surface of microcapsules by the complex. On the other hand, the surface of high NSO loaded microcapsules appeared irregular and bursting.

FTIR study confirmed the complex formation between κ-carrageenan and chitosan. The position of all notable peaks for polymers remained unchanged and a new band appeared. The appearance of this band at 1528.64 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.

Further, both in the physical mixture as well as NSO loaded microcapsules, the position of all notable bands of the chitosan-κ-carrageenan polyelectrolyte complex remained unaltered. This indicated the absence of any significant interaction between NSO and chitosan-carrageenan polyelectrolyte complex. Differential scanning

calorimetry (DSC) study also indicated the absence of any significant interaction between polyelectrolyte complex of κ-carrageenan-chitosan and NSO.

In order to study the effect of different crosslinkers, these 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 behaviour of NSO from encapsulated crosslinked microcapsules followed the order: tannic acid >genipin> glutaraldehyde. The interaction of the polyelectrolyte complex with these crosslinkers was studied. Glutaraldehyde was found to be the most effective crosslinker followed by genipin and tannic acid. Improvement in thermal stability was found maximum in the case of glutaraldehyde crosslinked microcapsules. Crosslinking improved thermal stability without affecting crystallinity. Roughness appeared on surface of microcapsules indicated the interaction between microcapsules and crosslinker.

(ii) In **κ-carrageenan-gelatin A** system, the yield of the coacervate was found to be dependent on the ratio of the two polymers and the pH of the medium. Viscosity and turbidity measurements were carried out to support the ratio of the two polymers that produced highest yield. The optimum ratio of gelatin to carrageenan and pH at which maximum coacervation occurred were 2:1 and 3.5 respectively at temperature 40 ^{0}C.

The optimized conditions were employed to prepare microcapsules containing neem (*Azadirachta Indica A.Juss.*) seed oil (NSO) using complex coacervation technique and genipin as crosslinker. The encapsulation efficiency and the release rates of NSO were dependent on the amount of crosslinker, oil loading and polymer concentration. With the increase in oil loading (%) (65.67-262.70), the encapsulation efficiency (%) (75.68 ± 1.01 to 91.12 ± 0.42), release rate and oil content (%) (30±0.4 to 66± 0.3) were found to increase throughout the range of oil concentration studied.

With the increase in crosslinker (genipin) concentration (0.1-0.8mmol), oil loading decreased (%) (131.35 to 119.07) for all but oil content (%) (45±1.0 to 51±0.7)

and encapsulation efficiency (%) (79.25 ±1.76 to 93.82±1.29) 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 carrageenan and gelatin. The release rate of oil was found to decrease as the percentage 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.

Both oil loading (%) (125.80-43.57) and oil content (%) (49±1.0 to 29±0.8) decreased with the increase in total polymer (1.5-4.5g) content but encapsulation efficiency (%) (87.95±1.79 to 95.55± 2.63) increased. With the increase in polymer content, more and more polymer was available to encapsulate the oil vesicles and thereby efficiency increased. The excess polymer after complete encapsulation would enhance the thickness of the microcapsule. 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 behavior.

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. FTIR spectroscopy and DSC study showed that there was no significant interaction between NSO and carrageenan-gelatin complex.

(iii) Complex coacervation of **gelatin A with sodium carboxymethyl cellulose** (SCMC) was studied as a function of pH and ratio of one polymer to the other. The efficiency of coacervation was measured by checking viscosity, coacervate yield (%) and turbidity of the mixture of gelatin A and SCMC solutions. Maximum coacervation was found to occur at pH 3.5 and SCMC: Gelatin ratio of 1.0: 2.33 at temperature 40 ^{0}C. Microencapsulation of NSO was carried out at this optimum ratio and pH.

Encapsulation efficiency, oil content and oil load of the microcapsules prepared under different conditions were evaluated. Release bevaviour of

the microcapsules were found to be dependent on the percentage of oil loading and the amount of crosslinker, glutaraldehyde.

The size of the microcapsules varied with the concentration of polymer and NSO as revealed by SEM study. With the decrease of the amount of polymer, the size of the microcapsules decreased. This might be due to the decrease of the thickness of the wall of the microcapsules. Again, the surface of the microcapsules having high NSO loading was appeared sticky and bursting as compared to the low NSO loading microcapsules.

Microcapsules were further characterized by FTIR spectroscopy, which showed that there was no significant interaction between the polymer and NSO.

***Part II* - Microencapsulation of Isoniazid, an antitubercular drug in three different polyelectrolyte complex systems-namely (i) chitosan-κ-carrageenan (ii) κ-carrageenan-gelatin A (iii) gelatin A-sodium carboxy methyl cellulose.**

In this part of work, the polymer systems optimized in Part I were used for preparation of microcapsules as well as hollow nanocapsules by different methods for encapsulation of drug, isoniazid.

(i) 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 by immersing in isoniazid solutions of different concentrations. Sonication and surfactant concentration controlled the particle size of the nanocapsules as indicated by the particle size analyzer. 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.

TEM study supported the observation obtained from particle size analyzer. The calculated diameter of a randomly chosen nanocapsule was 230 nm, which was close to that of average particle size (234nm) 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 little brighter.

The loading of drug into the nanocapsules was time dependent. The release rates of isoniazid were higher at acidic media (pH=1.2, HCl) compared to basic media (pH=7.4, phosphate buffer). FTIR study showed that there was no remarkable interaction between isoniazid and polyelectrolyte complex. Surface characteristics of the nanocapsules were studied by SEM. X-ray diffraction study showed that the dispersion of isoniazid was not occurred at molecular level.

(ii) Microspheres of **gelatin-A and κ-carrageenan** were prepared by using genipin, a naturally occurring crosslinker and sunflower oil as reaction medium. Varying concentrations of surfactant and polymer controlled the sizes of the microspheres. Studies indicated that surfactant (tween 80) had important role in preparation and stabilization of κ-carrageenan-gelatin microspheres using sunflower oil as dispersing medium. A matrix type product was formed in the absence of surfactant. SEM study showed that surfactant (0.33-1.0g/g of polymer) and polymer concentration (3.0 to 1.5 g) of varying amounts would form different sizes of spherical, free flowing microspheres. Higher amount of surfactant and lower polymer concentration favoured the formation of smaller microspheres. The microspheres thus prepared were used for encapsulation of isoniazid by absorption. The swelling behavior of microspheres was studied. In the basic medium (pH=7.4, phosphate buffer), the microspheres swelled more compared to that of acidic medium (pH=1.2, HCl).

The absorption of isoniazid into the microspheres was dependent on the concentration of isoniazid solution. Higher the concentration of isoniazid solution, higher was the encapsulation efficiency but lower was the stability of microspheres. Higher pH (pH=7.4) of the release medium facilitated the release of isoniazid more compared to lower pH (pH=1.2) medium. FTIR spectroscopy indicated the successful loading of

isoniazid into the microspheres. DSC and X-ray diffraction (XRD) results indicated a molecular level dispersion of isoniazid in the microspheres.

(iii) Microparticles of different sizes were prepared at the optimized ratio of **gelatin-A and sodium carboxy methyl cellulose** (SCMC). Effect of various factors like amount of surfactant (tween 80), concentration of polymer on the formation and size of the microparticles were investigated. Surfactant had very important role in stabilizing the microparticles formed in sunflower oil. A matrix like gel product was formed if surfactant was not added. But different sizes of microparticles were formed on addition of varying amount of surfactant. SEM study showed that with the increase of amount of tween-80, the sizes of the microparticles decreased. At higher concentration of surfactant, the aqueous phase is easily dispersed into finer droplets, owing to the higher activity of the surfactant, which resulted in a lower free energy of the system, and led to a smaller particle size.

Again, with the decrease of the amount of polymer, a decrease in the size of the microparticles was observed. In the presence of higher amount of polymer, the surfactant present might not be capable of covering all the surfaces of the microparticles properly. This resulted in the coalescence of some of the microparticles and led to the formation of larger microparticles. Besides this, the dispersive force of the stirrer became less efficient in presence of higher amount of polymer and as a result larger microparticles formed. Stirring speed also affected the nature and size of the microparticles. At lower stirrer speed (200 rpm), the agglomeration of particles was more compared to those of particles produced at higher stirrer speed (1500 rpm). Improper mixing of polymers at low stirrer speed resulted in agglomeration. Again, sizes of microparticles become finer at higher stirrer speed compared to lower stirrer speed.

These microparticles were used as carrier for isoniazid. The loading efficiency and release behaviour of loaded microparticles were found to be dependent on the amount of crosslinker used, concentration of drug and time of immersion. For similar extent of crosslinked (17.50 mmol/g of polymer) samples, maximum drug loading

efficiency (%) (60.7 ± 0.50) was observed at higher immersion time (48h) when immersed in higher concentration of isoniazid solution (20.0 g/100ml). The release rate of isoniazid was more at higher pH (pH=7.4) compared to that of at lower pH (pH=1.2). FTIR study indicated the successful incorporation of isoniazid into the microparticles. DSC study showed a molecular level dispersion of isoniazid in the microparticles. XRD study revealed the development of some crystallinity due to the encapsulation of isoniazid.

Chapter V - This chapter includes summary and conclusions of the present work. The salient outcomes of this thesis have been summarized.

➢ The pH and polymer ratio at which maximum complexation between **(i) κ-carrageenan-chitosan (ii) gelatin A-κ-carrageenan (iii) sodium carboxy methyl cellulose-gelatin A** for highest yield occurred were **(i) 4.5 and 1:0.36 (ii) 3.5 and 2:1** and **(iii) 3.5 and 1:2.33** respectively.

➢ NSO was microencapsulated in all the three systems successfully at the optimized conditions. The release rate of the oil was found to be dependent on various parameters such as oil content, crosslinker concentration and the amount of encapsulating polymer. Effects of various crosslinking agents on NSO encapsulated microcapsules were compared and glutaraldehyde was found to be most effective crosslinker followed by genipin and tannic acid. New bond formation and shifting of peaks in FTIR spectra confirmed the interaction between the complex forming polymers. Both FTIR and DSC study revealed no interaction between polymers and NSO in all the cases. SEM study showed the formation of spherical microcapsules having free flowing to bursting look depending on the extent of oil loading. The sizes of microcapsules increased with the increase of the concentration of the polymer.

➢ Using all the three polymer systems, microparticles were prepared by different techniques using greener solvent, water and sunflower oil. The microparticle formation was carried out at the optimized conditions of pH and ratio of the polymers. The formation and the size of the microparticles were also dependent on surfactant, polymer

concentration and stirring speed. Isoniazid was encapsulated successfully. Isoniazid concentration governed the absorption of isoniazid into the microparticles. Release rates of isoniazid were dependent on pH. FTIR study indicated the loading of isoniazid into the microparticles. DSC and XRD studies showed that isoniazid was dispersed in the microparticles.

Future scope

NSO and isoniazid were found to be successfully encapsulated within cross-linked polymers. The release of both NSO and isoniazid were found to be controlled if encapsulated within polymer microparticles. However, the present investigation is restricted to laboratory scale only. Further field evaluation for NSO microcapsules and release studies of isoniazid in animal models are needed for better understanding and optimization of the encapsulated products.

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INTRODUCTION

CHAPTER I

INTRODUCTION

1.1. Concept of controlled delivery

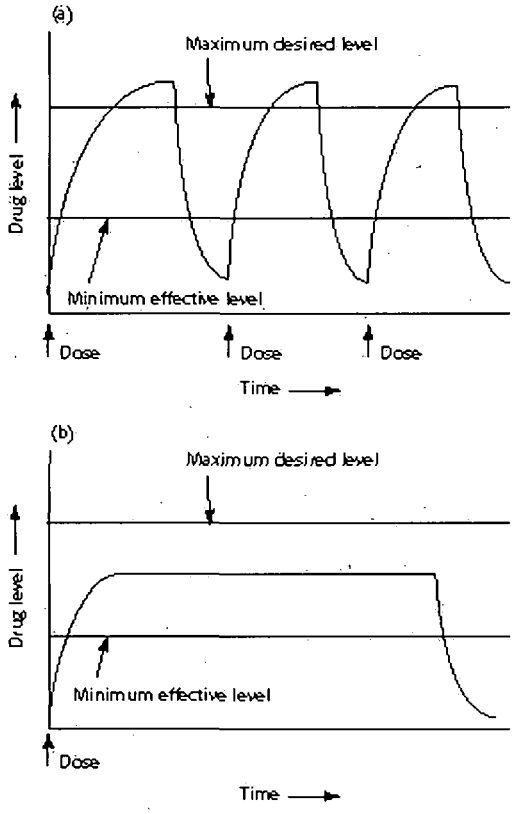
Recently a significant improvement can be experienced within the agricultural and pharmaceutical sectors regarding such environmentally friendly technologies that mainly target the production of healthy, nutritious, quality foodstuff as well as pharmaceutical products by taking environmental characteristics into account, and adapting to them. To achieve such goals mentioned above and to bring sustainable, environmentally human economical systems to the forefront, scientists today are more than ever before being challenged to provide environmentally benign, more economical, and more efficient products for the health and well being of mankind. "Controlled delivery" technologies have emerged as an approach with promise not only to utilize resources in the maximum efficient way but also for the prevention of pollution. Moreover, if the resource is natural or renewable polymer, then it will draw attention as more new, more economical and more eco-friendly source for use of mankind.

Controlled delivery may be defined as a technique or method in which active chemicals are made available to a specified target at a rate and duration designed to accomplish an intended effect [1]. Controlled delivery occurs when a polymer, natural or synthetic, is judiciously combined with an active agent in such a way that the active agent is released from the material in a predesigned manner. The release of the active agent may be constant or cyclic over a long period. It may be triggered by the environment or other external events. In any case, the purpose behind controlling the drug delivery is to achieve more effective therapies while eliminating the potential for both under and overdosing [2]. During the last decade, controlled release technology has received increasing attention in the face of a growing awareness that substances ranging from drugs to agricultural chemicals are frequently excessively toxic and sometimes ineffective when administered or applied by conventional means. Thus, conventionally administered drugs in the form of pills, capsules, injectables, and ointments are introduced into the body as pulses that usually produce large fluctuations of drug concentrations in the blood-stream and tissues and consequently,

unfavourable patterns of efficacy and toxicity. Conventional application of agricultural chemicals similarly provides an initial concentration far in excess to that required for immediate results in order to assure the presence of sufficient chemical for a practical time period. Such overdosing wastes much of the chemical's potential and all too often causes toxicity problems for nontarget organisms [1].

1.1.1. Why controlled delivery?

The goal of many of the original controlled-release systems was to achieve a delivery profile that would yield a high level of the active agent (drug, pesticide, fertilizer etc.) over a long period of time. With traditional devices level in the surrounding (soil or blood) follows the profile shown in Figure 1.1a, in which the level rises after each administration of the active agent and then decreases until the next administration. The key point with traditional administration is that the surrounding (soil or blood) level of the active agent should remain between a maximum value, which may represent a toxic level, and a minimum value, below which the drug/or pesticide is no longer effective. In controlled delivery systems designed for long-term administration, the active agent level in the soil or blood follows the profile shown in Figure 1.1b, remaining constant, between the desired maximum and minimum, for an extended period of time.



In recent years, controlled delivery formulations and the polymers used in these systems have become much more sophisticated, with the ability to do more than simply extend the effective release period for a particular active agent. For example, current controlled-release systems can respond to changes in the biological environment and deliver or cease to deliver active agents based on these changes.

Figure 1.1. Active agent levels in the surrounding medium with (a) traditional dosing and (b) controlled delivery dosing [2].

In addition, materials have been developed that should lead to targeted delivery systems, in which a particular formulation can be directed to the specific cell, tissue, or site where the active agent it contains is to be delivered. While much of this work is still in its early stages, emerging technologies offer possibilities that scientists have only begun to explore. Microencasulation and nanoencasulation are the peak methods used in the contemporary field of research.

Along with microcapsules, nanaocapsules are now getting considerable interest in the field of modern controlled release device. Several promising approaches for the preparation of hollow polymer particles have been developed. The current state of the art, therefore, provides a reasonable basis for developing such systems further towards applications. However, although various applications of these new materials, e.g., for encapsulation of drugs, enzymes or fragrances, are discussed in some papers [3] they are yet to be realized. Since the majority of the proposed applications are located in the life science area, most of the model polymers described up to now are not well-suited and nanocapsules composed of biocompatible materials are required. Generally, many of the described approaches are rather ineffective (e.g., they require very low concentrations) and hence a further challenge will be to scale up the production of the nanocapsules.

1.2. Basic components of controlled delivery formulations

The components of controlled delivery system include (a) the polymer matrix or matrices that regulate the release of the active components (b) the active agent and (c) the crosslinking agents. Varieties of active agents [4], polymers and crosslinking agents both natural and synthetic are being used for the development of controlled delivery formulations.

1.2.1. Polymers

Polymers, both natural and synthetic, are very much useful in preparing controlled delivery formulations. Most of the drugs, fertilizers, herbicides, pesticides and pheromones are low molecular weight compounds. Tremendous progress in polymer science and technology can make it possible to combine a low molecular active agent species physically or chemically to a polymer through a pre- or post polymerization reaction. In controlled delivery technique, the active agent is allowed to release from the polymer- active agent combination over a period of time, most often to a specific target. In physical combinations,

polymer acts as a rate-controlling device while in chemical combinations, it acts as a carrier for the active agent.

An important advantage of polymeric controlled delivery formulation is that the toxic natures of the chemicals are minimized. Many new pesticides are readily biodegradable and highly toxic. It poses great danger to non-target organisms also. But if it is encapsulated or distributed in a polymer, its toxicity will be much reduced, since the entire amount does not release at one time. Still another advantage is that the polymer combinations being solids are easy to handle [5].

The success of controlled delivery formulation relies on combining the active agent with the polymer in an economic manner, at the same time maintaining the desired release profile. These are often in opposition and one has to compromise in the ultimate cost/benefit ratio of controlled delivery formulations [6]. However there are many classes of polymers which can be effectively employed in controlled delivery formulations. The efficiency of controlled delivery formulations depends on the following polymer properties-

- Solubility and distribution characteristics with the active agent.
- Solubility and distribution characteristics with the environmental agents.
- Good compatibility with the environment i.e., it should be non-toxic.
- Good compatibility with the active agent i.e., it should not produce undesirable products.
- Stability in the environment i.e., it should not degrade during the course of action. Degradation is preferable after the completion of desired function. The degraded products should not harm the environment.
- Ease of fabrication.
- Cost.

A list of polymers that are used in controlled delivery formulations is shown in Table 1.1.

Table 1.1. Polymers used in controlled delivery formulations

Natural polymers	Synthetic polymers	Synthetic elastomers
• Carboxymethyl cellulose • Cellulose • Ethylcellulose • Gelatin • Gum Arabic • Starch • Bark • Methyl cellulose • Arabinogalactane • Zien • Nitrocellulose • Propylhydroxycellulose • Shellac • Proteins • Kraft lignin • Natural Rubber • Chitin and chitosan • Alginate • Guar gum • Waxes- paraffin • Carrageenan	• Polyvinyl alcohol • Polyvinyl acetate • Polyethylene • Polypropylene • Polystyrene • Polyacrylamide • Polyether • Polyamide • Polyurea • Epoxy • Ethylene vinyl acetate copolymer • Polyvinylidene chloride • Polyvinyl chloride • Polyvinyl chloride • Polyacrylate • Polyacrylonitrile • Chlorinated polyethylene • Actal copolymer • Polyurethane • Polyvinylpyrrolidone • Poly(p-xylene) • Polymethylmethacrylate	• Polybutadiene • Polyisoprene • Neoprene • Polysiloxane • Styrene-butadiene rubber • Silicone rubber • Hydrin rubber • Chloroprene • Butyl rubber • Acrylonitrile • Ethylene-propylene-diene terpolymer

1.2.1.1. Natural and biodegradable polymers

Natural polymers are promising biodegradable polymeric materials. These natural polymers do hold advantages over the synthetic polymers, generally because they are nontoxic, less expensive, and freely available. Although natural gums and their derivatives are used widely in pharmaceutical dosage forms, their use as biodegradable polymeric materials to deliver bioactive agents has been hampered by the synthetic materials in general. But, natural polymers can be modified to have tailor-made materials for controlled delivery systems and thus can compete with the synthetic biodegradable excipients available in the market.

Though controlled delivery technology do hold several advantages [7,8] over conventional formulations, there are some limits [5] and these limits can be overcome by using natural and biodegradable polymers. Although, a number of controlled delivery formulations for agrochemical applications have been developed, none of them, so far, has been exploited commercially to a large extent. They are successful only in laboratory field test but lesser extent in actual field trials. This is due to the cost consideration, technical difficulties and availability to the farmers. There is no doubt about their scale usage by farmers in future, if the prohibitive cost can be lowered by using (i) less costly, easily available active agent or/and (ii) applying simple economically favorable technique. In this respect, natural biodegradable polymeric materials are probably the materials of choice, though a better technique of application of these active agents is to be adopted for better performance and economic viability [9]. Several natural biodegradable polymers [10, 11] have been used for controlled release purposes in agricultural use.

Regarding their use in medicine, biomaterials can be divided mainly into three classes: scaffolds for tissue engineering, load bearing applications for surgery and drug delivery systems [12, 13]. Biomaterials should be biocompatible without any toxic, inflammatory, carcinogenic and immunogenic response. Accordingly, biomaterials can be divided into biotolerant, bioinert, biodegradable and bioactive materials [14].

In the present study, four natural polymers namely chitosan, κ-carrageenan, gelatine-A and sodium carboxymethyl cellulose have been used for preparation of controlled release systems.

1.2.1.1.1. Chitosan

Polysaccharides are distributed widely in nature with a broad range of molecular structure and properties. One of the most important naturally abundant and easily accessible polysaccharides is chitin (Figure 1.2). Chitin (2-acetoamido-2-dexoy-β-D-glucose) is a linear high molecular weight and crystalline polysaccharide, and like cellulose, is made of β-(1→4) linked D-glucose. Chitin is similar to cellulose in structure, but it has acetamide groups at the C-2 position in place of hydroxyl groups. Compared to cellulose, chitin has drawn less attention because of intractable bulk structure due to strong intra- and intermolecular hydrogen bonding.

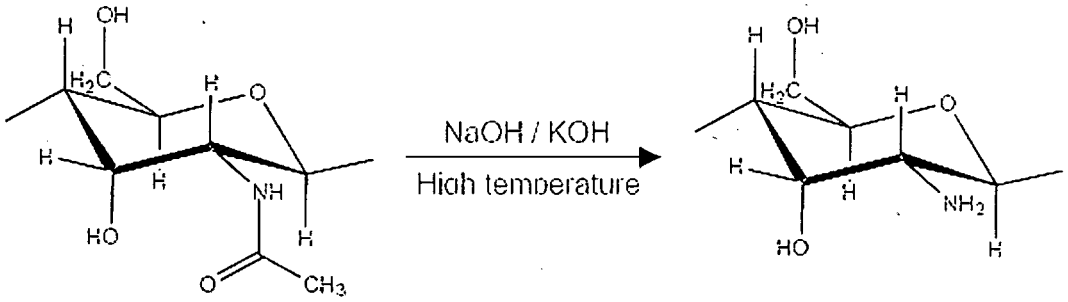


Figure 1.2. Chemical structure of chitin (left) and chitosan (right)

Chitin is commercially produced from arthropod shells (exoskeltons) and crustacean shells, such as crabs and shrimps, which are conveniently available as waste from seafood-processing industries [15]. Two other major components of these shells are proteins and calcium carbonate. Calcium carbonate can be removed by treatment in diluted hydrochloric acid. Proteins are decomposed in sodium hydroxide solution near 100°C. Chitin remains as a residue of colorless to off-white powdery material. The N-acetyl-D-glucosamine is the basic repeating unit of chitin. Although most of the C-2 amino groups are acetylated, there are some free amine groups. Three different crystalline forms (α, β, γ) of chitin have been reported [16, 17].The strong intermolecular hydrogen bonding is mainly responsible for limited solubility in common solvents.

When chitin is treated with 40~50% aqueous alkali at a high temperature (100-160°C) for a few hours, N-acetyl groups of chitin are removed. The resulting deacetylated chitin has various degrees of deacetylation (between 70 and 95%). This considerably deacetylated chitin is called chitosan and is soluble in dilute acetic acid solution. Thus, chitosan can be

considered block-type copolymers composed of N-acetyl-D-glucosamine and D-glucosamine residues linked together by β(1→4) glycosidic bonds [18,19]. The main chains can be depolymerized to some extent during the deacetylation, as shown by a considerable reduction in the molecular weight [20]. Chitosan's unique characteristics make it potentially useful in a variety of applications such as a drug carrier; for wound healing, implantation, and gene therapy because of the following advantages [21, 22].

- Chitosan is obtained from the second most abundant natural polymer chitin [23,24].
- Chitosan is nontoxic, biocompatible and biodegradable.
- Chitosan possesses more bioactivities: it has antacid and antiulcer acitivities that prevent or weaken drug irritation in the stomach [25].
- Organic solvents are not required in solubilization of chitosan.
- It is simple and easy to prepare chitosan microspheres of the desired size.

Chitosan oligomers can be prepared by cleaving the main chains with acidic or enzymatic degradation. The glycosidic linkages of chitosan are relatively stable against alkali, but cleaved with acid [20]. Hydrolysis using acids (such as hydrochloric acid and nitrous acid) is a common and fast method, but this procedure has some drawbacks (such as high cost and low yield). Enzyme degradation produces highly water-soluble low molecular weight chitosan using chitinase, chitosanase, and glucanase. This method is generally preferable over chemical treatment, because of the minimized alteration of reaction product and ease of hydrolysis control, but this process is very slow [26]. The enzymatic method is useful for preparing oligomers of a specific degree of polymerization, particularly dimer and higher ones with degrees of polymerization above 5 [20]. Other approach employs γ-ray irradiation. A rapid decrease of viscosity was observed when chitosan was irradiated in acetic acid solution. Chitosan oligomers exhibit significant physiological characteristics and functions, such as better biodegradability, blood compatibility, antifungal activity, antitumor activity, antimicrobial activity, and immuno-enhancing activity [27].

The nitrogen content of chitosan is generally 5 to 8% depending on the degree of deacetylation. Chitosan has nitrogen mostly in the form of primary aliphatic amino groups which predominate the chemistry of chitosan. The presence of amino groups in the chitosan molecules make them suitable for further chemical modification, which in turn allow extensive adjustment of the chemical and biological properties of the chitosan.

Chitosan is insoluble in either water or organic solvents, but is soluble in most aqueous dilute acids at a pH below 6.5. Aqueous solutions such as phosphoric, sulfuric, citric, and sebacic acids are not good solvents [20]. Acetic acid has been mostly used as a standard solvent for chitosan. The extent of solubility depends on the degree of deacetylation, concentration, and type of acid and pH. Chitosans with a relatively low degree of deacetylation (40%) have been found to be soluble only up to pH 9, whereas chitosans with a degree of deacetylation of about 85% have been found to be soluble only up to a pH of 6.5 (pKa 6.5) [19,24]. Solubility diminishes with increasing concentration of acid [20]. Upon dissolution, the amine groups of the polymers are protonated and the resultant soluble polysaccharide is positively charged. One of the interesting properties of chitosan is the ability to bind to negatively charged surfaces like mucosal membranes, to act as a bioadhesive molecule. The polycationic character and ability to interact with negatively charged molecules have drawn many researchers to study chitosan particles. Chemical as well as biological properties of chitosan are listed in the Table 1.2.

Table 1.2. Chemical and biological properties of chitosan

Chemical properties of chitosan	Biological properties of chitosan
• Cationic polyamine • High charge density at pH<6.5 • Adheres to negatively charged surfaces • Forms gels with polyanions • High molecular weight linear polyelectrolyte • Viscosity ranges from high to low • Form chelates with certain transition metals • Amiable for chemical modification • Highly reactive amine/hydroxyl groups.	• Biocompatible • Natural polymer • Biodegradable to normal body constituents • Safe and non-toxic • Haemostatic, bacteriostatic and fungistatic • Spermicidal • Anticarcinogen • Anticholestermic • Reasonable cost • Versatile.

Adapted from reference [28].

1.2.1.1.2. Carrageenans

Carrageenans are marine hydrocolloids obtained by extraction from some members of the class Rhodophyceae. The most important members of this class are *Chondrus crispus* and *Gigartina stellata.* There are three different types of carrageenans: κ-, ι-, and λ-carrageenan (Figure 1.3). All consist chiefly of the sulfated esters of D-galactose and 3,6-anhydro-D-galactose copolymers, linked α-1,3 and β-1,4 in the polymer. The λ-carrageenan does not

contain 3,6-anhydro galactose and is highly sulfated. It does not gel and is used as a thickening agent. The κ- and ι-carrageenan are very similar, except ι-carrageenan is sulfated at carbon-2. Both polymers swell and form gels. The κ-carrageenans form strong, rigid, and brittle gels. A very small amount of potassium ion is essential for this. The ι-carrageenan forms elastic gels that show thixotrophy, mainly in the presence of calcium ions [29]. Carrageenans are mainly used as gelling and thickening agents. Only a few studies have dealt with carrageenans for controlled-release tablets [30, 31, 32]. These studies dealt only with drug delivery from tablets on a hydraulic press or from tablets that contain the carrageenans in a mixture with other excipients. In a study of four natural hydrophilic gums formulated as minimatrices in hard gelatin capsules, it was concluded that carrageenan used in the study did not produce sufficient sustained release [33]. In past studies, the compaction and consolidation behavior of carrageenan were determined to prove their usefulness in tableting excipients for controlled-release tablets. The results indicated that the carrageenans were suitable tableting excipients for controlled-release tablets. The release behavior of model drugs diclofenac sodium and theophylline indicated that drug release was increased when water sorption and the extent of swelling decreased and viscosity increased [34-36].

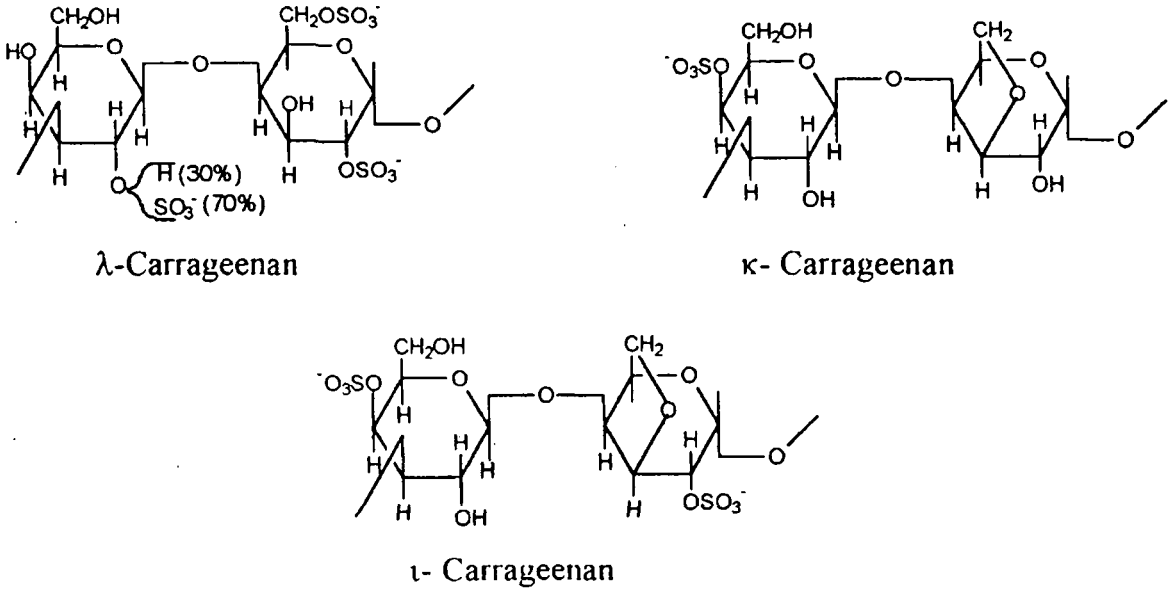


Figure 1.3. Structure of different types of carrageenans.

1.2.1.1.3. Gelatins

Gelatins are high molecular weight polypeptides derived from collagen, the primary protein component of animal connective tissues, such as bone, skin and tendon. The name gelatin is derived from the Latin *gelatus* which means firm or frozen. Although the term gelatin is sometimes used to refer to other gel formers, it is probably applied only to the collagen derived protein materials.

Modern technological applications of gelatin depend on its high solubility in hot water, polyampholyte character, availability in a wide range of viscosities, and thermally reversible gel formation. Gelatin forms thermally reversible gels with water and the gel melting temperature (< 35°C) is below body temperature, which gives gelatin products unique organoleptic properties and flavor release. The disadvantage of gelatin is that it is derived from animal hide and bone, and hence there are problems with regard to Kosher and Halal status and vegetarians also have objections to its use. Competitive gelling agents like starch, alginate, pectin, agar, carragenan etc., are all carbohydrates from vegetable sources.

There are two main types of gelatin: Type A with isoionic point of 7-9 is derived from collagen with exclusively acid pretreatment. Type B with isoionic point of 4.8 to 5.2 is the result of an alkaline pretreatment of the collagen. However, gelatin is sold with a wide range of special properties like gel strength to suit particular applications. Type A gelatin (dry and ash free) contains 18.5 % nitrogen, but due to the loss of amide groups, Type B gelatin contains only about 18 % nitrogen.

A typical chemical structure of gelatin is shown in Figure 1.4

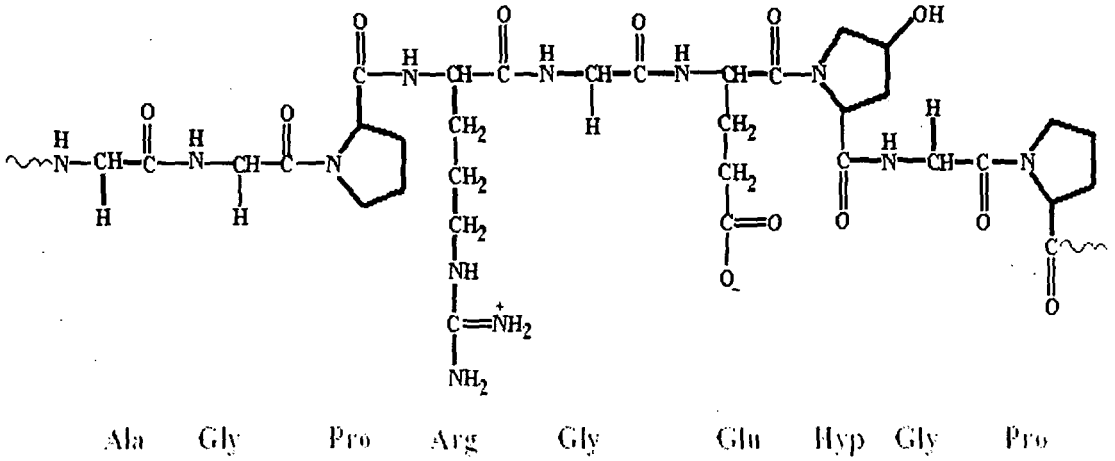


Figure 1.4. Structure of gelatin

Gelatin has been widely used as a fundamental material for microspheres [37], sealants[38], tissue adhesives[39] and carriers for controlled delivery systems [40-42]. Gelatin has also been widely used in combination with other polymers for encapsulation [43-44].

1.2.1.1.4. Sodium carboxy methyl cellulose

Cellulose is a linear polymer of β-anhydro glucose units. Each anhydro unit contains three hydroxyl groups. Cellulose ethers are cellulose derivatives prepared by etherification of these available hydroxyl groups (e.g., hydroxypropyl methylcellulose, hydroxypropyl cellulose, sodium carboxymethyl cellulose, methylcellulose, ethylcellulose, and cellulose acetate phthalate). Cellulose ethers are hydrophilic polymers that are quite popular in the design of controlled delivery dosage forms. They are biologically compatible and nontoxic. Apart from these advantages, their property of easy compression and the abilities to hydrate rapidly at body temperature and to accommodate a large percentage of the drug with negligible influence of the processing variables on the release rates are the main reasons for their popularity. The use of cellulose ethers in drug delivery for controlled-release dosage forms has been reviewed by many workers [45–47]. Carboxy methyl cellulose is a very useful hydrocolloid for the food industry.

Step one:

$$\text{Cell-OH} + \text{NaOH} \longrightarrow \text{Cell-ONa} + H_2O$$

Step two :

$$\text{Cell-ONa} + \text{Cl-CH}_2\text{-COONa} \longrightarrow \text{Cell-O-CH}_2\text{-COONa}$$

Figure 1.5. Reaction scheme for the manufacture of Carboxy methyl cellulose.

Figure 1.5 illustrates the reaction for the manufacture of Carboxy methyl cellulose [48]. It is essentially a two step process. In the first step, cellulose is suspended in alkali to open the bound cellulose chains, allowing water to enter. In the second step, the cellulose is reacted with sodium monochloroacetate to yield sodium carboxymethyl cellulose.

An idealized unit structure of Sodium Carboxy methyl cellulose is depicted in Figure 1.6. The Sodium carboxy methyl cellulose shown here has a D.S.(Degree of Substitution) of 1.0. If the remaining two hydroxyl groups on this unit became substituted, the D.S. would be 3.0. A D.S. of 3.0 is the theoretical maximum one could attain.

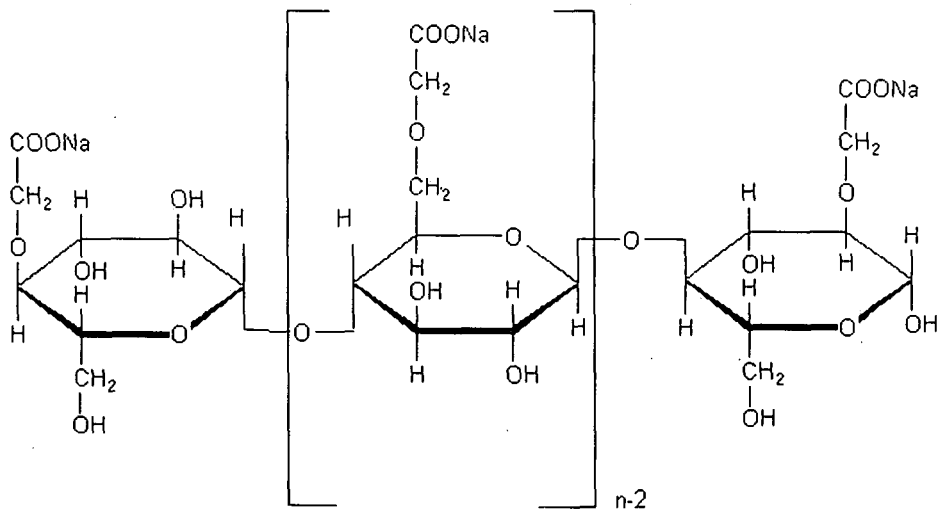


Figure 1.6. Structure of Sodium carboxy methyl cellulose [48]

1.2.2. Active Agents

The substance to be encapsulated or loaded is called active agent. It may be virtually any substance, natural or synthetic, that is soluble or entirely not soluble in the reaction medium or solvent. It may be solid, a hydrophobic or hydrophilic liquid, or a mixture of a solid and a hydrophobic or hydrophilic liquid. The major active agents include drugs/food products, agrochemicals and varieties of oils [4]. The loading substance may comprise of a purified or partially purified substance depending on the requirements of application.

1.2.2.1. Neem (*Azadirachta Indica A. Juss.*)

Neem is a fascinating tree. This plant may usher in a new era in pest control, provide millions with inexpensive medicines, cut down the rate of human population growth, and perhaps even reduce erosion, deforestation, and the excessive temperature of an overheated globe [49]. Neem played an important role in the life of ancient Indian people. The efficacy of neem as a medicine has been documented in several different ancient treatises. Neem has achieved a relatively wide distribution in the tropical areas of Asia, Africa, South America, and Oceania.

More than 135 compounds have been isolated from different parts of neem and several reviews have also been published on the chemistry and structural diversity of these compounds [50-58]. The compounds have been divided into two major classes: isoprenoids and others [57]. The isoprenoids include diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and csecomeliacins such as nimbin, salanin and azadirachtin. The nonisoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds, etc. The details of the chemistry of various compounds falling under these groups have already been reviewed [56,57].

Azadirachtin was first isolated based on its exceptional antifeedant activity in the desert locust, and this substance remains the most potent locust antifeedant discovered to date. Some entomologists concluded that neem had remarkable powers for controlling insects

that it would usher in a new era in safe, natural pesticides. Extracts from its extremely bitter seeds and leaves might be the ideal insecticides. They attack many pestiferous species; they seem to leave people, animals, and beneficial insects unharmed; they are biodegradable; and they appear unlikely to quickly lose their potency to a build up of genetic resistance in the pests. Neem seems likely to provide nontoxic and long-lived replacements for some of today's most suspect synthetic pesticides.

Childs et al. [59] reported that there were at least 12 brands of neem pesticides registered in India. Neem-based pesticides refer to those formulated pesticides containing azadirachtin as the major active compound. The most popular product currently seems to be 0.3% azadirachtin EC (emulsifiable concentrate) although some companies are producing products with up to 5% azadirachtin content [59].

Neem Seed Oil Components

The neem seed kernel is very rich in fatty acids often up to 50 percent of the kernel's weight. Neem seed oil is very bitter with a garlic/sulfur smell and contains vitamin E and other essential amino acids.Studies of the various components of the oil have been found the following fatty acids

- oleic acid - 52.8%
- stearic acid - 21.4%
- palmitic acid - 12.6%
- linoleic acid - 2.1%
- various lower fatty acids - 2.3%

The percentages vary from sample to sample depending on place and time of collection of the seeds. Neem oil is an excellent moisturizing oil that contains compounds with historical and scientific validity as medicinals. Use of the oil for cosmetics and medicines has been limited by its strongly bitter taste and sulfur/garlic smell.

Biologically active compounds from neem seed oil

Two types of insecticides can be obtained from seeds of the Indian neem tree, Azadirachta indica (Maliaceae) [60]. Neem oil, obtained by cold-pressing seeds, can be

effective against soft-bodied insects and mites but is also useful in management of phytopathogens. Apart from the physical effects of neem oil on pests and fungi, disulfides in the oil may likely to contribute to the bioactivity of the material. Neem seeds actually contain more than a dozen azadirachtin analogs, but the major form is azadirachtin. Seed extracts include considerable quantities of other triterpenoids, notably salanin, nimbin, and derivatives thereof. Besides azadirachtin, the role of all other biologically active compounds has also been in high esteem.

Neem seeds typically contain 0.2% to 0.6% azadirachtin by weight. From the crude, some tetranortriterpenes, including nimbin, nimbinin, nimbidinin, nimbolide and nimbidic acid have been isolated [61]. Sodium nimbidate, a soluble salt of nimbidin, obtained from neem seed showed significant dose dependent anti-inflammatory activity against acute paw oedema in rats and formalin-induced arthritis [62, 63].

Nimbidin, a component obtained from neem seed has shown antipyretic activity [64]. Oral administration of nimbidin demonstrated significant hypoglycaemic effect in fasting rabbits [65]. A significant antiulcer effect has been observed with nimbidin in preventing acetylsalicylic acid, indomethacin, stress or serotonin-induced gastric lesions as well as histamine or cysteamine-induced duodenal ulcers [66, 67]. Nimbidin has shown anti-gastric ulcer activity in gunea pig and rat [67]. It prevents the formation of ulcer and reduced the gastric acidity. The mechanism of action is attributed partially to the anti-peptic and anti-secretory activities. Pillai and Santhakumari [68] has reported that nimbidin isolated from the oil of the seed kernel suppressed the basal as well as the histamine and carbachol stimulated gastric acid output in rat. The spermicidal activity of nimbidin and nimbin (**1**) is reported in rats and human as early as 1959 [69, 70]. Nimbidin also shows antifungal activity by inhibiting the growth of *Tinea rubrum* [71]. In vitro, it can completely inhibit the growth of *Mycobacterium tuberculosis* and has been found to be bactericidal [71]. Diuretic activity is also reported for sodium nimbidinate in dogs [72].

Nimbolide (**2**) is reported to show antimalarial activity by inhibiting the growth of *Plasmodium* falciparum [73, 74]. Nimbolide also shows antibacterial activity against *S. aureus* and *S.* coagulase [75]. Gedunin (**3**), isolated from neem seed oil is reported to possess both antifungal [76] and antimalarial [74] activities. Azadirachtin (**4**), highly oxygenated C-secomeliacins isolated from neem seed and having strong antifeedant activity [77], has been

found to posses antimalarial property. It can inhibit to the development of malarial parasites [78]. Mahmoodin (**5**), a deoxygedunin isolated from seed oil, is reported to possess moderate antibacterial action against some strains of human pathogenic bacteria [57]. Table 1.3 shows the presence of some bioactive compounds from neem seed oil.

Table 1.3. Some bioactive compounds from neem seed oil

Neem compound	Biological activity	Reference
Nimbidin	Anti-inflammatory	62
	Antiarthritic	63
	Antipyretic	64
	Hypoglycaemic	65
	Antigastric ulcer	66, 67
	Spermicidal	70
	Antifungal	71
	Antibacterial	71
	Diuretic	72
Sodium nimbidate	Anti-inflammatory	62, 63
Nimbin (**1**)	Spermicidal	69
Nimbolide (**2**)	Antimalarial	73,74
	Antibacterial	75
Gedunin (**3**)	Antifungal	76
	Antimalarial	74
Azadirachtin (**4**)	Antimalarial	78
Mahmoodin (**5**)	Antibacterial	57

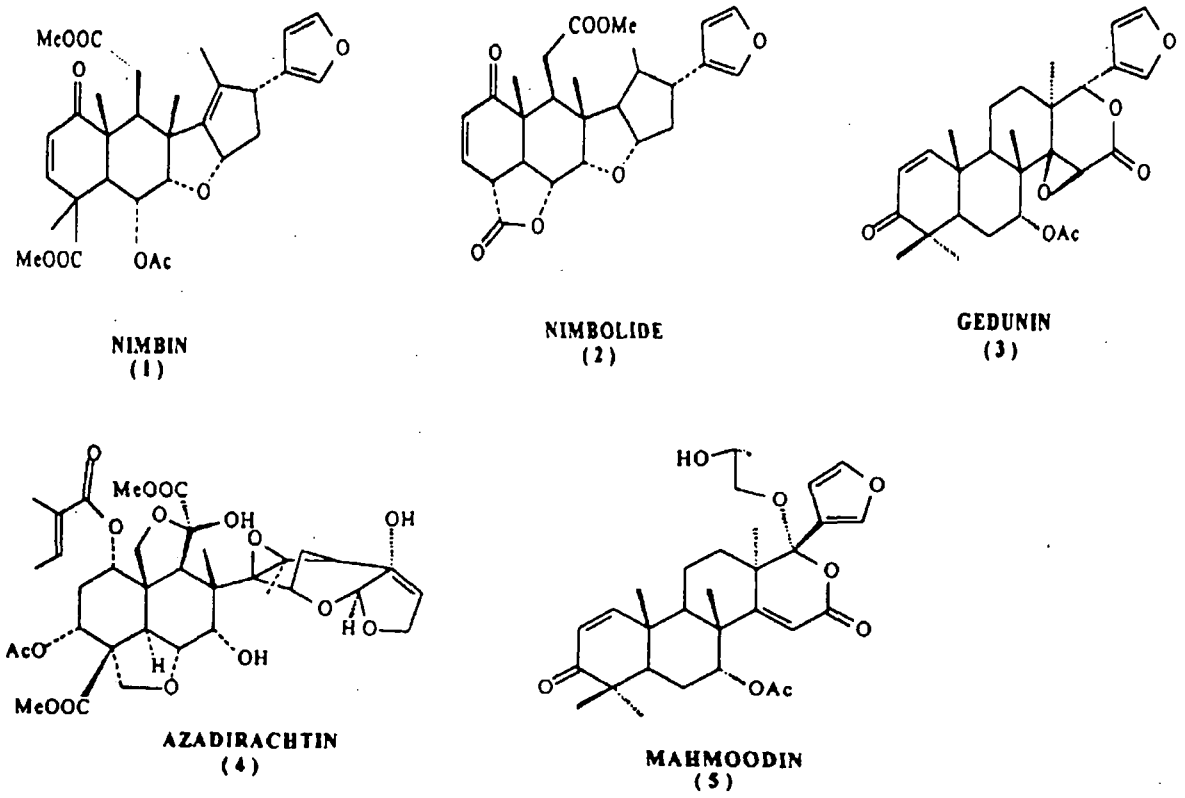


Figure 1.7. Major biologically active compounds present in neem seed oil

1.2.2.2. Isoniazid

4-Pyridinecarboxylic acid hydrazide is commonly known as isonicotinic acid hydrazide or isoniazid. Its *molecular formula is* $C_6H_7N_3O$. The structure of isoniazid is shown in Figure 1.8

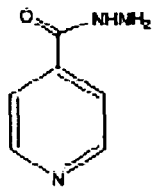


Figure 1.8. Structure of isoniazid

Chemical Properties, therapeutic index and toxicity

Isoniazid is not known to exhibit polymorphism or to form defined hydrates. Its synthesis results in orthorhombic crystals. Isoniazid is soluble to the extent of 125 mg/mL of

water at room temperature. Solubility in water varies as ~14% at 25°C, ~26% at 40°C; in ethanol: ~2% at 25°C, ~10% in boiling ethanol; in chloroform: ~0.1%. It is practically insoluble in ether and benzene. It has melting point 171.4°C. Isoniazid is one of the key active pharmaceutical ingredients (API) used in the combination treatment of tuberculosis (TB) recommended by the World Health Organization (WHO).

The WHO recommends a dosage range from 4 to 6 mg/kg, with the maximum daily dose not to exceed 300 mg. The 300 mg maximum daily dose is also used as preventive therapy for populations at high risk. At this dose, the antibiotic is generally well tolerated.

The most commonly occurring adverse effect in the treatment with isoniazid is hepatotoxicity. Serious toxic symptoms have been reported to occur at doses of 2–3 g or higher in adults. Doses of 10–15 g may be fatal without appropriate treatment [79].

Treatment of tuberculosis with antitubercular drugs

Tuberculosis is one of the various diseases that have afflicted the human race for centuries. While potentially curative treatments have been available for almost half a century, TB remains the leading cause of preventable deaths and hence continues to present a formidable challenge as a global health problem. Recent implementation of the World Health Organization's strategy (DOTS–directly observed therapy, short course) has been problematic, and TB remains a major burden in many developing countries. One of the major problems is non-compliance to prescribed regimens, primarily because effective chemotherapy of TB involves the daily administration of one or more drugs for a period of 6 months or longer. Clinical management of the disease is limited because of toxic side effects of drugs, degradation of drugs before reaching their target site, low permeability and poor patient compliance [80].

An important consideration in the treatment of TB is that the etiological agent, *Mycobacterium tuberculosis*, has the ability to persist intracellularly in the host macrophage for long periods of time. Optimum therapy, therefore, must depend upon the intracellular delivery of antimycobacterial agents for prolonged periods. This becomes even more important when one considers the ability of *M. tuberculosis* to persist in a dormant state, thus

giving rise to a large group of infected individuals who carry the organism in a subclinical state without having active disease [81].

1.2.3. Crosslinking agents

Crosslinking is the formation of chemical links between molecular chains to form a three dimensional network of connected molecules. The crosslinking is used to control and enhance the properties of the resulting polymer system or interface. Crosslinking of polymers improves the mechanical properties as well as control release behaviour. Depending on polymer and active agent properties, suitable crosslinkers are chosen for crosslinking.

A large number of crosslinking agents, both natural and synthetic, are known to crosslink different natural and synthetic polymers. Chemical cross-linking of chitosan by treatment with aldehydes such as formaldehyde, glutaraldehyde and glyceraldehydes is well known and is reported in most of the literature [82-85]. The cross-linking with dialdehydes occurred in mild conditions without any auxiliary additive. The main drawback of dialdehyde crosslinkers is that they are suspected to be toxic in human body [86]. Other chemical crosslinking agents include glyoxal [85,87], epichlorohydrin [83], sulfuric acid [82], sodium hexametaphosphate [85], sodium tripolyphosphate [88], diisocynate, carbodiimides, tannic acid etc. All these chemical crosslinking agents are relatively cytotoxic. Recently, biocompatible cross-linking agents have received much attention in the field of biomedical application. For example, enzyme-catalyzed cross-linking methods have been developed to cross-link protein-based biomaterials [89-91].

Transglutaminase and tyrosinase catalyze the glutamine and tyrosine residues in protein to link with the amino group of a lysine residue. However, it can not catalyze the cross-linking reaction of biopolymers lacking of glutamine and tyrosine residues. Genipin, a natural crosslinking agent, whose cytotoxicity, feasibility and biocompatibility have well been studied and reported [92]. Genipin can be obtained from its parent compound geniposide, which may be isolated from gardenia fruits. It has been reported that genipin can spontaneously react with amino acids or proteins to form dark blue pigments [93-95]. It is 10,000 times less toxic than glutaraldehyde [92].

The crosslinking of gelatin is used to extend the application of gelatins. The treatment of gelatin films with glutaraldehyde is receiving considerable importance because of their improvement in thermal resistance, mechanical properties and decrease in solubility in water. Two fundamental methods of crosslinking have been described for gelatin: physical and

chemical. Physical methods include UV irradiation and dehydrothermal treatment, although these are inefficient and make it difficult to control the crosslinking of the gelatin matrix [96].

Chemical crosslinking agents have been categorized into two types: non-zero length and zero length. Non-zero length crosslinkers are bifunctional and polyfunctional and operate by bridging free carboxylic acid residues or amine groups between adjacent protein molecules. Examples include aldehydes (i.e., formaldehyde, glutaraldehyde, glyceraldehydes), polyepoxides, and isocyanates [96], genipin [97].

Zero length crosslinking agents activate carboxylic acid residues to react directly with amine groups on adjacent protein chains. No intervening molecules are introduced between the crosslinked residues, so this process is able to achieve gelatin matrix crosslinking without integrating foreign molecules into the network. Crosslinking agents in this category include acylazides [96], water-soluble carbodiimides [98, 99].

Few generalized cross-linking reactions of gelatin, chitosan, carrageenan and sodium carboxymethyl cellulose, with different cross-linking agents are shown in Figure.1.9.

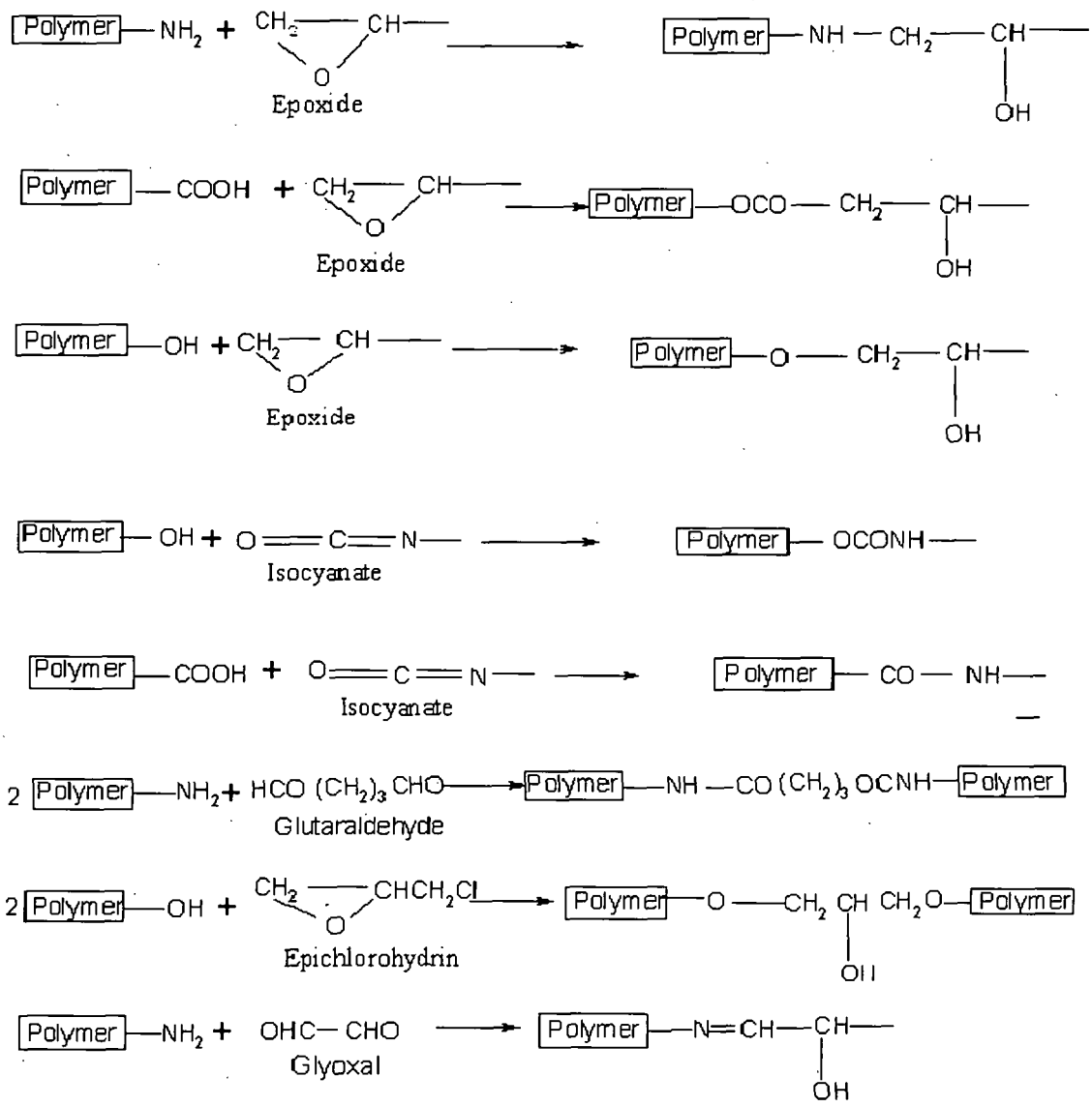


Figure 1.9. Cross-linking reactions of different functional group containing polymers (gelatin, carrageenan, chitosan, sodium carboxymethyl cellulose) with various cross-linkers.

In the present study, in order to improve the controlled release behaviour, the crosslinkers glutaraldehyde, tannic acid and genipin have been used for crosslinking of polymers.

1.3. Fabrication techniques of microparticles for controlled delivery formulations

The term 'microparticle' refers to a particle with a diameter of 1-1000 microns, irrespective of the interior structure [100]. Within the broad category of microparticles (Figure1.10), 'microspheres' specifically refer to spherical microparticles and 'microcapsules' applies to microparticles which have a core surrounded by a material which is distinctly different from that of the core. The core may be solid, liquid or even gas. A microparticle usually refers to a homogeneous mixture of the polymer and active agent, whereas microcapsules have at least one discrete domain of active agent.

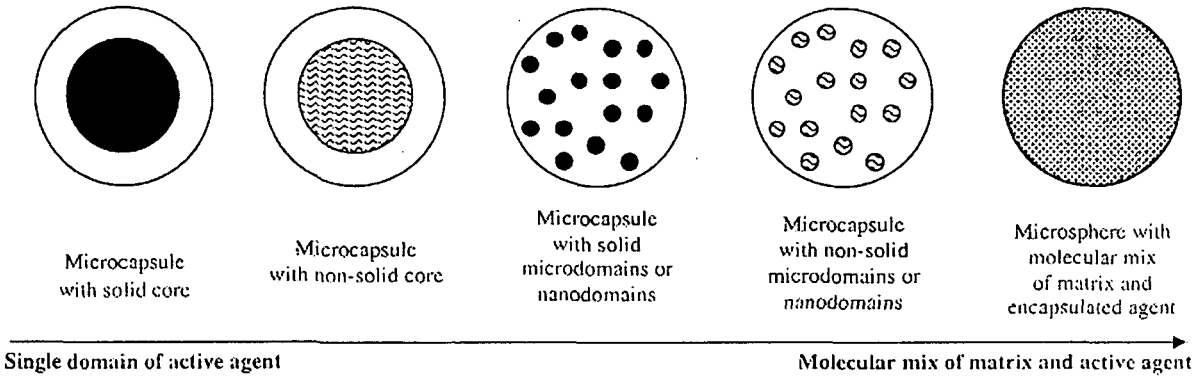


Figure 1.10. Different categories of microparticles [100]

1.3.1. Solvent evaporation and extraction based process

1.3.1.1. Phase separation or coacervation

IUPAC defined coacervation as: "The separation into two liquid phases in colloidal systems. The phase more concentrated in colloid component is the coacervate, and the other phase is the equilibrium solution." The first systematic approach of phase separation, that is, partial desolvation of a homogeneous polymer solution into a polymer-rich phase (coacervate) and the poor polymer phase (coacervation medium) was realized by Bungenberg and colleagues [101, 102]. These authors termed such a phase separation phenomenon "*coacervation*".

Currently, two methods for coacervation are available, namely simple and complex processes. The mechanism of microcapsule formation for both processes is identical, except for the way in which the phase separation is carried out. In simple coacervation a desolvation agent is added for phase separation, where as complex coacervation involves complexation between two oppositely charged polymers.

(a) Simple Coacervation

Aqueous solutions of water-soluble polymers are phase separated in aqueous media when sufficient salt is added to such solutions. This phenomenon is called simple coacervation. As long as phase separation produces a liquid polymer-rich phase, simple coacervation can be used to produce microcapsules [102]. Microcapsules with gelatin, or poly(vinyl alcohol) or different natural polymers shell have been produced in this manner.

(b) Complex coacervation

Complex coacervation occurs in aqueous media and is used to encapsulate water-immiscible liquids or water-insoluble solids [103]. Complex coacervation is carried out by mixing two oppositely charged polymers in a solvent (usually water); the process is shown schematically in Figure 1.11.

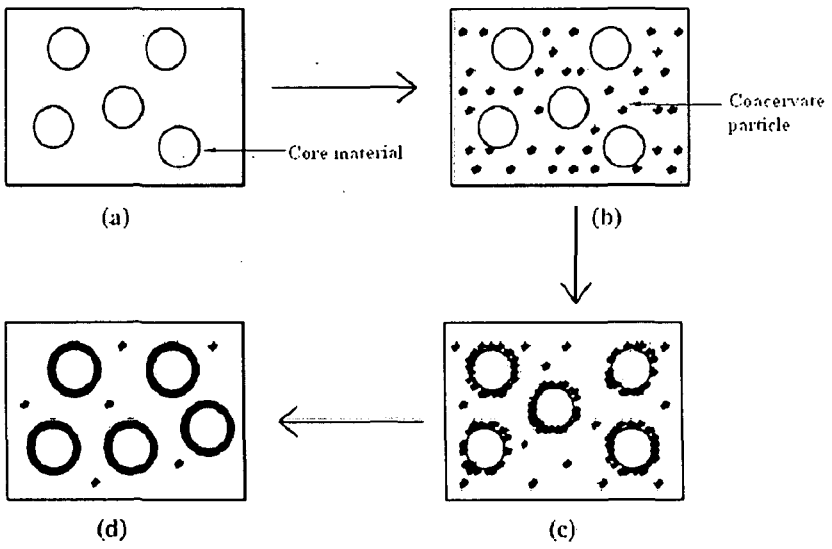


Figure 1.11. Schematic representation of complex coacervation process. (a) Core material dispersion in shell polymer solution; (b) separation of coacervate from solution; (c) coating of core material by microdroplet of coacervate; (d) coalescence of coacervate to form continuous shell around core particles.

The three basic steps in complex coacervation are: (i) preparation of the dispersion or emulsion; (ii) encapsulation of the core; and (iii) stabilization of the encapsulated particle. The core material (usually an oil) is first dispersed into a polymer solution (e.g., a cationic aqueous polymer). The second polymer (anionic, watersoluble) solution is then added to the prepared dispersion. Deposition of the shell material onto the core particles occurs when the two polymers form a complex. This process is triggered by the addition of salt or by changing the pH, temperature or by dilution of the medium. The shell thickness can be obtained as desired by controlled addition of the second polymer. Finally, the prepared microcapsules are stabilized by crosslinking, desolvation or thermal treatment. Complex coacervation is used to produce microcapsules containing fragrant oils, liquid crystals, flavors, dyes or inks as the core material. Porous microcapsules can also be prepared using this technique. When using this technique, certain conditions must be met to avoid agglomeration of the prepared capsules [104].

Figure 1.12 outlines one version of a complex coacervation encapsulation process. The first step is to disperse the core material in an aqueous gelatin solution. This is normally done at 40-60°C, a temperature range at which the gelatin forms a clear solution. After that, a polyanion or a negatively charged polymer like gum arabic added to the system, the pH and concentration of polymer are adjusted so that a liquid complex coaervate forms. The pH at which this occurs is typically between 4.0 and 4.5. Once the liquid coacervate forms, the system is cooled to room temperature. The gelatin in the coacervate gels thereby forming capsules with a very rubbery shell. In order to increase the strength of the water-swollen shell and create a gel structure that is not thermally reversible, the capsules normally are further cooled to approximately 10°C and treated with glutaraldehyde. The glutaraldehyde crosslinks the gelatin by reacting with amino groups located on the gelatin chain. Glutaraldehyde treated capsules can be dried to a free-flow powder or coated on a substrate and dried.

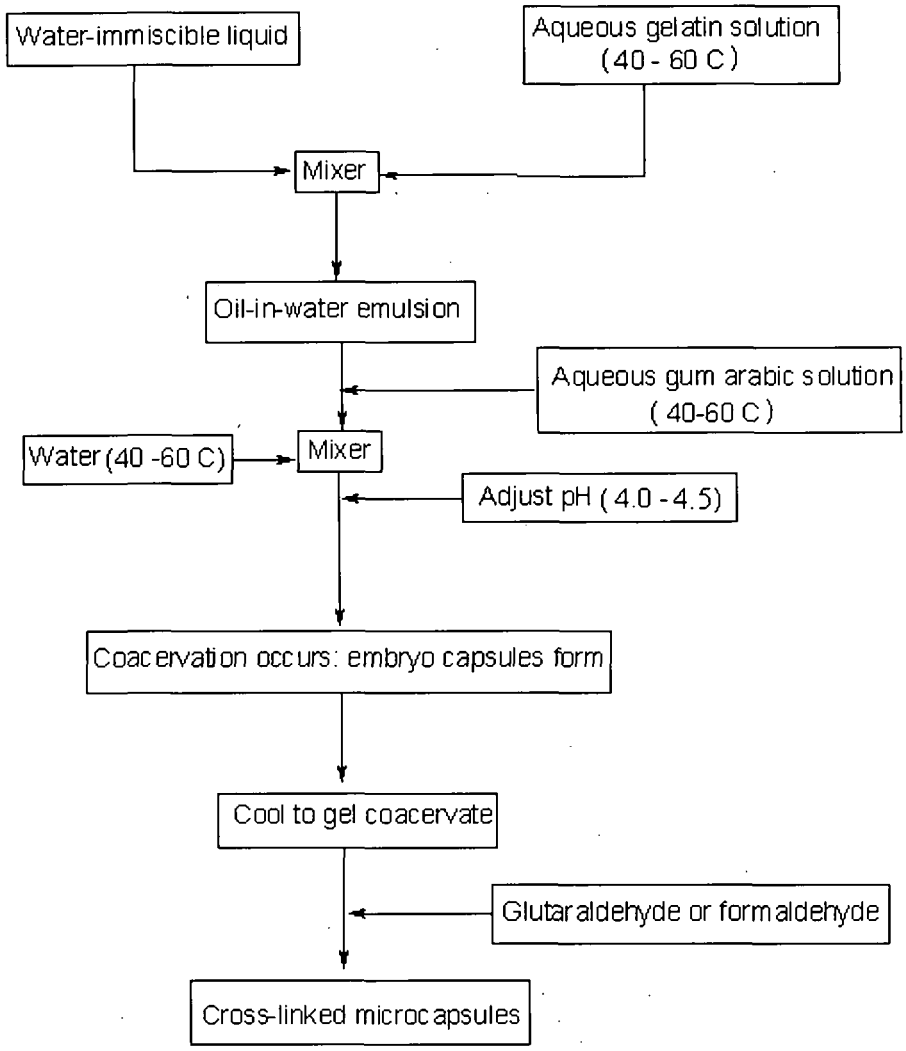


Figure 1.12. Typical encapsulation process based on the complex coacervation of gelatin with gum arabic.

1.3.2. Emulsion based process

1.3.2.1. Single emulsion process

This process involves oil-in-water (o/w) emulsification. The o/w emulsion system consists of an organic phase comprised of a volatile solvent with dissolved polymer and the drug to be encapsulated, emulsified in an aqueous phase containing a dissolved surfactant.

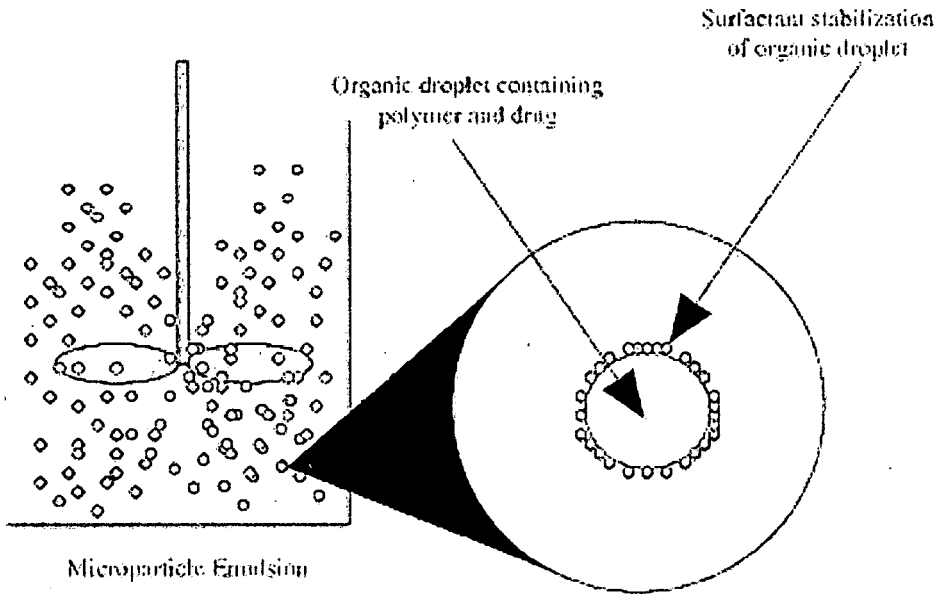


Figure 1.13. Encapsulation using oil-in-water emulsion technique [100]

A surfactant is included in the aqueous phase to prevent the organic droplets from coalescing once they are formed. The polymer-solvent-drug solution is emulsified (with appropriate stirring and temperature conditions) to yield an o/w emulsion. The emulsion is created by using a propeller or magnetic bar for mixing the organic and aqueous phases.

Once the emulsion is formed, it is subjected to solvent removal by either evaporation or extraction process to solidify the polymer droplets. In the case of solvent removal by evaporation, the emulsion is maintained at a reduced pressure or at atmospheric pressure and the stir rate is reduced to enable the volatile solvent to evaporate. The organic solvent leaches out of the droplet into the external aqueous phase before evaporating at the water air interface. In the case of extraction, the emulsion is transferred to a large quantity of water or other quench medium, into which the solvent associated with the oil droplets is diffused out. The rate of solvent removal by extraction depends on the temperature of quench medium, ratio of the emulsion volume to quench medium and the solubility characteristics of the polymer, the solvent and the dispersion medium. A high extraction will result in formation of particles with a high porosity that could lead to undesirable drug-release profiles [105, 106]. The solvent removal method by extraction is faster (generally <30 minutes) than the evaporation process and hence the microspheres made by this method are often more porous in comparison to those made by solvent evaporation method.

One of the disadvantages of the o/w emulsification process is the poor encapsulation efficiency with moderately water-soluble drugs. The drug diffuses out or partitions from the dispersed oil phase into the aqueous continuous phase and microcrystalline fragments of the hydrophilic drugs get deposited on to the microsphere surface [107] and dispersed in the polymer matrix. This results in poor trapping of the hydrophilic drug and initial rapid release of the drug (burst effect) [108]. The oil/water emulsification process is thus widely used to encapsulate lipid-soluble drugs. In order to increase the encapsulation efficiency of water soluble drugs, an oil-in-oil emulsion method was developed [109]. In this method, the drug may be dissolved or suspended in the oil phase before being dispersed in another oil phase.

1.3.2.2. Double emulsion process

The problem with encapsulating hydrophilic active agent is the loss of the active agent to the external aqueous phase during the formation of the microparticle. Along with the loss of active agent to the external phase, the remaining active agent may migrate to the surface of the droplet before solidifying. To minimize these problems, the organic droplets should be solidified into microparticles as quickly as possible following their formation [110]. This is achieved by using a viscous organic solution of polymer and active agent and a large secondary volume of water that attracts the organic solvent into the aqueous phase immediately, thus leaving the microparticle with the encapsulated active agent.The viscous dispersed phase minimizes the volume of organic solvent, facilitating its quick removal from the droplet and also makes it more difficult for the solid active agent particles/crystal to migrate to its surface, resulting in a more homogeneous distribution of the active agent within the particle. Another alternative to encapsulate hydrophilic active agent is to employ the water-in-oil-in water (w/o/w) emulsion process (Figure 1.14). An aqueous solution of the drug is added to an organic phase consisting of the polymer and organic solvent with vigorous stirring to form the first w/o emulsion. This emulsion is then dispersed in another aqueous phase containing more surfactant to form the w/o/w emulsion. The problem with this type of emulsion occurs when the inner emulsion is not sufficiently stabilized, resulting in loss of aqueous droplets containing drug to the external aqueous phase. The choice of surfactants that can be used to stabilize the inner emulsion is limited to materials that will dissolve in the organic solvent. Typically, the fatty acid esters of polyoxyethylene or sorbitan are used due to their high solubility in organic solvents and good biocompatibility.

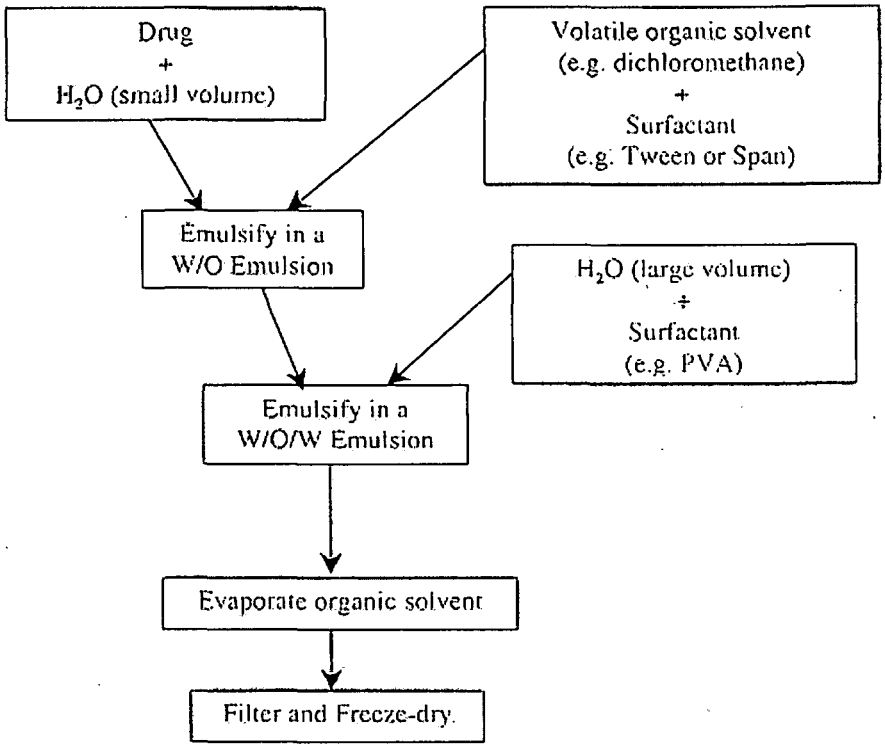


Figure 1.14. Schematic of w/o/w in-liquid drying process for microparticle preparation [100]

1.3.3. Other fabrication techniques of microencapsulation

Other processes of microencapsulation are also known which includes pan coating, sol-gel method, layer-by-layer (LBL) techniques, electrostatic encapsulation, etc. A steady stream of encapsulation technologies continues to appear in the patent literature. Some are simply modifications or improvements of the established technologies, where as others new technologies are confined to laboratory scale.

Thus, numerous preparation technologies available for the encapsulation of core material have been reported [111-114]. Some of the important processes used for microencapsulation are summarized in Table 1.4.

Table 1.4. Different techniques used for microencapsulation.

Chemical processes	Physical processes	
	Physico-chemical	Physico-mechanical
• Suspension, dispersion and emulsion polymerization • Polycondensation	• Coacervation • Layer-by-layer (L-B-L) assembly • Sol-gel encapsulation • Supercritical CO_2-assisted microencapsulation	• Spray-drying • Multiple nozzle spraying • Fluid-bed coating • Centrifugal techniques • Vacuum encapsulation • Electrostatic encapsulation

1.3.4. Matrix encapsulation technique

Controlled release products obtained by this technique lack a distinctive wall surrounding each particle of the active ingredient. The active agent is dispersed within a polymer and becomes entrapped within many small cells of a continuous matrix. The active ingredient may be dissolved or suspended in various polymers to yield ribbons, sheets or granules. Often an excipient is added to such formulations. Such excipients may be inorganic filler.

1.4.Classification of controlled delivery systems based on release mechanisms

The sought-after release profile from a controlled delivery system is the steady state release of active agent or a zero-order release mechanism kinetically. The attraction of such a system is that the rate of release is not affected by the amount of active agent released or not released at any moment [115]. However, many of the controlled delivery formulations do not fall under this category. Depending on the rate-controlling mechanism involved; the controlled delivery systems can be classified into several classes. According to Fan and Singh [116] the major release mechanisms involved in controlled delivery formulations are:

i) Diffusion

ii) Erosion or Chemical reaction controlled

iii) Swelling

iv) Osmosis

i) Diffusion-controlled systems

Here the rate-determining step is the diffusion of active agent through the polymer. The polymer-environment fluid interaction is practically nil, or polymer is seldom affected by the environment factors. Mainly two categories of diffusion-controlled devices are employed in controlled delivery formulations.

a) Reservoir systems

Here the active agent and polymer are physically combined or active agent is encapsulated in a spherical or cylindrical polymeric device. Micro-, macro- or nanoencapsulation coacervation and spray encapsulation are well-developed techniques and are employed in drug delivery systems [117]. Active agents release out to the environment by diffusion, through the micropores of the capsule walls. Ethylene vinyl acetate co-polymers, silicone rubber, polyethylene and polyurethane are commonly used to fabricate such systems [116].

b) Monolithic systems

Here the active agent is either dispersed heterogeneously or dissolved in the polymer. The polymer can be biodegradable or non-biodegradable. The dissolved or dispersed active agent releases out by diffusion. Release may take place by leaching in addition to diffusion, if there is interaction between polymer and the environment. If a soluble additive is incorporated in the polymer matrix, the environmental fluid can easily penetrate the matrix by dissolving the additive and interconnected channels will be formed, through which the release would be easy. These types of physical combinations need not be influenced by structure of the active agent or polymer. Hence this technique has a great applicability. A wide variety of active agents can be incorporated with a broad range of polymers to prepare controlled delivery formulations. Polymer matrix of silicone rubber, ethyl cellulose and hydroxyl propyl cellulose are widely used in this category [116].

ii) Erosion or Chemical reaction controlled system

Erosion-controlled system

The active agent is physically immobilized in the polymer matrix and release occurs only by erosion of the polymer. In an ideal system, polymer matrix undergoes surface erosion, releasing active agent at a rate proportional to the erosion rate. If erosion rate is

constant and the matrix dimension remains unchanged, a zero order release can be achieved. Poly (vinyl pyrrolidone) and coplymers of lactic and glycolic acids are used in preparing such systems [116].

Chemical reaction controlled system

From these chemical combinations, active agents are released only when the polymer active agent bond is cleaved, otherwise the polymer is to be degraded. When the active agent is a co-monomeric unit in polymer backbone, release may occur by polymer degradation and in such cases the release follow zero order kinetics.

iii) Swelling controlled delivery system

The active agent is dispersed or dissolved in a polymer matrix, in which it is unable to diffuse to any considerable extent. When the polymer gets into contact with an environment fluid which is compatible with it, swelling takes place and active agent present in the gel portion of the matrix diffuse out. Poly(hydroxyl methylmethacrylate), polyacrylamide and poly(ethylene glycols) are used in such systems [118].

iv) Osmosis controlled system

In this type of devices the driving force is the osmotic force. Usually such systems consist of a solid and water-soluble active agent, which is enclosed by a water-permeable, but active agent impermeable polymer membrane with a small opening. Water is transported into the core by permeation and hydrostatic pressure will be built up in the core and subsequently, the dissolved active agent comes out [116].

Apart from these common techniques, new concepts such as magnetic or ultra sound modulation, viable cell immobilization, microspheres and nanoparticles and targeted delivery are under thorough research in the controlled delivery field [116,119-120].

1.5. Advantages of controlled delivery technology

Controlled delivery technology offers several advantages [7, 8] over conventional formulations. These can be summarized as follows:

- Maintain of constant level of active agent: In conventional formulation, the active agent tends to release first at an overdose then undergoes to the local environment.

Controlled releases offer a solution for this problem by maintaining the concentration of active agent between the minimum effect and toxic level (Figure 1.1).

- Use of smaller dose: This includes more efficient utilization of active agents, resource saving, safety etc.
- Reduces the loss by limiting leaching, volatilization and degradation.
- Minimizes potential negative effect (if there any) associated with overdose.
- Economical because less active material is needed due to reduction of excessive amounts for a given time interval.
- Facilitation of handling and masking of any odour.
- Toxic material becomes chemically nontoxic when attached with polymers.
- Extention of the duration of the activity of less-persistent or non-persistent biocides unstable within the aquatic environment by protecting them from leaching and degradation, hence aiding the practical applications of these materials.
- Reduction of phytotoxicity by lowering the high mobility of the biocide in soil, hence reducing its residue in the food chain.
- Convenience: it converts liquids to solids; hence it results in easily transported materials with the reduction of flammability.

1.6. Disadvantages of controlled release technology

Though the advantages of controlled release are impressive, the merits of each application have to be examined individually, and the positive and negative effects weighed carefully before large expenditures for developmental work are committed. In other words, controlled release is not a panacea, and negative effects may, at times, more than offset advantages. Some of the disadvantages of controlled release or the areas that require a thorough appraisal include [5] .

- The cost of controlled release preparation and processing is substantially higher than the cost of standard formulations, but this could be compensated by minimizing the repeated applications.
- The fate of using excessive amounts of polymers as matrix and its effect on the environment is very important.This could be eliminated by using biodegradable

polymers and improving weight efficiency by using polymers that may be beneficial to the environment when degraded.

- The fate of polymer additives, such as plasticizers, stabilizers, antioxidants, fillers, etc. left behind, once application is over, may cause some impact on environment.
- The environmental impact of the polymer degradation products following heat, hydrolysis, oxidation, solar radiation and biological degradation. The cost, time, and probability of success in securing government registration of the product.

1.7. Applications of controlled delivery systems

Controlled delivery technology is used in a number of industries and for a number of types of products such as agricultural products (fertilizers, pesticides, soil nutrients, etc.), drugs and pharmaceuticals, fragrances and cosmetics. Controlled delivery formulations permit the application of a product containing a relatively concentrated amount of an ingredient which is referred to as the "active ingredient", which is then applied relatively slowly and over a relatively long period of time.

Controlled release drug delivery employs drug-encapsulating devices from which therapeutic agents may be released at controlled rates for long periods of time, ranging from days to months. Such systems offer numerous advantages over traditional methods of drug delivery, including tailoring of drug release rates, protection of fragile drugs and increased patient comfort and compliance. Polymeric microspheres find their applications as ideal vehicles for many controlled delivery applications due to their ability to encapsulate a variety of drugs.

Broad product areas in which controlled release applications have been made are shown in Table 1.5. However, the major effort in applying controlled release principles has been in the administration of drugs/ pharmaceuticals and the application of agrochemicals [6].

Table 1.5. Broad product areas in which controlled release has been applied

- Adhesives
- Antifouling agents
- Bacteria
- Blowing agents
- Catalysts
- Curing agents
- Detergents
- Drugs
- Dyes
- Flavours
- Foods
- Fuels
- Growth regulators
- Herbicides
- Insecticides
- Repellents
- Inks
- Metals
- Monomers
- Oils
- Perfumes
- Photographic agents
- Pigments
- Plasticizers
- Propellants
- Solvents
- Stabilizers
- Viruses
- Vitamins
- Paints

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CHAPTER II
LITERATURE REVIEW

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2.1. Natural pesticides

Four major types (pyrethrum, rotenone, neem and essential oils) along with three minor types (ryania, nicotine, and sabadilla) of botanical products used for pest control were reported in literature [1]. Isman [2] reported that pyrethrum accounted 80% of the global botanical insecticide market. Several plant derived compounds with pesticidal potential were discussed and reviewed in the literatures [3-7].

Shay et al. [8] studied the insecticidal and repellent properties of nine volatile constituents of essential oils against the American cockroach, *Periplaneta americana*. Alali et al. [9] studied the six compounds, representing the mono-tetrahydrofuran (THF) (gigantetrocin A, annomontacin), adjacent bis-THF (asimicin, parviflorin), and nonadjacent bis-THF (sylvaticin, bullatalicin) classes of annonaceous acetogenins, and compared them with technical grades of synthetic amidinohydrazone (hydramethylnon), carbamate (propoxur, bendiocarb), organophosphate (chlorpyrifos), and pyrethroid (cypermethrin) insecticides to determine their dietary toxicities to insecticide-resistant and insecticide-susceptible strains of the German cockroach, *Blattella germanica*.

Mugisha-Kamatenesi et al. [10] demonstrated that usage of botanical pesticides in field pest management was normal around Lake Victoria basin for the subsistence farmers. Mahfuz and Khanam reported [11] on the efficacy of seven different plant extracts viz. Acorus calamus rhizome, leaves of Datura fastuosa, Datura stramonium and seeds of D. stramonium, Corchorus capsularis, Aphanamixis polystachea and Jatropha curcas on Tribolium confusum adult.

Mishra et al. [12] isolated essential oils from leaves of Chenopodium ambrosioides, Cinnamomum zeylanicum, Citrus medica, Melaleuca lucadendron, Ocimum canum and O. grattissium. These oils demonstrated fungitoxicity against Aspergillus flavus at 200, 300, 400 and 500 ppm and most of them were shown to be more effective than synthetic fungicides

viz; Agrosan G.N., Copperoxychloride, Ceresan, Thiovit and Dithane M45. Asthana et al. [13] found the leaf extract of Ocimum adscendens to be fungitoxic against Aspergillus flavus. The volatile fungitoxic fraction was identified to be an essential oil, and was observed to be more active than some five synthetic fungicides tested.

Wang et al. [14] were able to isolate antifungal and larvicidal polyacetylenes for Artemisia borealis (B. campestris subsp. borealis). Dichloromethane extracts for the whole plant showed antifungal activity against Cladosporium cucumerinum.

Upadhyaya and Gupta [15] demonstrated the inhibitory effect of some medicinal plants on the growth of Curvularia lunata (Cochliobolus lunatus). Ethanol extracts of garlic followed by those of Ocimum santum, Datura alba and hemp were found to be most inhibitory to growth of the fungus. Aqueous extracts were less effective. Garlic extracts were shown to be inhibitory on the growth of a number of fungi [16].

From methanol extracts of twigs of Oxymitra velutina - a west african plant, 12 alkaloids; 5 aporphinoids including lysicamine, which is active against Bacillus subtilis, Botrytis cinerea, Saprolegnia asterophora and Rhizoctonia solani were isolated [17].

Yegen et al. [18] studied the fungitoxic effect of extracts of six selected plants from Turkey. Results indicated that aqueous and essential oils of Thymbra spicata, Satureja thymbra, Laura nobilis, Mentha spicata, Salvia futicasa and Inula viscosa were fungitoxic to Fusarium moniliforme, Rhizoctonia solani, Sclerotinia sclerotiorum and Phytophthora capsici. Kumar and Tripathi [19] screened leaf extracts of 18 plant species belonging to 11 families for their control of Pythium debaryanum, Fusarium oxysporum, R. solani and Sclerotium rolfsii.

2.1.1. Neem seed oil as pesticide

Pradhan et al. [20] reported the feeding deterrent property of neem seed kernel suspension against desert locust, *Schistocerca gregaria.* Subsequently, several bioactive ingredients were isolated from various parts of the tree, more notable being the isolation of meliantriol [21] and azadirachtin [22]. Both Meliantriol and Azadirachtin inhibited feeding of locust. In fact neem was recommended by most of the authors as a desirable method of pest

control and repellent as it did not cause harm to the people and the plant but selectively affected the insects [23].

Neem products affected different physiological processes in insects which were reported in literature [24]. Neem extract could influence over 200 species of insects. Many of which were resistant to modern pesticides. Active constituents like azadirachtin showed larvicidal and anti-feedant [25-27] activities.

It was reported that a spray of 1-percent neem oil in water "stopped 95 to 100 percent of the powdery mildew on hydrangeas, lilacs, and phlox." Locke and Larew demonstrated that neem oil could reduce damage caused by various pests, including spider mites. It was also reported that a 2-percent spray of neem seed oil applied directly to spider mite eggs resulted in an 87-percent mortality [28].

Neem oil provided effective control of rice plant hoppers like *Nilaparvatha lugens*, *Nephotettixs* spp. and *Sogatella furcifera*. A spray of 3% neem oil discouraged settling of hopper, *N. legens* on treated plants [29]. Similarly, 3% neem oil and 5% neem seed kernel extract were reported to control *Helicoverpa armigera* in bengal gram. Isman [30, 31] reported a significantly higher growth inhibition on grubs of rice seedlings while using the crude oils extracted from the seeds of neem, custard apple and china berry. An oil based neem formulation containing 300 ppm of Azadirachtin was used effectively against the desert locust, grasshoppers [32] and other lepidopteran pest [33]. Application of neem oil was also reported to reduce the incidence of plant viral diseases like yellow vein mosaic of okra, yellow mosaic of grain legumes, leaf curl of Chillies and Ragged-stunt virus of rice. Neem oil was reported to inhibit or reduce the transmission of Tungro virus and Tobacco mosaic virus [34].

The use of neem pesticides in cotton pest management was thoroughly reviewed by R.T. Gahukar [35]. Pesticides derived from neem (*Azadirachta indica A. Juss.*) controlled pests without having the nontarget toxicological effects associated with conventional pesticides [35]. Both azadirachtin and azadirachtin analogues were studied for antifeedant activity against the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) and azadirachtin was found to be the most active [36].

Neem oil (2%) inhibited normal growth and development of early third instar larvae of *S. litura* under laboratory conditions by affecting growth hormone systems [37]. Azadirachtin reduced pupation and adult eclosion in *S. litura* by 47% and 42% respectively [38, 39]. Several reports were found on using neem oil against mosquito and as larvicide [40-45].

Antifungal activity of neem oil for mycelia growth inhibition was studied and reported by Mohanty et al. [46]. Wanyika et al. [47] reported that pyrethrum-botanical oil (neem sedd oil, cotton seed oil and yellow oleander oils) blends against maize weevils was effective insecticide compared to pure pyrethrum based insecticide. Senthil-Nathan et al. [48] studied on the toxicity and physiological effects of neem pesticides applied to rice and found neem-based pesticides to be more effective to inhibit the growth and survival of N. lugens, the brown planthopper. When the synthetic insecticides chlorpyrifos (Termex®), cypermethrin+acetamiprid (Conquest®), and the natural insecticide neem (*Azadirachta indica* A. Juss) seed oil extract were used, the insecticides significantly reduced the populations of diamondback moth, *Plutella xylostella* L., and the cabbage head caterpillar, *Crocidolomia binotalis* Zeller [49]. Reports were also available on the advantages of neem oil based pesticides over other chemical pesticides [50].

2.2. Antitubercular drugs (Isoniazid and others)

Clinical studies of streptomycin, the first effective anti-TB agent, revealed the development of resistance to this agent. This problem was solved by the use of combination therapy with other agents discovered at around that time (notably, isoniazid and *para*-aminosalicylic acid). But some other problems were encountered. The major problem encountered was the patient adherence to a lengthy course of therapy associated with painful injections and toxic adverse effects [51]. Further, rifampicin, an anti-TB drug enabled the development of orally administered regimens that reduced the length of therapy from 18–24 months to a mere 6 months [52]. Thus, the most effective agents for the destruction of tubercle bacilli were isoniazid, pyrazinamide and rifampicin along with ethambutol [53].

Fluoroquinolones (ofloxacin, moxifloxacin, gatifloxacin and levofloxacin) are currently the only potential anti-TB agents that have entered advanced Phase II and III evaluation [54-58].

Isoniazid for latent tuberculosis treatment was studied by several authors [59-62] though toxic effects of isoniazid were also reported [63].

2.3. Polymers for controlled delivery formulations

2.3.1. Synthetic polymers

Several pesticides like sevin, dimethoate, ethyl trithion, methyl trithion, diazinon, malathion, chloropyriphos and temephos could be incorporated in plasticized poly(vinyl chloride) to obtain CR products [64].

El-Refaie and coworkers [65] prepared controlled release formulations based on crosslinked polyacrylamide derivatives. The release data of the herbicide 2,4-D in vitro from the formulations were described.

Micro-or macro encapsulation of active agents using polymers was one of the methods widely used for the preparation of CR products. Crosslinking of the polymer wall provided durable and storage stable capsules [66, 67]. Several controlled release pheromone formulations were also synthesized by microencapsulation.

Polymerizable derivatives of pesticides containing acid groups could be prepared by a reaction with alcohols having a vinyl group [68, 69]. Copolymers of vinyl 2,4-dichlorophenoxyacetate and trimethl amine methacrylamide were reported to be used for CR application [70]. Increased release of herbicide was obtained as the hydrophilic co-monomer content increased.

Kenawy and his group [71] prepared controlled release systems based on polyureas and poly(Schiff's bases). The effects of structure and temperature of the aqueous environment on the hydrolysis rate of the obtained polymer had been reported. Cheillini and

Akelah [72] synthesized polymeric herbicides containing 2,4-D and MCPA by modification of oligoethyleneoxylated styrene/divinylbenzene(DVB) resins. The release features for these systems were greatly affected by the pH.

Akelah et al. [73] reported chemical modifications of a series of polyamides containing hydroxyl groups with 2,4-D in the presence of dicyclohexylcarbodiimide(DCC) as a condensating agent to yield a series of polymer. They reported that the rates of release of 2,4-D from the formulations were mainly dependent on hydrophilicity, the pH and the temperature of the release medium.

Pesticides containing acid groups were converted to more reactive acid chlorides, which could react with polymers containing pendant hydroxyl or amino groups. Acylation of synthetic and natural polymers was possible in this manner [74-77]. Pentachlorophenol intercalated on mineral clay was reported by Akelah and Rehab [78]. The release of pentachlorophenol from the formulations was studied in different media at 30^0C and it was concluded that the release of pentachlorophenol from the formulations was dependent on the structure, swelling degree and the medium of release. Yan et al. [79] prepared crosslinked chitosan / poly (vinyl alcohol) blend with high mechanical strength.

Urea-formaldehyde microcapsules containing lemon oil were prepared by in situ interfacial polymerization. The particle size and their distribution under different experimental conditions were measured and reported [80]. Kulkarni et al. [81] reported the use of urea formaldehyde resin for the controlled release of diclofenac sodium. Bachtsi et al. [82] synthesized and studied the release behaviour of oil from oil containing poly(vinyl alcohol) microcapsules prepared by simple coacervation technique. Using of synthetic polymers for encapsulation was also found in several other reports [83, 84]. Starch urea formaldehyde matrix was used for encapsulation of agrochemicals [85]. The use of starch-g-poly (butyl acrylate) as a material for encapsulating carboxylic containing herbicides for controlled release were reported by Zhu et al. [86].

Reports were also available on synthesis of IPN beads of poly(vinylalcohol)-g-poly(acrylamide) with sodium alginate for the controlled release of cypermethrin pesticide [87].

Poly (DL-lactide-co-glycolide) (PLG) system was reported to be the most studied system for the delivery of isoniazid and all other anti-tubercular drugs (rifampin, pyrazinamide, and ethambutol) [88-90].Qurrat et al. [90] reported the development and chemotherapeutic potential of PLG microparticles containing rifampicin, isoniazid and pyrazinamide, which could be administered orally with sustained release of drug(s) to treat tuberculosis.

In a study, where similar biocompatible polymeric excipients of lactide and glycolide copolymers were used, improved formulations were evaluated individually and in combination with oral regimens of isoniazid for the treatment of *Mycobacterium tuberculosis* H37Rv-infected mice [91].

Pandey et al. demonstrated that poly(lactide-co-glycolide) (PLG) nanoparticles encapsulated with rifampin (RMP), isoniazid (INH), and pyrazinamide (PZA) provided sustained release of the antituberculosis drugs [92].Similar efficacy of the nanoparticle-bound drugs was also observed in guinea pigs [93]. At the same time, incorporation in microparticles was less effective and their drug loading capacity was lower [94, 95].

The behavior of polymeric nanoparticles in the gastrointestinal tract is influenced by their bioadhesive properties; adhesion of nanoparticles to the mucosa enhances the absorption of the associated drug, thus increasing its bioavailability. Thus lectins were found to improve mucoadhesion of the drug due to the biorecognition of the lectin-grafted carriers by glycosylated structures in the intestine [96].

Biodegradable PLGA microspheres for the delivery of novel subunit TB vaccine were prepared Kirby by et al. [97] by using modified double emulsion solvent evaporation method. They reported that liposomes were the more effective vaccine delivery systems compared to the microspheres.

Kim et al. [98] reported the synthesis of polyurethane microcapsules incorporated with isoniazid, through the interfacial polycondensation of tolylene 2,4-diisocyanate with different poly(ethylene glycols) in a water toluene emulsion.

Verma et al. [99] demonstrated the administration of dry powder inhalations (DPI) of microparticles containing anti-TB drugs to various laboratory animals, which could target drugs to macrophages while decreasing bioavailability to blood and blood-perfused organs. They suggested that polymeric microparticles prepared by spray drying process offered promises for treating pulmonary TB with reduced doses, lower dosing frequency and alleviated toxicity.

The effectiveness of pulmonary drug delivery employing nanoparticles was demonstrated in a number of studies [100]. The pharmacokinetics and antibacterial effect of the nanoparticle-bound antituberculosis drugs administered via respiratory route was investigated in guinea pigs [101]. Administration to infected guinea pigs of nebulized RMP, INH, and PZA coencapsulated in WGA-functionalized PLG nanoparticles was even more effective: three doses administered fortnightly for 45 days were sufficient to produce a sterilizing effect in lungs and spleen [102].

2.3.2. Natural polymers

Though the controlled delivery formulations regarding the synthetic polymers were extensive and encouraging, the recent trend shifted towards natural polymers [103, 104]. The utilization of starch as a polymer matrix for CR agrochemical was reported [105].

Pfister, Bahadir and Korte [106] used one system based on calcium alginate with a series of herbicides. Starch was used as an encapsulating material for S-ethyldipropylthiocarbamate (EPTC), atrazine and trifluralin [107-110]. Teft and Friend [111] synthesized controlled-release polymeric microspheres of herbicides Dicamba(DA) based on ethylcellulose , polyarylsulfone or a combination of the two.

Kulkarni et al. [112] reported the release kinetics and encapsulation efficiency of urea-formaldehyde (UF) crosslinked matrices of starch, guar gum (GG) and starch+guar gum (St+GG) for controlled release of solid (chloropyrifos) and liquid (neem seed oil) pesticides. In another report, Kulkarni and his group [113] claimed the synthesis of novel polymeric sodium alginate interpenetrating network (IPN) beads for the controlled release of chloropyrifos.

Marei et al. [114] compared carbofuran encapsulated controlled release formulation with the granular formulation in term of mobility of carbofuran and reported that leaching potential of alginate formulation decreased more than nine times compared with granular formulation. Chitosan gel beads and film were assessed for their ability to control the release of herbicide atrazine and fertilizer urea [115]. Elabahni et al. [116] developed a technique for encapsulation of herbicide inside ethyl cellulose microsphere and evaluated the shape and size of microspheres by scanning electron microscopy. Polysaccharides like cellulose, chitin, amylose and amylopectin were found to be useful natural polymers for the CR formulations of 2,4-dichlorophenoxyacetic acid and metribuzin [117]. CR formulation of kraft lignin and propachlor had been successfully prepared by Wilkins and Blackmore [118]. It was reported that rice husk lignin could be combined with 2,4- dichlorophenoxyacetic acid [70]. The application of lignin in CR formulations was reviewed by Wilkins [119].

A series of preformed polymers modified with pesticides were reported [70]. Chitin [120-122] as a naturally occurring polymer was used as carrier for herbicide metribuzin and the system showed slow release when polymer was directly attached to metribuzin.

Thimma and Tammishetti [123] studied the complex coacervation of gelatin with carboxymethyl guargum and applied it for microencapsulation of clove oil and sulphamethoxazole. Microcapsules containing fragnant oil were synthesized by in situ polymerization method and the microencapsulation efficiency and other physical properties were analyzed by Lee and his coworkers [124]. Chang et al. [125] studied the encapsulation of wheatgerm oil and evening primrose oil using sodium alginate by emulsification method. Rosenblat et al. [126] studied the effect of electrolytes, stirring rate and surfactant concentration on coacervation and microencapsulation process of gelatin. Sun et al. [127] prepared a series of gelatin microspheres by emulsification –coacervation method and studied the influence of preparation parameters like concentration of gelatin, emulsifier, emulsifying time, stirring speed etc. on particle size, surface morphology and dispersion of gelatin microspheres. Shu and Zhu [128] studied the interaction of chitosan with three kinds of anion (tripolyphosphate, citrate and sulphate) by turbidimetric titration and reported that the electrostatic interaction took place in a certain region of solutions. Chitosn microspheres were prepared using sodium sulphate as precipitant. The microspheres were loaded with drugs and the loading property was investigated by spectrophotometry. The loaded microsphere were characterized by SEM and DSC [129].

Microencapsulation of hexadecane in a vegetable protein by salting out method and the effect of different process parameters on microcapsules characteristics were investigated by Mauguet et al. [130]. Iwanga [131] and coworkers studied the release rate of insulin from gelatin microspheres with crosslinking densities. The release rate of insulin showed initially a burst effect followed by a slow release phase regardless of the crosslinking density.

Tomida et al. [132] suggested that κ-carrageenan-chitosan membrane spherical capsules could release theophylline as a model drug from the capsules. Polyelectrolyte complexation of chitosan and κ-carrageenan was studied by Tapia et al. [133]. They 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 The appearance of a new band at 1528.64 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 between the two polymers.

Piyakulawat and coworkers [134] observed and reported the appearance of irregular and rough surfaces while studying the SEM micrographs of glutaric acid crosslinked chitosan-carrageenan beads.

Fourier transform infrared (FTIR) spectroscopic study of acid soluble collagen and gelatin from skins and bones of young and adult Nile perch (Lates niloticus) was carried out by Muyona et al. [135]. Characterizations of fish gelatin films added with gellan and κ-carrageenan was studied by Pranoto et al. [136]. In the complex of gelatin and carrageenan, a slight shift of the peak of amide I was observed and reported [136]. The shifting of the slulphonic acid absorption band to higher wave number due to interaction between carrageenan and gelatin was reported by Li et al. [137] during studying of electrosynthesis of κ carrageenan – gelatin complex.

Liu et al. [138] studied on a novel pH- sentisitive gelatin-DNA semi-interpenetrating polymer network hydrogel. They studied the swelling of gelatin-DNA semi interpenetrating network at different pH. Agnihotri et al. [139] studied the swelling behavior and reported that

chitosan microspheres with higher crosslinking produced lower swelling. The microsphere wall became compact due to crosslinking and as result swelling decreased.

While studying the DSC and XRD analysis of carvedilol drug encapsulated within alginate microspheres Patil et al. [140] reported that drug was molecularly dispersed in the microspheres.

Kulkarni et al. [141] reported on the study of glutaraldehyde crosslinked sodium alginate beads containing liquid pesticide NSO for soil application. Moretti et al. [142] reported that glutaraldehyde crosslinked microcapsules of essential oils (NSO and other) had high loading efficiency and could be used for insect pest control.

Riyajan and Sakdapipanich [143] reported on the release of the neem seed oil pesticide encapsulated within natural rubber (NR) layer, glutaraldehyde–alginate gel capsules. The optimization of the properties of the neem containing beads was achieved by changing variables such as the extent of crosslinking, the amount of loading and NR layer. The SEM study indicated that the walls of the beads were smooth and nonporous. The swelling results indicated that swelling of the polymeric beads decreased with increasing exposure time to glutaraldehyde and reduced the rate of release of the pesticide.

Alginate–chitosan microspheres used for encapsulating three frontline anti-tuberculous drugs (ATDs), rifampicin, isoniazid and pyrazinamide, were reported [144]. It was reported that the drug encapsulation efficiency ranged from 65% to 85% with a loading of 220–280 mg of drug per gram microspheres. Administration of a single oral dose of the microspheres to guinea pigs resulted in sustained drug levels in the plasma for 7 days and in the organs for 9 days.

Deol et al. [145] reported the efficacies of isoniazid and rifampin encapsulated in lung-specific stealth liposomes which were evaluated by injecting liposomal drugs and free drugs into tuberculous mice twice a week for 6 weeks. Lung specific liposomes was developed and investigated in animal models of tuberculosis [146]. Sterilizing effect was achieved when the drugs were loaded in solid lipid nanoparticles (SLN) [147,148].

2.4. Crosslinking Agents

Kumbar et al. [149] studied the effect of various crosslinking agents on the release behaviour of diclofenac sodium encapsulated chitosan microspheres. The effect of crosslinking agent on the release of lactic acid from gelatin microsphere was studied by Dinarvand et al. [150]. Varieties of crosslinking agents like glutaraldehyde [151], formaldehyde [152], epoxy compounds [153] were reported to be employed for improving controlled release behaviour of controlled release polymers. Genipin, a natural crosslinker whose cytotoxicity, feasiblility and biocompatibility were studied and reported [154,155]. Sung et al. [154] reported that genipin was 10,000 times less toxic than glutaraldehyde. Genipin crosslinked alginate-chitosan microcapsules for live cell encapsulation was reported by Chen et al. [156]. In another study, Chen et al. [157] investigated the fluorogenic characteristics of chitosan-genipin reaction for microencapsulation purposes.

2.5. Objectives and plan of work

With the ever escalating world population, there will always be an increased need to boost agricultural production which claims that the need for pesticides is absolute and hence there exists an urgent necessity to improve the efficiency of product for longer time. This could be achieved by using and applying controlled release technology. Many of the controlled release formulations are highly efficient in sustaining the release of the biologically active components. It should be recognized that the polymeric material to be used in controlled release must degrade to some fashion before there can be any environmental impact in the chemical, biochemical or biological sense. If polymers for use in controlled release are completely inert or their degradation rates are measured in geologic time, the cumulative aspect will be a matter of concern. However it is encouraging to use naturally occurring polymers or degradable synthetic polymers.

The use of synthetic pesticides has undoubtedly resulted in increased crop production. However, these chemicals are hazardous both to man and the environment. The natural pesticides though freely available, the potential and efficiency of natural pesticides have not been properly utilized. Natural pesticides are ecofriendly, safe and lesser toxic compared to synthetic pesticides. In this context, neem seed oil which already had proved its potential as

effective natural pesticide draws special attention. But due to its liquid nature the application of it to the soil is limited. Moreover, this oil shows larvicidal and mosquito repellent activity. For utilization of this potent natural biopesticide to soil as well as in water it needs solid form which can be achieved by microencapsulation technique. Very few reports are available regarding the microencapsulation of neem seed oil. There is enough scope to do further work in this area.

Tuberculosis is one of the various diseases that have afflicted the human race for centuries. TB remains the leading cause of preventable deaths and hence continues to present a formidable challenge as a global health problem. One of the major problems is non-compliance to prescribed regimens, primarily because effective chemotherapy of TB involves the daily administration of one or more drugs for a period of 6 months or longer. Clinical management of the disease is limited because of toxic side effects and degradation of drugs before reaching their target site, low permeability and poor patient compliance. Thus, the drawbacks of conventional chemotherapy necessitate the development of a delivery or carrier system to release drugs slowly over extended time periods, which will also allow reduction in frequency and dosing numbers. This can be achieved by microencapsulation and controlled delivery technology developed by using natural polymers.

Keeping in view all the above backgrounds, the aim of the present work was set and had been undertaken. In the present research work controlled release polymeric systems for neem seed oil (NSO), a natural pesticide and isoniazid, an antitubercular drug, were developed and characterized. The polymeric materials used were naturally occurring polymers such as κ-carrageenan, chitosan, gelatin-A and sodium carboxymethyl cellulose. Microencapsulation by complex coacervation technique was used for the synthesis of controlled release formulations as it can help to tailor make the polymeric wall with specific desired properties. The polymeric wall is designed to permit controlled release of the encapsulated material under desired conditions. The release of the active agents can be controlled by crosslinking of the polymeric wall. Both synthetic and naturally occurring crosslinking agents will be used. Synthetic crosslinking agent used is glutaraldehyde and natural crosslinking agents used are tannic acid and genipin. Distilled water and sunflower oil (a vegetable oil) will be used as solvent and medium for the encapsulation process.

The plan of work has been divided into the followings-

- To optimize the ratio between the two polymers and pH for maximum yield.
- To develop and optimize the microencapsulation of active agents (NSO and Isoniazid) in crosslinked natural polymers for controlled delivery application.
- To study the effect of various parameters like concentration of active agents, polymer and crosslinking agent on percent encapsulation, active agent content and release rate.
- To characterize the encapsulated product by FTIR spectroscopy, differential scanning calorimetry (DSC), thermogravimetry (TGA), scanning electron microscopy (SEM), transmission electron microscopy (TEM) and X-ray diffractometry (XRD).

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CHAPTER III
EXPERIMENTAL

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EXPERIMENTAL

This chapter covers the materials and methods, which includes the raw materials used, techniques for sample preparation, release studies and characterization of the products.

3.1. Materials Used

The chemicals used in the present study and the manufacturers are listed below:

Materials	Suppliers
(i) Carrageenan (predominantly κ)	Sigma-Aldrich Inc., USA
(ii)Chitosan (Medium molecular weight)	Sigma-Aldrich Inc., USA
(iii) Gelatin Type-A	Sigma-Aldrich Inc., USA
(iv) Sodium carboxy methyl cellulose (medium molecular weight)	Rankem, India
(v) Neem Seed Oil (cold pressed)	Ozone biotech., Faridabad, India
(vi) Tween 80	E-Merck, India
(vii) Glacial Acetic acid	E-Merck , India
(viii) Isoniazid	Sigma-Aldrich Inc., USA
(ix) Hydrochloric acid (35%)	E-Merck, India
(x) Sodium Hydroxide (pellets)	E-Merck, India
(xi) Tannic acid	E-Merck, India
(xii) Genipin (Mw=226.23)	Challenge Bioproducts Co., Taiwan
(xiii) Glutaraldehyde 25%(w/v)	E-Merck, Germany
(xiv) Disodium hydrogen phosphate	E-Merck, India
(xv) Pottasium dihydrogen phosphate	E-Merck, India

Other reagents used for the study were of analytical grade.

3.2. Methods

3.2.1. Complex coacervation

Complex coacervation is a process where the phase separation is induced by the interaction of two oppositely charged macromolecules. It can be experimentally monitored by measuring the coacervate yield, relative viscosity and turbidity. Complex coacervation depends on several factors like ratio of one polymer to other, pH [1], temperature,etc.

Measurement of turbidity, viscosity and coacervate yield

In order to optimize the ratio of two oppositely charged polymers and pH for maximum complexation, the measurements of turbidity, viscosity and coacervate yield (%) are essential. The mixing of the polymers in different ratios (or pH) would produce solutions of different turbidity. The optimal ratio at which complete phase separation occurred between the polymers was the point where the solution would have the maximum turbidity and the supernatant would have the minimum 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 30^0C. Polymer in the supernatant solution would be either negligible or absent when the interaction between the two oppositely charged polymers 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 two oppositely charged polymers in different ratios (or pH) was measured gravimetrically. The coacervates remained after decantation of supernatants were washed with distilled water and then dried at 40^0C till the attainment of constant weight.

3.2.1.1. Complex coacervation of κ-carrageenan and chitosan

(a) Optimisation for pH

Solutions of chitosan (0.5% w/v) and κ-carrageenan (0.5% w/v) were prepared in 0.3% acetic acid/sodium acetate buffer at two different pH namely 4 and 5. This range

(4.0-5.0) was below the pH at which precipitation of chitosan occurred. The stability of carrageenan would also not be much affected at this pH. Both solutions were mixed in different proportions to make 30 ml. The mixtures were incubated at 40 ^{0}C for 24 hours. It was then centrifuged at 2500 rpm for 1 hr. The supernatant solution was separated, viscosity and turbidity were measured. The supernatant solution did not exhibit any significant difference in both viscosity and turbidity at pH 4 and 5.This indicated that the interaction between chitosan and carrageenan would remain similar within the pH range 4.0-5.0. The determination of optimum ratio between chitosan and carrageenan was, therefore, done at pH 4.5. This pH was maintained for preparing of chitosan and carrageenan solution used for subsequent experiments.

(b) Optimisation of chitosan and κ-carrageenan ratio

Turbidity measurements were done to confirm the optimum ratio between chitosan and carrageenan to form an insoluble polyelectrolyte complex. Solutions of pure chitosan (0.5% w/v), carrageenan (0.5% w/v) and the supernatants from the mixture of both at different ratios were scanned in the range 200-400 nm employing UV spectrophotometer. Both the solution of pure chitosan and carrageenan showed no peak within the scanned range. But the supernatant of the mixture of chitosan and carrageenan showed a peak at 341 nm due to the development of turbidity caused by the interaction between the polymers. Therefore, all the turbidity measurements reported were done at 341 nm.

(c)Microencapsulation of NSO in κ-carrageenan-chitosan complex

In order to optimize the encapsulation process, a series of experiments were conducted by varying the parameters like temperature during formation and hardening of microcapsules, time and temperature for completion of cross linking reaction. The optimized process are described as follows –

In a beaker, known amount of (100ml) 0.3-0.85% (w/v) of κ-carrageenan solution was taken. This polymer solution was stirred by mechanical stirrer under high agitation at 70±1^{0}C. This temperature was maintained throughout the experiment. To this, neem seed oil (0.68-2.04 g) was added under high agitation to form an emulsion. A known amount of (36 ml) chitosan solution of 0.3-0.85% (w/v) was added to the beaker drop wise to attain complete phase separation. However, the weight ratio of κ-carrageenan to chitosan was maintained at 1: 0.36 during all the experiments to get maximum yield because at this ratio, interaction between chitosan

and carrageenan took place completely as judged by the viscosity and turbidity measurements. The beaker containing the microcapsules was left to rest at this temperature for approximately 15 minutes. The system was then brought to 5-10^{0}C to harden the microcapsules. The cross linking of the polymer capsule was achieved by slow addition of certain amount of glutaraldehyde/tannic acid/genipin. The temperature of the beaker was then raised to 45°C and stirring was continued for another 3-4 hrs to complete the crosslinking reaction. The beaker was then cooled to room temperature. The microcapsules were filtered through 300-mesh nylon cloth, washed with 0.1% Tween 80 surfactant solution to remove any oil adhered to the surface of microcapsules followed by distilled water, dried and stored inside a refrigerator in a glass bottle.

(d) Encapsulation of isoniazid in κ-carrageenan-chitosan complex

In a beaker, known amount of (100ml) 0.35% (w/v) of carrageenan solution was taken. This polymer solution was stirred by mechanical stirrer under high agitation at 70±1^{0}C. In a separate beaker tween 80 (0.1g-0.3g) was dissolved in minimum amount of distilled water by heating. It was then mixed with a stipulated amount of NSO (1g) by stirring. This Tween 80 added NSO was mixed with carrageenan solution at 60^{0}C(approx.) under continuous stirring. This mixture was then sonicated for 90 minutes(approx.). After completion of sonication, this was stirred by mechanical stirrer under high agitation at 70±1^{0}C.This temperature was maintained throughout the experiment. Chitosan solution of 0.35%(w/v) was added dropwise to this by a syringe to attain complete phase separation. The beaker, containing the nanocapsules was left to rest at this temperature for 15 minutes. The system was then brought to 5-10^{0}C to harden the microcapsules. The pH of the system was then brought to 7.0 by addition of sodium hydroxide solution as this pH favoured maximum crosslinking with genipin. Now, the cross linking of the polymer nanocapsules were achieved by 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 hrs 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 free from alkali.

Wet crosslinked nanocapsules were directly transferred to a beaker containing sufficient amount of acetone and left for 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

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, washed with water to remove any isoniazid adhered to its surface. Finally, it was dried in a vacuum oven at room temperature (30 ± 1^0C).

3.2.1.2. Complex coacervation of κ-carrageenan and gelatin-A

(a) Optimisation of κ-carrageenan and gelatin-A ratio

Solutions of gelatin (0.5% w/v) and carrageenan (0.5% w/v) were prepared in acetic acid/sodium acetate buffer at two different pH namely 3.5 and 4. Both solutions were mixed in different proportions to make 45 ml. The mixtures were incubated at 40 ^{0}C for 24 hours.

20 ml of 0.5% carrageenan solution was taken in a beaker and titration was carried out by adding gelatin solution of 0.5% dropwise. The change in absorbance due to turbidity was monitored continuously at a particular wavelength using UV spectrophotometer at 490nm. Solutions of both the polymers show a very low turbidity and hence low absorbance at 490nm and this makes possible to study the phase behaviour in mixed gelatin-carrageenan solutions.

The mixture solutions prepared at different ratios of the polymers were kept in beakers for 12 hours and the supernatants were decanted separately. The viscosity of the supernatant solution and % coacervate yield were measured.

(b) Optimisation for pH

For monitoring the effect of variation of pH, κ-carrageenan (1%) and gelatin-A (1%) solutions were prepared in distilled water. 10ml of the carrageenan solution was mixed with 20 ml of the gelatin solution so that the weight ratio between the two polymers remained (carrageenan:gelatin A) as 1:2. This ratio was obtained from the optimization study between gelatin-A and κ-carrageenan. The same mixture of gelatin-A and κ-carrageenan was taken in several beakers separately and the pH of the mixture solutions of different beakers was kept

different by adding 2.5% glacial acetic acid solution. The amount of precipitate and turbidity in different beakers would be different depending on the pH of the solutions. The appearance of turbidity was monitored by using UV spectrophotometer at wavelength of 490nm. The coacervate % yield was measured gravimetrically.

(c) Microencapsulation of NSO in the complex

In a beaker, known amount of (100ml) 0.5-1.5% (w/v) of κ-carrageenan solution was taken. This polymer solution was stirred by mechanical stirrer under high agitation at 70±1^{0}C. This temperature was maintained throughout the experiment. To this, neem seed oil (1-4g) was added under high agitation to form an emulsion. A known amount of (200ml) 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 as judged by the coacervate % yield and viscosity measurements. The pH of the mixture was then brought down to 3.5 by adding 2.5% (v/v) of the glacial acetic acid solution. At that pH the yield was maximum as confirmed by % yield and turbidity measurements. The beaker containing the microcapsules was left to rest at this temperature for approximately 15 minutes. The system was then brought to 5-10^{0}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%). The temperature of the beaker was then raised to 45°C and stirring was continued for another 3-4 hrs 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% 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.

(d) Microencapsulation of isoniazid in carrageenan-gelatin complex

In a beaker, known amount of (350 ml) sunflower oil was taken. Under stirring condition, (25-50) ml of κ-carrageenan solution (2%(w/v)) at temperature 60±1^{0}C was added to the beaker containing sunflower oil at the same temperature to form an emulsion. (1-3) g of the tween 80 dissolved in 10 ml of water was added to the beaker to stabilize the emulsion.

A known amount of (25-50ml) gelatin A solution of 4% (w/v) was added slowly to the beaker. The optimum ratio of carrageenan to gelatin and pH at which complete phase separation 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 for approximately 15 minutes. The system was then brought to 5-10^{0}C to harden the microspheres. The cross linking 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 hrs 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 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 min). The isoniazid filled microspheres were 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 bottle in a refrigerator.

3.2.1.3. Complex coacervation of Gelatin-A and SCMC

(a) Optimization of ratio between the gelatin-A and SCMC

The ratio between the gelatin and SCMC was optimized by measuring the coacervate yield (%), turbidity and viscosity of the supernatant. Solutions of SCMC (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 45ml. The mixtures were incubated at 40^{0}C for 24 hours, and turbidity was measured. The supernatant solution was separated. Coacervate yield(%) of the precipitate and viscosity of the supernatant were measured.

(b) Optimization for pH

To optimize the pH, solutions of gelatin (0.5% w/v) and SCMC (0.5% w/v) were prepared in DDI. Both the solutions were mixed in the ratio of 2.33:1.0 (obtained from

optimization study). The pH of the mixing solution was varied from 2.0-5.5 by using 2.5% glacial acetic acid. Maximum coacervate yield (%) was measured.

(b) Microencapsulation of NSO in gelatin A-SCMC complex

In a beaker, a known amount of (100ml) 0.5-4.0% (w/v) of gelatin A solution was taken. This polymer solution was stirred by mechanical stirrer under high agitation at 45±1^{0}C.To this, NSO (1.5-7.0g) was added under high agitation to form an emulsion. A known amount of (100ml) SCMC solution of 0.214-1.714% (w/v) was added to the beaker drop wise. However, the percentage of SCMC was maintained 30% of the total polymer during all the experiments. At this percentage, and pH of 3.5, interaction between gelatin and SCMC took place maximum as judged by 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. The beaker containing the microcapsules was left to rest at this temperature for approximately 15 minutes. The system was then brought to 5-10^{0}C to harden the microcapsules. The cross linking of the polymer capsule was achieved by slow addition of certain amount of glutaradehyde solution. The temperature of the beaker was then raised to 45°C and stirring was continued for another 3-4 hrs 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% 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.

(c) Microencapsulation of isoniazid in gelatin A-SCMC complex

To a beaker containing (150-350 ml) of sunflower oil, (15-50) ml of gelatin-A solution (2-4%w/v) was added under stirring condition (200 – 1500 rpm approx) at 60±1^{0}C to form an emulsion. (0-1) g of the Tween 80, dissolved in 10 ml of water was added to the beaker to stabilize the emulsion. (15-50ml) of SCMC solution of concentration 0.857-1.714% (w/v) was added to the beaker drop wise to attain complete phase separation. However, the weight ratio of SCMC to gelatin was maintained at 1:2.33 during all the experiments. The pH of the mixture was then brought down to 3.5 by adding 2.5% (v/v) of the glacial acetic acid solution. The beaker containing the microparticles was left to rest at this temperature for approximately

15 minutes. The system was then cooled down slowly for 30 mins to bring down the temperature to 5-10^{0}C by replacing the hot water from water bath with ice cluster. This was done to harden the microparticles. The cross linking of the polymer microparticles was achieved by slow addition of certain amount of glutaraldehyde (4.375-17.50 mmol/g of polymer) solution. The temperature of the beaker was then allowed to rise to room temperature and stirring was continued for another 10-11 h to complete the crosslinking reaction. The microparticles were filtered through 300-mesh nylon cloth, washed with acetone to remove oil, if any, adhered to the surface of microparticles. This was further washed with distilled water, and freeze-dried. The dried microparticles were then dipped in aqueous isoniazid solution (0.5%-20%, w/v) for different times (0.5 – 48 h), filtered through 300-mesh nylon cloth, and quickly washed with water to remove the surface adhered isoniazid. The isoniazid-encapsulated microparticles were again freeze-dried and stored in a glass bottle in refrigerator.

3.3. Preparation of calibration curve

3.3.1. Calibration curve of NSO

A calibration curve is required for the determination of release rate of oil from the microcapsules. It was found that 0.1 gm 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% 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 /100ml, a prominent peak at 254 nm was noticed. The absorbance values at 254nm 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.

3.3.2. Calibration curve of isoniazid

A calibration curve is required for the determination of release rate of isoniazid from the microparticles. A known concentration of isoniazid in DDI water was scanned in the range of 200-500 nm using UV visible spectrophotometer. For isoniazid having concentration in the range 0.001 to 0.01 gm/100ml, a prominent peak at 261 nm was noticed. The

absorbance values at 261nm 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.

3.4. Determination of % oil loading, oil content, encapsulation efficiency and isoniazid loading efficiency

3.4.1. Oil loading (%), oil content (%) and encapsulation efficiency (%)

A known amount of accurately weighed NSO 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 [2].

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

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

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

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

3.4.2. Isoniazid loading efficiency

A known amount of accurately weighed isoniazid loaded microparticles was grounded in a mortar, transferred with precaution to a volumetric flask containing 100ml of water (having pH=7.4, maintained by phosphate buffer solution) and kept for overnight with continuous stirring to dissolve the isoniazid in the microparticles. The solution was collected and the isoniazid inside the microparticles was determined employing UV spectrophotometer. The loading efficiency (%), was calculated by using the calibration curve and the following formulae

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

where

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

w_2 = weight of microparticles

3.5. Release characteristics

3.5.1. Release characteristics of NSO loaded microcapsules

Oil release studies of encapsulated oil were done by using UV–visible spectrophotometer (UV-2001 Hitachi). A known quantity of microcapsules was taken into a known volume of 0.1% 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.

3.5.2. Release characteristics of isoniazid loaded micro/nano capsules

Isoniazid release studies from the isoniazid-encapsulated microparticles were carried out by using UV–visible spectrophotometer (UV-2001 Hitachi). A known quantity of microparticles was taken into a known volume of water having different pH (pH=1.2 and 7.4). This pH was maintained by using HCl and phosphate buffer solution. The content was shaken from time to time and the temperature maintained throughout was 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.

3.6. Water uptake or swelling studies

The swelling behavior of NSO free polymer complex was carried out in DDI water. Known amount (w_0) of the NSO free crosslinked products 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_t).

The swelling behavior of isoniazid free microparticles were studied in two systems at pH=1.2 (0.1N HCl) and pH=7.4 (phosphate buffer).The microspheres/microparticles were immersed in either 0.1 N HCl at pH 1.2 or phosphate buffer at pH 7.4. The diameters or the weights of swollen microparticles were determined after a stipulated time period.

The changes of the diameter of the microparticles were determined using a microscope with a micrometer. The swelling ratio for each sample determined at time t was calculated using the following equation [3,4].

$$S_w = D_t / D_0$$

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 % water uptake for each sample determined at time 't' was caculated using the following equation [5].

$$\text{Water uptake (\%)} = [\ (W_t - W_0) / W_0\ ] \times 100$$

where W_t is the weight of the microparticles (or polymer complex) after allowing to swell for a time (t), and W_0 is the initial weight of the microparticles (or polymer complex) before swelling. The experiments were performed in triplicate and represented as a mean value.

3.7. Particle size analysis

Particle size measurements were carried out using 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 measurement of particle size. The particle size analyzer measured and reported the mean size of the nanocapsules.

3.8. Transmission electron microscopy (TEM) 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.

3.9. Scanning electron microscopy (SEM) study

The samples were deposited on a brass/copper holder and sputtered with gold (or platinum). Surface characteristics and size of the microcapsules / microspheres were studied using scanning electron microscope (model JEOL, JSM-6360 or JSM-6390) at an accelerated voltage of 5-20 kv and at room temperature.

3.10. Fourier transform infrared (FTIR) spectroscopy study

FTIR spectra were recorded using KBr pellet in a Nicholet (model Impact-410) spectrophotometer. Microcapsules/microspheres/microparticles or nanocapsules, physical mixtures of polymer with active component, active component alone and polyelectrolyte complex of the polymers were finely grounded with KBr and FTIR spectra were recorded in the range of 4000-400cm^{-1}.

3.11. Thermal properties study

Thermal properties of active agent containing microcapsules/microparticles, microcapsules/microparticles without active agent, active agent alone, individual polymers and polyelectrolyte complexes were evaluated by employing thermogravimetric analyzer (TGA) and differential scanning calorimeter (DSC). TGA study was carried out using thermogravimetric analyzer (model TA 50, shimadzu) at a heating rate of 10^0C/min up to 600°C. DSC study was done in a differential scanning calorimeter (model DSC-60, shimadzu) at a heating rate of 10^0C/min up to 400°C. Both the study was done under nitrogen atmosphere.

3.12. X-ray diffraction (XRD) study

X-ray diffractograms of active agent, active agent loaded microcapsules/microparticles with or without crosslinker were recorded on an X-ray diffractometer (Model MiniFlex, Rigaku corporation, Japan). The samples were scanned between $2\theta = 10^0$ to 50^0 at the scan rate of 4^0 /min.

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CHAPTER IV
RESULTS AND DISCUSSION

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4.1. Microencapsulation of Neem Seed Oil (NSO), a potent natural pesticide, in three different polyelectrolyte complex systems, namely (i) chitosan- κ-carrageenan (ii) κ-carrageenan-gelatin A (iii) gelatin A-sodium carboxy methyl cellulose (SCMC).

In this part of work, the author has chosen complex coacervation (oil in water) technique for the microencapsulation of neem seed oil (NSO). Optimisations of polyelectrolyte complexations of three different systems as well as microencapsulation of NSO in those systems were studied. The effects of various parameters like oil loading, polymer concentration, crosslinker type and concentration on the microcapsule properties have been explained.

4.1.1. Chitosan- κ-carrageenan system for microencapsulation of NSO

4.1.1.1. Optimization of the system by viscosity and turbidity measurement

Figure 4.1.1.1(a) shows the change in supernatant viscosity with variation in percentage of chitosan in chitosan–carrageenan mixture. Each value represents the mean of three values. Viscosity was found to decrease initially, reaching a minimum value, and after that it increased with the increase in the percentage of chitosan. The minimum viscosity observed when the percentage of chitosan in the mixture was in between 30-40. Similar type of observation was reported by Tapia et al. [1]. At this percentage of chitosan (36%), both the polymers probably reacted completely 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 chitosan in the supernatant.

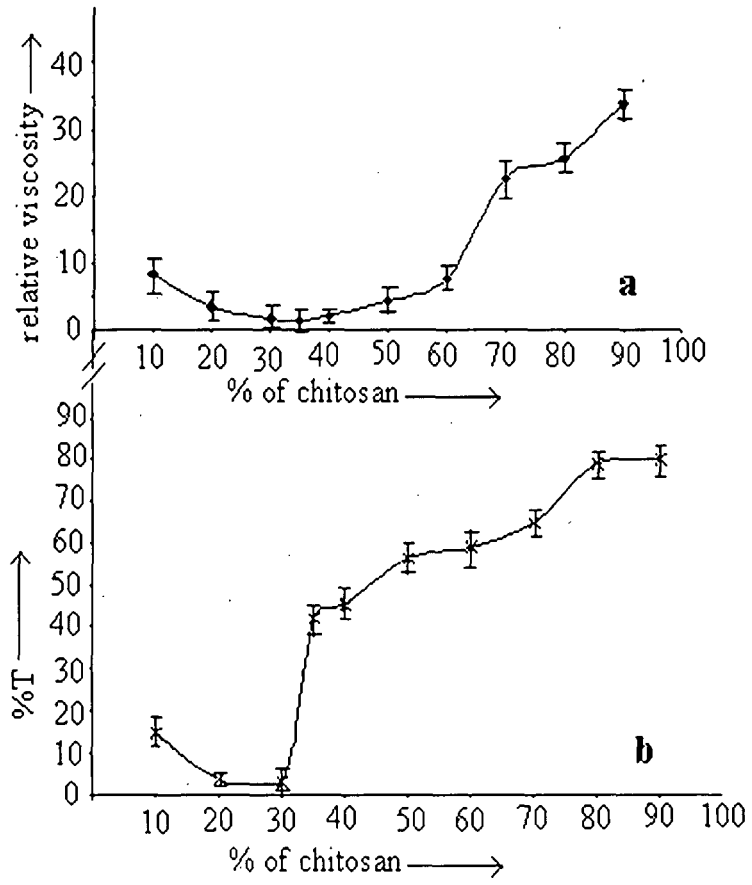

Figure 4.1.1.1 **(a)** Effect of variation of chitosan concentration in chitosan-carrageenan mixture on relative viscosity of supernatant.

(b) Effect of variation of chitosan concentration in chitosan-carrageenan mixture on (%) transmittance of supernatant

The plot of % transmittance against % of chitosan is presented in Figure 4.1.1.1(b). Each value represents the mean of three values. The % transmittance showed a decreasing trend initially followed by an increasing trend latter. The minimum % transmittance occurred when the % of chitosan in the mixture was in between 30-40 and also in between these points the transmittance increased sharply. The reason for this could be explained as before. The maximum turbidity developed when the interaction between chitosan and carrageenan was maximum. The higher the turbidity, the lower was the transmittance. The % of increased chitosan latter would decrease the turbidity and hence transmittance increased.

4.1.1.2. Scanning electron microscopy study

SEM photographs of neat carrageenan + chitosan complex and NSO loaded microcapsules are presented in Figure 4.1.1.2. Photographs of neat carrageenan + chitosan complex (fig. a) appeared more regular and free flowing compared to NSO loaded microcapsules(fig. b and c). The surface of high NSO loaded microcapsules (fig. c) appeared more irregular and bursting in comparison with that of low NSO loaded microcapsules (fig. b). Similar observation was reported in the literature [2]. Fig. d represents the photograph of the sample after release of NSO. Levels of NSO loading in both the samples(c and d) were almost similar. Bursting look observed in loaded samples was found to decrease after releasing of NSO. SEM photographs at different magnification did not change either the irregularity nature or free flowing characteristics of the particles. Different magnifications were used to get better clarity of the photographs.

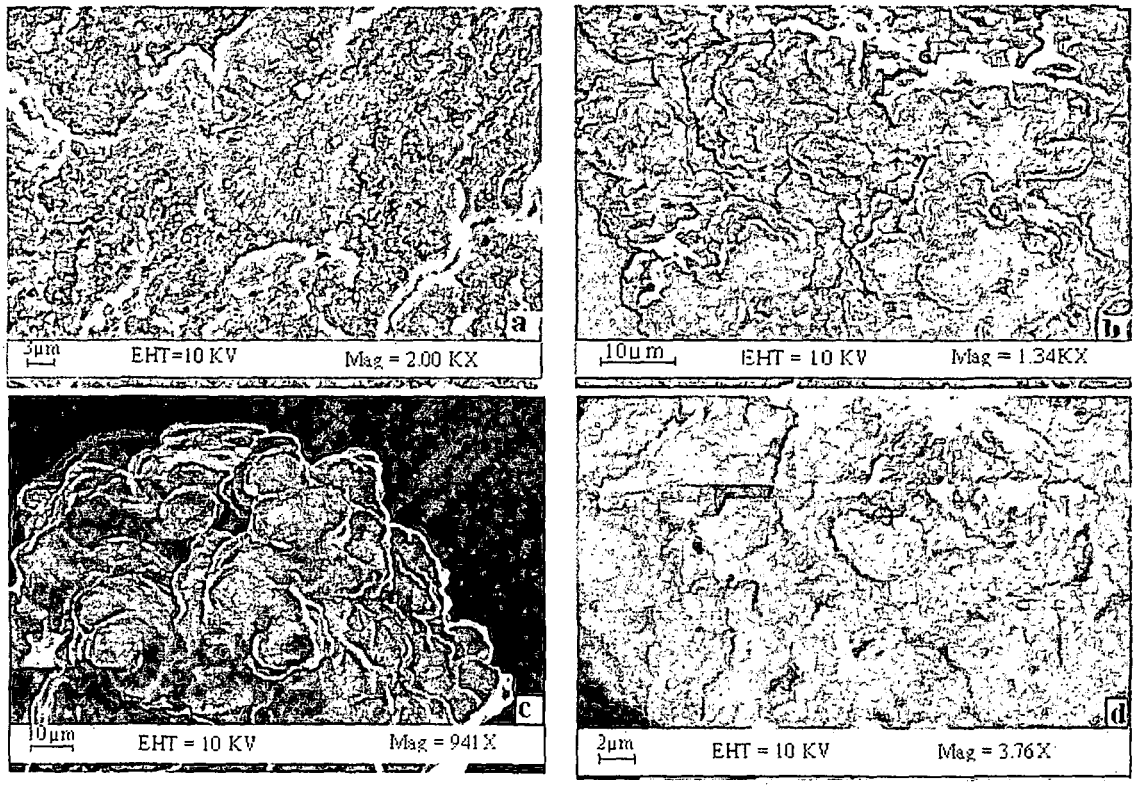

Figure 4.1.1.2. Scanning electron micrographs a) neat carrageenan+chitosan complex b) microcapsules loaded with low percentage of NSO c) microcapsules loaded with high percentage of NSO d) microcapsules loaded with high percentage of NSO(after release of NSO).

4.1.1.3. 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 4.1.1.1 and Figure 4.1.1.3. All experiments were carried out in triplicate and the results presented were the average value. 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 vesicles. The polymer present in the mixture was enough to encapsulate these vesicles. The dispersion force became progressively difficult as the oil load increased. This would develop large oil vesicles and as a result encapsulation efficiency would increase. As the amount of polymer was fixed, therefore, the polymers would encapsulate all the large oil vesicles 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 [3], 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 vesicles indicating that there was an abundance of the encapsulating polymer for the oil present. With the increase in oil load (%), the number of oil vesicles in the microcapsule increased which resulted in an increase in oil content. An increase in oil content (%) of microcapsules due to increase in oil loading was supported by SEM study. The surface characteristics of the microcapsules were found to change as oil content (%) varies.

Table 4.1.1.1. Effect of variation of oil loading, polymer and glutaraldehyde concentration on the behaviour of microcapsules.

[chitosan: 0.018-0.306g; carrageenan:0.30-0.85g; water:136ml; NSO: 0.68-2.04g; glutaraldedyde: 1.5-12.5 mmol; temperature: 70±1°C]

Sample formulations			Oil load (%)	Oil Content (%)	Encapsulation efficiency (%)
Total polymer (gm)	Glutaraldehyde (mmol)	NSO (gm)			
0.68	2.5	0.68	73.12	31±1.0	73.39±2.37
0.68	2.5	1.36	146.23	48±1.3	80.82±2.18
0.68	2.5	2.04	219.35	59±0.5	85.897±0.728
0.68	1.5	1.36	163.85	41±2.0	66.02±3.22
0.68	7.5	1.36	95.10	48±0.74	98.47±1.51
0.68	12.5	1.36	70.4	40±1.0	96.78±2.40
0.408	1.875	2.04	342.56	61±0.9	78.80±1.169
1.156	4.625	2.04	126.04	46±2.3	82.49±4.13

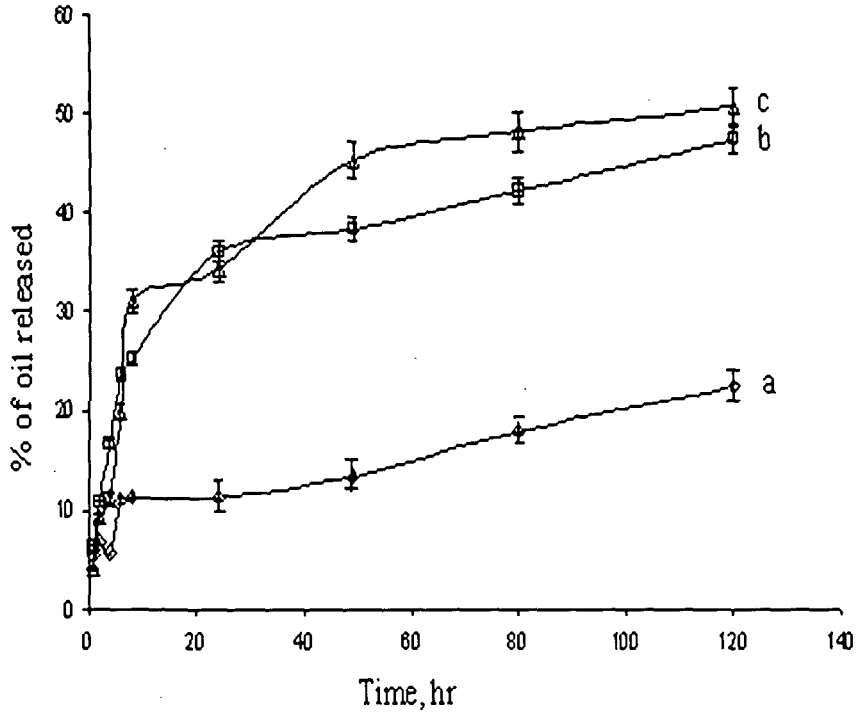

Figure 4.1.1.3. Effect of variation of oil loading on release profile

[a: polymer 0.68 gm; crosslinker 2.5 mmol; NSO 0.68 gm, b: polymer 0.68 gm; crosslinker 2.5 mmol ; NSO 1.36gm, c: polymer 0.68 gm; crosslinker 2.5 mmol ; NSO 2.04 gm]

4.1.1.4. 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 4.1.1.1 and Figure 4.1.1.4. The trends of oil loading (%) and oil content (%) shown in the table were as per expectation. With the increase in glutaraldehyde concentration, oil loading decreased for all as expected. But oil content and encapsulation efficiency increased. A decrease in trend in oil content was observed in the case of 12.5 mmol concentration of glutaraldehyde. 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 glutaraldehyde and polyelectrolyte complex of carrageenan and chitosan.

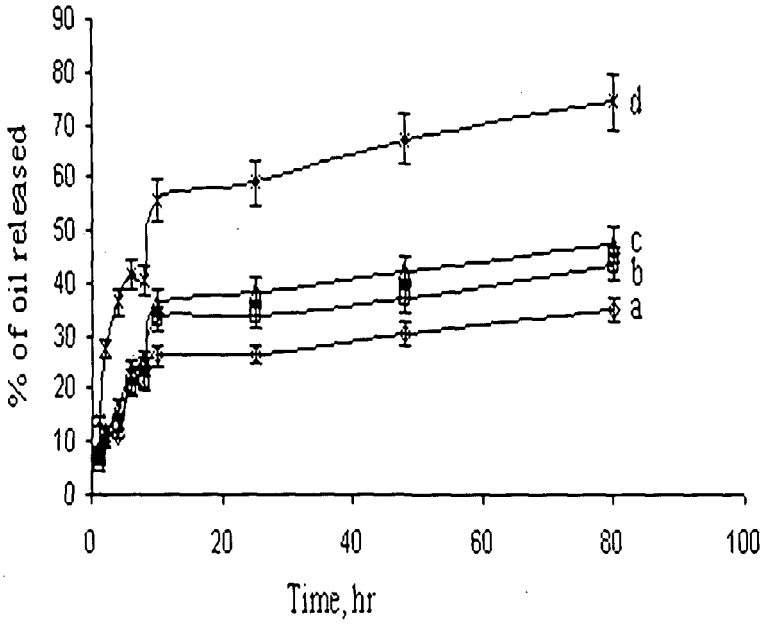

Figure 4.1.1.4. Effect of variation of crosslinker concentration on release profile.

[a: polymer 0.68 gm; crosslinker 12.5 mmol ; NSO 1.36 gm, b: polymer 0.68 gm; crosslinker 7.5 mmol ;NSO 1.36gm c: polymer 0.68 gm; crosslinker 2.5 mmol ; NSO 1.36 gm, d: polymer 0.68 gm; crosslinker 1.5 mmol ; NSO 1.36 gm]

The release rate of oil was found to decrease as the % of glutaraldehyde 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 [4].

4.1.1.5. Effect of variation of polymer concentration

Table 4.1.1.1 shows the results of the effect of variation of total polymer concentration on oil loading, oil content and encapsulation efficiency. In all the studied experiments, the ratio of polymer to cross-linker was kept fixed. As expected, both oil loading (%) and oil content (%) decreased with the increase in total polymer content. Encapsulation efficiency increased initially and then leveled off. With the increase in polymer content, more and more polymer would be available to encapsulate the oil vesicles

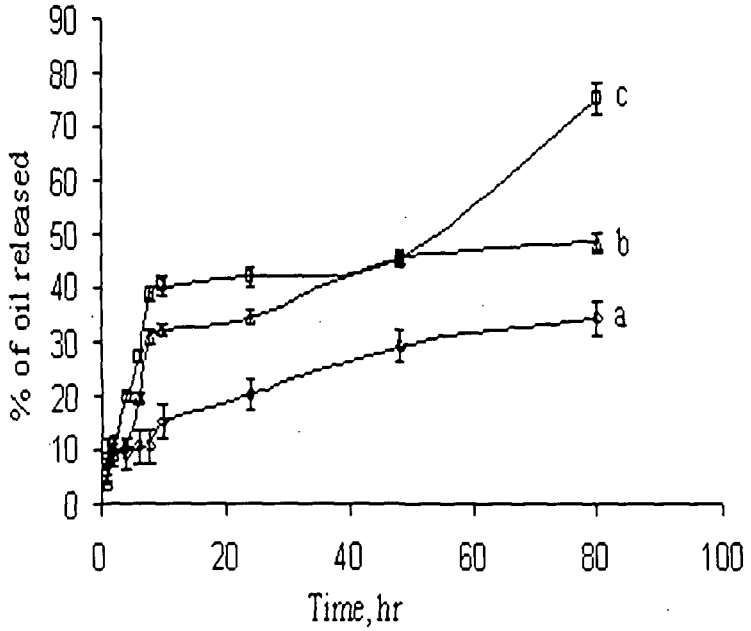

Figure 4.1.1.5. Effect of variation of polymer concentration on release profile [a: polymer 1.156 gm; crosslinker 4.625 mmol; NSO 2.04 gm, b: polymer 0.68 gm; crosslinker 2.5 mmol ; NSO 2.04 gm, c: polymer 0.408 gm; crosslinker 1.875 mmol ; NSO 2.04 gm]

and thereby efficiency increased. The excess polymer after complete encapsulation would enhance the thickness of the microcapsule. The release profile is shown in fig 4.1.1.5. 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 behavior [2].

4.1.1.6. Fourier transform infrared (FTIR) study

FTIR spectra of Chitosan (curve-a), Carrageenan (curve-b), chitosan-carrageenan polyelectrolyte complex (curve-c), NSO(curve-d), physical mixture of (NSO+ chitosan-carrageenan polyelectrolyte complex)(curve-e), and NSO loaded chitosan-carrageenan microcapsules (curve-f) are shown in Figure 4.1.1.6. The spectrum of chitosan showed a strong absorption band at 1635.33 cm^{-1} assigned to NH bending. The other notable peaks

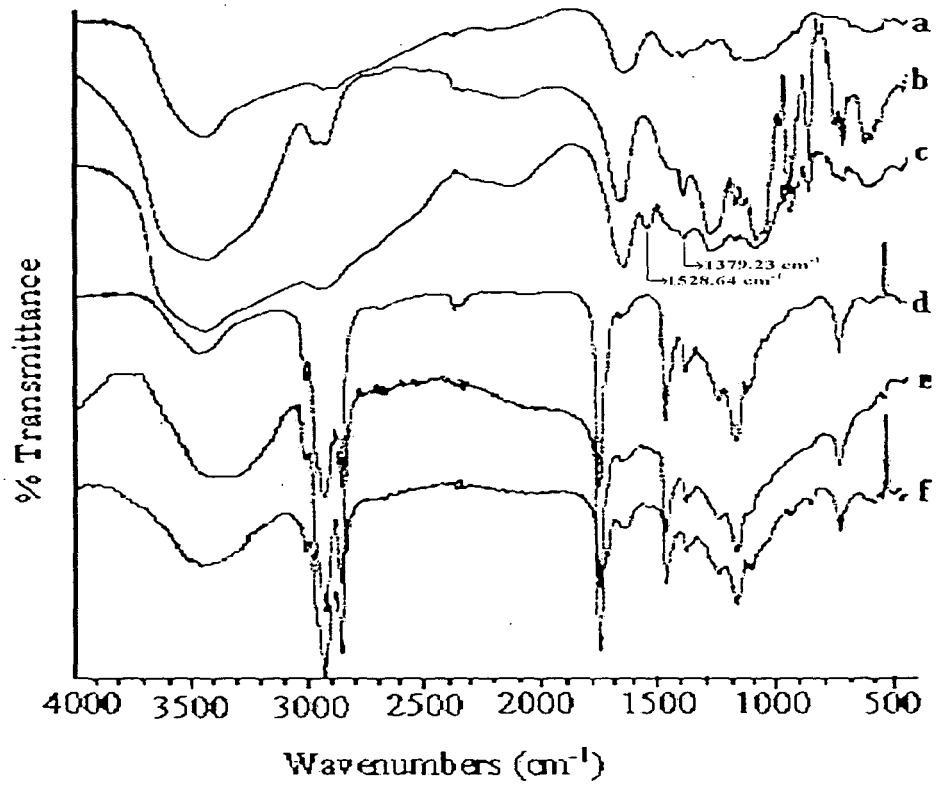

Figure 4.1.1.6. FTIR spectra of a) chitosan b) carrageenan c) polyelectrolyte complex of chitosan and carrageenan d) NSO e) physical mixture of (NSO+ polyelectrolyte complex of chitosan and carrageenan) f) NSO loaded microcapsules.

appeared at 3435, 2920, 1425 and 1384, 1330, 1170, 1075, and 1030 cm^{-1}, were due to O-H + N-H stretching vibration, CH_3 symmetric + CH_2 asymmetric vibration, CH_3 +CH_2 bending vibration, vibration of C-N group, C-O-C asymmetric vibration, C-O(-C-OH-) vibration, C-O(-CH_2 -OH-) vibration respectively . All these above peaks appeared in the spectrum of chitosan were observed in the spectrum of carrageenan except the peaks corresponding to nitrogen atom related groups. Besides this, the other notable 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. The appearance of a new band at 1528.64 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 [5]. The absorption bands appeared in the spectrum of NSO at 1745.90, 1463.04 and 1163.85 cm^{-1} were due to carbonyl stretching, CH_2

asymmetric deformation and C-C stretching vibration. The position of these bands in the physical mixture as well as in the NSO loaded microcapsules remained almost unchanged indicating the absence of any significant interaction between NSO and chitosan-carrageenan polyelectrolyte complex.

4.1.1.7. Thermal property study

DSC thermograms of neat chitosan-carrageenan polyelectrolyte complex (curve-a), NSO (curve-b), NSO loaded microcapsules(curve-c) and physical mixtures of (NSO+ chitosan-carrageenan polyelectrolyte complex)(curve-d) are shown in Figure 4.1.1.7. In the physical mixture, the ratio of NSO to neat chitosan-carrageenan polyelectrolyte complex was kept similar to that of microcapsules loaded with NSO. The endotherm appeared in all the thermograms except that of oil at around 100^0C was due to the removal of moisture. The thermogram of NSO showed an endothermic peak at around 220°C which might be due to the decomposition of organo sulphur compounds present in the neem seed oil. Both the NSO loaded microcapsules and physical mixture of complex and NSO did not show any remarkable difference in their thermograms. In both the thermograms, the endothermic peak due to NSO appeared almost in the similar position. These results indicated that there was no significant interaction between NSO and chitosan-carrageenan complex. The results also suggested a low compatibility in thermal properties in the relation between NSO and chitosan-carrageenan polyelectrolyte complex.

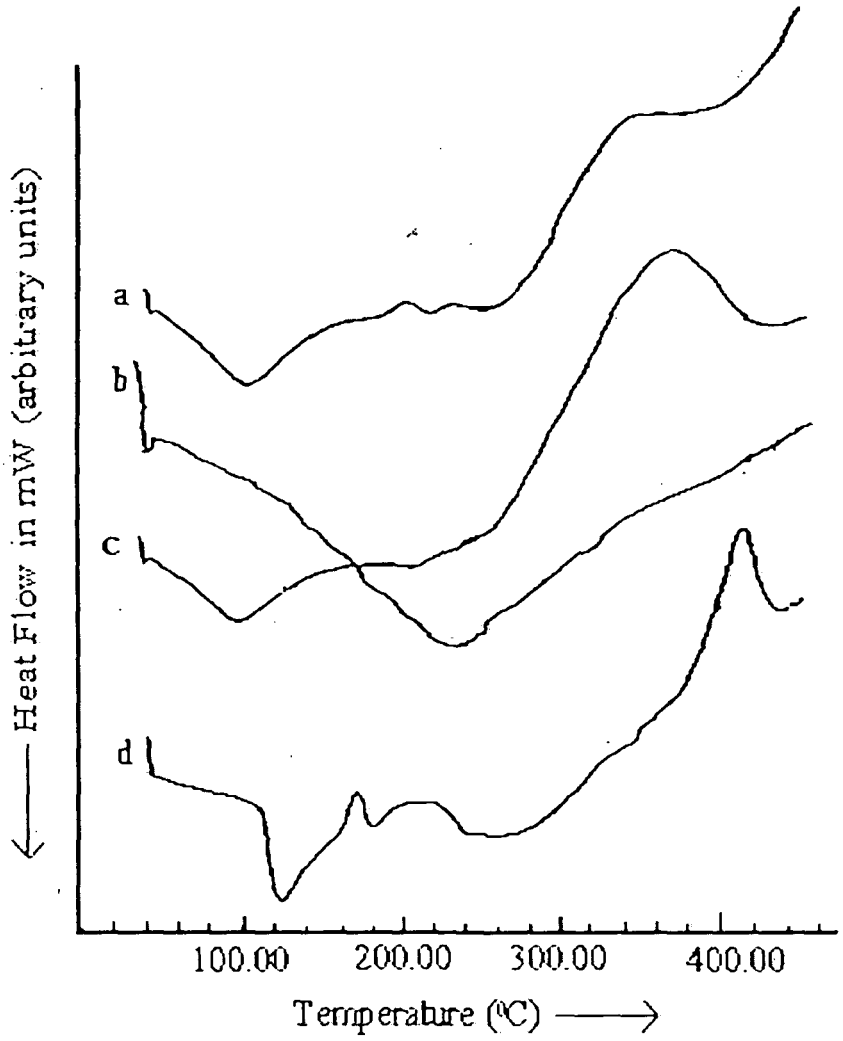

Figure 4.1.1.7. DSC thermograms of a) neat chitosan-carrageenan polyelectrolyte complex b) NSO c) NSO loaded microcapsules d) physical mixture of (NSO+ chitosan-carrageenan polyelectrolyte complex)

4.1.1.8. Comparison of effect of different crosslinkers on various parameters

Effect of crosslinkers on oil loading, oil content, encapsulation efficiency and release rate

The effect of variation of crosslinker type and concentration on oil loading (%), oil content (%), encapsulation efficiency (%) and release rate are shown in the Table 4.1.1.2 and Figure 4.1.1.8 (a-c). 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.

Table 4.1.1.2. Effect of variation of type and concentration of crosslinker on the behaviour of microcapsules. [chitosan : 0.1g; carrageenan : 0.5g; water: 136ml; NSO: 2.04 g; crosslinker: 0.2-0.8mmol; temperature: 70±1^{0}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.

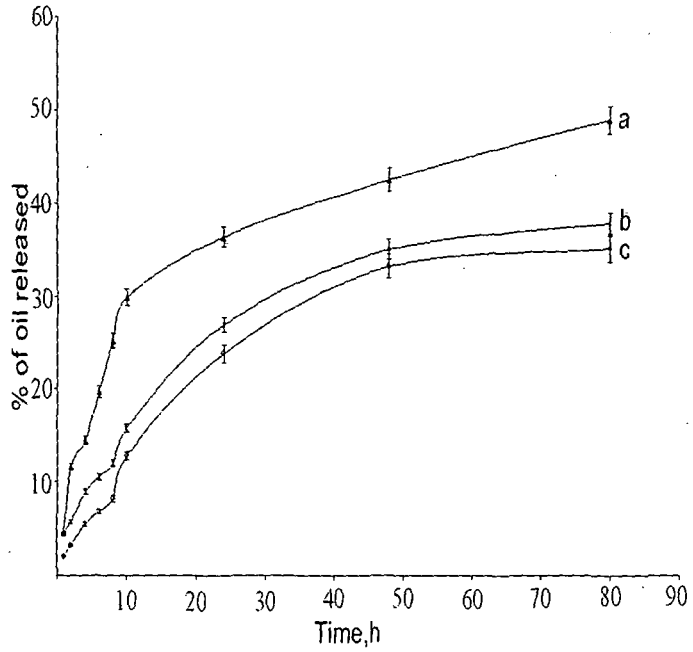

Figure 4.1.1.8.(a) Oil release profiles of microcapsules crosslinked with 0.2mmol a) tannic acid b) genipin c) glutaraldehyde

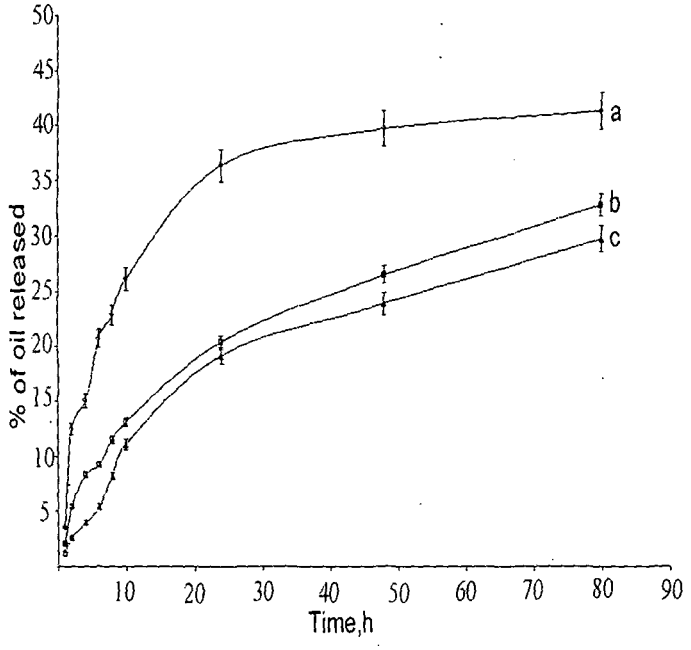

Figure 4.1.1.8.(b) Oil release profiles of microcapsules crosslinked with 0.4mmol a) tannic acid b) genipin c) glutaraldehyde

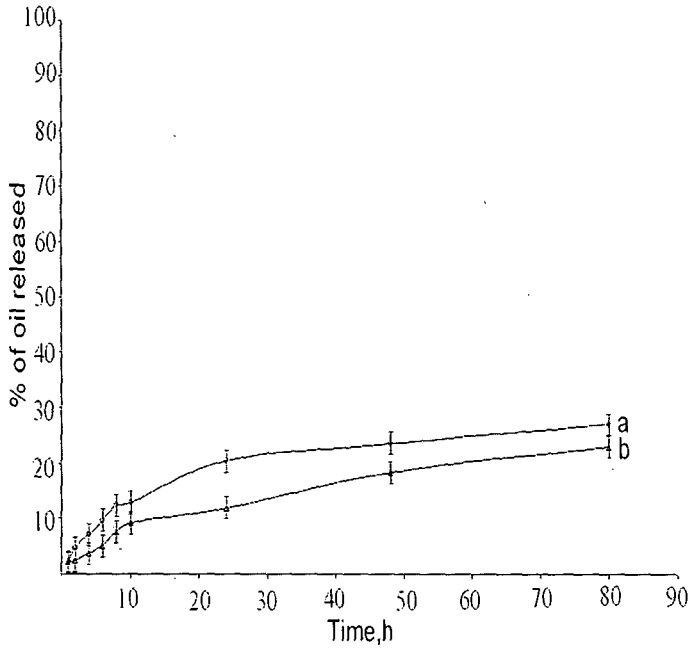

Figure 4.1.1.8.(c) Oil release profiles of microcapsules crosslinked with 0.8mmol a) genipin b) glutaraldehyde

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 [6]. Tannic acid possessed large number of free phenolic hydroxyl groups, which could form strong hydrogen bonds with proteins and carbohydrates [7]. Tannic acid might also form complex with proteins [8]. 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 [9]. The crosslinking intensity would be less due to the 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 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 [10].

Water uptake studies

The results of swelling experiments of different crosslinked products with different crosslinkers are shown in Figure 4.1.1.9 The crosslinked products were allowed to swell in water at room temperature for 40 hrs. 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.

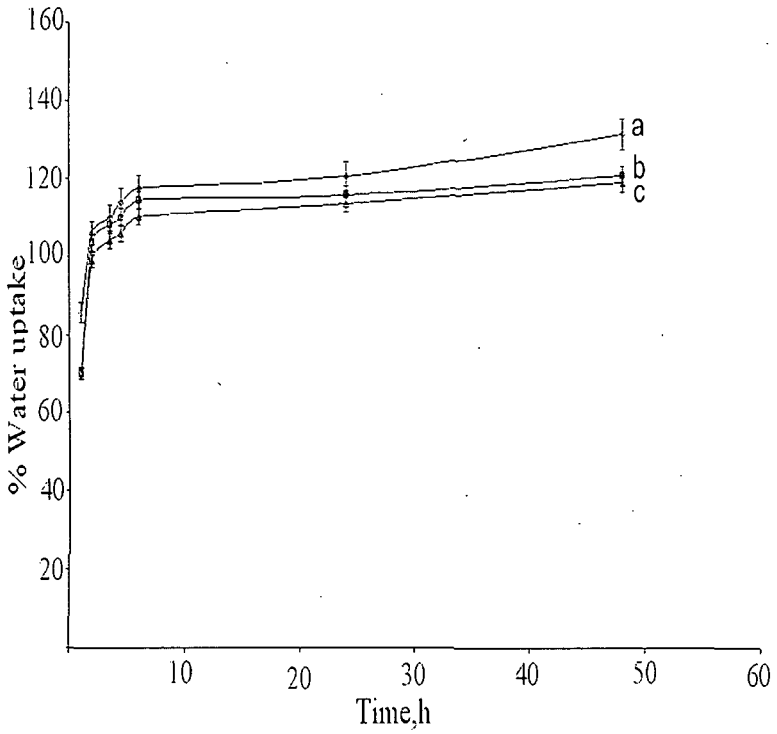

Figure 4.1.1.9. Percentage water uptake of matrices crosslinked with a) tannic acid b) genipin c) glutaraldehyde

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 4.1.1.10.The spectrum of chitosan showed a strong absorption band at 1635.33 cm^{-1} 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 [5]. 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.

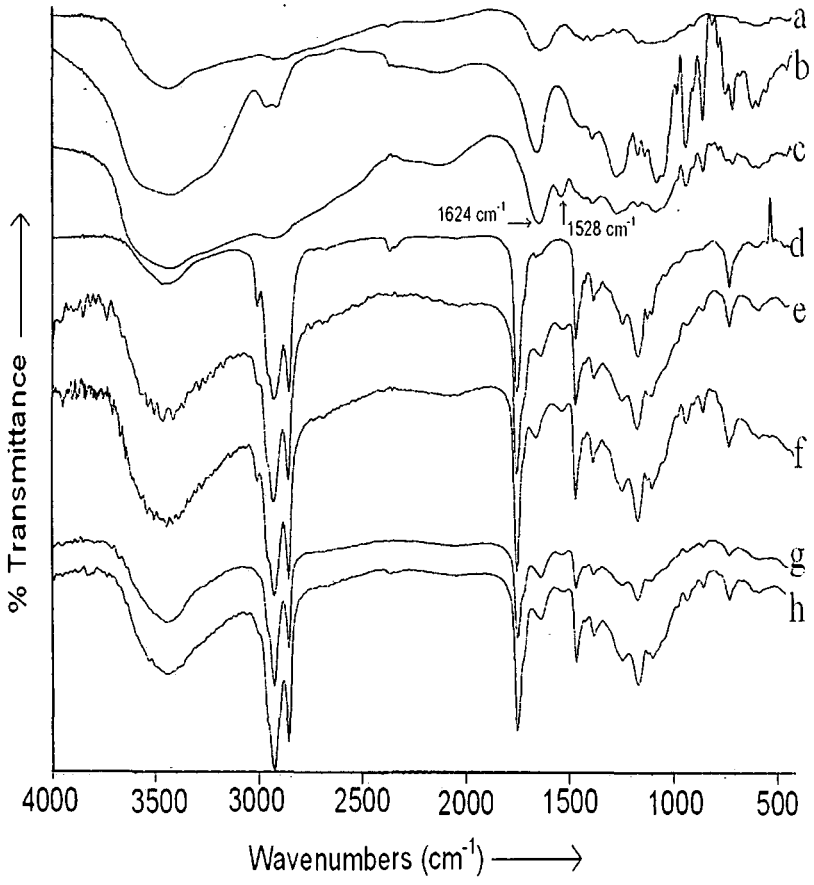

Figure 4.1.1.10. FTIR spectra of a) chitosan b) carrageenan c) polyelectrolyte complex of chitosan and carrageenan d) NSO e) NSO loaded uncrosslinked microcapsules f) NSO loaded tannic acid crosslinked microcapsules g) NSO loaded genipin crosslinked microcapsules h) NSO loaded glutaraldehyde crosslinked microcapsules

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 4.1.1.3. 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.

Scanning electron microscopy study

Figure 4.1.1.11 shows the scanning electron micrographs of chitosan-carrageenan complex (Fig. a) NSO loaded crosslinked microcapsules (Fig.b, c, d) and uncrosslinked microcapsules (Fig. e) respectively. The photograph of chitosan-carrageenan complex appeared powdery. Similarly, photograph of NSO loaded uncrosslinked (Fig.e) appeared spherical, smooth and agglomerated. Partly spherical and partly bean like structure were observed in glutaraldehyde crosslinked microcapsules (Fig. b). In the case of genipin crosslinked microcapsules (Fig. c), 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. d) were spherical and smooth. The roughness appeared on the surface of the microcapsules might be due to the interaction between chitosan-carageenan microcapsules and crosslinker. Piyakulawat and coworkers [11] observed and reported the appearance of irregular and rough surfaces while studying the SEM micrographs of glutaric acid crosslinked chitosan-carrageenan beads. The micrographs were taken at different magnification in order to get better clarity of the samples.

able 4.1.1.3. Temperature of decomposition at different weight loss (%) of carrageenan-chitosan complex ıd oil containing crosslinked microcapsules

Sample particulars	Cross linker (mmol)	Temperature of decomposition (T_D) (^{0}C) at different weight loss (%)							Residue (%) at 500 (^{0}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

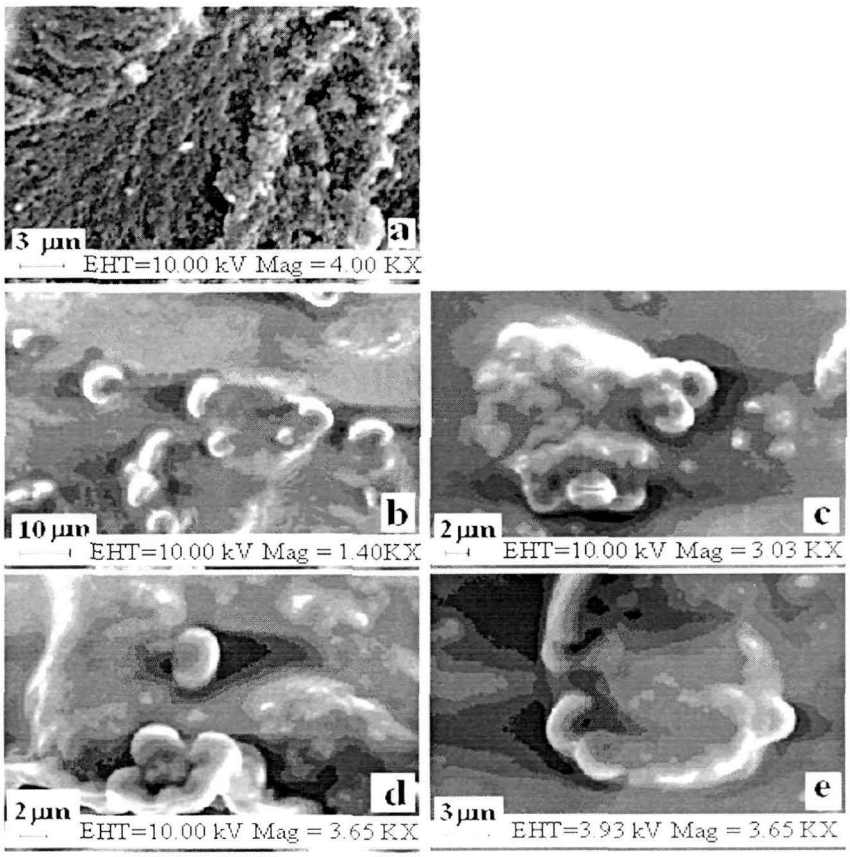

Figure 4.1.1.11. Scanning electron micrographs of a) carrageenan-chitosan complex b) glutaraldehyde crosslinked microcapsules c) genipin crosslinked microcapsules d) tannic acid crosslinked microcapsules e) uncrosslinked microcapsules

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 4.1.1.12. Chitosan showed its characteristic peak at $2\theta=20^0$. This was similar to that of reported in the literature [12]. Similarly carrageenan showed sharp peaks at $2\theta=29^0$ and 41^0 respectively. Bhise and coworkers [13] observed the appearance of multiple peaks in the diffractogram of κ-carrageenan between 2θ values of 15^0 to 30^0. Meena and coworkers [14] reported the formation of broad peak at around $2\theta=20^0$. In the uncrosslinked microcapsule, a broad peak appeared at 22.4^0.This showed that the uncrosslinked microcapsule was amorphous in nature compared to either chitosan or carrageenan. Further 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 [15]. 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 coworkers [14].

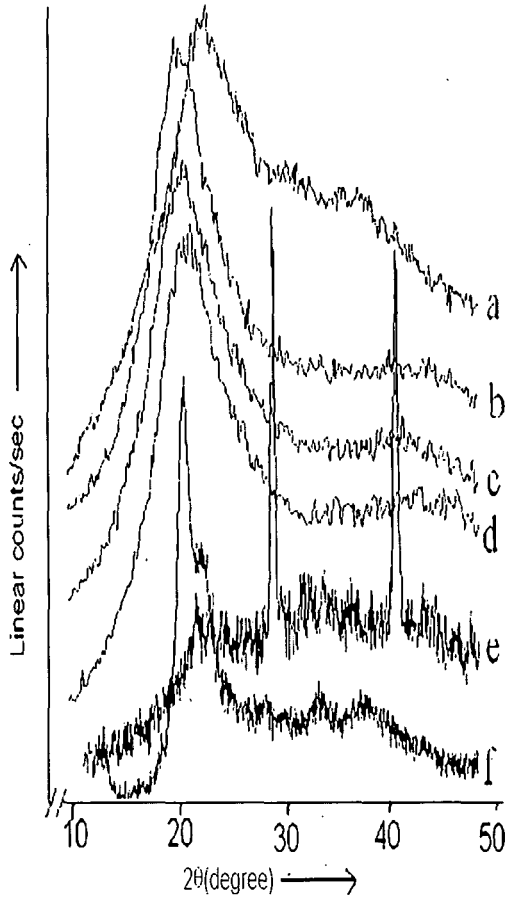

Figure 4.1.1.12. X-ray diffractograms of NSO loaded chitosan-carrageenan microcapsules a) uncrosslinked b) glutaraldehyde crosslinked c) genipin crosslinked d) tannic acid crosslinked e) neat carrageenan f) neat chitosan

4.1.2. κ-carrageenan – gelatin-A system for microencapsulation of NSO

4.1.2.1. Turbidity, viscosity and coacervate yield

20 ml of the 0.5% solution of carrageenan was titrated by 0.5% solution of gelatin. The plot of absorbance (%) against volume of gelatin (ml) was presented in Figure 4.1.2.1(a). 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 4.1.2.1(b) 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 presented in Figure 4.1.2.1(c). The trend was similar to that of turbidity measurement and could be explained as before.

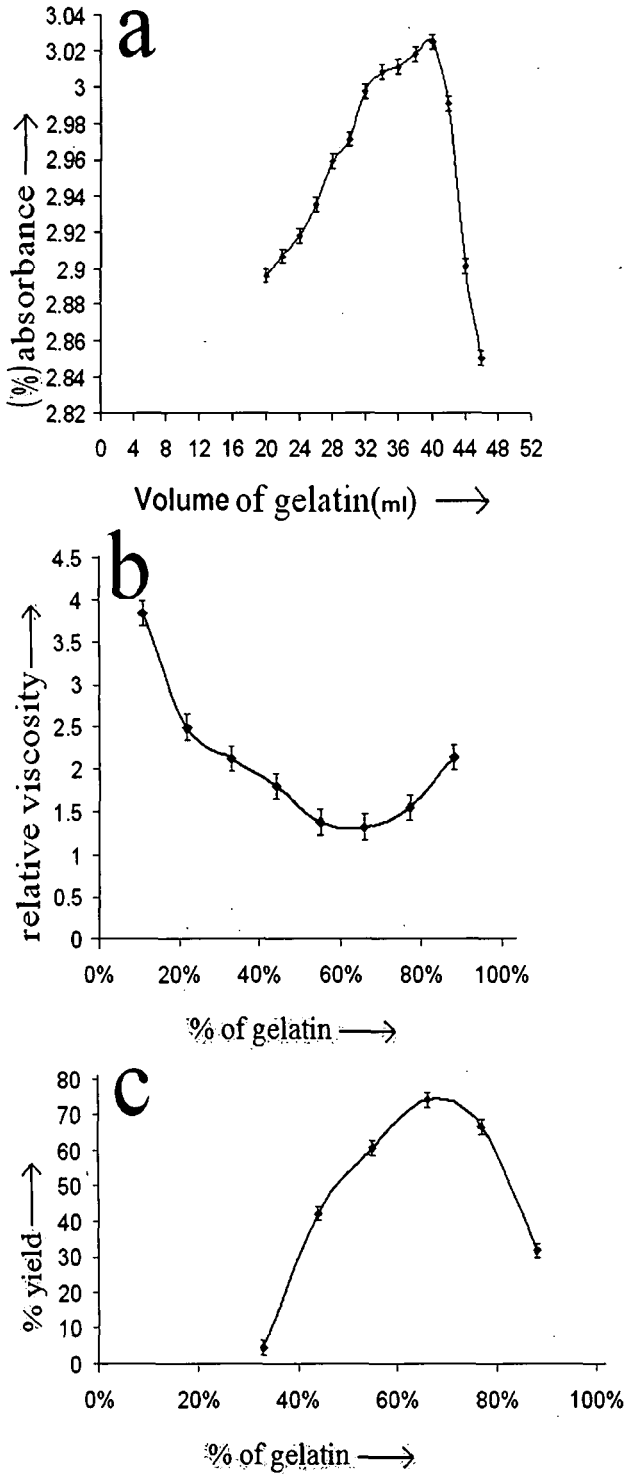

Figure 4.1.2.1. Effect of variation of gelatin concentration in gelatin - carrageenan mixture on **(a)** turbidity of the mixture solution **(b)** relative viscosity of the supernatant. **(c)** coacervate yield (%)

4.1.2.2. Effect of variation of pH

The effect of variation of pH (2.5-5.0) on turbidity was shown in fig.4.2.2 (a), 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 of given earlier.

This finding was further confirmed by the plotting the coacervate % yield against pH. The plot was shown in fig.4.1.2.2 (b). The % yield was highest at pH 3.5 and the trend was similar to that of absorbance (%) against pH plot.

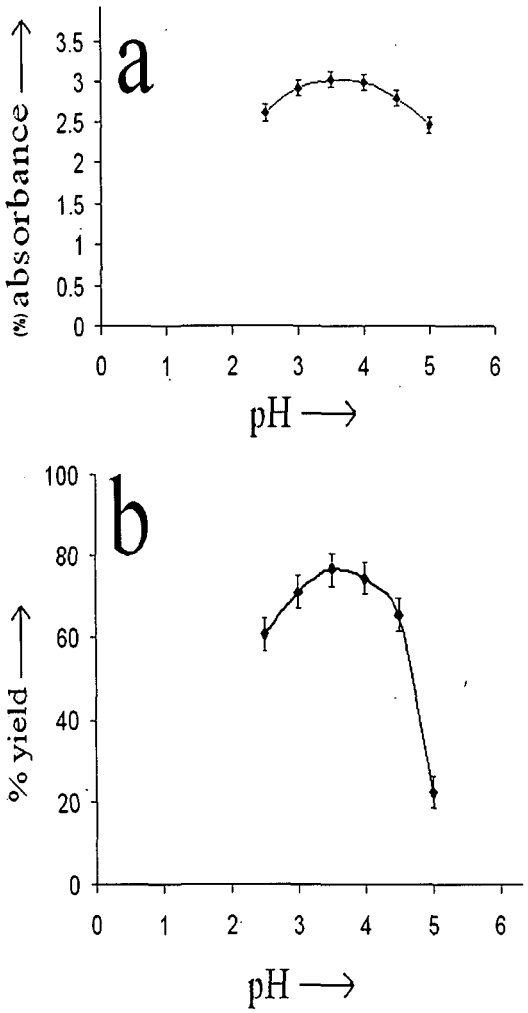

Figure 4.1.2.2. Effect of variation of pH on **(a)** turbidity **(b)** coacervate yield.(%)

4.1.2.3. Scanning electron microscopy study

SEM photographs of neat carrageenan + gelatin complex and NSO loaded microcapsules are presented in Figure 4.1.2.3. Photographs of neat carrageenan + gelatin complex (fig. a) appeared agglomerated with no definite structure. In contrast, the NSO loaded samples (fig. b,c,d) were having free flowing spherical shape. With the increase of the amount of polymer concentration (fig. b to fig. d) the size of the microcapsules increased. This might be due to the increase of the thickness of the wall of the microcapsules. Zhuo et al.[16]. reported similar observations during studying the particle size of polyurea microcapsules by interfacial polymerisation of polyisocyanates. Again, the surface of the microcapsules having high NSO loading (fig. e) appeared sticky and agglomerated compared to the low NSO loading (fig. b).

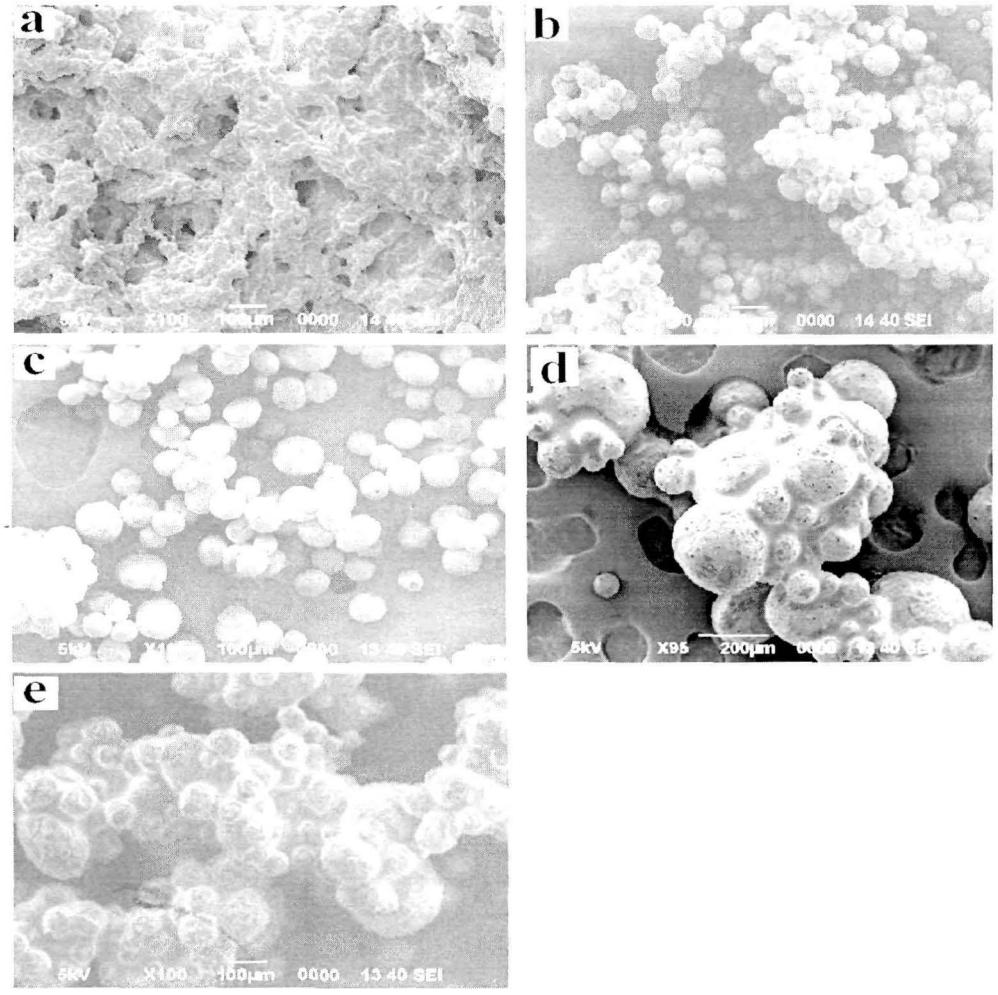

Figure 4.1.2.3. Scanning electron micrographs **(a)** neat carrageenan + gelatin complex; *microcapsules loaded with* **(b)** NSO =2g, *polymer* =1.5g **(c)** NSO =2g, *polymer* = 3g **(d)** NSO =2g, polymer = 4.5g **(e)** NSO=4g, polymer =1.5g.

4.1.2.4. 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 4.1.2.1 and Figure 4.1.2.4. 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 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.

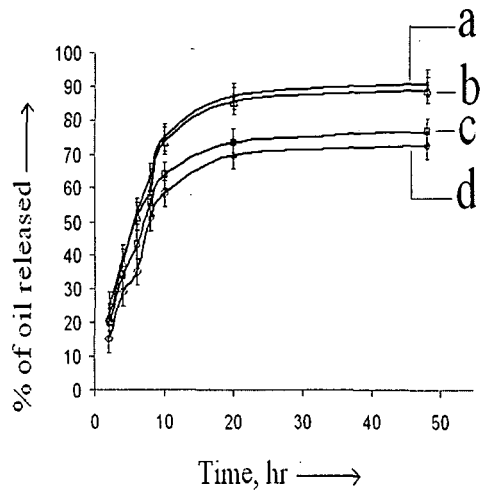

Figure 4.1.2.4. Effect of variation of oil loading on release profile [**a**:polymer 1.5 gm; crosslinker 0.1mmol; NSO 4 g, **b**: polymer 1.5 g; crosslinker 0.1 mmol ; NSO 3 g, **c**: polymer 1.5 g; crosslinker 0.1 mmol ; NSO 2 g; **d**: polymer 1.5 g; crosslinker 0.1 mmol; NSO 1 g]

Table 4.1.2.1. Effect of variation of oil loading, polymer and genipin concentration on the behaviour of microcapsules.[carrageenan: 0.5-1.5 g ; gelatin A :1.0-3.0 g ; water:200ml ; NSO: 1.0-4.0 g ; genipin: 0.1-0.8 mmol; temperature: 70±1°C]

Sample formulations						
Carrageen -an (g)	Gelatin A (g)	Genipin (mmol)	NSO (g)	Oil load (%)	Oil Content (%)	Encapsulation efficiency (%)
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

4.1.2.5. 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 4.1.2.1 and Figure 4.1.2.5. 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 carrageenan and gelatin. 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 [10].

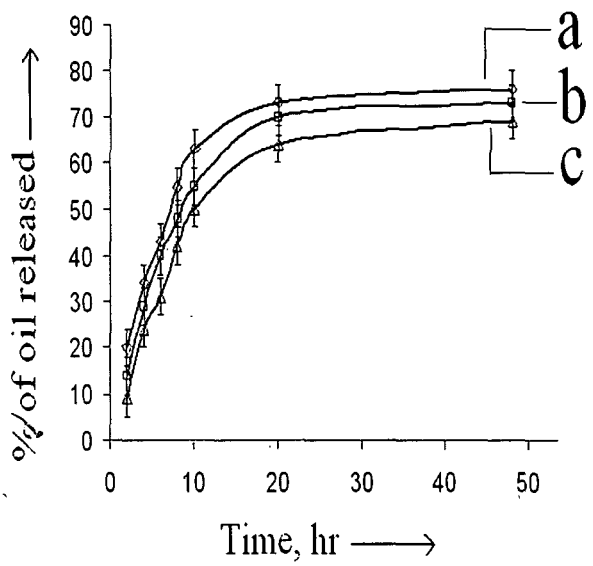

Figure 4.1.2.5. Effect of variation of crosslinker concentration on release profile.
[**a**: polymer 1.5 g; crosslinker 0.1 mmol ; NSO 2.0 g, **b**: polymer 1.5 g; crosslinker 0.4 mmol ; NSO 2.0 g **c**: polymer 0.68 g; crosslinker 0.8 mmol ; NSO 2.0 g]

4.1.2.6. Effect of variation of polymer concentration

Table 4.1.2.1 and Figure 4.1.2.6 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 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 Figure 4.1.2.6. 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 [10].

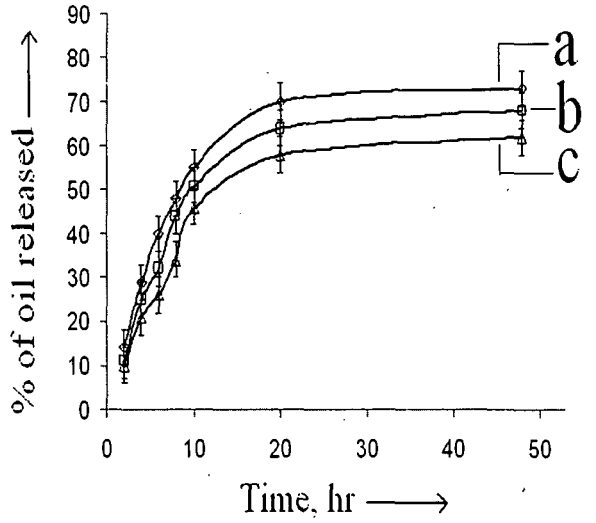

Figure 4.1.2.6. Effect of variation of polymer concentration on release profile

[**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]

4.1.2.7. Fourier transform infrared (FTIR) 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 Figure 4.1.2.7. The spectrum of carrageenan showed absorption bands at 3423 cm^{-1}, 2910 cm^{-1}, 1643 cm^{-1}, 1434 cm^{-1}, 1379 cm^{-1}, 1265 cm^{-1} and 846 cm^{-1} which were due to O-H stretching vibration, CH_3 symmetric + CH_2 assymetric vibration, N-H bending, 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 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 1600-1700 cm^{-1} is the most important peak for IR analysis of the secondary structure of protein like gelatin [17]. In the complex of gelatin and carrageenan, a slight shift of the peak of amide I from 1630.44 cm^{-1} 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. [18]. 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 [19]. However this type of shifting was not observed in this case. The absorption band appeared in the spectrum of NSO at 1743 cm^{-1}, 1456 cm^{-1} and 1163 cm^{-1} were due to the carbonyl stretching, CH_2 assymetric 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.

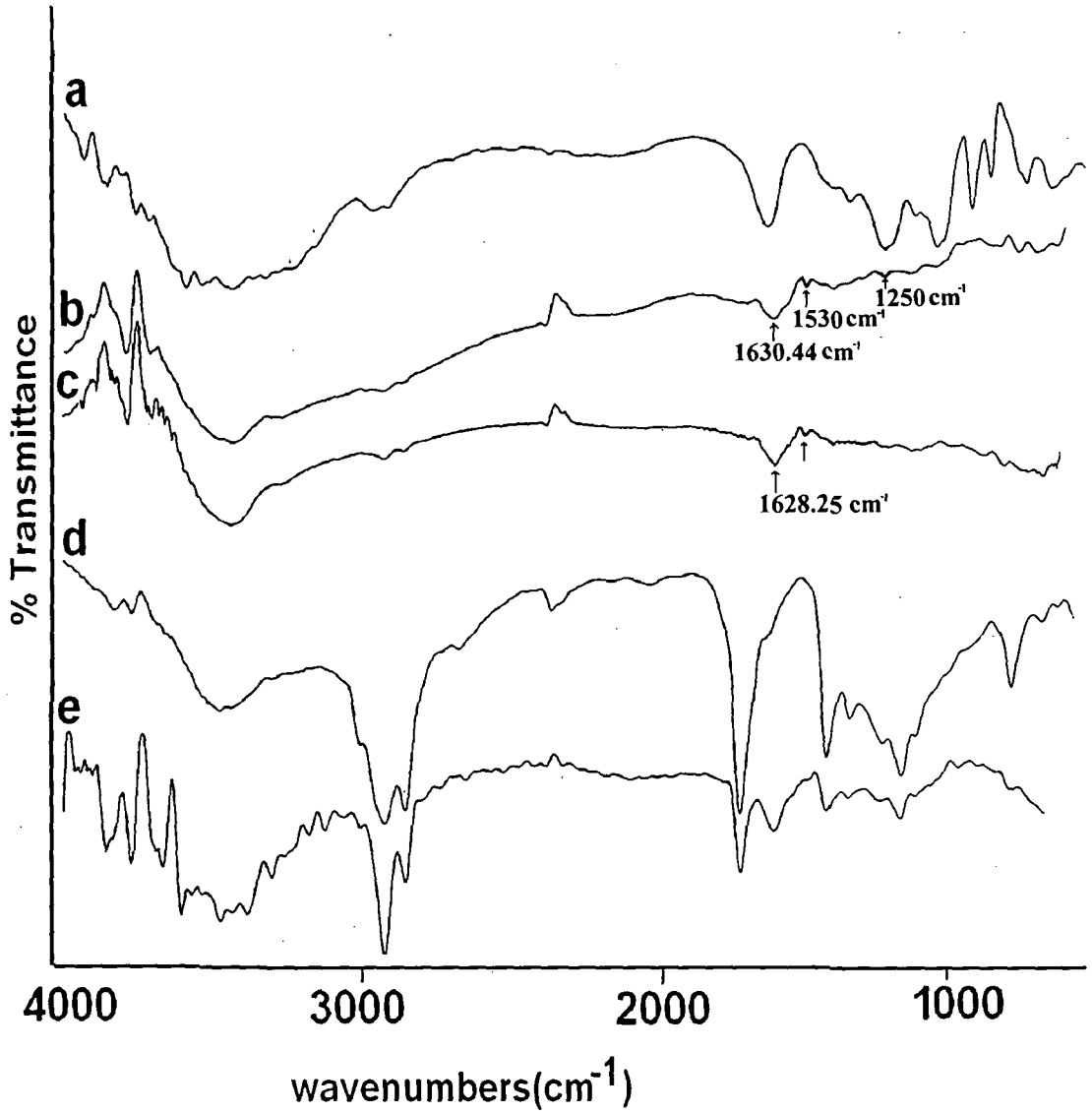

Figure 4.1.2.7. FTIR spectra of **a**) carrageenan **b**) gelatin **c**) gelatin-carrageenan complex **d**) NSO **e**) NSO loaded microcapsules

4.1.2.8. 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 Figure 4.1.2.8. 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 ^{0}C were due to removal of moisture. The thermograms of NSO showed an endothermic peak at around 220 ^{0}C which might be due to the decomposition of various organo sulphur compounds present in the neem seed oil. Both NSO loaded microcapsules and physical mixture showed two endothermic peaks corresponding to NSO and carrageenan-gelatin complex in the range 218-225 ^{0}C and 305-315 ^{0}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.

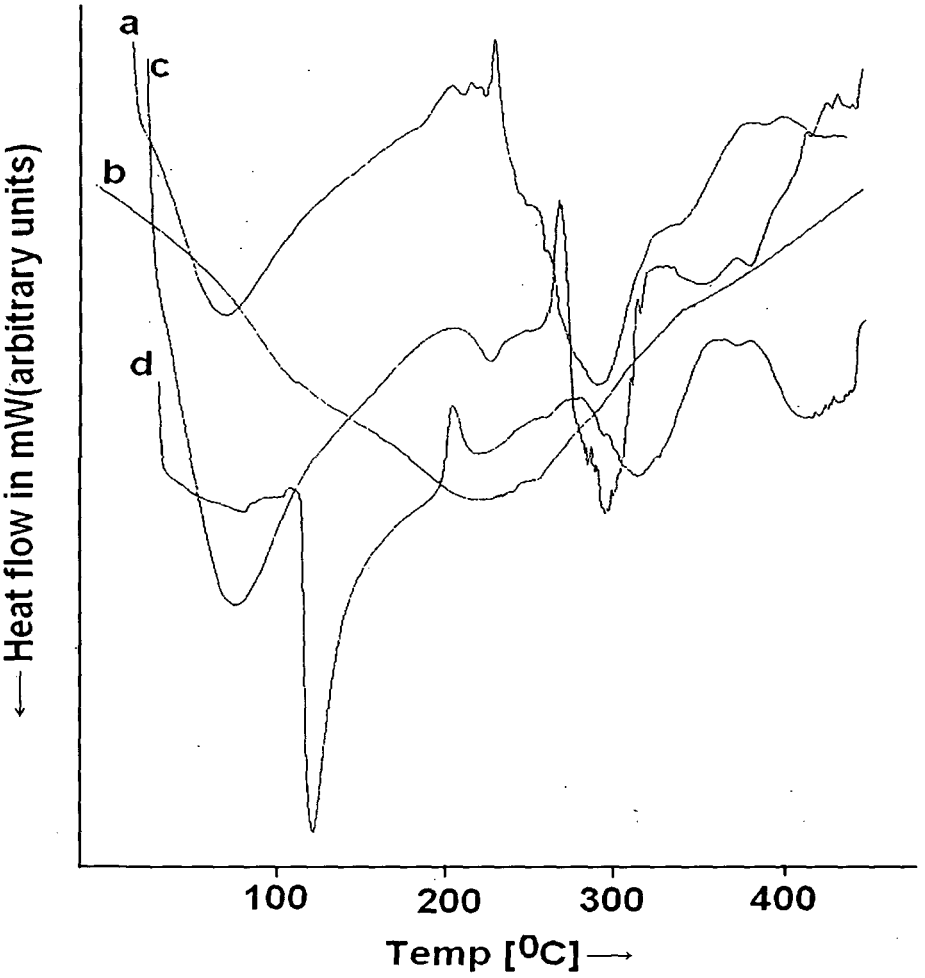

Figure 4.1.2.8. DSC thermograms of **(a)** carrageenan-gelatin complex, **(b)** NSO, **(c)** NSO loaded microcapsules and **(d)** physical mixture of (NSO+carrageenan-gelatin complex)

4.1.3. Gelatin A - SCMC system for microencapsulation of NSO

4.1.3.1. Viscosity and coacervate yield

The plot of coacervate yield (%) against % of SCMC is presented in figure 4.1.3.1(a). The % yield increased initially, reaching maximum and then decreased later. The maximum yield occurred when the % of SCMC in the mixture was 30%. The yield increased due to

increase in interaction between gelatin A and SCMC. The maximum yield found when the interaction between gelatin A and SCMC was maximum.

Figure 4.1.3.1(b) shows the change in supernatant viscosity with variation in percentage of SCMC in gelatin–SCMC mixture. Viscosity was found to decrease initially, reaching a minimum value, and after that it increased with the increase in the percentage of SCMC. The minimum viscosity observed when the percentage of SCMC in the mixture was 30%. At this percentage of SCMC, 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 SCMC in the supernatant.

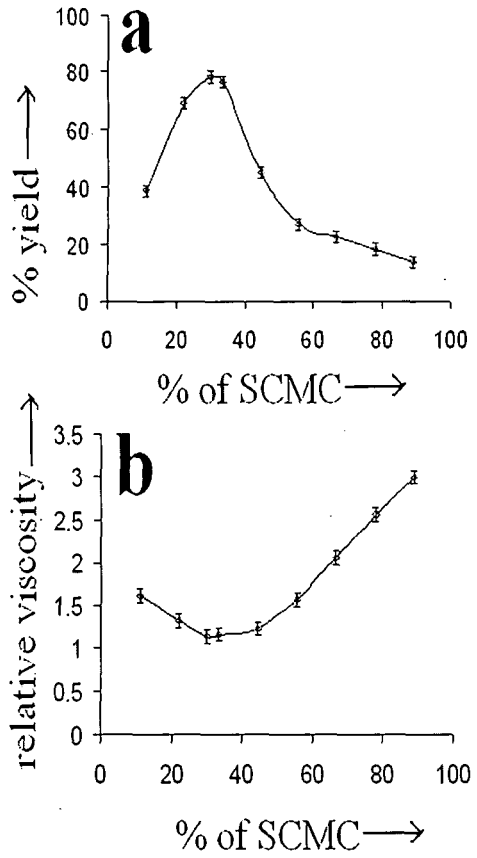

Figure 4.1.3.1. Effect of variation of SCMC concentration in gelatin - SCMC mixture on **(a)** turbidity of the mixture solution **(b)** relative viscosity of the supernatant **(c)** coacervate yield (%)

4.1.3.2. Effect of variation of pH

The effect of variation of pH (2.0-5.5) on % yield was plotted in fig.4.3.2; which was plotted as the coacervate yield (%) against pH. The coacervate yield increased initially with the increase of pH, reached maximum at pH 3.5 and then again decreased. This implied that the coacervation between the two polymers was highest at this pH. The explanation for this was similar to that of given earlier.

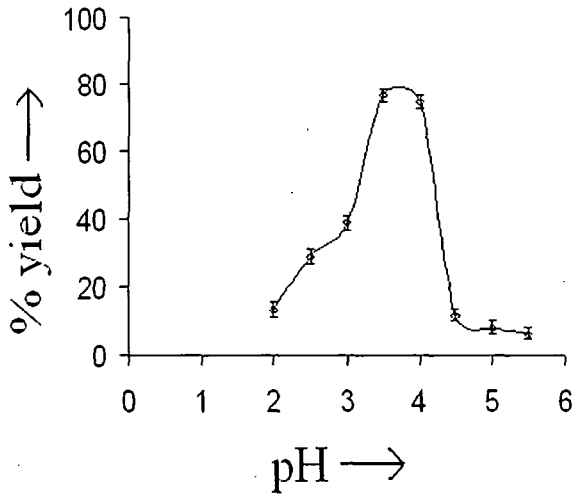

Figure 4.1.3.2. Effect of variation of pH on coacervate yield (%)

4.1.3.3. Scanning electron microscopy study

SEM photographs of neat SCMC + gelatin complex and microcapsules loaded with varying amount of NSO are presented in Figure 4.1.3.3. Photographs of neat SCMC + gelatin complex (fig. a) appeared like a sheet with no definite structure. In contrast, the NSO loaded samples (fig. b, d) were having free flowing spherical shape. With the decrease of the amount of polymer (fig. b and d) the size of the microcapsules decreased. This might be due to the decrease of the thickness of the wall of the microcapsules, which in turn would increase the release rate of NSO. Again, the surface of the microcapsules having high NSO loading (fig. c) appeared sticky and bursting as compared to the low NSO loading (fig. b) microcapsules.

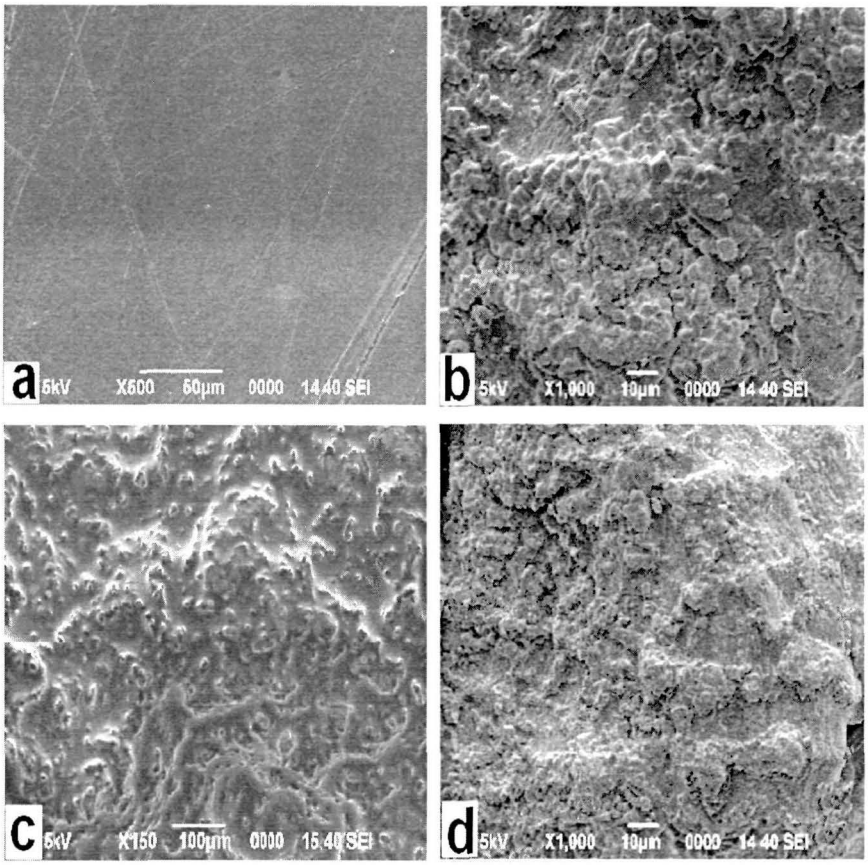

Figure 4.1.3.3. Scanning electron micrographs **(a)** neat SCMC + gelatin complex; microcapsules loaded with **(b)** NSO =3.0g, polymer =2.857g **(c)** NSO =7g, polymer = 2.857g **(d)** NSO =3g, polymer = 0.714g

4.1.3.4. 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 4.1.3.1 and Figure 4.1.3.4.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 sufficient 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 [3, 20], 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 increase in oil load (%), the number of oil droplets in the microcapsule increased which resulted in an increase in oil content.

Table 4.1.3.1. Effect of variation of oil loading, polymer and glutaraldehyde concentration on the behaviour of microcapsules.

[Gelatin: 0.5-4.0g; SCMC: 0.214-1.714g; water: 200ml; NSO: 1.5-7.0g; glutaraldedyde: 5.0 –20.0 mmol; temperature: 45±1°C]

Sample formulations					
Total polymer (g)	Glutaraldehyde (mmol)	NSO (g)	Oil load (%)	Oil Content (%)	Encapsulation efficiency (%)
2.857	12.5	1.5	36.52	20±1.0	74.76±3.74
2.857	12.5	3.0	73.04	37±0.50	87.76±1.07
2.857	12.5	5.0	121.74	51±0.80	92.89±1.45
2.857	12.5	7.0	170.44	59±0.87	93.61±1.38
2.857	5.0	3.0	89.36	36±0.95	76.28±2.01
2.857	20.0	3.0	61.76	37±0.76	96.90±1.99
0.714	5.0	3.0	247.11	51±1.20	71.63±1.69
5.714	5.0	3.0	48.20	31±1.3	95.23±3.99

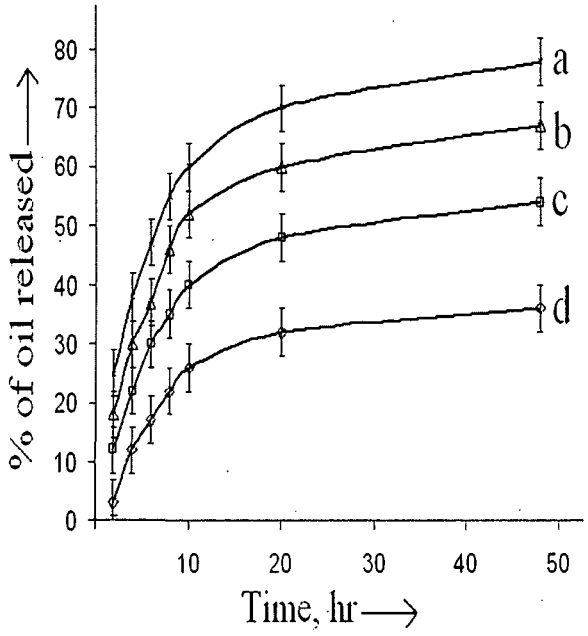

Figure 4.1.3.4. Effect of variation of oil loading on release profile [**a**: polymer = 2.857 gm, crosslinker = 12.5 mmol, NSO = 7 g, **b**: polymer = 2.857 g, crosslinker = 12.5 mmol ; NSO = 5 g, **c**: polymer = 2.857 g, crosslinker = 12.5 mmol, NSO = 3 g, **d**: polymer = 2.857 g, crosslinker = 12.5 mmol, NSO = 1.5 g]

4.1.3.5. 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 4.1.3.1 and Figure 4.1.3.5. The trends of oil loading (%) and oil content (%) shown in the table were as per expectation. With the increase in glutaradehyde 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 glutaraldehyde and polyelectrolyte complex of SCMC and gelatin. The possible interaction of SCMC-gelatin complex with glutaraldehyde is shown in Figure 4.1.3.6. The release rate of oil was found to decrease as the % of glutaraldehyde 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 [10].

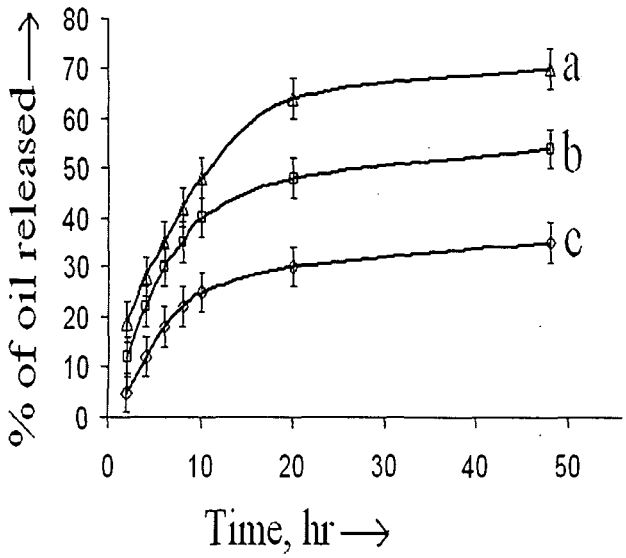

Figure 4.1.3.5. Effect of variation of crosslinker concentration on release profile [**a**: polymer = 2.857 g, crosslinker = 5 mmol, NSO = 3.0 g, **b**: polymer = 2.857 g, crosslinker = 12.5 mmol , NSO = 3.0 g **c**: polymer = 2.857 g, crosslinker = 20 mmol, NSO = 3.0 g]

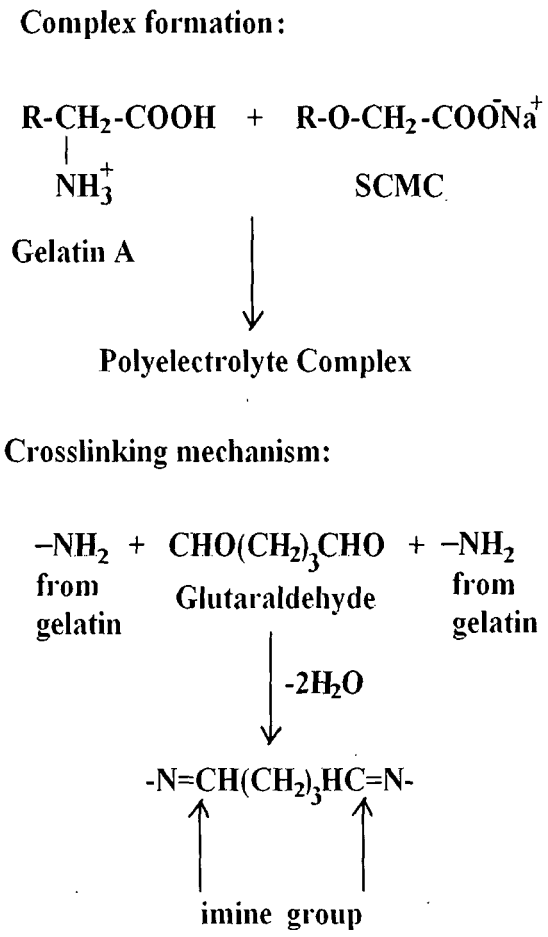

Figure 4.1.3.6. Probable reaction scheme for interaction between SCMC and gelatin and glutaraldehyde with SCMC-gelatin complex.

4.1.3.6. Effect of variation of polymer concentration

Table 4.1.3.1 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 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 Figure 4.1.3.7. 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 behavior [10].

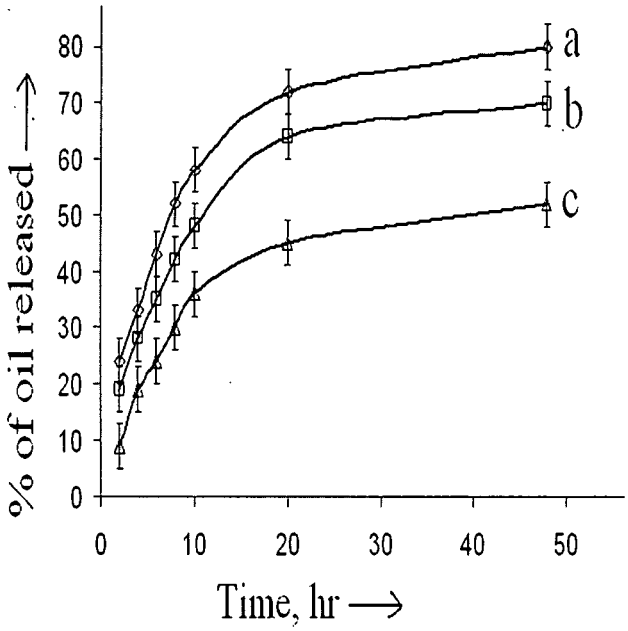

Figure 4.1.3.7. Effect of variation of polymer concentration on release profile [**a**: polymer = 0.714 g, crosslinker = 5 mmol, NSO 3.0 g, **b**: polymer = 2.857 g, crosslinker = 5 mmol , NSO = 3.0 g, **c**: polymer = 5.714 g, crosslinker = 5 mmol, NSO = 3.0 g]

4.1.3.7. Fourier transform infrared (FTIR) study

The spectra of SCMC (curve-a), gelatin-A (curve-b), SCMC-gelatin complex (curve-c), NSO (curve-d), NSO loaded crosslinked SCMC-gelatin microcapsules (curve-e) are shown in Figure 4.1.3.8. The spectrum of SCMC showed absorption bands at 3364 cm^{-1}, 2942 cm^{-1}, 1627 cm^{-1}, 1422 cm^{-1} and 1063 cm^{-1} which were due to O-H stretching vibration, CH_3 symmetric+ CH_2 assymetric vibration, C-O stretching band for cellulose, CH_3 + CH_2 bending vibration and strong C-O stretching band for ethers. 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 1600-1700 cm^{-1} is the most important peak for IR analysis of the secondary structure of protein like gelatin [17]. In the complex of gelatin and SCMC, a slight shift of the peak of amide I from 1630.44 cm^{-1} to 1628.25 cm^{-1} was observed. This indicated that the negatively charged carboxy methyl groups might associate with positively charged gelatin. Similar type of observation was reported by Pranoto et al. [18] while studying the interaction between carrageenan and gelatin. The absorption band appeared in the spectrum of NSO at 1743 cm^{-1}, 1456 cm^{-1} and 1163 cm^{-1} were due to the carbonyl stretching, CH_2

assymetric 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 SCMC gelatin complex.

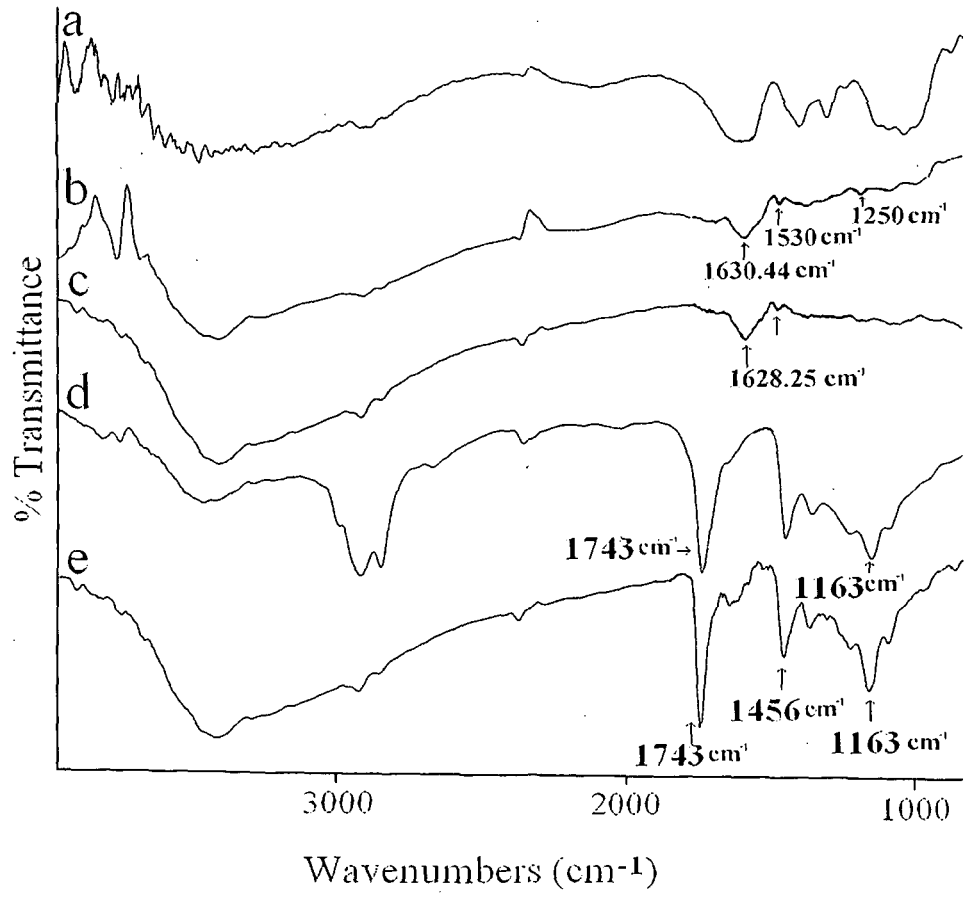

Figure 4.1.3.8. FTIR spectra of **a**) SCMC **b**) gelatin **c**) gelatin-SCMC complex **d**) NSO **e**) NSO loaded microcapsules

4.2. Microencapsulation of Isoniazid, an antitubercular drug in three different polyelectrolyte complex systems-namely (i) chitosan-κ-carrageenan (ii) κ-carrageenan-gelatin A (iii) gelatin A-sodium carboxy methyl cellulose.

In this part of work, the polymer systems optimized in *Part-I* were used for preparation of microcapsules/nanocapsules as well as microparticles by different methods for encapsulation of drug, isoniazid. The variations in the size of the micro/nano capsules or microparticles with variation in surfactant (tween 80) concentrations were discussed. The release behaviors of drug from the microparticles and nanocapsules at two different pH media 1.2 and 7.4 with different crosslinker concentrations have been explained.

4.2.1. Chitosan-κ-carrageenan system for encapsulation of Isoniazid

4.2.1.1. Particle size analysis

Effects of variation of surfactant amount on the average particle size of the capsules are shown in Table 4.2.1.1. 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 [21] with the increase in the concentration of surfactant and sonication time.

Table 4.2.1.1. Effect of variation of amount of surfactant on particle size [Carrageenan : 0.35g; Chitosan : 0.13g; NSO: 1g; Sonication:90 minutes; Temperature: 60±1^{0}C,Total volume:136ml]

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

4.2.1.2. Transmission electron microscopy study

Transmission electron micrographs of NSO encapsulated samples are shown in Figure 4.2.1.1. Micrograph (a) represented the capsules without addition of surfactant. Micrographs fig.4.2.1.1 (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/100ml of polymer solution. Micrograph for the sample prepared by using surfactant and without any sonication was shown in fig. 1b. The micrograph shown in fig. 4.2.1.1e was magnified further in order to get some clear idea about the size of the capsule. This was shown in fig. 1f. 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 (fig.4.2.1.1 c-e). The calculated diameter of a randomly chosen nanocapsule was 230 nm, which was close to that of average particle size (234nm) 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 little brighter. Similar

observation was reported by Navi et al. [22] 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 was in agreement with the results obtained from particle size analyzer.

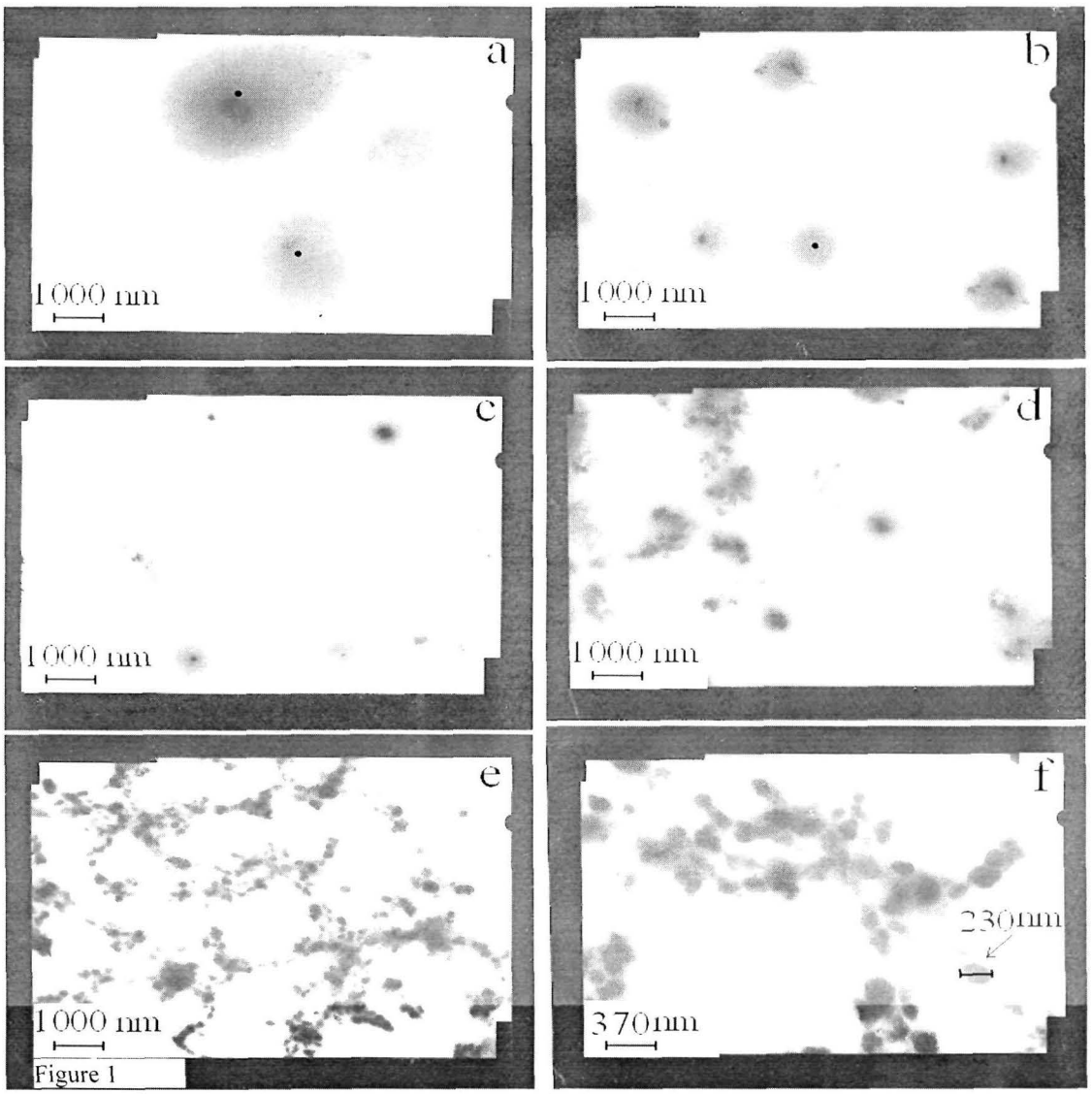

Figure 4.2.1.1. Transmission electron micrographs of NSO loaded nanocapsules prepared (a) without surfactant (b) 0.1g Tween 80 (c) 0.1g Tween 80 and sonication (d) 0.2g Tween 80 and sonication (e) 0.3g Tween 80 and sonication (f) 0.3g Tween 80 and sonication (higher magnification)

4.2.1.3. 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 4.2.1.2. 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 was 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 to till attainment of equilibrium governed the encapsulation (%).

Table 4.2.1.2 : Effect of variation of immersion time in isoniazid solution and concentration of crosslinker on percent encapsulation [Carrageenan:0.35g; Chitosan:0.13g; NSO:1g; Genipin:0.1-0.4 mmol; Total volume:136ml; Temperature:30±1^{0}C]

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

4.2.1.4. Effect of variation of cross-linker concentration

The effect of variation of cross-linker concentration on encapsulation (%) and release rate is shown in the Table 4.2.1.2 and Figure 4.2.1.2. The encapsulation (%) increased as the

concentration of genipin was varied from 0.1 to 0.4 mmol. The increase in encapsulation (%) could be due to the improvement of drug retention capacity of the nanocapsules caused by the formation of crosslinking. The crosslinking reaction took place between genipin and polyelectrolyte complex of carrageenan and chitosan. Further the percent encapsulation increased initially at a rapid rate and later at a slow rate. As the concentration of the crosslinker increased, the crosslinking reaction approached towards saturation due to which encapsulation decreased at later stage. The release rate of 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 six hours, and then almost leveled off. The nanocapsule wall became compact as degree of crosslinking increased. This resulted in the decrease of diffusion rate through the nanocapsule wall [10]. The swelling capacity of the capsules decreased as crosslinker increased. Agnigotri et al. reported similar type of phenomenon of swelling of chitosan microparticles [23]. The decrease in release rate could be explained by considering the combining effect of swelling and diffusion.

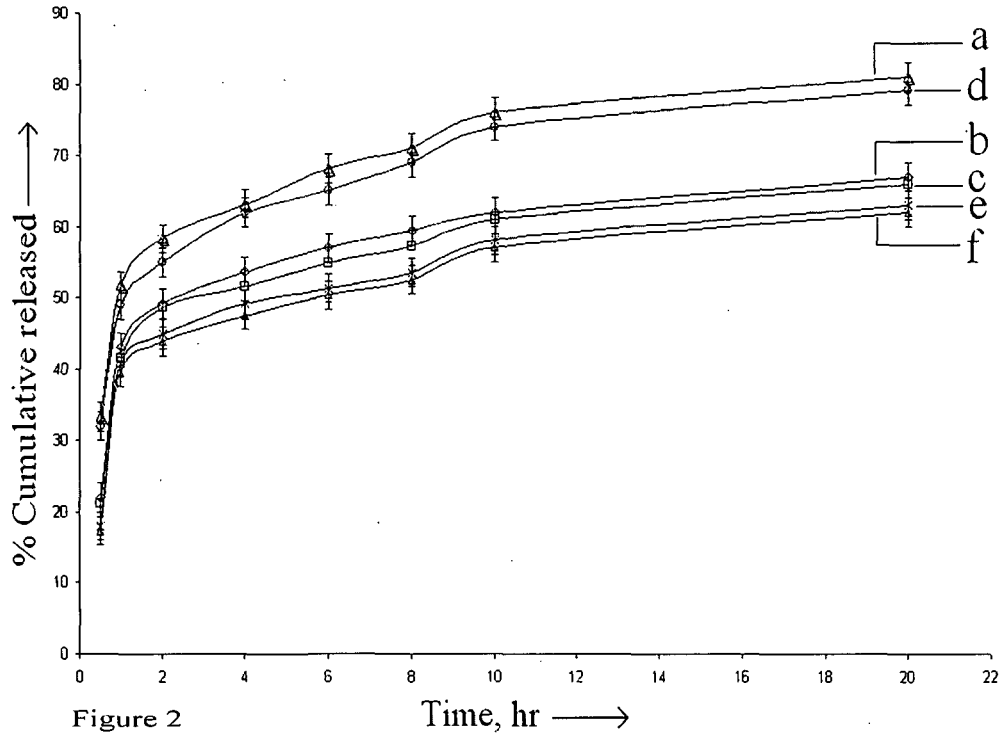

Figure 2

Figure 4.2.1.2. Effect of variation of crosslinker concentration and pH of the medium on release rate of isoniazid (a) 0.1mmol genipin, pH=1.2 (b) 0.2mmol genipin, pH=1.2 (c) 0.4mmol genipin, pH=1.2 (d) 0.1mmol genipin, pH=7.4 (e) 0.2mmol genipin, pH=7.4 (f) 0.4mmol genipin, pH=7.4

4.2.1.5. Effect of variation of pH

Figure 4.2.1.2 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 rate were fast initially upto 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 polyeletrolyte complex, the swelling did not significantly changed on altering the pH of the system as reported by Piyakulawat et al. [11]. The swelling of chitosan and carrageenan gel was not occurred below pH of 9.0 [24]. Thus the difference in release profile in both 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 was reported in the literature [25]. Therefore, the higher release rate of isoniazid at acidic pH may be attributed to its higher solubility at lower pH.

4.2.1.6. Fourier transform infrared (FTIR) study

FTIR spectra of neat polyelectrolyte complex of chitosan and carrageenan (curve a), NSO (curve b), NSO loaded chitosan-carrageenan nanocapsules (curve c), NSO free nanocapsules (curve d), isoniazid (curve e) and isoniazid loaded chitosan-carrageenan nanocapsules (curve f), were shown in Figure 4.2.1.3. In curve (a), the band appeared at 1528 cm^{-1} due to NH_3^+ groups confirmed the formation of strong polyelectrolyte complex between chitosan and carrageenan. The absorption bands appeared 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) were 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 (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), suggested the successful loading of isoniazid in the hollow nanocapsules. Similar type of IR spectral pattern for isoniazid and isoniazid containing capsules were reported by Kim et al.[26].

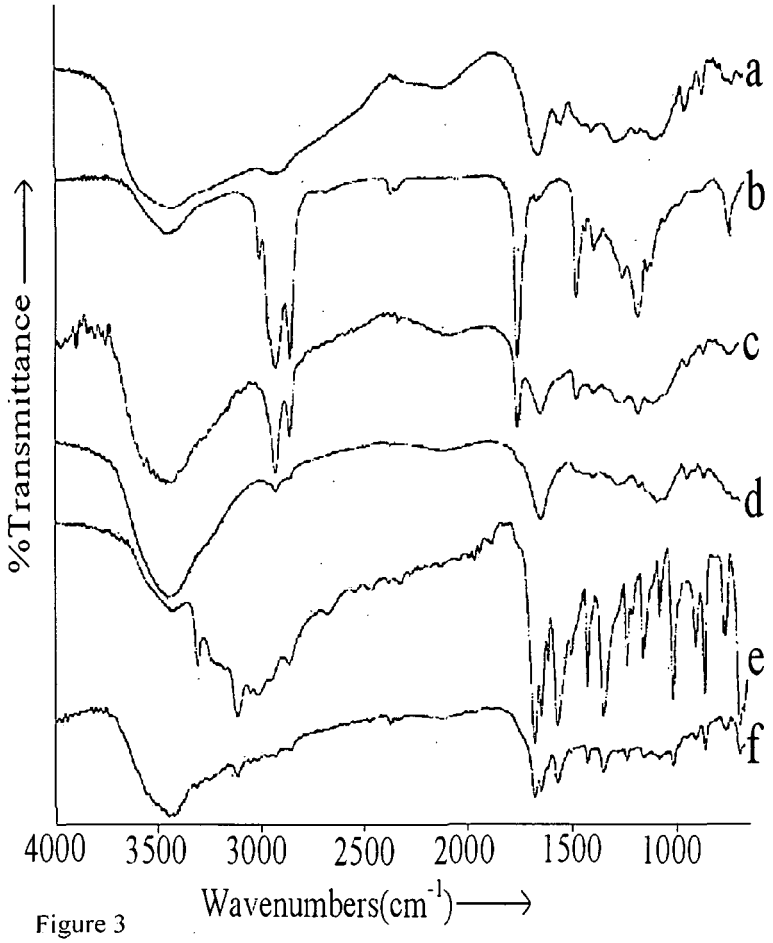

Figure 3

Figure 4.2.1.3. 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.

4.2.1.7. Scanning electron microscopy study

The scanning electron micrographs of the samples prepared by using varying amount of surfactants (Tween 80, 0.1g-0.3g) are shown in Figure 4.2.1.4 (a-c). Figure-d represents capsules after removal of NSO. Figure-e shows the micrograph of isoniazid loaded capsules.The size of the capsules were found to decrease as the amount of surfactant increased.TEM study also confirmed these findings. Micrograph -c shows the capsules in agglomerated form. Figure-d represents the micrograph of the sample after complete removal of NSO. In this case the agglomeration was lost but the capsules seemed to be little collapsed and deformed after the removal of NSO. Figure-e was the micrograph of the sample loaded with isoniazid. The surface of isoniazid loaded nanocapsules appeared to be collapsed. Similar type of observation was reported in the literature [27].

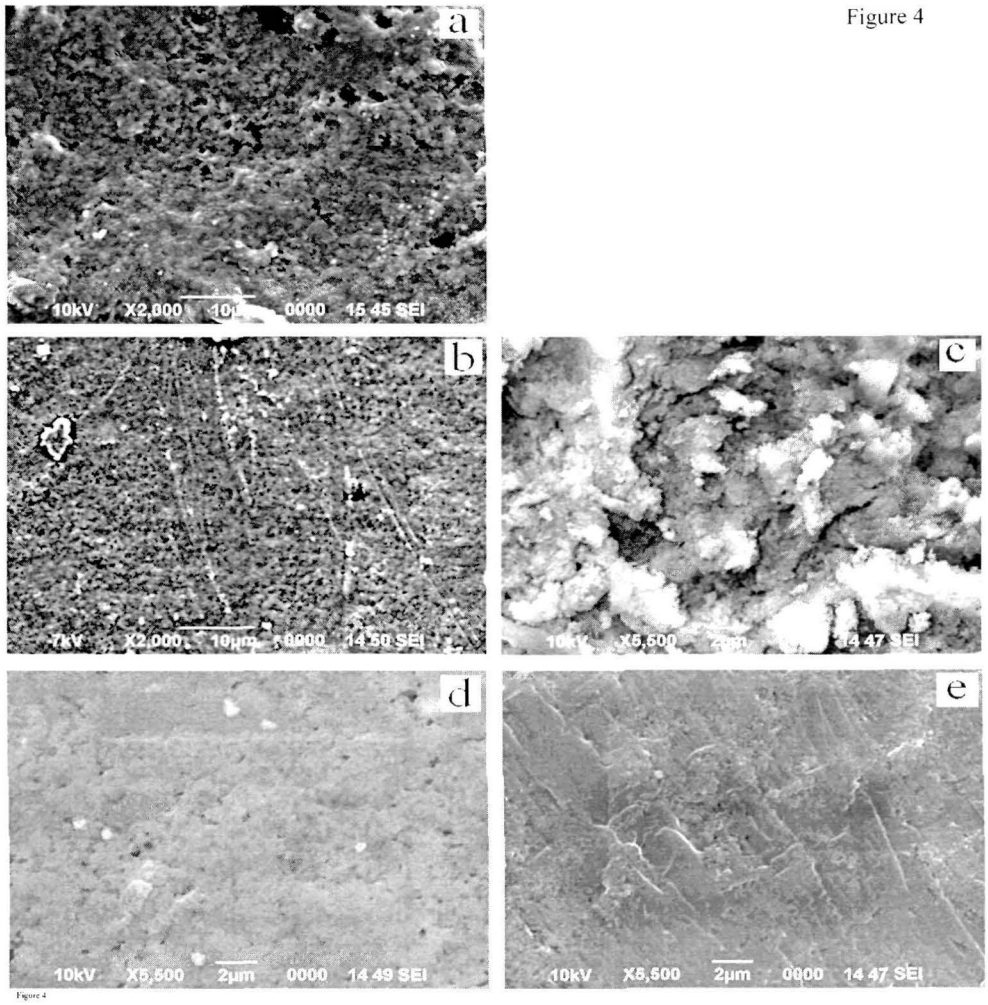

Figure 4.2.1.4. Scanning electron micrographs of NSO loaded nanocapsules prepared by using (a) 0.1g Tween 80 and sonication (b) 0.2g Tween 80 and sonication (c) 0.3g Tween 80 and sonication (d) nanocapsules after removal of NSO (e) isoniazid loaded nanocapsules

4.2.1.8. 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 4.2.1.5. 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 2θ = 11.8, 16.8,25.4 and 34.2. Fukuoka et al. also reported the X-ray diffractogram of isoniazid having the peaks in this region [28]. 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.

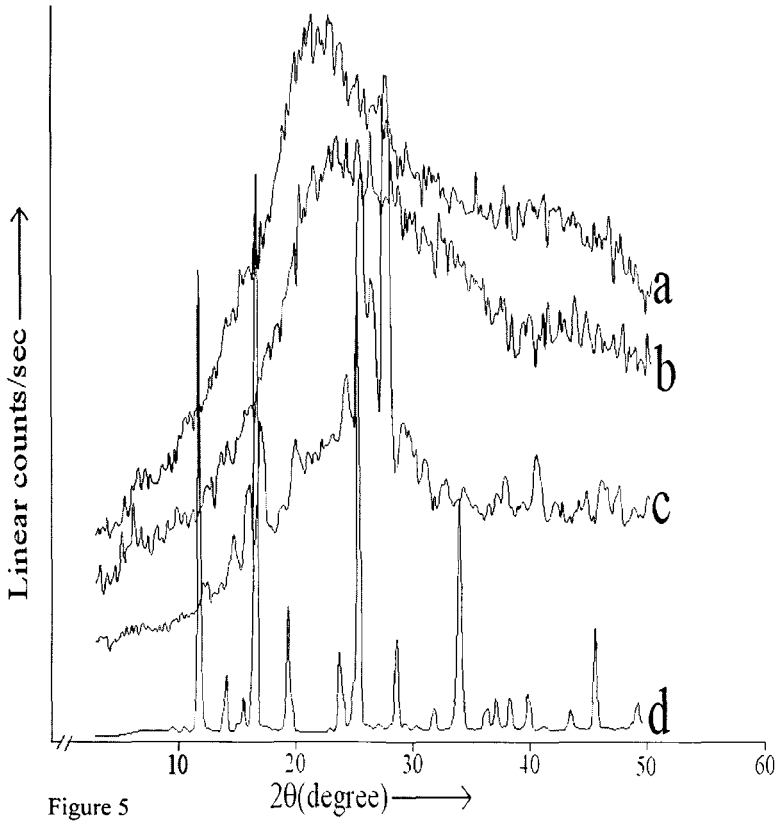

Figure 5

Figure 4.2.1.5. X-ray diffractograms of (a) NSO loaded nanocapsules (b) NSO free nanocapsules (c) isoniazid encapsulated nanocapsules (d) isoniazid

4.2.2. κ-carrageenan- gelatin-A system for encapsulation of Isoniazid

4.2.2.1. 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 4.2.2.1 represents the SEM 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 0.33g to 1.0 g/g of polymer (fig.a-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 due to the higher activity of the surfactant. This would lead to decrease the interfacial free energy of the system and provide mechanical barrier to coalescence [29]. Interfacial polymerization of polyisocyanates produced smaller microcapsules of polyurea by using higher concentration of emulsifier was reported [16].

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

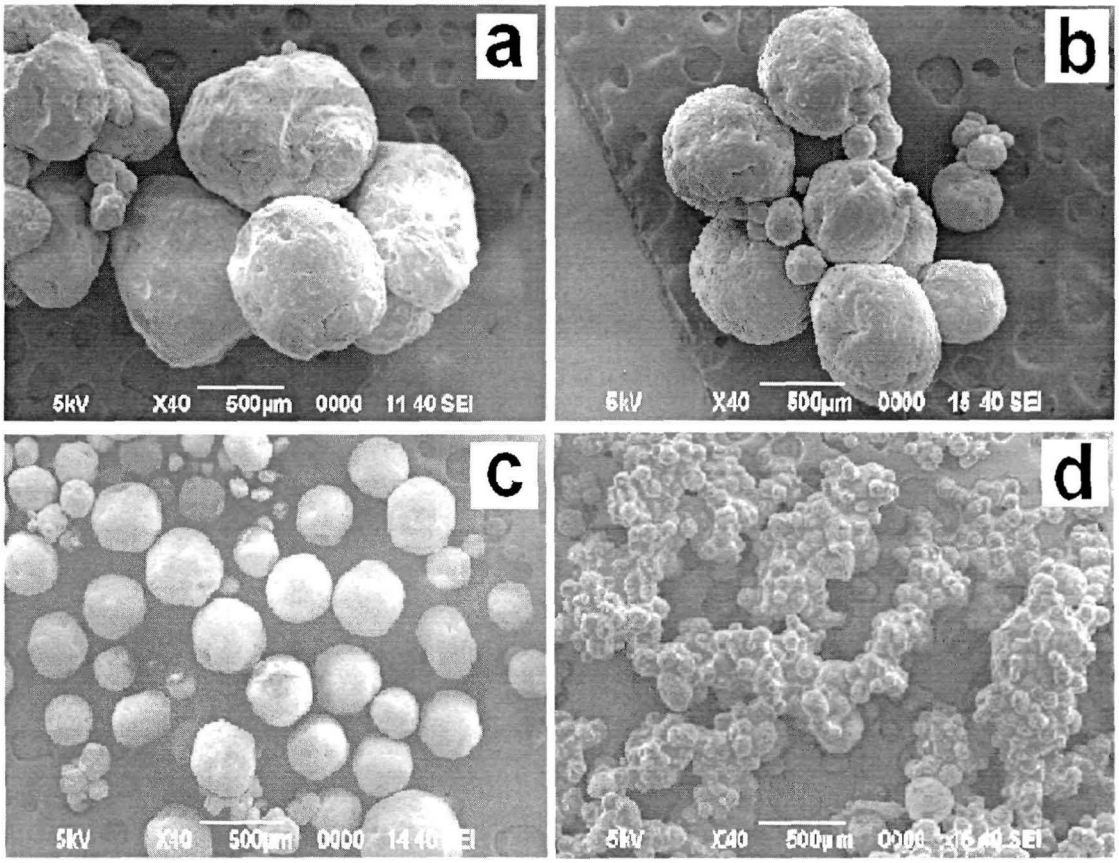

Figure 4.2.2.1. Scanning electron micrographs of microcapsules prepared by using **(a)** tween 80 **:** 0.33g/g of polymer; polymer :3.0 g **(b)** tween 80 **:** 0.55g/g of polymer; polymer: 3.0g **(c)** tween 80 **:**1.0g/g of polymer; polymer: 3.0 g **(d)** tween 80 **:**1.0g/g of polymer; polymer: 1.5g

4.2.2.2. Swelling and stability study

The plot of variation of swelling ratio of microsphere against time of immersion is shown in Figure 4.2.2.2. 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 due to the decomplexation between carrageenan and gelatin. Similar observations were reported by Liu et al. during studying the swelling of gelatin-DNA semi-interpenetrating network at different pH [30]. Further, microsphere with higher crosslinking showed lower swelling ratio. The lower swelling ratio might be due to the formation of more compact wall caused by the formation of

crosslinking [23]. The probable interaction between carrageenan and gelatin and crosslinking is shown in Figure 4.2.2.3.

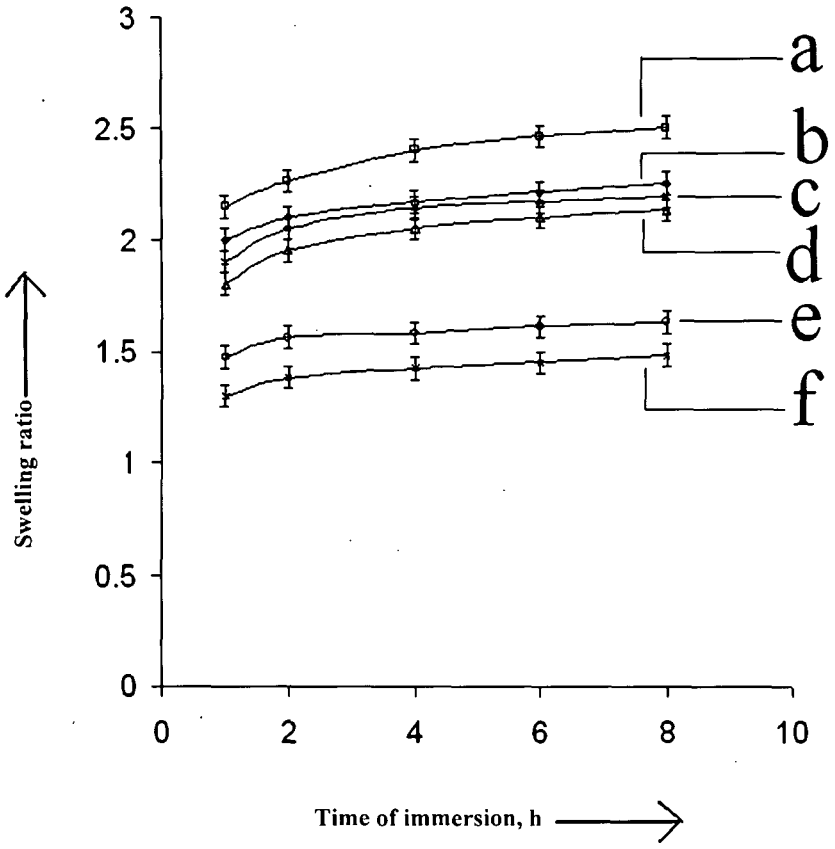

Figure 4.2.2.2. Effect of variation of time of immersion on swelling ratio of microspheres [At pH: 7.4, polymer: 3.0 g **a:** crosslinker: 0.2 mmol; **c**: crosslinker: 0.4 mmol; **e**: crosslinker: 0.8 mmol. At pH:1.2, polymer: 3.0 g **b:** crosslinker: 0.2 mmol; **d**: crosslinker: 0.4 mmol; **f**: crosslinker: 0.8 mmol]

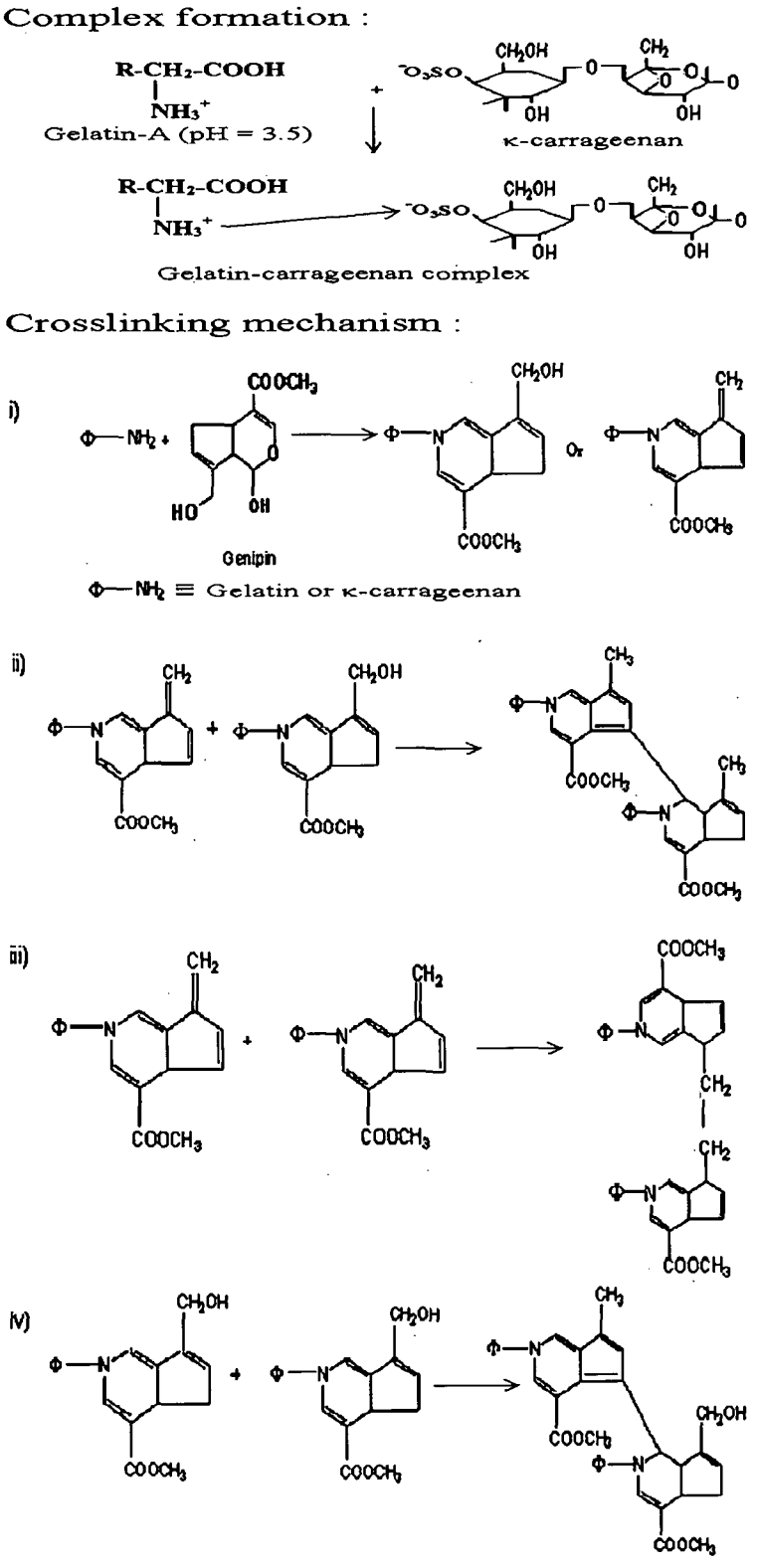

Figure 4.2.2.3. Probable reaction scheme for interaction between carrageenan and gelatin, and genipin with carrageenan-gelatin complex.

4.2.2.3. 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 4.2.2.1. 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 due to 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 [25]. Higher percent encapsulation and basic nature of isoniazid might help to decompose rapidly the carrageenan-gelatin complex which in turn would decrease the stability of the microspheres.

Table 4.2.2.1. Effect of variation of isoniazid concentration and crosslinking on stability of microspheres

Isoniazid concentration(w/v) %	Amount of genipin (mmol)	Stability of the microspheres (min)
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

The effect of variation of isoniazid concentration on percent encapsulation is shown in Table 4.2.2.2. 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 due to the more absorption of isoniazid. The decrease in encapsulation (%) might be attributed to the formation of more compact wall in the microspheres due to crosslinking, which led to decrease in the diffusion rate of isoniazid.

Table 4.2.2.2. Effect of variation of isoniazid concentration on percent encapsulation

Amount of polymer(g)	Concentration of isoniazid (%) (w/v)	Time of immersion (min)	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

4.2.2.4. Effect of variation of cross-linker concentration on release rate of isoniazid

The effect of variation of cross-linker concentration (0.067-0.267 mmol/g of polymer), on release rate at pH=1.2 and 7.4 are shown in Figure 4.2.2.4 and Figure 4.2.2.5 respectively. Microspheres having approximately similar loading of isoniazid were considered for the study of the release rate at different pH.

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

Further, the amount of isoniazid release at lower pH (pH=1.2) was less compared to that of at higher pH (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.

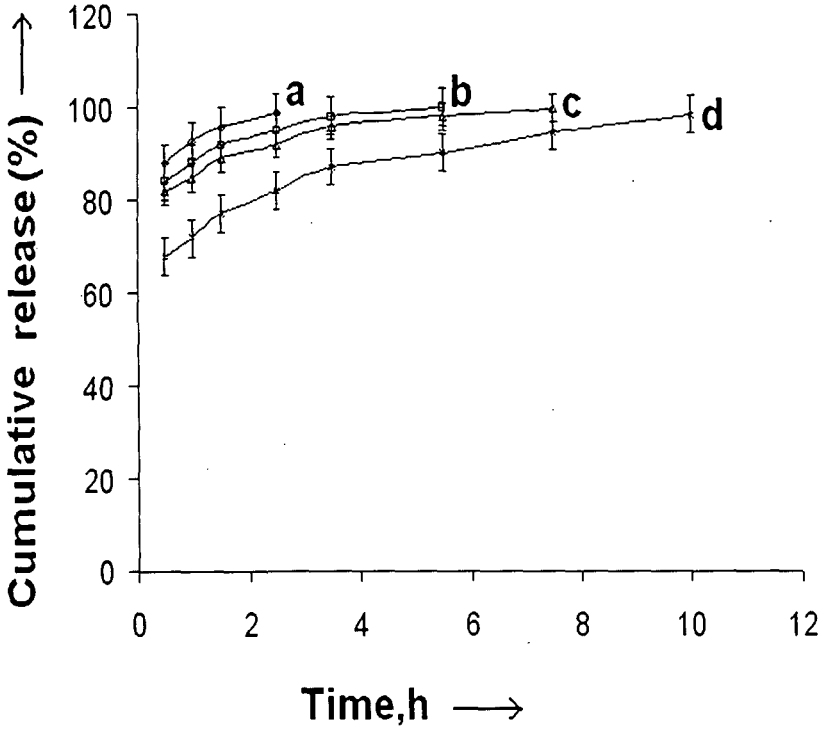

Figure 4.2.2.4. Effect of variation of crosslinker concentration on release profile at pH: 1.2 [**a**: polymer: 3.0 g; crosslinker: 0 mmol, **b**: polymer: 3.0 g; crosslinker: 0.2 mmol **c**: polymer: 3.0 g; crosslinker: 0.4 mmol, **d**: polymer: 3.0 g; crosslinker: 0.8 mmol]

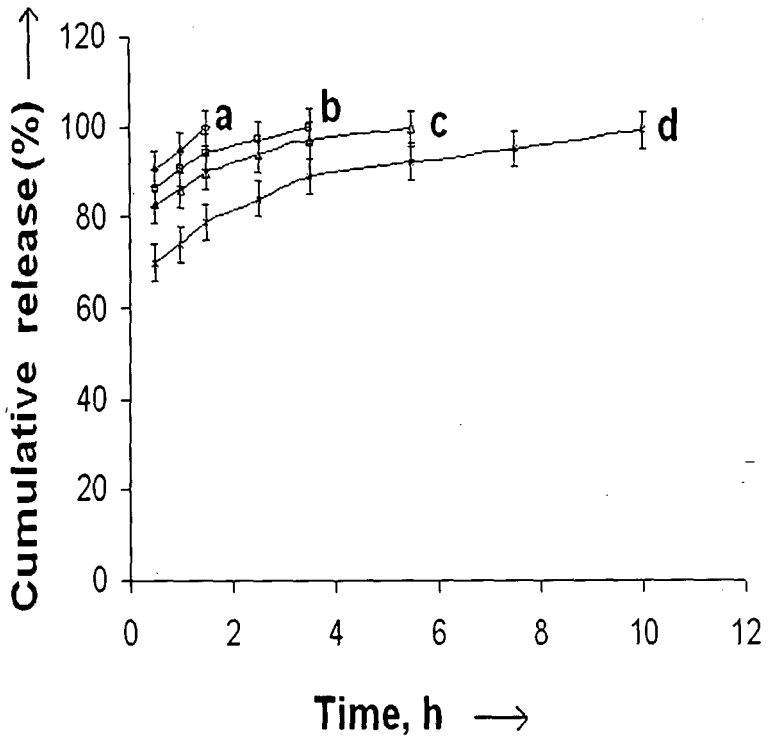

Figure 4.2.2.5. Effect of variation of crosslinker concentration on release profile at pH: 7.4 [**a**: polymer: 3.0 g; crosslinker: 0 mmol, **b**: polymer: 3.0 g; crosslinker: 0.2 mmol **c**: polymer: 3.0 g; crosslinker: 0.4 mmol, **d**: polymer: 3.0 g; crosslinker: 0.8 mmol]

4.2.2.5. Fourier transform infrared (FTIR) study

The spectra of carrageenan (curve-a), gelatin-A (curve-b), carrageenan-gelatin complex (curve-c), Isoniazid (curve-d), isoniazid loaded crosslinked carrageenan-gelatin microspheres (curve-e) are shown in Figure 4.2.2.6. The spectrum of carrageenan showed absorption bands at 3423 cm^{-1}, 2910 cm^{-1}, 1643 cm^{-1}, 1434 cm^{-1}, 1379 cm^{-1}, 1265 cm^{-1} and 846 cm^{-1} which were due to O-H stretching vibration, CH_3 symmetric+ CH_2 assymetric vibration, N-H bending, 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 3421 cm^{-1} (NH- stretching), 1630.44 cm^{-1} (amide-I, CO and CN stretching),1530 cm^{-1} (amide-II), and 1250 cm^{-1} (amideIII). Among the absorption bands, the amide I band between 1600-1700 cm^{-1} is the most important peak for IR analysis of the secondary structure of protein like gelatin [17]. In the complex of gelatin and carrageenan, a slight shift of the peak of amide I from 1630.44 cm^{-1} 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* [18].The probable interaction between carrageenan and gelatin is shown in Figure 4.2.2.3. A shift 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 [19]. However this type of shifting was not observed in this case. In the spectrum (shown as curve-d) 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 due to N-H bending of the secondary amide group. Moreover, multiple bands appeared between 1407 cm^{-1} to 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 IR spectral pattern for isoniazid and isoniazid containing capsules were reported by Kim et al. [26].

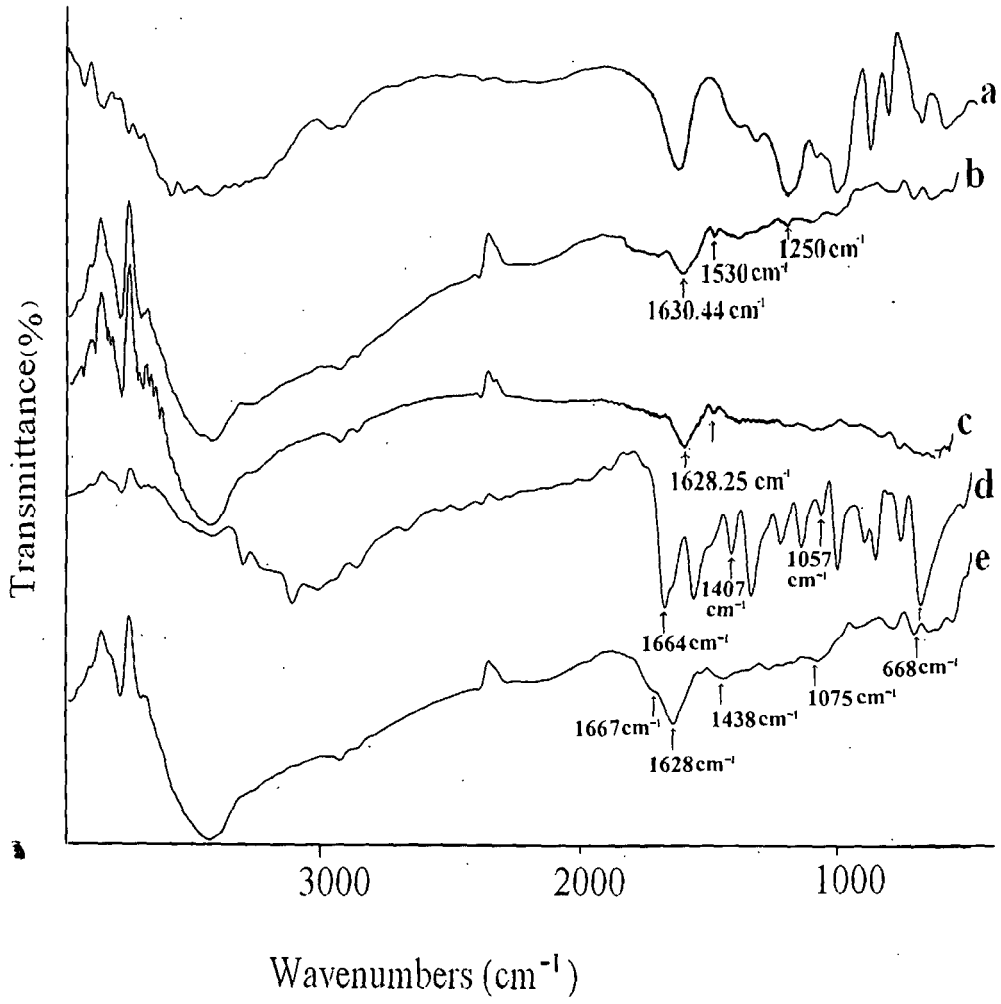

Figure 4.2.2.6. FTIR spectra of **a)** carrageenan **b)** gelatin **c)** gelatin-carrageenan complex **d)** isoniazid **e)** isoniazid loaded microcapsules.

4.2.2.6.Thermal property study

DSC thermograms of carrageenan-gelatin microspheres (curve-a), isoniazid (curve-b), isoniazid loaded microspheres (curve-c) are shown in Figure 4.2.2.7. The endotherm appeared in all the thermograms except isoniazid at around 100 ^{0}C were due to removal of moisture. The thermograms of isoniazid showed an endothermic peak due to melting at around 190 ^{0}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 [31].

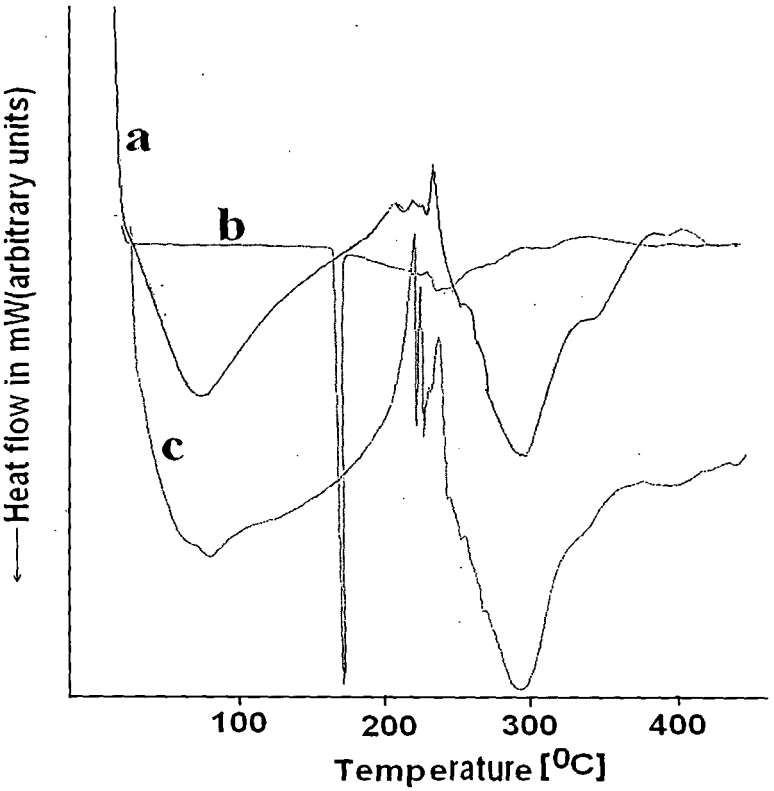

Figure 4.2.2.7. DSC thermograms of **(a)** carrageenan-gelatin complex, **(b)** isoniazid **(c)** isoniazid loaded microcapsules

4.2.2.7. X-ray diffraction studies

X-ray diffractograms of carrageenan-gelatin microspheres (curve-a), isoniazid loaded microspheres (curve-b), isoniazid (curve-c) are shown in Figure 4.2.2.8. Isoniazid showed multiple sharp peaks at 2θ varying from 12 to 50^0 which were due to the crystalline nature of

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 [31].

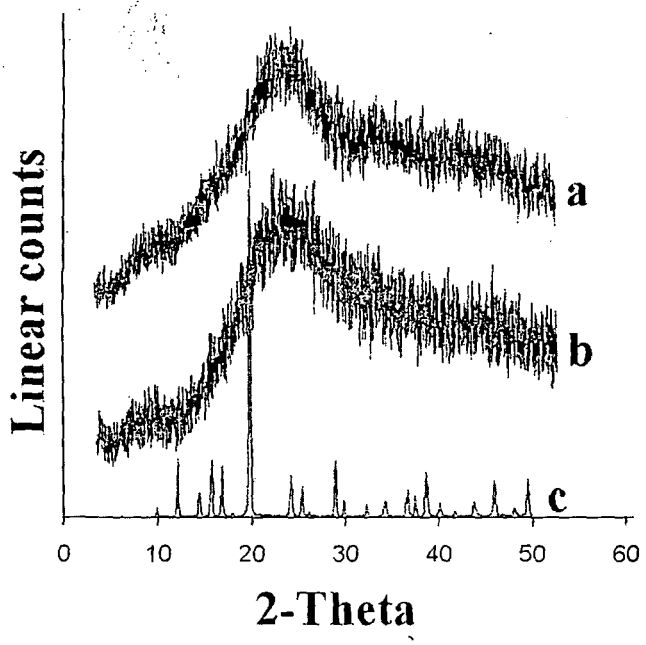

Figure 4.2.2.8. X-ray diffractograms of **(a)** carrageenan-gelatin microspheres **(b)** isoniazid loaded microspheres **(c)** pure isoniazid .

4.2.3. Gelatin A-SCMC system for encapsulation of Isoniazid

4.2.3.1. Effect of variation of amount of surfactant and polymer concentration on size of the microparticles

The formation and particle size of each micro particle depends on the size of the dispersed droplet, which is determined by the surfactant (tween 80) used and the emulsifying conditions. For the system of SCMC-gelatin, surfactant tween-80 had important role in stabilizing the micro particles formed in sunflower oil. A matrix gel like product was formed if surfactant was not added. But different sizes of micro particles were formed on addition of varying amount of surfactant. SEM photographs of the micro particles are shown in Figure 4.2.3.1. With the increase of amount of tween-80 from 0.22g to 1.16 g/g of polymer, the sizes

of the micro particles decreased as shown in Figure (a-c). At higher concentration of surfactant, the aqueous phase is easily dispersed into finer droplets, owing to the higher activity of the surfactant, which would result in a lower free energy of the system, and lead to a smaller particle size. Landfester also reported the same type of phenomena that with the increase of surfactant, the size of the droplets in miniemulsions decreased [21].

Again, with increase of amount of polymer from 0.857g to 1.714 g, an increase in the size of the micro particles was observed (Figure c-e). In the presence of higher amount of polymer, the surfactant present might not be capable of covering all the surfaces of the micro particles properly. This resulted in the coalescence of some of the micro particles and led to the formation of larger micro particles. Besides this, the dispersive force of the stirrer became less efficient in presence of higher amount of polymer and as a result larger micro particles formed (Figure c-e).

Stirring speed also affected the nature and size of the micro particles. At low stirrer speed (200 rpm approx.), the agglomeration of particles was more (Figure f) compared to those of particles produced at higher stirrer speed (1500 rpm approx) (Figure d). Improper mixing of polymers at low stirrer speed might be responsible for the observed agglomeration. Zhuo et al. reported similar observations during studying the particle size of polyurea microcapsules by interfacial polymerisation of polyisocyanates [16].

All the micro particles were spherical in shape except the particles produced at low stirrer speed. The microparticles arising from low stirrer speed had no definite structure probably due to agglomeration. Again some roughness appeared on the surfaces of all the micro particles (Figure a-d). The surface of the micro particles prepared at higher polymer and surfactant concentration (Figure e) showed least roughness compared to those of other particles.

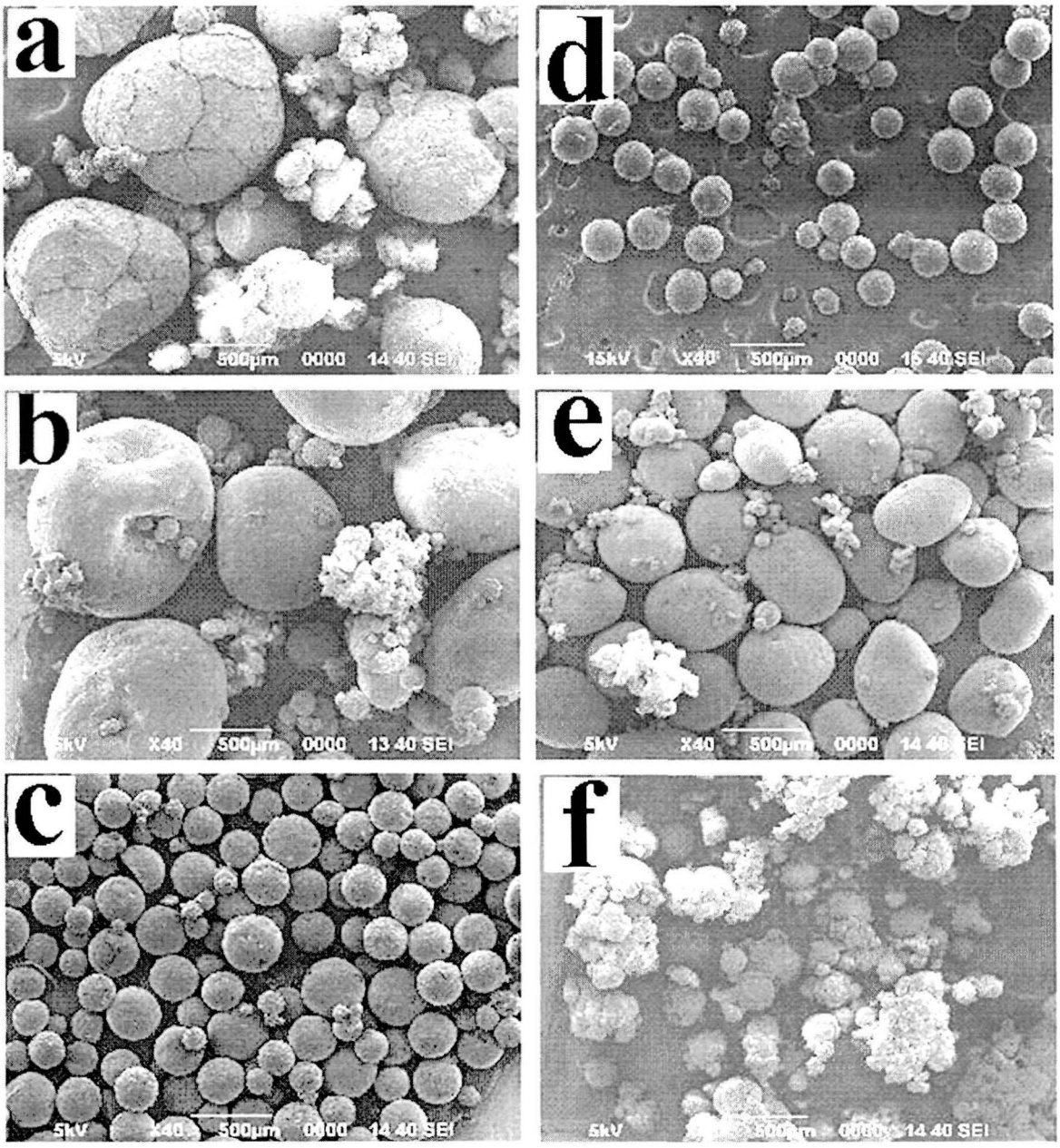

Figure 4.2.3.1. Scanning electron micrographs of microparticles prepared by using **(a)** tween 80 =0.22g/g of polymer, total polymer = 0.857.0 g **(b)** tween 80 =0.46g/g of polymer, total polymer = 0.8570 g **(c)** tween 80 =1.16g/g of polymer, total polymer = 0.857g **(d)** tween 80 =1.16g/g of polymer, total polymer =1.428g **(e)** tween 80 =1.16g/g of polymer, total polymer =1.714g **(f)** tween 80 = 1.16g/g of polymer, total polymer =0.857(low stirrer speed)

4.2.3.2. Water uptake study

The water uptake of micro particles was plotted against time of immersion in water (Figure 4.2.3.2). The water uptake increased rapidly upto 2 hour, then slowly till 5 hours as shown and finally almost levelled off. Micro particles with higher crosslinking showed lesser water uptake than the micro particles with low crosslinking. This was due to the formation of more compact wall [23] caused by crosslinking. Probable reaction scheme for complexation between the two polymers and crosslinking with glutaraldehyde is shown in Figure 4.2.3.3. Again, water uptake was more at higher pH compared to lower pH. Microparticles formed by the complexation between gelatin A and SCMC became more stable probably at lower pH. At higher pH, the tendency to decomplexation between gelatin and SCMC might be responsible for the higher water uptake capacity. Similar findings were reported by Liu et al. [30] during studying the swelling behaviour of gelatin-DNA semi-interpenetrating polymer network at different pH.

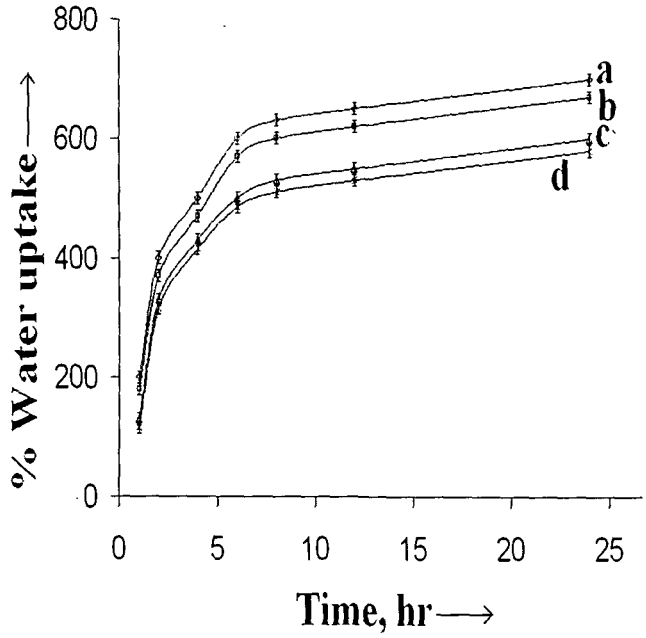

Figure 4.2.3.2. Effect of variation crosslinker amount and pH on water uptake [**a**: polymer = 2.857 g; crosslinker = 4.375 mmol/g of polymer; pH=7.4, **b**: polymer = 2.857 g; crosslinker =4.375 mmol/g of polymer; pH=1.2 , **c**: polymer =2.857 g; crosslinker = 17.50 mmol/g of polymer; pH=7.4, **d**: polymer=2.857 g; crosslinker = 17.50 mmol/g of polymer; pH=1.2.

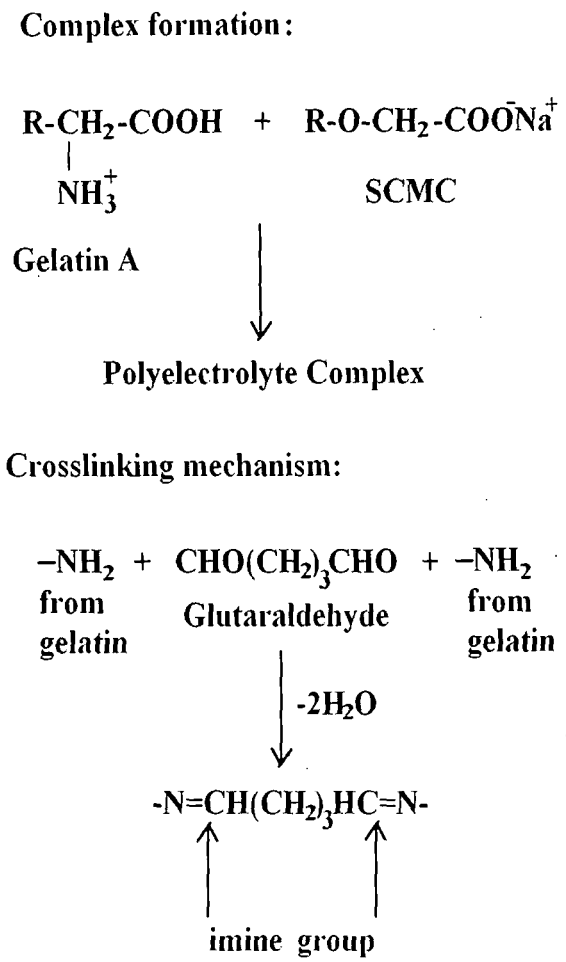

Figure 4.2.3.3. Probable reaction scheme for interaction between SCMC and gelatin and glutaraldehyde with SCMC-gelatin complex.

4.2.3.3. Effect of variation drug concentration and immersion time on loading efficiency

The effect of variation of concentration of isoniazid and immersion time of micro particles on loading efficiency is shown in Table 4.2.3.1. At a fixed immersion time, the loading efficiency was found to increase with the increase in the concentration of isoniazid. An increase in loading efficiency was also observed as immersion time increased. Again, higher the amount of crosslinker in the micro particles, the lower was the loading efficiency. The increase in loading efficiency was due to the more diffusion of isoniazid into the micro particles. The decrease in loading efficiency might be attributed to the formation of more compact wall due to crosslinking that led to decrease in diffusion rate of isoniazid.

Table 4.2.3.1. Effect of variation of isoniazid concentration and immersion time on loading efficiency.

Concentration of glutaraldehyde (mmol)/g of polymer	Concentration of isoniazid g/100ml	Time of immersion (h)	Loading efficiency (%)
4.375	0.5	1.5	0.33 ± 0.01
	1.0		0.99 ± 0.12
	3.0		14.33 ± 0.11
	5.0		28.66 ± 0.17
	7.0		44.0 ± 0.21
	10.0		51.0 ± 0.43
	20.0		58.11 ± 0.55
10.50	0.5	1.5	0.33 ± 0.01
	1.0		0.66 ± 0.11
	3.0		14.0 ± 0.16
	5.0		23.11 ± 0.18
	7.0		40.21 ± 0.35
	10.0		46.33 ± 0.39
	20.0		50.00 ± 0.52
17.50	0.5	1.5	0.33 ± 0.01
	1.0		0.66 ± 0.13
	3.0		14.0 ± 0.32
	5.0		22.0 ± 0.47
	7.0		38.79 ± 0.58
	10.0		42.11 ± 0.59
	20.0		46.23 ± 0.63
17.50	5.0	0.5	19.0 ± 0.25
		1.0	28.0 ± 0.31
		2.0	35.0 ± 0.42
		4.0	36.0 ± 0.41
		6.0	36.45 ± 0.35
		8.0	37.12 ± 0.32
		17.0	46.0 ± 0.46
		28.0	58.0 ± 0.38
		48.0	58.50 ± 0.31
4.375	20.0	48.0	63.2 ± 0.53
10.50	20.0	48.0	62.0 ± 0.61
17.50	20.0	48.0	60.7 ± 0.50

Again, at a fixed isoniazid concentration, the loading efficiency increased with time of immersion upto a certain time and after that it remained constant. But, high and low crosslinked micro particles showed more or less similar loading efficiency when immersed in similar concentration of isoniazid solution for a longer period. Longer immersion time allowed the micro particles to become saturate with isoniazid solution.

4.2.3.4. Effect of variation of cross-linker on release rate of isoniazid

The effect of variation of crosslinker concentration (4.375-17.50 mmol/g of polymer) on release rate at pH 1.2 and 7.4 is shown in Figure 4.2.3.4. Micro particles having approximately similar loading were chosen for the study of the release rate at different pH. The release rate of isoniazid was found to decrease with the increase in the amount of crosslinker in the microparticles. In all the cases, the release was fast initially, reaching maximum and levelled off finally. The compact microparticle wall was responsible for the decrease in release rate as explained earlier.

Further, the percentage of isoniazid release at lower pH (pH=1.2) was less compared to that of at higher pH (pH=7.4). The lower and higher release rate in lower and higher pH respectively might be explained by considering the tendency of complexation and decomplexation between gelatin A and SCMC as discussed earlier.

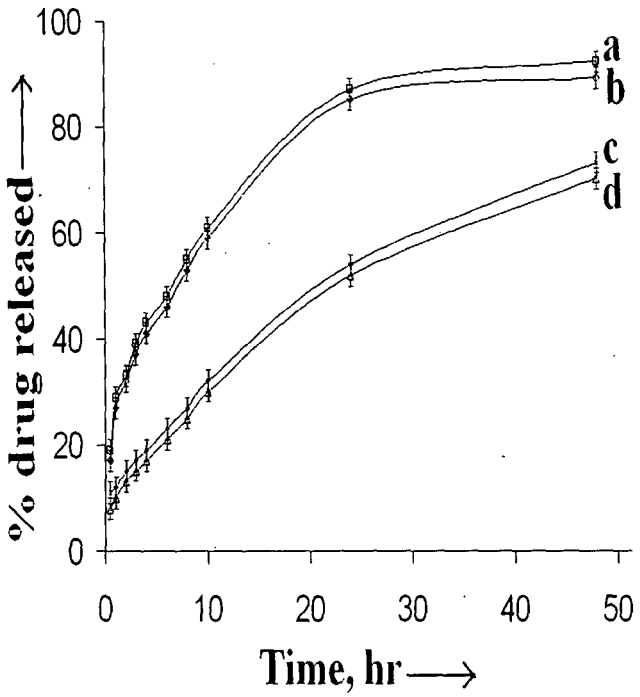

Figure 4.2.3.4. Effect of variation of crosslinker concentration and pH on release profile [**a**: polymer = 2.857 g; crosslinker = 4.375 mmol/g of polymer; pH =7.4, **b**: polymer =2.857 g; crosslinker = 4.375 mmol/g of polymer; pH=1.2 , **c**: polymer =2.857 g; crosslinker = 17.50 mmol/g of polymer; pH=7.4, **d**: polymer =2.857 g; crosslinker = 17.50 mmol/g of polymer; pH=1.2

4.2.3.5. Fourier transform infrared (FTIR) study

The spectra of SCMC (curve-a), gelatin-A (curve-b), SCMC-gelatin complex (curve-c), Isoniazid (curve-d), isoniazid loaded crosslinked SCMC-gelatin micro particles (curve-e) are shown in fig.4.2.3.5. The spectrum of SCMC showed absorption bands at 3364 cm^{-1}, 2942 cm^{-1}, 1627 cm^{-1}, 1422 cm^{-1}, 1063 cm^{-1} which were due to O-H stretching vibration, CH_3 symmetric+ CH_2 assymetric vibration, C-O stretching band for cellulose, CH_3 + CH_2 bending vibration and strong C-O stretching band for ethers. 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 1600-1700 cm^{-1} is the most important peak for IR analysis of the secondary structure of protein like gelatine [17]. In the complex of gelatin and SCMC, a slight shift of the peak of amide I from 1630.44 cm^{-1} to 1628.25 cm^{-1} was observed. This

indicated that the negatively charged carboxy methyl groups might associate with positively charged gelatin. Similar type of observation was reported by Pranoto et al. [18].while studying the interaction between carrageenan and gelatin. The probable interaction between SCMC and gelatin is shown in Figure 4.2.3.3. In the spectrum (shown as curve-d) of isoniazid, the carbonyl absorption (amide I band) appeared at 1664 cm^{-1}. The amide II band that occurred at 1555.90 cm^{-1} was due to N-H bending of the secondary amide group. Moreover, in the spectrum of isoniazid, multiple bands appeared between 1400 cm^{-1} to 668 cm^{-1}. Some of the characteristic bands of isoniazid appeared in the isoniazid loaded micro particles (curve e), suggested the successful loading of isoniazid in the micro particles. Similar type of IR spectral pattern for isoniazid and isoniazid containing capsules were reported by Kim et al. [26].

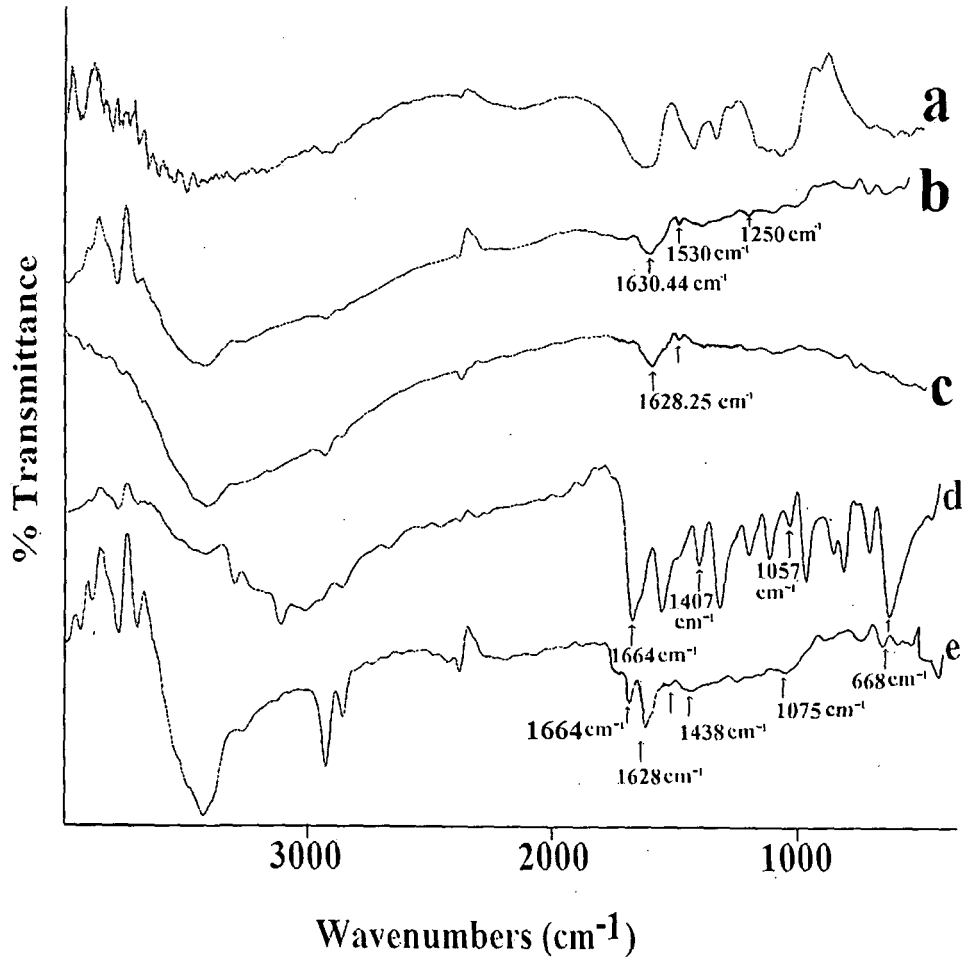

Figure 4.2.3.5. FTIR spectra of **a**) carrageenan **b**) SCMC **c**) gelatin-SCMC complex d) isoniazid **e**) isoniazid loaded microcapsules.

4.2.3.6. Thermal property study

DSC thermograms of SCMC-gelatin complex (curve-a), isoniazid (curve-b), isoniazid loaded micro particles (curve-c) are shown in Figure 4.2.3.6. The endotherm appeared in all the thermograms except isoniazid at around 100 ^{0}C were due to removal of moisture. The thermograms of isoniazid showed an endothermic peak due to melting at around 190 ^{0}C. There was no characteristic peak of isoniazid in the thermogram of isoniazid loaded micro particles. These results indicated that isoniazid was dispersed in the microparticles. Similar observation was reported by Patil et al. during DSC analysis of carvedilol drug encapsulated within alginate microspheres [31].

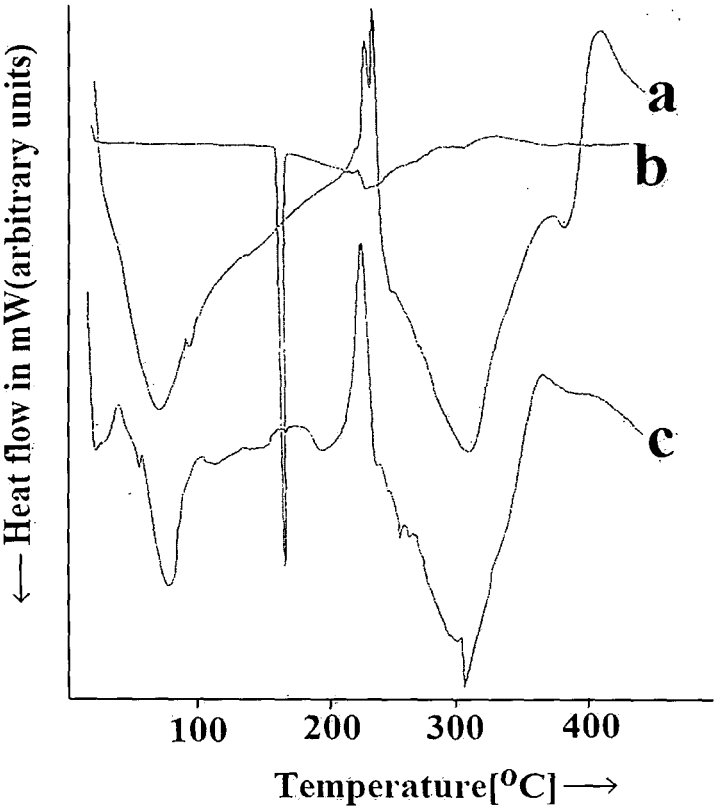

Figure 4.2.3.6. DSC thermograms of **(a)** SCMC-gelatin complex, **(b)** isoniazid **(c)** isoniazid loaded microcapsules

4.2.3.7. X-ray diffraction (XRD) study

X-ray diffractograms of gelatin-SCMC micro particles (curve-a), isoniazid loaded micro particles (curve-b), isoniazid (curve-c) are shown in Figure 4.2.3.7. Isoniazid showed multiple sharp peaks at 2θ varying from 12 to 50^0 which were due to the crystalline nature of isoniazid. Appearance of some of these peaks in the diffractograms of isoniazid loaded micro particles indicated development of some crystallinity due to the encapsulation of isoniazid.

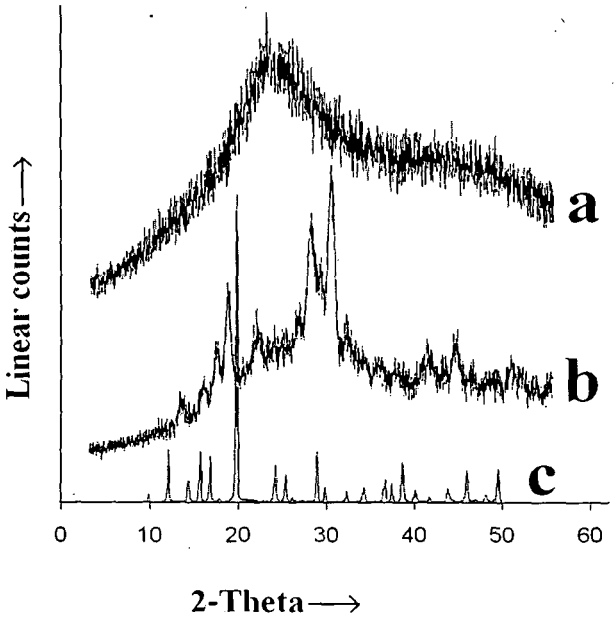

Figure 4.2.3.7. X-ray diffractograms of **(a)** microspheres without isoniazid **(b)** isoniazid **(c)** isoniazid loaded microspheres.

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CHAPTER V
SUMMARY AND CONCLUSIONS

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5.1. Summary and conclusions

The salient features that come out of the present study could be summarized as follows-

In the present experimental work, it was found that Neem *(Azadirachta Indica A.Juss.)* Seed Oil (NSO) could be encapsulated successfully inside glutaraldehyde, tannic acid or genipin cross-linked polyelectrolyte complexes of carrageenan-chitosan, gelatin-carrageenan and SCMC-gelatin microcapsules. The microcapsules were prepared by varying pH of the polymer solution and using complex coacervation technique.

The pH and polymer ratio at which maximum complexation between (i) κ-carrageenan-chitosan (ii) gelatin A-κ-carrageenan (iii) sodium carboxy methyl cellulose-gelatin A for highest yield occurred were (i) 4.5 and 1:0.36 (ii) 3.5 and 2:1 and (iii) 3.5 and 1:2.33 respectively. These optimizations were carried out by measuring % coacervate yield, turbidity and viscosity.

Microencapsulation of Neem Seed Oil

In all the studied complexes, the oil loading (%), oil content (%), encapsulation efficiency (%) and release rate of oil were dependent on various factors like amount of oil, concentration of polymer, type and concentration of crosslinker.

In general, encapsulation efficiency increased as oil load increased. Similarly encapsulation efficiency was found to increase when concentration of polymer (κ-carrageenan-chitosan, gelatin A-κ-carrageenan, sodium carboxy methyl cellulose-gelatin A) or cross-linker (glutaraldehyde, genipin, tannic acid) increased. At higher polymer concentration, the availability of the polymer was more to encapsulate oil droplets and

thereby efficiency increased. Higher crosslinker concentration (glutaraldehyde /tannic acid/ or genipin) increased the cross-linking which improved the oil retention capacity.

The faster or slower release rate of oil by the microcapsules prepared by varying different conditions could be explained on the basis of either decrease or increase in the thickness and compactness of microcapsule wall formed due to addition of polymer and crosslinker. Higher oil loading decreased the thickness of microcapsule wall whereas higher concentration of polymer increased the thickness of the wall. This in turn would control the release rate. Microcapsules wall became more compact as the amount of cross-linker increased. This led to decrease in the release rate.

Effects of various crosslinking agents (glutaraldehyde, genipin, tannic acid) on NSO encapsulated microcapsules of carrageenan-chitosan complex were compared and glutaraldehyde was found to be most effective crosslinker followed by genipin and tannic acid.

FTIR spectroscopy indicated that there was no significant interaction between the polymer complexes and NSO. But interaction was observed between the polymers undergoing polyelectrolyte complexation during complex formation. New bond formation and shifting of peaks in FTIR spectra confirmed the interaction between the complex forming polymers. Both FTIR and DSC study revealed no interaction between polymers and NSO in all the cases.

Thermal stability of chitosan-carrageenan microcapsules (as suggested by thermogravimetric analysis) was found to improve with the increase in the concentration of crosslinker. In case of various crosslinkers, glutaraldehyde crosslinked samples showed highest thermal stability followed by genipin and tannic acid.

SEM study showed the formation of spherical microcapsules having free flowing to bursting look depending on the extent of oil loading. SEM micrographs of microcapsules prepared at higher oil loading appeared oily, agglomerated and having a bursting look compared to those of microcapsules prepared at lower oil loading. The sizes of microcapsules increased with the increase of the concentration of the polymer.

Microencapsulation of Isoniazid

Genipin crosslinked NSO loaded nanocapsules of κ-carrageenan-chitosan polyelectrolyte complex were prepared. NSO was removed from nanocapsules and then loaded with isoniazid by immersing in isoniazid solutions of different concentrations. Sonication and surfactant concentration controlled the particle size of the nanocapsules as indicated by the particle size analyzer. 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.

TEM study supported the observation obtained from particle size analyzer. The calculated diameter of a randomly chosen nanocapsule was 230 nm, which was close to that of average particle size (234nm) 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 little brighter.

The loading of drug into the nanocapsules was time dependent. The release rates of isoniazid at lower pH (pH=1.2; HCl) were more compared to higher pH (pH=7.4, phosphate buffer). FTIR study showed that there was no remarkable interaction between isoniazid and polyelectrolyte complex. Surface characteristics of the nanocapsules were studied by SEM. X-ray diffraction study showed that the dispersion of isoniazid was not occurred at molecular level.

Microparticles were prepared by using gelatin A- κ-carrageenan and SCMC-gelatin A polyelectrolyte complex and following emulsification process. Sunflower oil was used as reaction medium. The microparticle formation was carried out at the optimized conditions of pH and ratio of the polymers. The formation and the size of the microparticles were dependent on surfactant, polymer concentration and stirring speed. SEM study showed that the sizes of the microparticles decreased with the increase of amount of tween-80. At higher concentration of surfactant, the aqueous phase is easily dispersed into finer droplets, owing to the higher activity of the surfactant, which resulted in a lower free energy of the system, and led to a smaller particle size.

Again, with the decrease of the amount of polymer, a decrease in the size of the microparticles was observed. In the presence of higher amount of polymer, the surfactant present might not be capable of covering all the surfaces of the microparticles properly. This resulted in the coalescence of some of the microparticles and led to the formation of larger microparticles. Besides this, the dispersive force of the stirrer became less efficient in presence of higher amount of polymer and as a result larger microparticles formed. Stirring speed also affected the nature and size of the microparticles. At lower stirrer speed (200 rpm), the agglomeration of particles was more compared to those of particles produced at higher stirrer speed (1500 rpm). Improper mixing of polymers at low stirrer speed resulted in agglomeration. Again, sizes of microparticles become finer at higher stirrer speed compared to lower stirrer speed.

Isoniazid was encapsulated successfully within these microparticles. Isoniazid concentration governed the absorption of isoniazid into the microparticles. In case of gelatin-carrageenan system, the microparticles were unstable when immersed in isoniazid solution. But crosslinking improved the stability of these microparticles. For similar extent of crosslinked microparticles, maximum drug loading efficiency was observed when immersed in higher concentration of isoniazid solution for a longer time.

Release rates of isoniazid for both gelatin-carrageenan and SCMC-gelatin microparticles were dependent on pH and amount of crosslinker. The release rate of isoniazid was more at higher pH (pH=7.4) compared to that of at lower pH (pH=1.2). Similarly, higher extent of crosslinking decreased the release rate. FTIR study indicated the loading of isoniazid into the microparticles. DSC and XRD studies showed that isoniazid was dispersed in the microparticles.

5.2. Future scope

- NSO was found to be encapsulated successfully within cross-linked polymer complex. The release of NSO was found to be controlled by the use of natural polymer systems. However, the present investigation is restricted to laboratory scale only. Further field evaluation is needed for commercialization of the products.

- Polyelectrolyte micro/nanocapsules were prepared by using different natural polymers. It is also possible to provide more than one layer of polymers by layer-by-layer self assembly technique or by other preferable techniques for required controlled release of the active agent. Further study relating to controlling of the particle size, their distribution and thickness of capsule wall membrane may allow to produce monodisperse particles which may potentially open up many opportunities in other biomedical and pharmaceutical applications.

- Both micro as well as nano particulate therapy (controlled delivery) holds great potential for treating tuberculosis, with reduced drug dosage and dosing frequency, leading to less systematic side effects and improved patient compliance. Thus, each of the sustained release anti-tubercular delivery systems holds promise for reducing the dosing frequency and improving patient compliance. Till more potent drugs become available, these systems could be cost-effective, feasible and save valuable life and resources in the management of tuberculosis. However, there remains a challenge for micro/nano particulate drug delivery and that is to understand the fate of particles and their interactions with biological systems. In this respect, a thorough study is necessary which may shower some light on the interaction of the remaining polymer after drug delivery with the biological system. This may help further to design the delivery system in a better way.

- The main obstacle to the wider use of controlled release product will remain the high cost of these materials compared to those of conventional products. In case of agricultural use, the use of cheaper natural polymer waste materials such as sawdust, park, starch, baggase, rice husk lignin and pine-kraft lignin seems to be a viable way to provide the loading of bioactive species. Other possible trends to reduce cost are to increase weight efficiency by increasing the content of the active ingredients and to use the polymer itself in a positive way rather than as an inert vehicle. One possibility is to make the polymer backbone that has a dual function as an active ingredient and as a carrier for the bioactive agent.

- The particle shape and size, thickness of polymer coating, crosslinking density, loading percentage, etc. have to be taken into account for designing a particular controlled release formulation. There yet remain considerable amounts of art to

coacervative microencapsulation. In coacervation, the kind of addition and the rate of and the order of addition are extremely critical. These have to be taken care in order to better understand the process and to get better product.

Publications

Papers Published

1. "A novel microencapsulation of Neem (*Azadirachta Indica A.Juss.*) Seed Oil (NSO) in polyelectrolyte complex of κ-carrageenan and chitosan." Nirmala Devi and T.K.Maji. *Journal of Applied Polymer Science*, 113(3), 2009, 1576-1583.
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A Novel Microencapsulation of Neem (*Azadirachta indica A. Juss.*) Seed Oil (NSO) in Polyelectrolyte Complex of κ-Carrageenan and Chitosan

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ABSTRACT: Microcapsules containing neem (*Azadirachta Indica A. Juss.*) seed oil (NSO) were prepared by encapsulation of natural liquid pesticide NSO in a polyelectrolyte complex of κ-carrageenan and chitosan. The optimum ratio between carrageenan and chitosan to form a stable polyelectrolyte complex was found as 1 : 0.36. The microencapsulation method for NSO loading was also optimized. SEM study demonstrated that the surface of the microcapsules became more irregular as oil loading increased. The release rates of NSO were studied by varying the percentage of oil loading, concentration of cross-linking agent, and polymer concentration. Fourier transform infrared spectroscopy (FTIR) study confirmed the complex formation between κ-carrageenan and chitosan. Differential scanning calorimetry (DSC) and FTIR study indicated the absence of any significant interaction between polyelectrolyte complex of κ-carrageenan -chitosan and NSO. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 113: 1576–1583, 2009

Key words: *microencapsulation; neem seed oil; polyelectrolyte complex; cross-linking agent; characterization*

INTRODUCTION

Botanical insecticides have long been touted as attractive alternatives to synthetic chemical insecticides for pest management because botanicals reputedly pose little threat to the environment or human health.[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] But, NSO, as it is a liquid, cannot be used in soil. Microencapsulation technique seems to be the best technique to design the liquid pesticide to a solid form and for controlled delivery of the oil in the soil for long duration efficiently.

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. Chitosan has attracted much attention because of its biocompatibility, antibacterial fungal, and antimicrobial properties.[4,5] Moreover chitosan on degradation, when used for controlled release formulation for delivery of NSO, may produce nitrogen which can enhance the quality of soil.

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Carrageenans, a naturally occurring high-molecular-weight polysaccharides, are made up of repeating units of galactose and 3,6 anhydrogalactose. They cosists of sulpfate esters of galactose and 3,6 anhydrogalactose joined by alternating α-1,3 and β-1,4 glycosidic linkages. Three types of carrageenans namely iota (ι), kappa (κ), and lambda (λ) with one, two, and three sulpfate groups are available. Both iota and kappa carrageenans are able to produce gels which can govern the release behavior of mixtures. Carrageenas have been investigated for use in controlled release tablets.[6] Both carrageenans and chitosan are little bit expensive but considering their multiple advantages, they can be used as an matrix for controlled release of NSO.

When two oppositely charged polyelectrolytes are mixed in an aqueous solution, a complex is formed by the electrostatic attraction between polyelectrolytes. Both chitosan and carrageenan can react to form polyelectrolyte complex. Various neutral polymers (e.g. hydroxypropylmethylcellulose [HPMC]), cationic polymer (e.g.,chitosan), and anionic polymers (κ-carrageenan, sodium alginate) have been used in the form of polyelectrolyte complexes[7] such as sodium alginate-chitosan,[8–12] polyacrylic acid-chitosan,[13] and chitosan-carrageenan[14,15] for the design of controlled release formulations. Several reports addressing the use of polyelectrolyte complex of chitosan and κ-carrageenan for controlled release of drugs have been cited.[12,14] Sakiyama et al.[15] studied the swelling behavior and other properties of this

polyelectrolyte complex. But there is little information regarding the use of this polyelectrolyte complex for encapsulation of agrochemicals. To improve the controlled release behavior, glutaraldehyde has been reported to be used as a cross-linker.[16,17]

The aim of this work is to evaluate the possibility of using chitosan κ-carrageenan complex for encapsulation of NSO and to study the release characteristic of NSO from glutaraldehyde cross-linked microcapsules prepared under various conditions.

EXPERIMENTAL

Materials

Carrageenan Type I, containing predominantly κ-and lesser amount of λ-carrageenan was purchased from Sigma-Aldrich Inc. (USA). Chitosan, medium molecular weight with brookfield viscosity ~ 200 cps 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) were used without further purification. The core material, cold pressed NSO was a gift sample of Ozone Biotech., Faridabad, India. Double-distilled deionised (DDI) water was used throughout the study. Other reagents used were of analytical grade.

Optimisation of chitosan and carrageenan ratio

The optimum ratio between chitosan and carrageenan was judged from the measurement of turbidity and viscosity of the supernatant solution. The complex formation between polyelectrolytes is very much dependent on pH.[18] Mixing of both chitosan and carrageenan solution at different ratio would change the pH which might affect the reaction between polyelectrolytes. For that buffer solution was used to prepare both the solution of chitosan and carrageenan.

Solutions of chitosan (0.5% w/v) and carrageena (0.5% w/v) were prepared in 0.3% acetic acid/sodium acetate buffer at two different pH namely 4 and 5. This range (4.0–5.0) was below the pH at which precipitation of chitosan occurred. The stability of carrageenan would also not be much affected at these pH. Both solutions were mixed in different proportions to make 30 mL. The mixtures were incubated at 40°C for 24 h. It was then centrifuged at 2500 rpm for 1 h. The supernatant solution was separated, viscosity and turbidity were measured. The supernatant solution did not exhibit any significant difference in both viscosity and turbidity at pH 4 and 5.This indicated that the interaction between chitosan and carrageenan would remain similar within the pH range 4.0–5.0. The determination of optimum ratio between chitosan and carrageenan was, therefore, done at pH 4.5. This pH was maintained for preparing of chitosan and carrageenan solution used for subsequent experiments.

Turbidity measurements were done to confirm the optimum ratio between chitosan and carrageenan to form an insoluble polyelectrolyte complex. Solutions of pure chitosan (0.5% w/v), carrageenan (0.5% w/v) and the supernatants from the mixture of both at different ratios were scanned in the range 200–400 nm employing UV spectrophotometer. Both the solution of pure chitosan and carrageenan showed no peak within the scanned range. But the supernatant showed a peak at 341 nm. Therefore, all the turbidity measurements reported were done at 341 nm.

Microencapsulation procedure

In order to optimize the encapsulation process, a series of experiments were conducted by varying the parameters like temperature during formation and hardening of microcapsules, time and temperature for completion of cross linking reaction. The optimized process are described as follows:

In a beaker, known amount of (100 mL) 0.3–0.85% (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, NSO (0.68–2.04 g) was added under high agitation to form an emulsion. A known amount of (36 mL) chitosan solution of 0.3–0.85% (w/v) was added to the beaker drop wise to attain complete phase separation. However, the weight ratio of carrageenan to chitosan was maintained at 1 : 0.36 during all the experiments. At this ratio, interaction between chitosan and carrageenan took place completely as judged by the viscosity and turbidity measurements. The beaker containing the microcapsules was left to rest at this 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 glutaraldehyde (2.5–12.5 mmol/g of polymer). The temperature of the beaker was then raised to 45°C and stirring was continued for another 3–4 h to complete the cross-linking reaction. The beaker was then cooled to room temperature. The microcapsules were filtered through 300-mesh nylon cloth, washed with 0.1% Tween 80 surfactant solution to remove any oil adhered to the surface of microcapsules followed by distilled water, dried and stored inside a refrigerator in a glass ampule.

Measurements

Calibration curve of oil

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% 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 by knowing the absorbance value.

Viscosity and turbidity measurement

The viscosity of the supernatant solution arising from the mixing of chitosan and carrageenan was measured by using an Ubbelohde viscometer at 30°C. The optimal ratio between chitosan and carrageenan was obtained when both polymers would react completely to form an insoluble complex. At this stage, polymer in the supernatant solution would be either absent or negligible. This would make the supernatant viscosity similar or close to that of solvent viscosity.

Because of the mixing of chitosan and carrageenan solution in different ratios, turbidity appeared was different in different solutions. The optimal ratio at which complete phase separation occurred between chitosan and carrageenan solution was the point where the supernatant would have the maximum turbidity. The change in transmittance due to turbidity was monitored continuously at 341 nm wavelength using UV spectrophotometer.

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% 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[17]

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

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

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

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 cross-linker.

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.

Oil release studies

Oil release studies of encapsulated oil were done by using UV–visible spectrophotometer (UV-2001 Hitachi). A known quantity of microcapsules (0.2–0.3 g) was placed into a known volume of 0.1% 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.

Fourier transform infrared (FTIR) study

FTIR spectra were recorded using KBr pellet in a Nicolet (model Impact-410) spectrophotometer. Chitosan, carrageenan, polyelectrolyte complex of chitosan–carrageenan, NSO, NSO loaded microcapsules, and physical mixture of (NSO+ polyelectrolyte complex of chitosan–carrageenan) were each separately finely grounded with KBr and FTIR spectra were recorded in the range of 4000–400 cm^{-1}. The scanning was done thirty times before taking the final spectra.

Thermal property study

Thermal properties of chitosan–carrageenan polyelectrolyte complex, NSO, NSO loaded microcapsules and physical mixtures of (NSO+ chitosan-carrageenan polyelectrolyte complex) were evaluated using differential scanning calorimeter (DSC). The sample (6.0 mg approx.) was taken in an aluminium pan and sealed. The study was done in a differential scanning calorimeter (model DSC-60, shimadzu) at a heating rate of 10°C/min up to 400°C under nitrogen atmosphere.

RESULTS AND DISCUSSION

Viscosity and turbidity measurement

Figure 1(a) shows the change in supernatant viscosity with variation in percentage of chitosan in

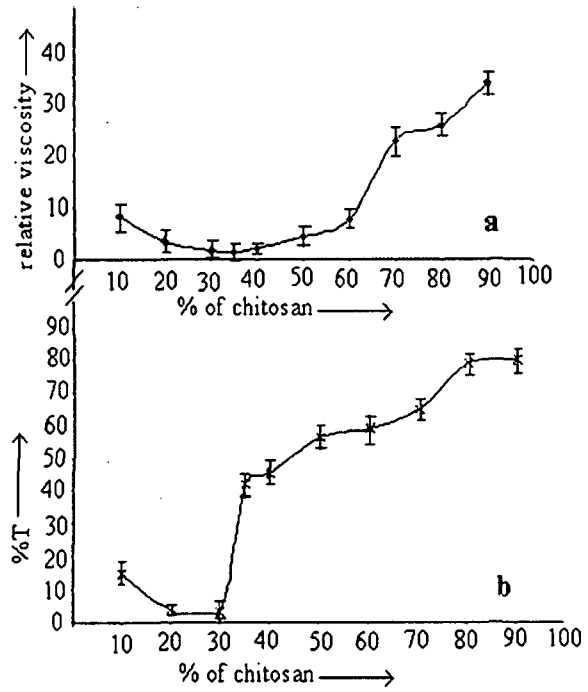

Figure 1 (a) Effect of variation of chitosan concentration in chitosan–carrageenan mixture on relative viscosity of supernatant. (b) Effect of variation of chitosan concentration in chitosan carrageenan mixture on (%) transmittance of supernatant.

chitosan–carrageenan mixture. Each value represents the mean of three values. Viscosity was found to decrease initially, reaching a minimum value, and after that it increased with the increase in the percentage of chitosan. The minimum viscosity observed when the percentage of chitosan in the mixture was in between 30 and 40. Similar type of observation was reported by Tapia et al.[12] At this percentage of chitosan (36%), both the polymers probably reacted completely 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 chitosan in the supernatant.

The plot of % transmittance against % of chitosan is presented in Figure 1(b). Each value represents the mean of three values. The % transmittance showed a decreasing trend initially followed by an increasing trend latter. The minimum % transmittance occurred when the % of chitosan in the mixture was in between 30 and 40 and also in between these points the transmittance increased sharply. The reason for this could be explained as before. The maximum turbidity developed when the interaction between chitosan and carrageenan was maximum. The higher the turbidity, the lower was the transmittance. The % of increased chitosan latter would decrease the turbidity and hence transmittance increased.

Scanning electron microscopy study

SEM photographs of neat carrageenan + chitosan complex and NSO loaded microcapsules are presented in Figure 2. Photographs of neat carrageenan + chitosan complex [Fig. 2(a)] appeared more regular and free flowing compared to NSO loaded microcapsules[Fig. 2 (b,c)]. The surface of high NSO loaded microcapsules [Fig. 2(c)] appeared more irregular and bursting in comparison with that of low NSO loaded microcapsules [Fig. 2(b)] Similar observation was reported in the literature.[18] Figure 2(d) represents the photograph of the sample after release of NSO. Levels of NSO loading in both the samples (c and d) were almost similar. Bursting look observed in loaded samples was found to decrease after releasing of NSO.

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 I and Figure 3. All experiments were carried out in triplicate and the results presented were the average value. 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 vesicles. The polymer present in the mixture was enough to encapsulate these vesicles. The dispersion force became progressively difficult as the oil load increased. This would develop large oil vesicles and as a result encapsulation efficiency would increase. As the amount of polymer was fixed, therefore, the polymers would encapsulate all the large oil vesicles 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,[19] 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 vesicles indicating that there was an abundance of the encapsulating polymer for the oil present. With the increase in oil load (%), the number of oil vesicles in the microcapsule increased which resulted in an increase in oil content. An increase in oil content (%) of microcapsules due to increase in oil loading was supported by SEM study. The

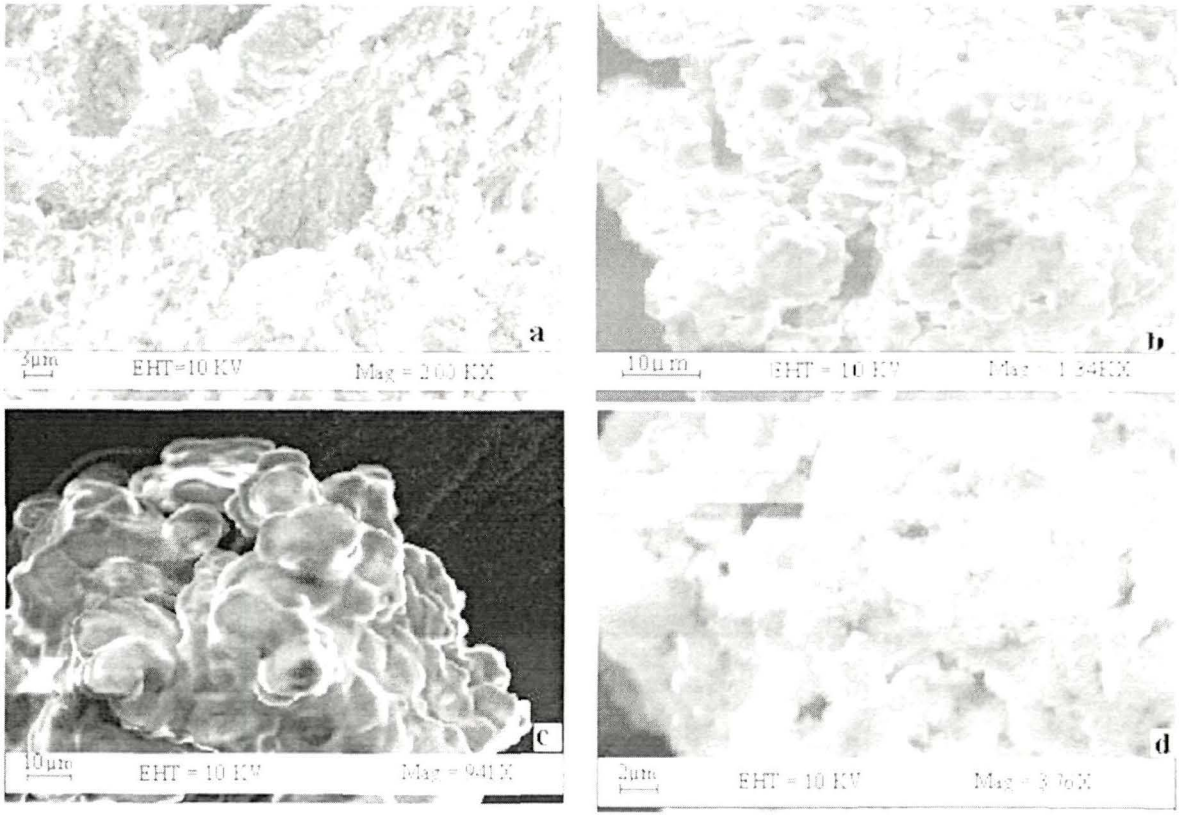

Figure 2 Scanning electron micrographs (a) neat carrageenan + chitosan complex, (b) microcapsules loaded with low percentage of NSO, (c) microcapsules loaded with high percentage of NSO, (d) microcapsules loaded with high percentage of NSO (after release of NSO).

surface characteristics of the microcapsules were found to change as oil content (%) varies.

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 I and Figure 4. The trends of oil loading (%) and oil content (%) shown in the table were as per expectation. With the increase in glutaraldehyde concentration, oil loading decreased for all as expected. But oil content and encapsulation efficiency increased. A decrease in trend in oil content

TABLE I
Effect of Variation of Oil Loading, Polymer and Glutaraldehyde Concentration on the Behavior of Microcapsules

Sample formulations					
Total polymer (g)	Glutaraldehyde (mmol)	NSO (g)	Oil load (%)	Oil content (%)	Encapsulation efficiency (%)
0.68	2.5	0.68	73.12	31 ± 1.0	73.39 ± 2.37
0.68	2.5	1.36	146.23	48 ± 1.3	80.82 ± 2.18
0.68	2.5	2.04	219.35	59 ± 0.5	85.897 ± 0.728
0.68	1.5	1.36	163.85	41 ± 2.0	66.02 ± 3.22
0.68	7.5	1.36	95.10	48 ± 0.74	98.47 ± 1.51
0.68	12.5	1.36	70.4	40 ± 1.0	96.78 ± 2.40
0.408	1.875	2.04	342.56	61 ± 0.9	78.80 ± 1.169
1.156	4.625	2.04	126.04	46 ± 2.3	82.49 ± 4.13

Chitosan: 0.018–0.306 g; carrageenan: 0.30–0.85 g; water: 136 mL; NSO: 0.68–2.04 g; glutaraldedyde: 1.5–12.5 mmol/g of polymer; temperature: (70 ± 1)°C.

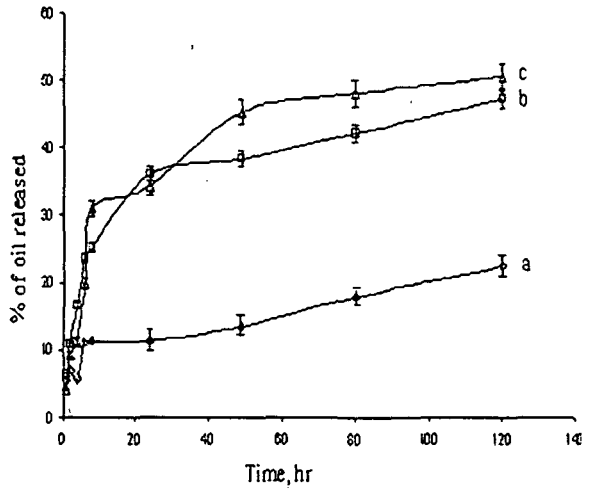

Figure 3 Effect of variation of oil loading on release profile [a: polymer 0.68 g; cross-linker 2.5 mmol; NSO 0.68 g, b: polymer 0.68 g; cross-linker 2.5 mmol; NSO 1.36 g, c: polymer 0.68 g; cross-linker 2.5 mmol; NSO 2.04 g].

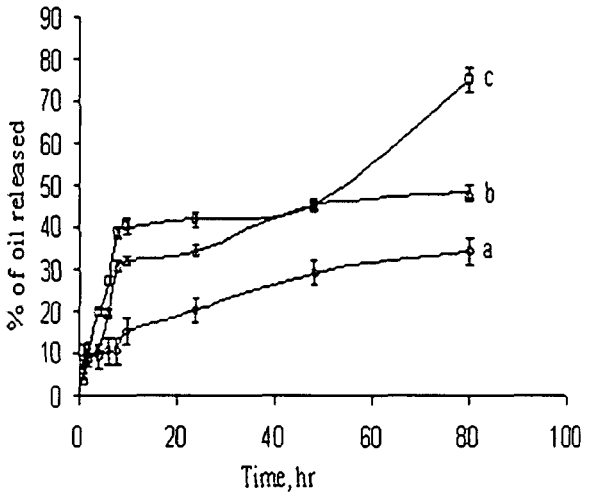

Figure 5 Effect of variation of polymer concentration on release profile [a: polymer 1.156 g; cross-linker 4.625 mmol; NSO 2.04 g, b: polymer 0.68 g; cross-linker 2.5 mmol; NSO 2.04 g, c: polymer 0.408 g; cross-linker 1.875 mmol; NSO 2.04 g].

was observed in the case of 12.5 mmol concentration of glutaraldehyde.

The increase in encapsulation efficiency (%) could be due to the improvement of oil retention capacity of the microcapsules caused by the formation of cross-linking. The cross-linking reaction took place between glutaraldehyde and polyelectrolyte complex of carrageenan and chitosan. The release rate of oil was found to decrease as the % of glutaraldehyde increased. The microcapsule wall became compact as degree of cross-linking increased. This resulted in the decrease of diffusion rate through the microcapsule wall. Similar findings were cited in the literature.[20]

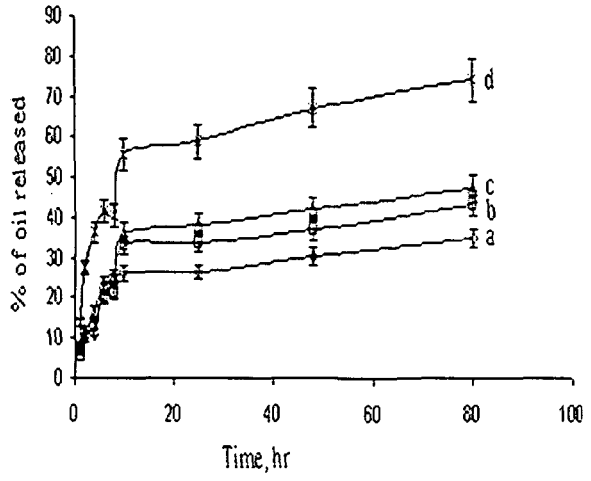

Figure 4 Effect of variation of cross-linker concentration on release profile [a: polymer 0.68 g; cross-linker 12.5 mmol; NSO 1.36 g, b: polymer 0.68 g; cross-linker 7.5 mmol; NSO 1.36 g c: polymer 0.68 g; cross-linker 2.5 mmol; NSO 1.36 g, d: polymer 0.68 g; cross-linker 1.5 mmol; NSO 1.36 g].

Effect of variation of polymer concentration

Table I shows the results of the effect of variation of total polymer concentration on oil loading, oil content, and encapsulation efficiency. In all the studied experiments, the ratio of polymer to cross-linker was kept fixed. As expected, both oil loading (%) and oil content (%) decreased with the increase in total polymer content. Encapsulation efficiency increased initially and then leveled off. With the increase in polymer content, more 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. The release profile is shown in Figure 5. 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 behavior.[18]

FTIR study

FTIR spectra of Chitosan (curve-a), Carrageenan (curve-b), chitosan-carrageenan polyelectrolyte complex (curve-c), NSO(curve-d), physical mixture of (NSO+ chitosan–carrageenan polyelectrolyte

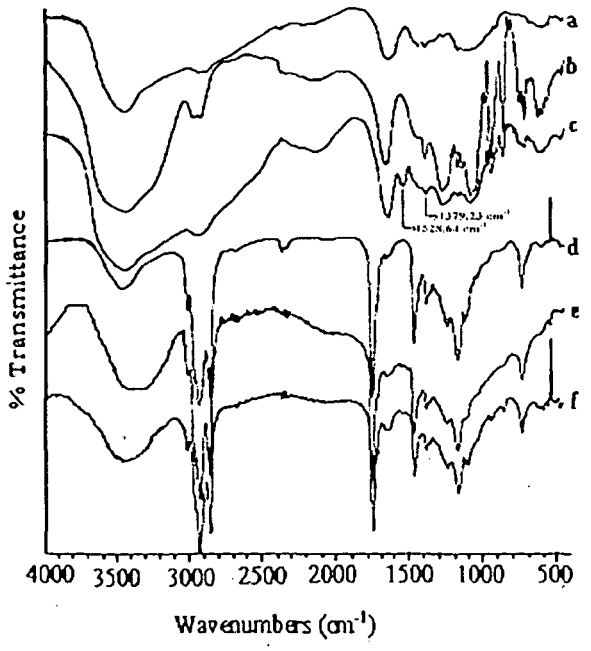

Figure 6 FTIR spectra of (a) chitosan (b) carrageenan (c) polyelectrolyte complex of chitosan and carrageenan (d) NSO (e) physical mixture of (NSO+ polyelectrolyte complex of chitosan and carrageenan) (f) NSO loaded microcapsules.

complex) (curve-e), and NSO loaded chitosan–carrageenan microcapsules (curve-f) are shown in Figure 6. The spectrum of chitosan showed a strong absorption band at 1635.33 cm^{-1} assigned to NH bending. The other notable peaks appeared at 3435, 2920, 1425 and 1384, 1330, 1170, 1075, and 1030 cm^{-1}, were due to O—H + N—H stretching vibration, CH_3 symmetric + CH_2 asymmetric vibration, CH_3 +CH_2 bending vibration, vibration of C—N group, C—O—C asymmetric vibration, C—O(—C—OH—) vibration, C—O(—CH_2—OH—) vibration respectively. All these above peaks appeared in the spectrum of chitosan were observed in the spectrum of carrageenan except the peaks corresponding to nitrogen atom related groups. Besides this, the other notable 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. The appearance of a new band at 1528.64 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.[12] The absorption bands appeared in the spectrum of NSO at 1745.90, 1463.04, and 1163.85 cm^{-1} were due to carbonyl stretching, CH_2 asymmetric deformation, and C—C stretching vibration. The position of these bands in the physical mixture as well as in the NSO loaded microcapsules remained almost unchanged indicating the absence of any significant interaction between NSO and chitosan–carrageenan polyelectrolyte complex.

Thermal property study

DSC thermograms of neat chitosan–carrageenan polyelectrolyte complex (curve-a), NSO (curve-b), NSO loaded microcapsules (curve-c), and physical mixtures of (NSO+ chitosan–carrageenan polyelectrolyte complex) (curve-d) are shown in Figure 7. In the physical mixture, the ratio of NSO to neat chitosan–carrageenan polyelectrolyte complex was kept similar to that of microcapsules loaded with NSO. The endotherm appeared in all the thermograms except that of oil at around 100°C was due to the removal of moisture. The thermogram of NSO showed an endothermic peak at around 220°C. Both the NSO loaded microcapsules and physical mixture of complex and NSO did not show any remarkable difference in their thermograms. In both the thermograms, the endothermic peak due to NSO appeared almost in the similar position. These results indicated that there was no significant interaction between NSO

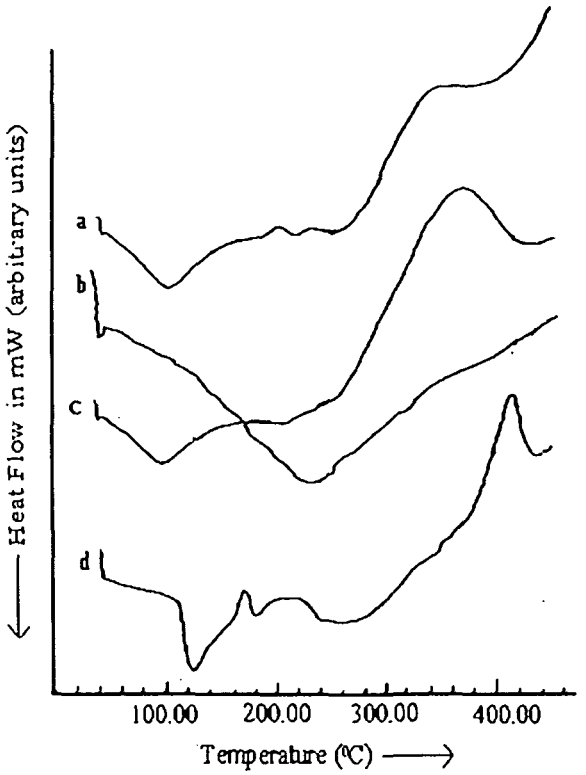

Figure 7 DSC thermograms of (a) neat chitosan–carrageenan polyelectrolyte complex (b) NSO (c) NSO loaded microcapsules (d) physical mixture of (NSO+ chitosan–carrageenan polyelectrolyte complex).

and chitosan–carrageenan complex. The results also suggested a low compatibility in thermal properties in the relation between NSO and chitosan–carrageenan polyelectrolyte complex.

CONCLUSION

The release rate of NSO was found to be dependent on polymer concentration, cross-linker concentration, and oil content. The encapsulation efficiency, oil content, and release rate of NSO increased with the increase in oil loading. The higher the polymer and cross-linker concentration, the lower was the release of NSO from microcapsules. SEM study showed a change in the surface characteristics of the microcapsules due to variation of NSO loading. FTIR results confirmed the formation of chitosan–carrageenan polyelectrolyte complex. It also showed that there was no interaction between chitosan–carrageenan polyelectrolyte complex and NSO. DSC study showed the poor compatibility in thermal properties in the relation between chitosan–carrageenan polyelectrolyte complex and NSO. All these results indicated that chitosan–carrageenan polyelectrolyte complex could be used as an efficient matrix for encapsulation of NSO.

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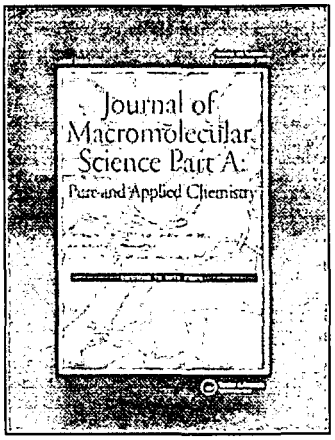

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Effect of Crosslinking Agent on Neem (*Azadirachta Indica A. Juss.*) Seed Oil (NSO) Encapsulated Microcapsules of κ-Carrageenan and Chitosan Polyelectrolyte Complex

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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 *i*-carrageenan. κ-carrageenan has one sulfate group per two galactose residues (produces a weak gel which suffer syneresis), *i*-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

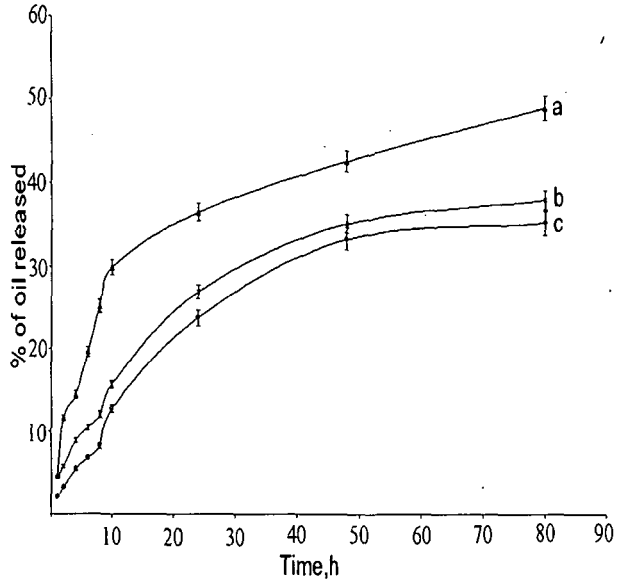

Fig. 1. Oil release profiles of microcapsules crosslinked with 0.2 mmol a) tannic acid, b) genipin, c) glutaraldehyde.

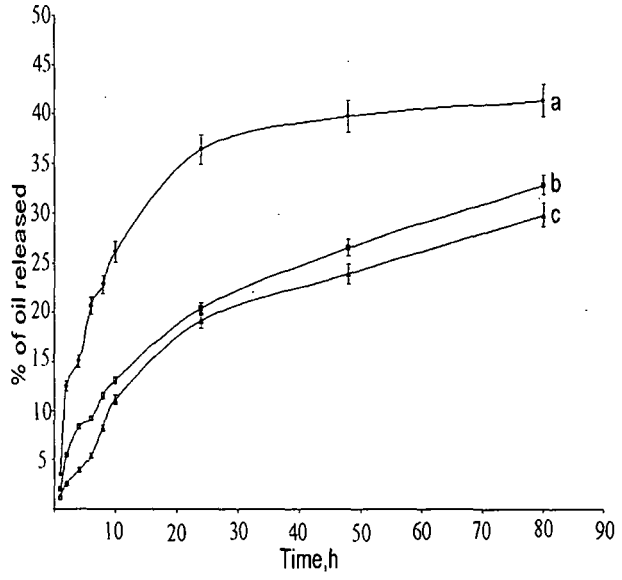

Fig. 2. Oil release profiles of microcapsules crosslinked with 0.4 mmol a) tannic acid, b) genipin, c) glutaraldehyde.

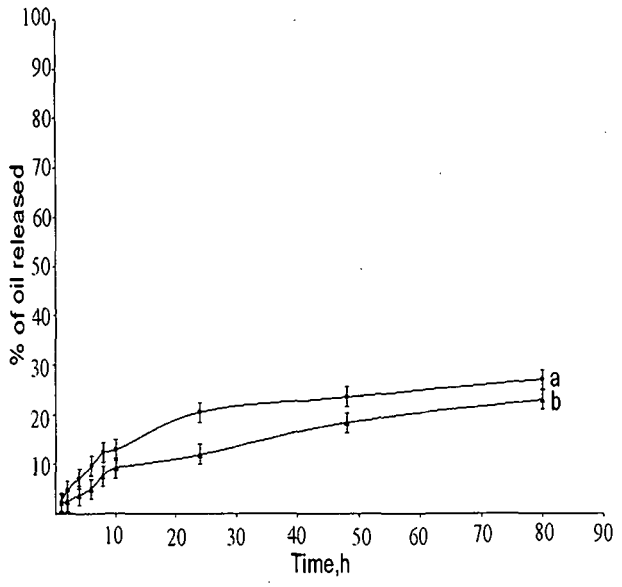

Fig. 3. Oil release profiles of microcapsules crosslinked with 0.8 mmol a) genipin, b) glutaraldehyde.

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).

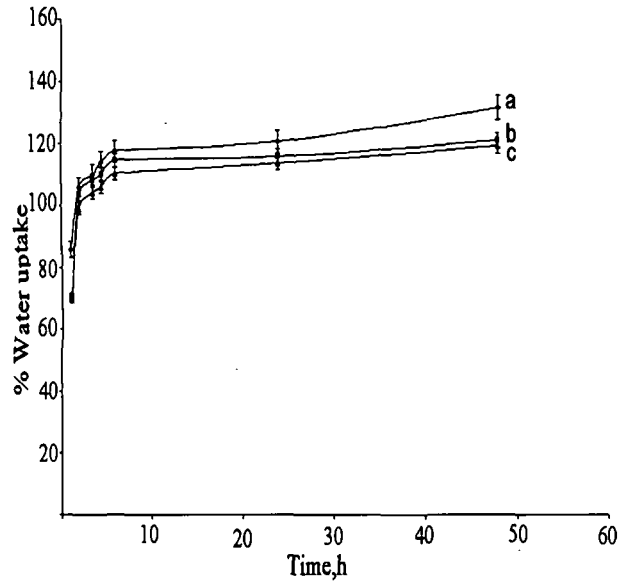

Fig. 4. Percentage water uptake of matrices crosslinked with a) tannic acid, b) genipin, c) glutaraldehyde.

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}

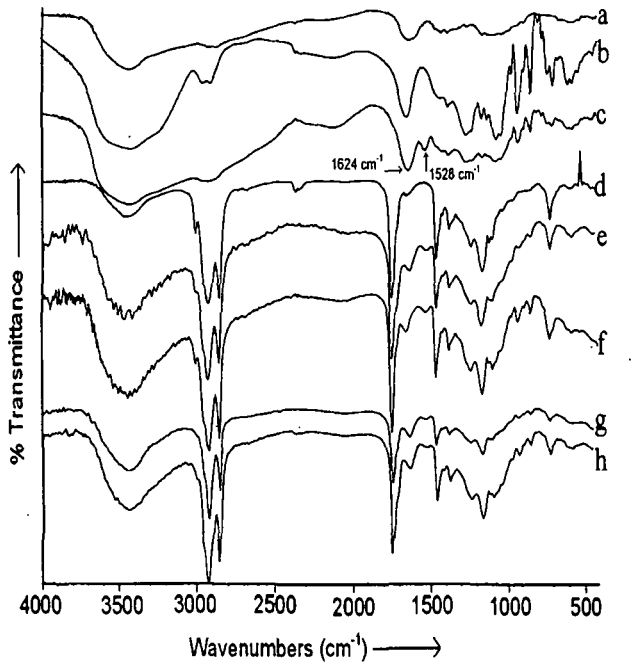

Fig. 5. FTIR spectra of a) chitosan; b) carrageenan; c) polyelectrolyte complex of chitosan and carrageenan; d) NSO; e) NSO loaded uncrosslinked microcapsules; f) NSO loaded tannic acid crosslinked microcapsules; g) NSO loaded genipin crosslinked microcapsules; h) NSO loaded glutaraldehyde crosslinked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

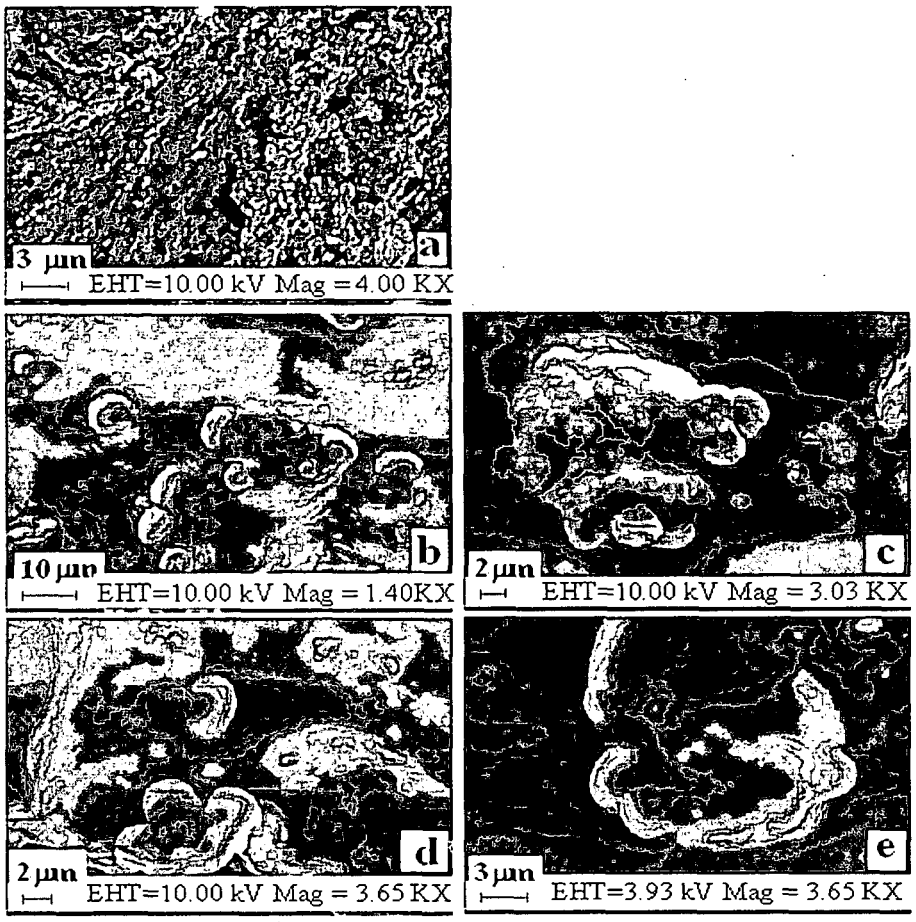

Fig. 6. Scanning electron micrographs of a) carrageenan-chitosan complex; b) glutaraldehyde crosslinked microcapsules; c) genipin crosslinked microcapsules; d) tannic acid crosslinked microcapsules; e) uncrosslinked microcapsules.

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 $2\theta = 20°$. This was similar to that reported in the literature (25). Similarly, carrageenan showed sharp peaks at $2\theta = 29°$ 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 $2\theta = 20°$. 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).

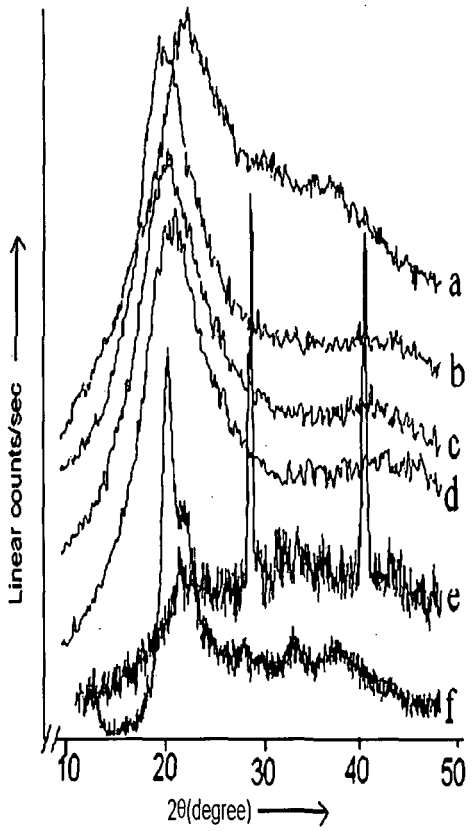

Fig. 7. X-ray diffractograms of NSO loaded chitosan-carrageenan microcapsules a) uncrosslinked; b) glutaraldehyde crosslinked; c) genipin crosslinked; d) tannic acid crosslinked; e) neat carrageenan; f) neat chitosan.

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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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

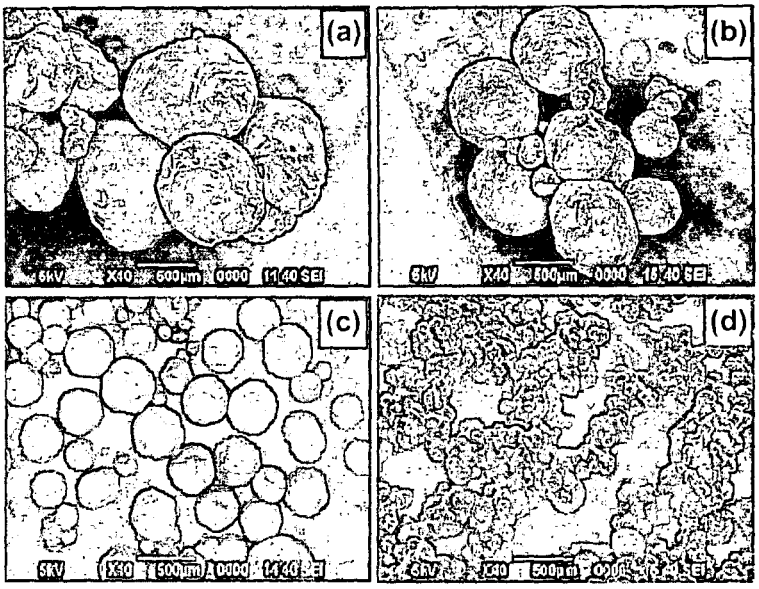

Figure 1. Scanning electron micrographs of microcapsules prepared by using (a) Tween 80: 0.33 g/g of polymer, polymer: 3.0 g; (b) Tween 80: 0.55 g/g of polymer, polymer: 3.0 g; (c) Tween 80: 1.0 g/g of polymer, polymer: 3.0 g; (d) Tween 80: 1.0 g/g of polymer, polymer: 1.5 g.

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].

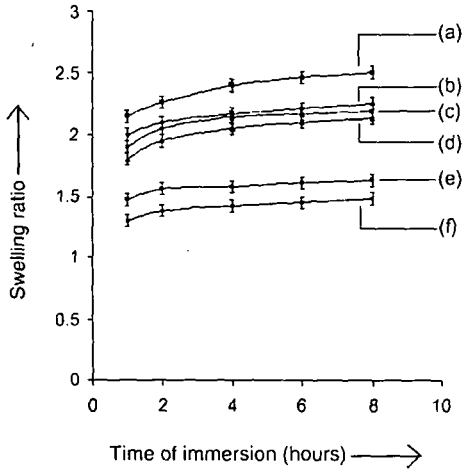

Figure 2. Effect of variation of time of immersion on swelling ratio of microspheres [at pH 7.4, polymer: 3.0 g: (a) crosslinker: 0.2 mmol; (c) crosslinker: 0.4 mmol; (e) crosslinker: 0.8 mmol. At pH 1.2, polymer: 3.0 g: (b) crosslinker: 0.2 mmol; (d) crosslinker: 0.4 mmol; (f) crosslinker: 0.8 mmol].

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.

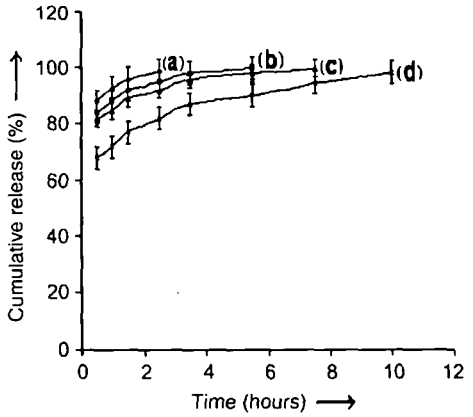

Figure 3. Effect of variation of crosslinker concentration on release profile at pH 1.2. [(a) polymer: 3.0 g, crosslinker: 0 mmol; (b) polymer: 3.0 g, crosslinker: 0.2 mmol; (c) polymer: 3.0 g, crosslinker: 0.4 mmol; (d) polymer: 3.0 g, crosslinker: 0.8 mmol].

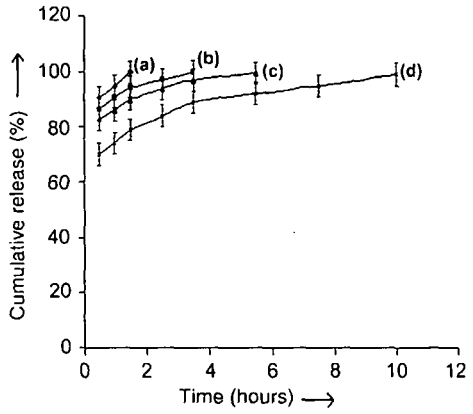

Figure 4. Effect of variation of crosslinker concentration on release profile at pH 7.4. [(a) polymer: 3.0 g, crosslinker: 0 mmol; (b) polymer: 3.0 g, crosslinker: 0.2 mmol; (c) polymer: 3.0 g, crosslinker: 0.4 mmol; (d) polymer: 3.0 g, crosslinker: 0.8 mmol].

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)

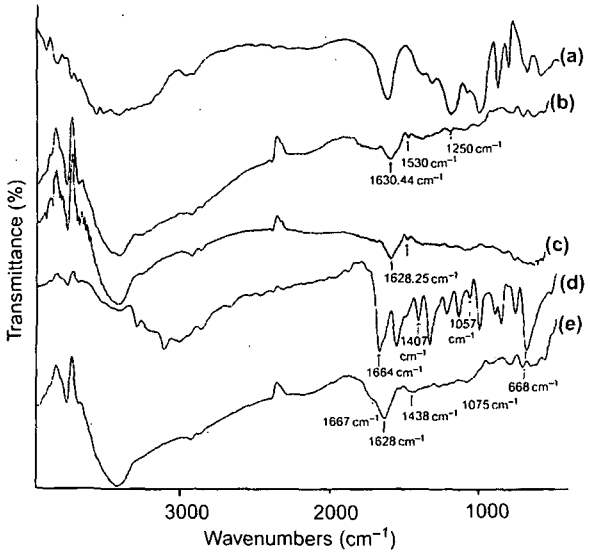

Figure 5. FTIR spectra of (a) carrageenan, (b) gelatin, (c) gelatin-carrageenan complex, (d) isoniazid, and (e) isoniazid-loaded microcapsules.

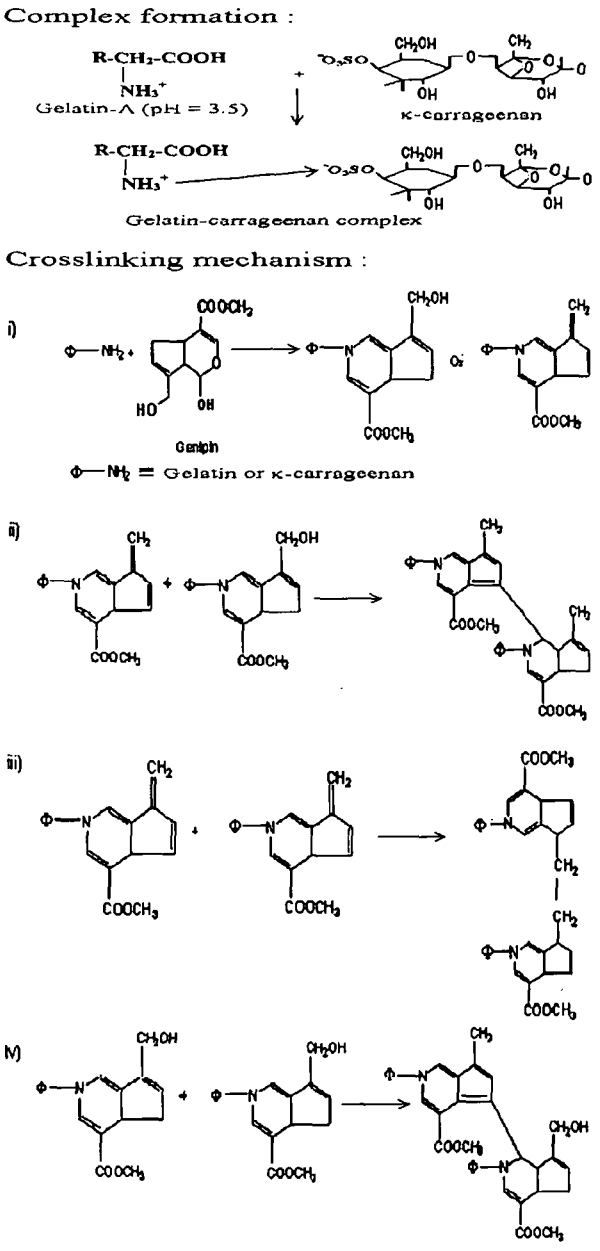

Figure 6. Probable reaction scheme for interaction between carrageenan and gelatin, and genipin with carrageenan-gelatin complex.

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

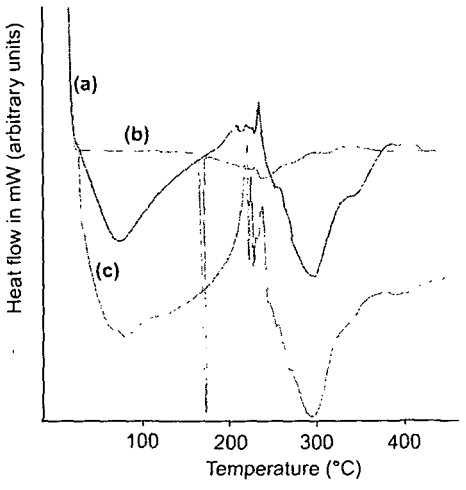

Figure 7. DSC thermograms of (a) carrageenan-gelatin complex, (b) isoniazid, and (c) isoniazid-loaded microcapsules.

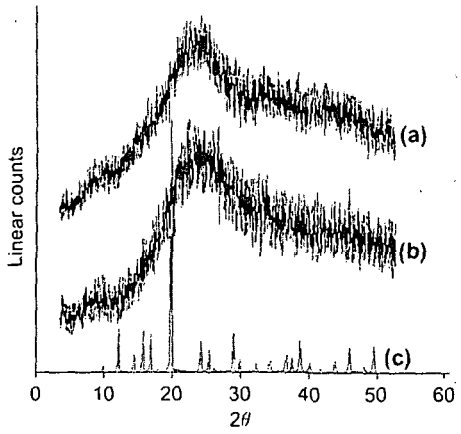

Figure 8. X-ray diffractograms of (a) carrageenan-gelatin microspheres, (b) isoniazid-loaded microspheres, and (c) pure isoniazid.

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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Research Article

Preparation and Evaluation of Gelatin/Sodium Carboxymethyl Cellulose Polyelectrolyte Complex Microparticles for Controlled Delivery of Isoniazid

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Abstract. The ratio of gelatin to sodium carboxymethyl cellulose (SCMC) at which maximum yield was obtained was optimized. This optimized ratio of gelatin to SCMC along with other parameters was used to prepare microparticles of different sizes. Vegetable oil was used as emulsion medium. Effect of various factors like amount of surfactant, concentration of polymer on the formation, and size of the microparticles was investigated. These microparticles were used as carrier for isoniazid. Among different cross-linkers, glutaraldehyde was found to be the most effective cross-linker at the temperature and pH at which the reaction was carried out. The loading efficiency and release behavior of loaded microparticles were found to be dependent on the amount of cross-linker used, concentration of drug, and time of immersion. Maximum drug loading efficiency was observed at higher immersion time. The release rate of isoniazid was more at higher pH compared to that of at lower pH. The sizes of the microparticles were investigated by scanning electron microscope. In all the cases, the microparticles formed were found spherical in shape except to those at low stirring speed where they were agglomerated. Fourier transform infrared study indicated the successful incorporation of isoniazid into the microparticles. Differential scanning calorimetry study showed a molecular level dispersion of isoniazid in the microparticles. X-ray diffraction study revealed the development of some crystallinity due to the encapsulation of isoniazid.

KEY WORDS: characterization; gelatin; isoniazid; microparticle; sodium carboxy methyl cellulose.

INTRODUCTION

Tuberculosis is one of the various diseases that have afflicted the human race for centuries. Approximately one third of the world population is infected with *Mycobacterium tuberculosis* (TB), resulting in more than eight million new cases and two million deaths annually (1). While potentially curative treatments have been available for almost half a century, TB remains the leading cause of preventable deaths and hence continues to present a formidable challenge as a global health problem. Recent implementation of the World Health Organization's strategy (directly observed therapy, short course) has been problematic, and TB remains a major burden in many developing countries. One of the major problems is noncompliance to prescribed regimens, primarily because effective chemotherapy of TB involves the daily administration of one or more drugs for a period of 6 months or longer. Clinical management of the disease is limited because of toxic side effects of drugs, degradation of drugs before reaching their target site, low permeability, and poor patient compliance (2). Thus, the drawbacks of conventional chemotherapy necessitate the development of a delivery or carrier system to release drugs slowly over extended time periods, which would also allow reduction in frequency and dosing numbers.

An important consideration in the treatment of TB is that the etiological agent, *M. tuberculosis*, has the ability to persist intracellularly in the host macrophage for long periods of time. Optimum therapy, therefore, must depend upon the intracellular delivery of antimycobacterial agents for prolonged periods. This becomes even more important when one considers the ability of *M. tuberculosis* to persist in a dormant state, thus giving rise to a large group of infected individuals who carry the organism in a subclinical state without having active disease (3).

Properly devised delivery techniques should theoretically circumvent these problems by positioning effective drugs within host macrophages, thus giving direct access to dormant organisms that presumably would be within macrophages or in the surrounding lymphatic area (4). In the case of a drug that is effective against actively multiplying mycobacteria, this would be advantageous because the drug would continually be available for prolonged periods at the site in the event the organism underwent any multiplication cycle. Microsphere technology has the capability of accomplishing these goals by achieving intracellular delivery of antimycobacterial drugs and allowing programmed controlled release over a prolonged period (5).

Various carrier systems such as liposomes (6,7), polymers (8–10), and microcontainers (11) are used as antitubercular drug carriers. Although the experience with synthetic poly-

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mers is extensive and encouraging (12–14), the recent trend has been to shift towards natural polymers (15). The major advantage of natural polymers includes their low cost and compatibility with the encapsulation of wide range of drugs, with minimal use of organic solvents (16). Furthermore, bioadhesion, stability, safety, and their approval for human use by the US FDA are additional advantages (15,17).

Gelatin is an amphoteric protein and is positively charged below its isoelectric point. Sodium carboxymethyl cellulose (SCMC) is an anionic derivative of cellulose. They are, thus, expected to interact and form polyelectrolyte complex under controlled conditions. The use of gelatin–sodium carboxymethyl cellulose interpenetrating polymer network for controlled delivery of drugs has been cited in the literature (18). The purpose of this study was to prepare and evaluate the polyelectrolyte complex of gelatin A and SCMC as new controlled drug release system for isoniazid. Cross-linking agents are generally employed in order to increase the efficiency of controlled-release system. Varieties of cross-linking agents have been tried. Citric acid did not produce sufficient cross-linking at the temperature (room temperature) at which the reaction was carried out. Genipin, a naturally occurring cross-linker, was found efficient at higher pH (6.0–7.0). Glutaraldehyde was found efficient at the pH (3.5) at which the reactions were carried out. Besides, this process involves water as a solvent and a vegetable oil (sunflower oil) as emulsion medium to eliminate the organic solvent, particularly the most popularly used paraffin oil (19,20). Another purpose of this study was to investigate the optimal conditions for the formation of microparticles of desired size and hence the dependence of drug encapsulation efficiency and release on the reaction conditions.

MATERIALS AND METHODS

Materials

SCMC (medium viscosity) was purchased from Rankem (India). Gelatin type A was purchased from Sigma-Aldrich Inc. (USA). Glacial acetic acid (E. Merck, India), Tween 80 (E. Merck, India), and glutaraldehyde 25% *w/v* (E. Merck, Germany) were used as received. Isoniazid was purchased from Sigma-Aldrich Inc. (USA). Edible-grade refined sunflower oil was purchased from local market. Double-distilled deionized (DDI) water was used throughout the study. Other reagents used were of analytical grade.

Microparticle Preparation

Polyelectrolyte Complexation Conditions

Formation of a polyelectrolyte depends on several parameters such as pH of the polymer solutions, ratio between the polymers, temperature, etc. The optimal conditions for the formation of polyelectrolyte complex of gelatin/SCMC were evaluated by determining the percent yield at various ratios of SCMC to gelatin and at various pH conditions. All the successive experiments were performed at this optimal pH and polymer ratio. The optimum ratio of SCMC to gelatin and pH at which maximum complexation (judged by the percent yield) occurred were 1.0:2.33 and 3.5, respectively.

Preparation Procedure of Microparticles and Microencapsulation

To a beaker containing 150–350 ml of sunflower oil, 15–50 ml of gelatin A solution (2–4% *w/v*) was added under stirring condition (200–1,500 rpm approximately) at 60±1°C to form an emulsion. Zero gram to 1 g of the Tween 80, dissolved in 10 ml of water, was added to the beaker to stabilize the emulsion. Fifteen milliliters to 50 ml of SCMC solution of concentration 0.857–1.714% (*w/v*) was added to the beaker dropwise to attain complete phase separation. However, the weight ratio of SCMC to gelatin was maintained at 1:2.33 during all the experiments. The pH of the mixture was then brought down to 3.5 by adding 2.5% (*v/v*) of the glacial acetic acid solution. The beaker containing the microparticles was left to rest at this temperature for approximately 15 min. The system was then cooled down slowly for 30 min to bring down the temperature to 5–10°C by replacing the hot water from water bath with ice cluster. This was done to harden the microparticles. The cross-linking of the polymer microparticles was achieved by slow addition of a certain amount of glutaraldehyde (4.375–17.50 mmol/g of polymer) solution. The temperature of the beaker was then allowed to rise to room temperature, and stirring was continued for another 10–11 h to complete the cross-linking reaction. The microparticles were filtered through 300-mesh nylon cloth, washed with acetone to remove oil, if any, and adhered to the surface of microparticles. This was further washed with distilled water and freeze-dried. The dried microparticles were then dipped in aqueous isoniazid solution (0.5–20%, *w/v*) for different times (0.5–48 h), filtered through 300-mesh nylon cloth, and quickly washed with water to remove the surface-adhered isoniazid. The isoniazid-encapsulated microparticles were again freeze-dried and stored in a glass bottle in refrigerator.

Calibration Curve of Isoniazid

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

Loading Efficiency

Microparticles were first grounded in a mortar; 0.3 g of accurately weighed grounded materials was transferred with precaution to a volumetric flask containing 100 ml of phosphate buffer solution (pH=7.4, ionic strength 0.01 at 30°C) and kept overnight with continuous stirring to dissolve the isoniazid in the microparticles. The solution was allowed to settle down, filtered, and collected. The isoniazid inside the

microparticles was determined, employing UV spectrophotometer. The loading efficiency (%) was calculated by using the calibration curve and the following formula

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

where

Nomenclature

w_1 amount of isoniazid encapsulated in a known amount of microparticles
w_2 weight of microparticles

Drug Release Studies

Isoniazid release studies from the isoniazid-encapsulated microparticles were carried out with the help of absorbance readings by using UV-visible spectrophotometer (UV-2001 Hitachi); 0.3 g of microparticles was taken into a known volume (100 ml) of dissolution media (pH=1.2 and 7.4). The pH of the medium was maintained by using HCl and phosphate buffer solution. The content was shaken from time to time, and the temperature maintained throughout was 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 dissolution medium was returned to the container.

Water Uptake Study

The swelling behavior of SCMC–gelatin microparticles was studied in two systems at pH=1.2 (0.1 N HCl) and pH= 7.4 (phosphate buffer). Microparticles (0.3 g) were taken in a pouch made of nylon cloth. The empty pouch was first conditioned by immersing it in either 0.1 N HCl (pH 1.2) or phosphate buffer (pH 7.4) for different time periods (1–24 h). The pouch containing the microparticles was immersed in a similar way in either 0.1 N HCl (pH 1.2) or phosphate buffer (pH 7.4) for the similar time periods. The weights of wet microparticles at a definite time period were determined by deducting the respective conditioned weight of the empty nylon pouch from this.

The water uptake (%) was determined by measuring the change of the weight of the microparticles. The percentage of water uptake for each sample determined at time t was calculated using the following equation.

$$\text{Water uptake}(\%) = [(W_t - W_0)/W_0] \times 100$$

where W_t is the weight of the wet microparticles after allowing to swell for a time (t), and W_0 is the initial weight of the microparticles before swelling. The experiments were performed in triplicate and represented as a mean value.

Scanning Electron Microscopy Study

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

Fourier Transform Infrared Study

Fourier transform infrared (FTIR) spectra were recorded in the range of 4,000–400 cm^{-1} using KBr pellet in a Nicholet (model Impact-410) spectrophotometer. Gelatin A, SCMC, polyelectrolyte complex of gelatin A and SCMC, isoniazid, and isoniazid-containing microparticles were each separately finely grounded with KBr and thus kept ready for taking spectra.

X-ray Diffraction Study

X-ray diffractograms of gelatin A–SCMC microparticles, isoniazid, and microparticles with isoniazid encapsulation were recorded on an X-ray diffractometer (Model MiniFlex, Rigaku Corporation, Japan). The samples were scanned between 2θ=3° and 50° at a scan rate of 4°/min.

Thermal Property Study

Thermal properties of SCMC–gelatin microparticles, isoniazid, and isoniazid-loaded microparticles were evaluated by employing differential scanning calorimeter (DSC).The samples were hermetically sealed in a pan, and a 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 studies were done under nitrogen atmosphere.

RESULTS AND DISCUSSION

Effect of Variation of Amount of Surfactant and Polymer Concentration on Size of the Microparticles

The formation and particle size of each microparticle depend on the size of the dispersed droplet, which is determined by the surfactant (Tween 80) used and the emulsifying conditions. For the system of SCMC–gelatin, surfactant Tween-80 had an important role in stabilizing the microparticles formed in sunflower oil. A matrix gel-like product was formed if the surfactant was not added. But different sizes of microparticles were formed on addition of varying amounts of surfactant. SEM photographs of the microparticles are shown in Fig. 1. With the increase of amount of Tween-80 from 0.22 g to 1.16 g/g of polymer, the sizes of the microparticles decreased as shown in Fig. 1a–c. At higher concentration of surfactant, the aqueous phase is easily dispersed into finer droplets, owing to the higher activity of the surfactant, which would result in a lower free energy of the system and lead to a smaller particle size. Landfester also reported the same type of phenomena that, with the increase of surfactant, the size of the droplets in miniemulsions decreased (21).

Again, with increase of the amount of polymer from 0.857 to 1.714 g, an increase in the size of the microparticles was observed (Fig. 1c–e). In the presence of higher amount of polymer, the surfactant present might not be capable of covering all the surfaces of the microparticles properly. This

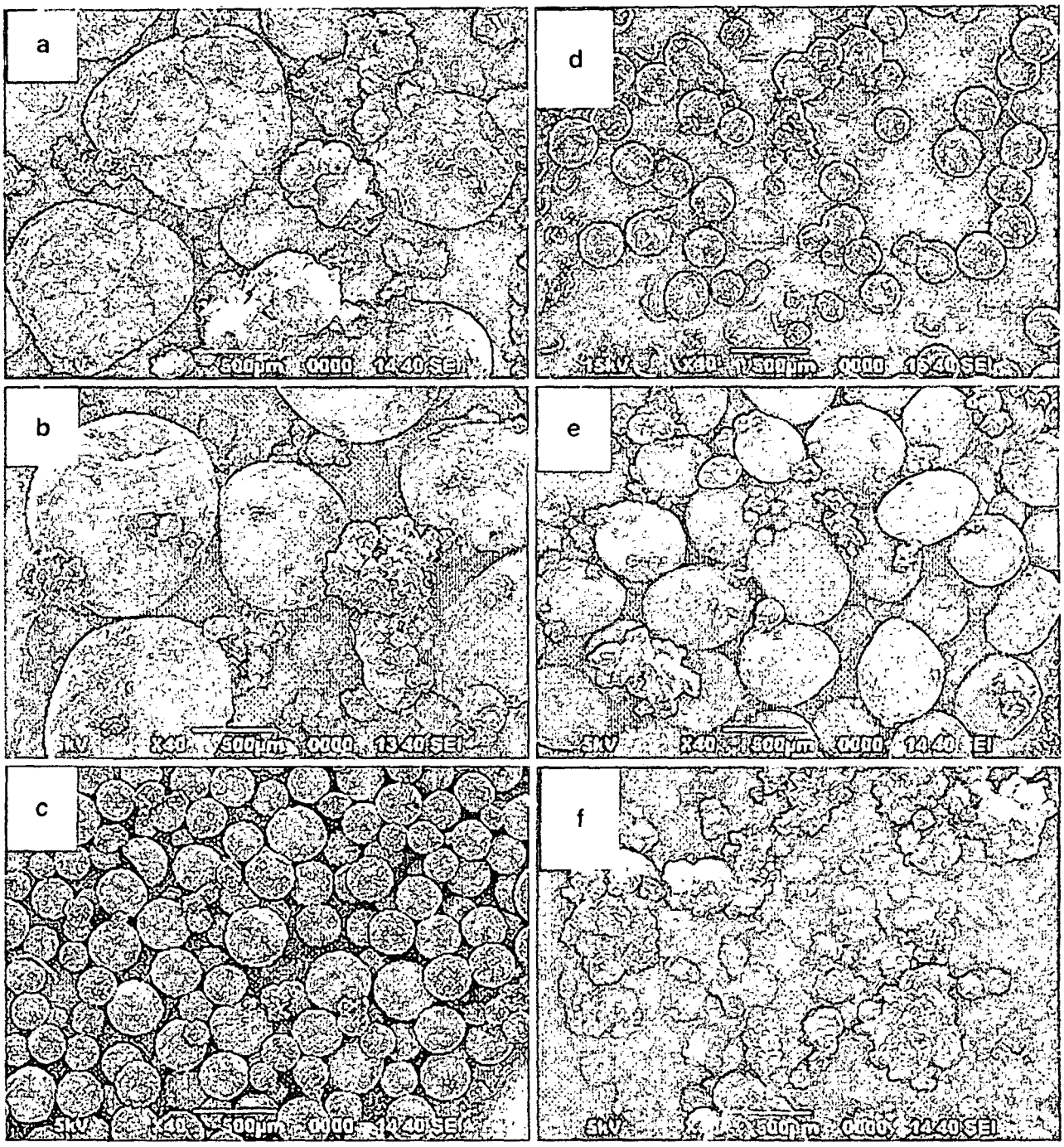

Fig. 1. Scanning electron micrographs of microparticles prepared by using **a** Tween 80=0.22 g/g of polymer, total polymer=0.857.0 g; **b** Tween 80=0.46 g/g of polymer, total polymer=0.8570 g; **c** Tween 80=1.16 g/g of polymer, total polymer=0.857 g; **d** Tween 80=1.16 g/g of polymer, total polymer=1.428 g; **e** Tween 80=1.16 g/g of polymer, total polymer=1.714 g; **f** Tween 80=1.16 g/g of polymer, total polymer=0.857 (low stirrer speed)

resulted in the coalescence of some of the microparticles and led to the formation of larger microparticles. Besides this, the dispersive force of the stirrer became less efficient in the presence of higher amount of polymer and as a result larger microparticles were formed (Fig. 1c–e).

Stirring speed also affected the nature and size of the microparticles. At low stirrer speed (200 rpm approximately), the agglomeration of particles was more (Fig. 1f) compared to those of particles produced at higher stirrer speed (1,500 rpm approximately; Fig. 1d). Improper mixing of polymers at low stirrer speed might be responsible for the observed agglomeration. Zhuo *et al.* reported similar observations during the study of the particle size of polyurea microcapsules by interfacial polymerization of polyisocyanates (22).

All the microparticles were spherical in shape except the particles produced at low stirrer speed. The microparticles

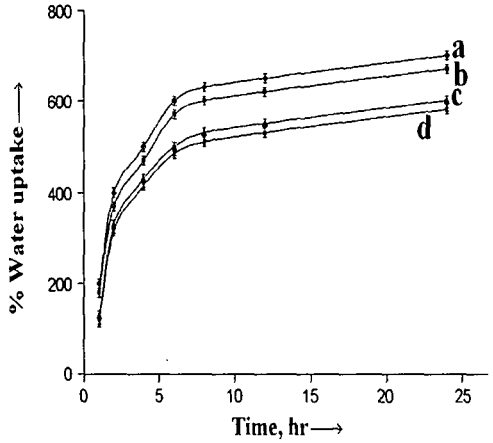

Fig. 2. Effect of variation cross-linker amount and pH on water uptake (*a*: polymer=2.857 g, cross-linker=4.375 mmol/g of polymer, pH=7.4; *b*: polymer=2.857 g, cross-linker=4.375 mmol/g of polymer, pH=1.2; *c*: polymer=2.857 g, cross-linker=17.50 mmol/g of polymer, pH=7.4; *d*: polymer=2.857 g, cross-linker=17.50 mmol/g of polymer, pH=1.2)

arising from low stirrer speed had no definite structure probably due to agglomeration. Again, some roughness appeared on the surfaces of all the microparticles (Fig. 1a–d). The surface of the microparticles prepared at higher polymer and surfactant concentration (Fig. 1e) showed least roughness compared to those of other particles.

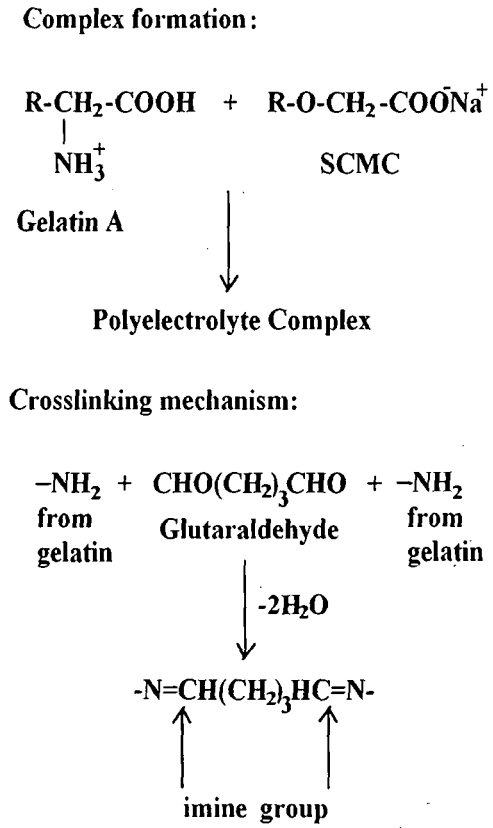

Fig. 3. Probable reaction scheme for interaction between SCMC and gelatin and glutaraldehyde with SCMC–gelatin complex

Water Uptake Study

The water uptake of microparticles was plotted against time of immersion in water (Fig. 2). The water uptake increased rapidly up to 2 h then slowly until 5 h as shown and finally almost leveled off. Microparticles with higher cross-linking showed lesser water uptake than the microparticles with low cross-linking. This was due to the formation of more compact wall (23) caused by cross-linking. Probable reaction scheme for complexation between the two polymers and cross-linking with glutaraldehyde is shown in Fig. 3. Again, water uptake was more at higher pH compared to lower pH. Microparticles formed by the complexation between gelatin A and SCMC became more stable probably at lower pH. At higher pH, the tendency to decomplex between gelatin and SCMC might be responsible for the higher water uptake capacity. Similar findings were reported by Liu *et al.* (24) during the study of the swelling behavior of gelatin–DNA semi-interpenetrating polymer network at different pH.

Table I. Effect of Variation of Isoniazid Concentration and Immersion Time on Loading Efficiency

Concentration of glutaraldehyde (mmol/g of polymer)	Concentration of isoniazid (g/100 ml)	Time of immersion (h)	Loading efficiency (%)
4.375	0.5	1.5	0.33±0.01
	1.0		0.99±0.12
	3.0		14.33±0.11
	5.0		28.66±0.17
	7.0		44.0±0.21
	10.0		51.0±0.43
	20.0		58.11±0.55
10.50	0.5	1.5	0.33±0.01
	1.0		0.66±0.11
	3.0		14.0±0.16
	5.0		23.11±0.18
	7.0		40.21±0.35
	10.0		46.33±0.39
	20.0		50.00±0.52
17.50	0.5	1.5	0.33±0.01
	1.0		0.66±0.13
	3.0		14.0±0.32
	5.0		22.0±0.47
	7.0		38.79±0.58
	10.0		42.11±0.59
	20.0		46.23±0.63
17.50	5.0	0.5	19.0±0.25
		1.0	28.0±0.31
		2.0	35.0±0.42
		4.0	36.0±0.41
		6.0	36.45±0.35
		8.0	37.12±0.32
		17.0	46.0±0.46
		28.0	58.0±0.38
		48.0	58.50±0.31
4.375	20.0	48.0	63.2±0.53
10.50	20.0	48.0	62.0±0.61
17.50	20.0	48.0	60.7±0.50

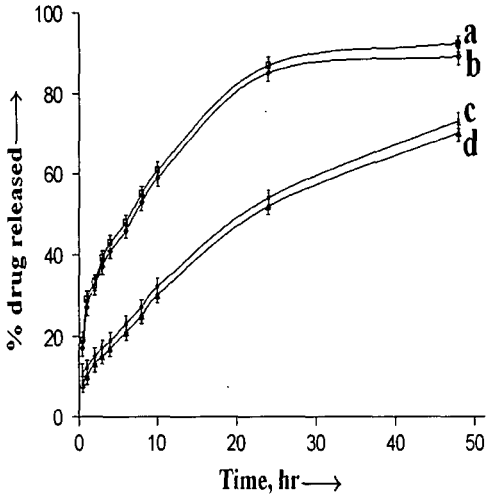

Fig. 4. Effect of variation of cross-linker concentration and pH on release profile (*a*: polymer=2.857 g, cross-linker=4.375 mmol/g of polymer, pH=7.4; *b*: polymer =2.857 g, cross-linker=4.375 mmol/g of polymer, pH=1.2; *c*: polymer=2.857 g, cross-linker=17.50 mmol/g of polymer, pH=7.4; *d*: polymer=2.857 g, cross-linker=17.50 mmol/g of polymer, pH=1.2)

Effect of Variation Drug Concentration and Immersion Time on Loading Efficiency

The effect of variation of concentration of isoniazid and immersion time of microparticles on loading efficiency is shown in Table I. At a fixed immersion time, the loading efficiency was found to increase with the increase in the concentration of isoniazid. An increase in loading efficiency was also observed as immersion time increased. Again, the higher the amount of cross-linker in the microparticles, the lower was the loading efficiency. The increase in loading efficiency was due to more diffusion of isoniazid into the microparticles. The decrease in loading efficiency might be attributed to the formation of more compact wall due to cross-linking that led to a decrease in diffusion rate of isoniazid.

Again, at a fixed isoniazid concentration, the loading efficiency increased with time of immersion up to a certain time and after that it remained constant. But high and low cross-linked microparticles showed more or less similar loading efficiency when immersed in similar concentration of isoniazid solution for a longer period. Longer immersion time allowed the microparticles to become saturated with isoniazid solution.

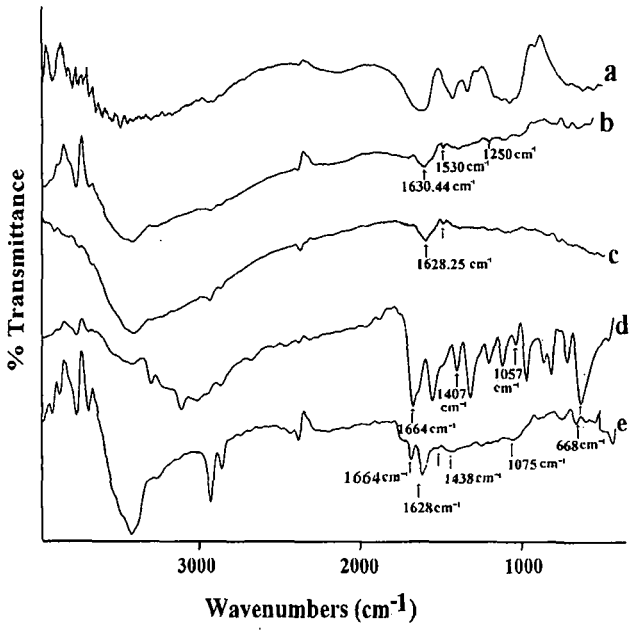

Fig. 5. FTIR spectra of *a* carrageenan, *b* SCMC, *c* gelatin–SCMC complex, *d* isoniazid, *e* isoniazid-loaded microcapsules

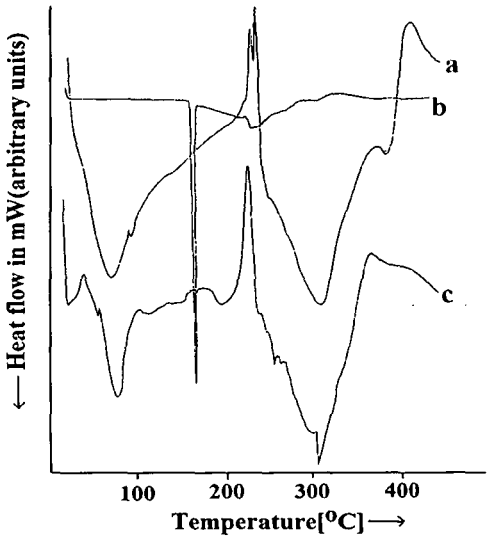

Fig. 6. DSC thermograms of *a* SCMC–gelatin complex, *b* isoniazid, *c* isoniazid-loaded microcapsules

Effect of Variation of Cross-linker on Release Rate of Isoniazid

The effect of the variation of cross-linker concentration (4.375–17.50 mmol/g of polymer) on release rate at pH 1.2 and 7.4 is shown in Fig. 4. Microparticles having approx-

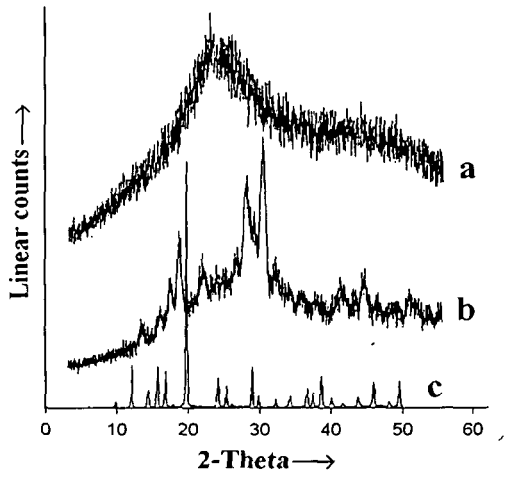

Fig. 7. X-ray diffractograms of *a* microspheres without isoniazid, *b* isoniazid, *c* isoniazid-loaded microspheres

imately similar loading were chosen for the study of the release rate at different pH. The release rate of isoniazid was found to decrease with the increase in the amount of cross-linker in the microparticles. In all the cases, the release was fast initially, reaching maximum, and leveled off finally. The compact microparticle wall was responsible for the decrease in release rate as explained earlier.

Further, the percentage of isoniazid release at lower pH (pH=1.2) was less compared to that of at higher pH (pH= 7.4). The lower and higher release rate in lower and higher pH, respectively, might be explained by considering the tendency of complexation and decomplexation between gelatin A and SCMC as discussed earlier.

Fourier Transform Infrared Study

The spectra of SCMC (curve a), gelatin A (curve b), SCMC–gelatin complex (curve c), isoniazid (curve d), isoniazid-loaded cross-linked SCMC–gelatin microparticles (curve e) are shown in Fig. 5. The spectrum of SCMC showed absorption bands at 3,364, 2,942, 1,627, 1,422, 1,063 cm^{-1} which were due to O–H stretching vibration, CH_3 symmetric + CH_2 asymmetric vibration, C–O stretching band for cellulose, CH_3 + CH_2 bending vibration, and strong C–O stretching band for ethers. The notable absorption bands for gelatin A appeared at 3,421 cm^{-1} (NH stretching), 1,630.44 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 (25). In the complex of gelatin and SCMC, a slight shift of the peak of amide I from 1,630.44 to 1,628.25 cm^{-1} was observed. This indicated that the negatively charged carboxy methyl groups might associate with positively charged gelatin. A similar type of observation was reported by Pranoto *et al.* (26) while studying the interaction between carrageenan and gelatin. The probable interaction between SCMC and gelatin is shown in Fig. 3. In the spectrum (shown as curve d) of isoniazid, the carbonyl absorption (amide I band) appeared at 1,664 cm^{-1}. The amide II band that occurred at 1,555.90 cm^{-1} was due to N–H bending of the secondary amide group. Moreover, in the spectrum of isoniazid, multiple bands appeared between 1,400 and 668 cm^{-1}. Some of the characteristic bands of isoniazid appeared in the isoniazid-loaded microparticles (curve e), suggesting the successful loading of isoniazid in the microparticles. A similar type of IR spectral pattern for isoniazid and isoniazid-containing capsules was reported by Kim *et al.* (12).

Thermal Property Study

DSC thermograms of SCMC–gelatin complex (curve a), isoniazid (curve b), and isoniazid-loaded microparticles (curve c) are shown in Fig. 6. The endotherm appeared in all the thermograms except isoniazid at around 100°C, which was due to removal of moisture. The thermograms of isoniazid showed an endothermic peak due to melting at around 190°C. There was no characteristic peak of isoniazid in the thermogram of isoniazid-loaded microparticles. These results indicated that isoniazid was dispersed in the microparticles. A similar observation was reported by Patil *et al.* during DSC analysis of carvedilol drug encapsulated within alginate microspheres (27).

X-ray Diffraction Study

X-ray diffractograms of gelatin–SCMC microparticles (curve a), isoniazid-loaded micro particles (curve b), and isoniazid (curve c) are shown in Fig. 7. Isoniazid showed multiple sharp peaks at 2θ, varying from 12° to 50°, which were due to the crystalline nature of isoniazid. Appearance of some of these peaks in the diffractograms of isoniazid-loaded microparticles indicated development of some crystallinity due to the encapsulation of isoniazid.

CONCLUSIONS

The optimum conditions for maximum complexation between carrageenan and gelatin occurred at SCMC to gelatin ratio of 1.0:2.33 and pH 3.5. Microparticles of various sizes were prepared by varying surfactant and polymer concentration. Isoniazid concentration governed the absorption of isoniazid into the microparticles. Higher pH medium facilitated the release of isoniazid more compared to lower pH. FTIR study indicated the loading of isoniazid into the microparticles. DSC studies showed that isoniazid was dispersed in the microparticles. X-ray study indicated the development of some crystallinity due to encapsulation of isoniazid.

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