# CROSSLINKED CHITOSAN AS A CARRIER FOR CONTROLLED DRUG DELIVERY

A Thesis presented

by

**S. R. JAMEELA**

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in partial fulfilment of the requirements
for the degree of
Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES & TECHNOLOGY
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# DECLARATION

I, S. R. Jameela hereby declare that I had personally carried out the work depicted in the thesis entitled "CROSSLINKED CHITOSAN AS A CARRIER FOR CONTROLLED DRUG DELIVERY" except where external help sought are acknowledged.

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This is to certify that Smt. S. R. JAMEELA in the division of Polymer Chemistry of this Institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph. D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The work relating to her thesis entitled "**CROSSLINKED CHITOSAN AS A CARRIER FOR CONTROLLED DRUG DELIVERY**" was carried out under my direct supervision.

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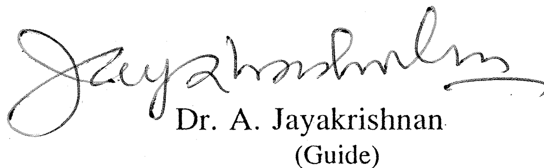

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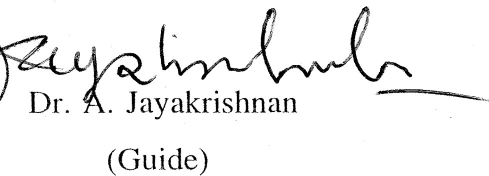

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**DEDICATED TO**

**MY BELOVED PARENTS**

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# SYNOPSİS

# SYNOPSIS

Controlled release delivery systems are designed to maintain the drug concentration within therapeutic range over the desired treatment interval. The use of biodegradable polymers in drug delivery systems allows the implantation of the delivery device without the need for its removal after the delivery is completed. For biodegradable drug carriers, their degradation products *in vivo* should be non-toxic and preferably should be readily eliminated from the body. The use of polymeric microspheres as carriers for the sustained delivery of many therapeutic substances has been well documented in the literature. Unlike other geometries, microspheres have the advantage that they can be injected using a hypodermic needle. The release rate of the active agent from microspheres can easily be manipulated by changing their size, the polymer-drug ratio and crosslinking density in the case of crosslinked spheres. Also, other routes of administration such as oral route is possible using microspheres to sustain the delivery of orally administered drugs.

Chitosan is a natural polysaccharide second only in abundance to cellulose. A large number of biomedical applications have been envisaged for chitosan. Chitosan is susceptible to enzymes such as lysozyme and the degradation products

are amino sugars present in the body and therefore non-toxic. In the field of drug delivery, chitosan has already received much attention as a binder for tablets. The amino functions present on this polysaccharide can be utilised for crosslinking chitosan using a number of chemical crosslinking agents. A dialdehyde such as glutaraldehyde is an excellent reagent to crosslink proteins and polysaccharides having amino functions. In this thesis, the possibility of fabricating chitosan microspheres having good spherical geometry, strength and desired size using glutaraldehyde as the crosslinking agent was investigated. Such microspheres were examined for the sustained delivery of many micro and macromolecules. The possibility of developing photocrosslinkable matrix from chitosan was also explored with a view of regulating the drug release from the matrix by photo irradiation.

The introductory chapter gives an overview of the fundamentals of controlled release, polymeric microspheres for controlled drug delivery, the literature on chitosan as a drug carrier and the aim and scope of the study.

Chapter 2 discusses the experimental procedures involved in the investigation. This consists of characterisation of chitosan with respect to its molecular weight and degree of deacetylation, preparation of glutaraldehyde crosslinked chitosan microspheres, their characterisation using different techniques such as scanning electron microscopy, particle size analysis, swelling studies, implantation studies to evaluate the tissue response and biodegradation. The methods for preparing various drug-loaded microspheres and methods for following drug release *in vitro* and *in vivo* in different animal models are also described. Experimental techniques for the

preparation of photocrosslinkable chitosan and its characterisation are also included in this chapter.

In Chapter 3.1 the results obtained on the preparation and evaluation of glutaraldehyde crosslinked chitosan microspheres are discussed. The size, surface morphology, internal structure and particle size of chitosan microspheres prepared are analysed with respect to the techniques employed in their preparation.

Chapter 3.2 discusses the results of the degradation studies of glutaraldehyde crosslinked chitosan microspheres. *In vitro* degradation studies were conducted by exposing microspheres in lysozyme solutions of different concentrations at 37°C. Surface topography changes examined using scanning electron microscopy did not suggest any significant degradation for crosslinked microspheres *in vitro* even after incubation for many weeks. *In vivo* degradation studies were conducted by implantation of microspheres fabricated from 74% deacetylated chitin in rat muscle and explantation over different periods of time up to one year. Histological analysis of tissue containing the microspheres did not suggest any adverse tissue reactions due to microspheres and showed that the microspheres were undergoing biodegradation in rat muscle although slowly. Scanning microscopic examination of explanted microspheres showed that the microspheres were degraded in the tissue by a surface erosion mechanism rather than by bulk erosion. Biodegradation was not complete even after one year suggesting that glutaraldehyde crosslinked chitosan microspheres could possibly function as a long acting biodegradable drug delivery vehicle. In view of the fact that chitosan having different degrees of deacetylation may biodegrade at different rates, biodegradation studies were also

conducted using chitosan which was acetylated further. However, no significant difference in the degradation profiles was observed.

Chapter 3.3 deals with the results obtained using mitoxantrone, a novel anticancer agent encapsulated in glutaraldehyde crosslinked chitosan microspheres. Mitoxantrone was loaded into chitosan microspheres at an initial loading of 5 and 10% and the *in vitro* release of the drug was examined in phosphate buffer at 37°C. The study showed that drug release from microspheres could be controlled by changing the extent of crosslinking in a very elegant manner. Drug release was also examined from microspheres of different size and drug payload. The anti-tumour activity of mitoxantrone-loaded chitosan microspheres was evaluated in mice bearing Ehrlich ascites carcinoma by following the animal survival time and change in body weight. The study showed that the microsphere modality was therapeutically many times more effective than administration of the free drug at several times the LD50 of mitoxantrone.

Chapter 3.4 discusses the studies carried out using the anti-fertility steroid, progesterone-loaded chitosan microspheres. *In vitro* release of progesterone from microspheres was examined in phosphate buffer as a function of crosslinking density of microspheres and particle size. *In vivo* bioavailability of progesterone was examined in a rabbit model by intramuscular injection of microspheres loaded with progesterone. The study showed that while the steroid administered in the free form disappeared within 5 days from the animal, the microencapsulated form was able to sustain a serum progesterone concentration of 1-2 ng/mL for about 5 months without peaking to toxic levels at any time unlike the free drug. The data suggested

that chitosan microspheres would be useful as a carrier for the prolonged delivery of antifertility steroids.

Chapter 3.5 discusses the *in vitro* and *in vivo* evaluation of chitosan microspheres loaded with an oral drug, diclofenac sodium. *In vitro* release of the drug was examined from microspheres of different loadings in simulated intestinal fluid at 37°C. The effect of crosslinking density of microspheres, drug payload and size of microspheres on *in vitro* release is discussed. Bioavailability of the drug from microspheres was assessed in a rabbit model after oral administration. The study showed that microspheres could sustain a plasma concentration for about 12 h.

Chapter 3.6 comprises the results of the work done using chitosan microspheres loaded with macromolecules such as bovine serum albumin (BSA) and diphtheria toxoid (DT). Both macromolecules were loaded into preformed glycine quenched chitosan microspheres by passive absorption from aqueous solutions of the proteins. *In vitro* release of BSA from microspheres into phosphate buffer showed an initial 'burst' followed by a gradual increase in the release rate with time for several days. Modulation of the release was attempted by coating the BSA-loaded microspheres using poly(lactic acid) and paraffin oil. The poly(lactic acid) coating given to microspheres resulted in extending the release of entrapped BSA up to 2 months. Immunogenicity studies were carried out in rats using DT passively loaded into preformed chitosan microspheres. The antibody response was compared with DT given on alum as control. While the titres obtained with the microsphere modality was rather low compared to DT given on alum as control, the response was rather uniform during the period of the study. The results of this study are analysed

critically and possible future potential of chitosan microspheres as a vaccine carrier is highlighted.

Chapter 3.7 deals with an attempt to prepare a derivative of chitosan that would crosslink on photo irradiation. The idea was to produce a chitosan having azide functions that could be crosslinked using ultraviolet light. 1-chloro- 2,3-epoxy propane was converted to 1-azido-2,3-epoxy propane by treatment with sodium azide in the presence of a phase transfer catalyst. The azidated epoxide was then treated with chitosan in the presence of a mineral acid to obtain chitosan bearing azide functions which was characterised using infrared spectroscopy. Films were cast from azidated chitosan in the presence of a model drug such as theophylline and subsequently crosslinked using ultraviolet irradiation. The release of theophylline from uncrosslinked films and films crosslinked by irradiation was examined in simulated biofluids.

Finally, chapter 4 contains the summary, conclusions and future prospects of the investigations reported in this thesis.

# CHAPTER 1
# INTRODUCTION

# INTRODUCTION

## 1.1 Need for Controlled Release

A controlled release system is simply any system that regulates or controls the release of a bioactive agent. Controlled release systems regulate the availability of the active agent to fairly narrow ranges in order to elicit the desired response, while reducing potential dangerous side effects to acceptable levels (Gebelein and Carraher, 1985). In the agriculture field, a controlled delivery system can be used for the controlled release of pesticides and fertilizers. Its potential applications in pharmaceutical field are numerous. When a drug is administered by conventional dosage form, the systemic drug level far exceeds the therapeutic level for a brief period and then gradually declines to an ineffective level at which point repeated administration becomes necessary. Often, the initial maximum concentration is above the therapeutically desirable level thereby increasing the risk of side effects. On the other hand, minimum concentration may be below the therapeutically effective level. In this way conventional dosage form can result in a drug regimen in which the patient oscillates between alternating periods of drug overdose and drug inefficacy. A controlled release system ideally smooths the peaks and valleys in the

drug concentration in blood providing a more effective therapy by releasing the drug in a controlled, predictable and predetermined fashion (Juliano, 1980; Robinson, 1978). A schematic representation of drug concentration in blood by controlled release and conventional dosage forms is shown in the Fig. 1.1.

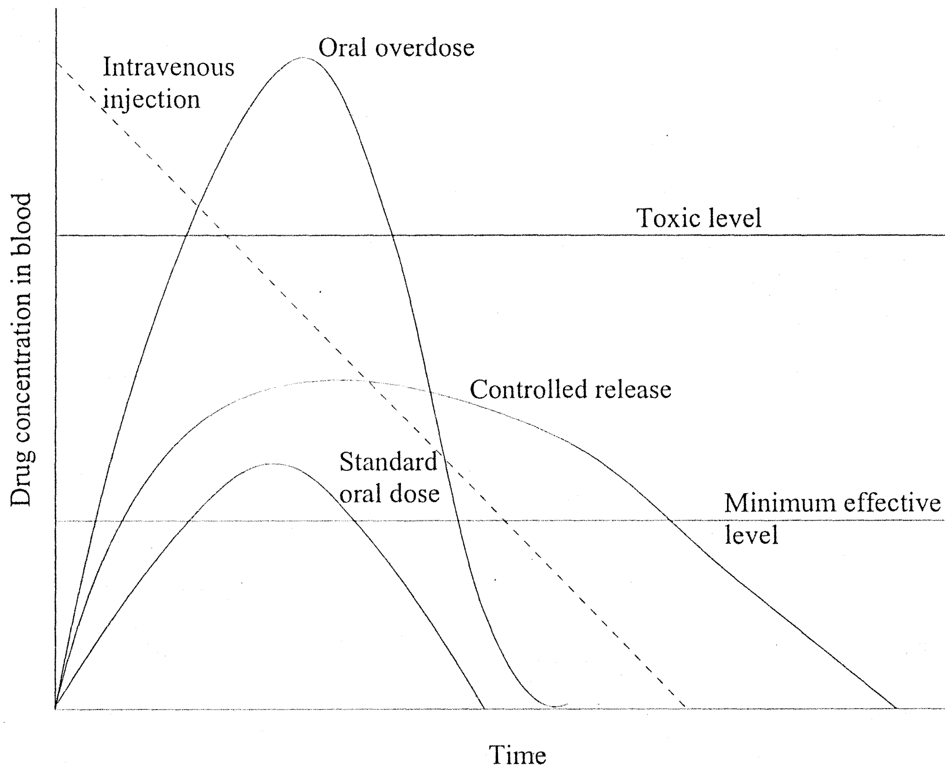



Fig. 1.1 Relationship between drug concentration and time

Thus, controlled drug delivery has the following potential advantages compared with conventional drug delivery.

(1) Avoids patient compliance problem.

(2) Employs less total drug.

(3) Minimises or eliminates local and systemic side effects.

(4) Minimises drug accumulation with chronic dosing.

(5) Improves efficiency in treatment.

Thus, proper drug delivery system leads to more prompt cure.

## 1.2 Mechanism of Drug Release

The release rate of a dissolved or dispersed drug from a polymer film or tablet introduced in a specific environment, strongly depends on the nature of the diffusion and sorption involving the polymer/environment system and the polymer/drug system. Theoretically, three different mechanisms of drug release can be identified. They are: (1) solvent controlled, (2) diffusion controlled and, (3) chemically controlled release.

### 1.2.1 Solvent Controlled System

Polymer swelling and osmosis are the two primary solvent controlled mechanisms of drug release. Swelling controlled system includes mostly hydrogels. In biodegradable hydrogels, drugs are usually in contact with water and thus drug solubility is an important factor in drug release. Upon contact with an aqueous dissolution medium, a distinct interface (front) is observed, which corresponds to the water penetration front into the polymer and separates the glassy state of the material from the rubbery (gel-like) state. A sharp boundary separates the essentially unpenetrated core from a uniformly swollen shell. The polymer relaxation and swelling are driven by the osmotic stress generated at the moving boundary by the presence of the penetrant and remains constant as long as constant local concentration persists (Sarti and Apicella, 1980; Sarti *et al.*, 1984; Thomas

and Windle, 1981). The rate of swelling depends on the hydrophilic/ hydrophobic balance of the polymer matrix and the degree of crosslinking (Brondsted and Kopecek, 1991).

### 1.2.2 Diffusion Controlled System

The dissolved species will diffuse from a matrix which does not actively interact with the external environment according to an ordinary diffusion law. In such a case, the concentration profile in the matrix decreases with time leading to the progressive reduction of the release rate. Under this category controlled release systems are of two types, viz., the reservoir systems and the matrix systems.

#### 1.2.2.1 Reservoir System

A typical reservoir system consists of non-degradable rate limiting polymeric membrane, separating a core of drug from the biological environment (Good and Lee, 1984) . Reservoir systems have been formulated as capsules, microcapsules, hollow fibers or tubes with sealed end. In reservoir systems consisting of non-degradable polymers, the rate of drug release is strictly controlled by the rate of drug diffusion through the polymeric membrane. An implantable reservoir type contraceptive device consisting of the steroid levonorgestrel within a polycaprolactone cylinder under the trade name Capronor™ has been formulated and tested clinically in many countries (Pitt, 1990a). Here, the drug release is diffusion controlled, lasts over a year and the polycaprolactone membrane completely degrades after 3 years from the date of implantation.

#### 1.2.2.2 Matrix System

Here the drug is uniformly distributed within the polymeric phase (Cardinal, 1984). For a matrix system fabricated from a degradable polymer, the release mechanism will be governed by a combination of diffusion and polymer degradation.

### 1.2.3 Chemically Controlled System

If the rate of drug release is mainly controlled by the rate of polymer degradation or rate of cleavage of a drug covalently bound to the polymer backbone it is said to be chemically controlled system. Thus, two main types of chemically controlled systems are matrix systems based on degradable polymers and the pendent chain system.

#### 1.2.3.1 Matrix System Based on Biodegradable Polymers

The use of biodegradable polymers in drug delivery systems allows the implantation of the device without the need for its removal after the drug has been released (Heller, 1984). The desirable characteristics of polymer system used for drug delivery , whether natural or synthetic, are minimal effect on biological system after introduction into the body, *in vivo* degradation at a well defined rate to non-toxic and readily excreted degradation products, and absence of toxic endogenous impurities or residual chemicals used in their preparations.

#### 1.2.3.2 Pendent Chain System

This consists usually of a degradable or nondegradable polymeric backbone to which a pharmacologically active agent is covalently attached through reactive pendent chain. A spacer may be placed between the drug and the polymer backbone (Harris, 1984).

## 1.3 Biodegradation

Biodegradation has been defined in various ways by different investigators (Park *et al.*, 1993). It has been defined as change in surface properties or loss of mechanical strength (Lemm *et al.*, 1981), assimilation by microorganisms (Potts *et al.*, 1973), degradation by enzymes (Huang *et al.*, 1979; Swift, 1992), backbone breakage and subsequent reduction in the average molecular weight of the polymer (Ratner *et al.*, 1988; Hergenrother *et al.*, 1992), or extraction of low molecular weight materials leading to surface defects (Ratner *et al.*, 1988). Degradation can occur by each of the above mechanism alone or in combination with one another. Biodegradation can occur on many different structural levels ie; molecular, macromolecular, microscopic and macroscopic depending on the mechanism (Marchant *et al.*, 1984). The material degrades into less complex products through biodegradation and this occurs through four different mechanisms: solubilisation, charge formation followed by dissolution, hydrolysis and enzyme catalysed degradation (Gilding, 1981).

Biodegradation of polymers is expected to take place in four stages. Hydration, loss of strength, loss of mass integrity and mass loss (Kronenthal, 1975). The

hydration of polymers depends on the hydrophilicity of the polymer. The hydration results from disruption of secondary and tertiary structures stabilized by van der Waals forces and hydrogen bonds. If the interaction between polymer chains is great, it may not dissolve in water. For example, intermolecular hydrogen bonding between hydroxyl groups on the glucose residues of cellulose is so strong that cellulose is insoluble in water (Mac Greger and Greenwood, 1980). During and after hydration, the polymer chains may become water-soluble and/or the polymer backbone may be cleaved by chemical or enzyme-catalyzed hydrolysis to result in the loss of polymer strength. For crosslinked polymers, the polymer strength may be reduced by cleavage of either the polymer backbone, crosslinker, or pendent chains. Further cleavage of the polymer chains leads to the loss of mass.

## 1.4 Polymeric Microspheres as Drug Carriers

In controlled release technology, polymeric microspheres have been extensively investigated as drug carriers (Davis *et al.*, 1984; Guiot and Couvreur, 1986). Microspheres offer several advantages over large implants of equal mass and drug load. The greater surface area of multiple small particles allows for higher release rates. It also provides flexibility in route of administration. For example, drug loaded microspheres can be reconstituted in a suitable vehicle and can be administered by a single injection. Other physical forms of drug delivery devices such as rods, cylinders and slabs require surgery for implantation and therefore are less patient compliant.

The oral route of administration is the most preferred route when systemic drug effects are sought since self-administration is facilitated. It is usually

the least expensive and is typically the most reliable and safest method of self medication. Gastro-intestinal (GI) absorption of orally administered drug depends on physicochemical properties of the drug and dosage form, the physiological functions of the GI tract, the metabolic activity of the lumen microflora etc. Limitation of this route of administration is low bioavailability due to brief transit time through GI tract ($\sim$12 h) and the severe chemical conditions in the stomach.

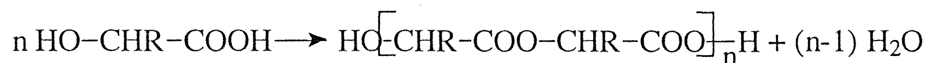
The parenteral route includes subcutaneous, intramuscular, intravenous, and intraperitoneal routes. Parenteral route offers challenges as well as opportunities for sustained, controlled release. Here the system must be sterile, and they must also be biocompatible and safe. This route is particularly attractive for delivery of peptides and proteins due to their poor oral absorption and requirement for frequent injectable dosing regimens (Floy *et al.*, 1993). Here, first pass effect associated with oral delivery is obviated. The drawbacks of this route is that injections are painful, can lead to infection, and repeated intravenous injections can lead to severe vascular problems.

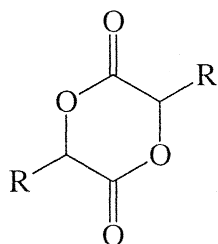
## 1.5 Microspheres Based on Synthetic Polymers

### 1.5.1 Polyester Microspheres

The polyesters are by far the most widely studied synthetic polymers for drug delivery. They are easly prepared as homo- and copolymers in a wide range of molecular weights. Among these, the most extensively studied are the poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) and their copolymers of various compositions.

PLA and PGA can be prepared by the direct polycondensation of lactic acid/and glycolic acid respectively or by the ring opening melt condensation of the cyclic diester of lactide or glycolide.




R = H for glycolide
 = $CH_3$ for lactide

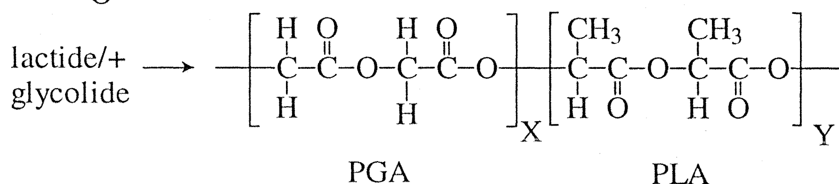



Fig. 1.2. Scheme for the preparation of polylactide/glycolide polymers

The degradation mechanism for all polyesters is reported to be due to homogeneous erosion by random hydrolytic chain scission (Baker, 1987). Effect of enzymes on the biodegradation of these polymers is a subject of controversy. Microspheres of these polymers are prepared by dispersion/emulsion technique, followed by solvent removal (Sato *et al.*, 1988; Jeffery *et al.*, 1991). The process involves dissolving the drug and the polymer in a common solvent usually methylene chloride and dispersing this in a continuous phase to form microdispersions. Solvent removal from the microdispersion is accomplished by one of the three solvent removal techniques: solvent evaporation method or solvent extraction or freez drying. If the drug incorporated is insoluble in the solvent

used, drug is distributed as discrete particles throughout the microsphere. Many conventional pharmaceutical agents formulated in lactide /glycolide microspheres for controlled release have been widely studied for almost two decades (Jackanicz *et al.*, 1973; Wise *et al.*, 1978; Moulding, 1987; Ramtoola *et al.*, 1992; Lalla and Sapna, 1993; Giordano *et al.*, 1993; Le Corre *et al.*, 1995). Most successful lactide/glycolide formulations in regard to clinical results have been those of Beck and coworkers (Beck *et al.*, 1979, 1980, 1981, 1983; Beck and Tice, 1983). Recently several workers have reported on these microspheres for the controlled release of steroids like leuprorelin acetate (Toguchi, 1992; Okada *et al.*, 1994), and norethisterone (Zhou *et al.*, 1993). Other reported works are those in which it is used for the controlled release of various antimalarial drugs (Tsakala *et al.*, 1988, 1990), anticancer drugs (Bissery *et al.*, 1985; Yamada *et al.*, 1991; Ike *et al.*, 1991) antibiotics (Tice *et al.*, 1984), antiinflammatory agents (Tice *et al.*, 1985) and anesthetics (Williams *et al.*, 1984). These microspheres also seem to have considerable potential in delivering proteins and polypeptides (Uchida *et al.*, 1994; O'Hagan *et al.*, 1993a, 1993b; Yan *et al.*, 1994; Gander *et al.*, 1995). In controlled vaccine delivery, this matrix has been used by several investigators for the controlled release of vaccines such as staphylococcal enterotoxin B (Eldridge *et al.*, 1991), diphtheria toxoid (Singh *et al.*, 1991), hepatitis B surface antigen (Nellore *et al.*, 1992), tetanus toxoid (Alonso *et al.*, 1993, 1994), vibrio cholera antigens (Chandrasekhar *et al.*, 1994), and HIV-1 (Cleland *et al.*, 1994).

Another polyester that has been investigated is poly(ε-caprolactone) (PCL). (Pitt *et al.*, 1979, 1980, 1981, Jaffe *et al.*, 1981). PCL is prepared by the ring opening polymerisation of the monomer caprolactone.

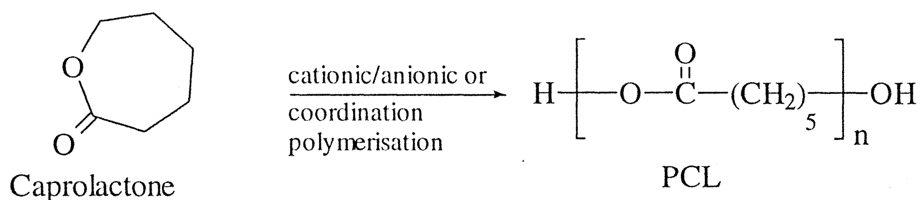
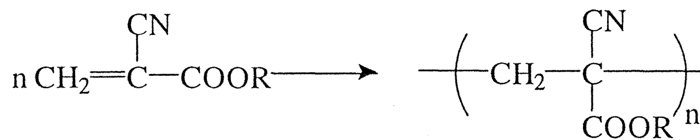



Fig. 1.3. Scheme for the preparation of poly(ε-caprolactone)

This polyester is biocompatible and biodegradable to non-toxic degradation products and can be blended with many different commercial polymers over a wide range of composition (Ory *et al.*, 1983; Vion *et al.*, 1986). In comparison with PLA or PGA, PCL has a very low Tg of around -60°C and is rubbery at room temperature. This unusual property contributes to the very high permeability for PCL for many molecules (Engelberg and Kohn, 1991). Use of this polymer in controlled drug delivery applications has been reviewed by Pitt (1990a). The degradation of PCL is significantly slower than that of PLA or PGA and therefore is more suitable for long term delivery of therapeutic substances. The biodegradation of PCL has been extensively studied in the past 20 years and several reviews are available (Pitt and Schindler, 1983; Pitt, 1990a). Dordunoo *et al* (1995) encapsulated taxol in PCL microspheres and tested for angiogenesis inhibition using chick chorioallantotic membrane model. Pitt *et al* (1979) studied the progesterone release from PCL and found that the release is diffusion controlled. Song *et al* (1987) studied norgestrel release from the microspheres of poly(caprolactone-*b*-lactic acid) with different block length and found that the PCL gives diffusion controlled release and the lactic acid segment gives an erosion controlled release.

Polycyanoacrylates are another class of polyesters used as drug carriers. They are prepared by the polymerisation of cyanoacrylate monomers.




R = H or other alkyl groups

Fig. 1.4. Scheme for the preparation of polycyanoacrylate

Microspheres of these polymers were prepared by emulsion polymerisation of the monomers (Thies and Bissery, 1984). Polycyanoacrylates are biodegradable with the rate of breakdown being dependent on the length of the alkyl chain. Under physiological conditions, degradation occurs mainly by enzymatic process (Couvreur *et al.*, 1984). Several compounds such as adriamycin, actinomycin D and insulin have been incorporated into polyalkylocyanoacrylate nanoparticles of different formulations (Couvreur *et al.*, 1984). Couvreur *et al* (1979) studied the correlation between the degradation of nanoparticles and *in vitro* release of actinomycin D. A good correlation was found between the drug release and degradation in a wide range of buffer at different pHs. Use of these nanoparticles in topical drug delivery with $C^{14}$-labelled poly(hexyl-2-cyanoacrylate) has been attempted by Wood *et al* (1985). Li *et al* (1986) determined the efficiency of encapsulation of a model lipid soluble compound, progesterone, by poly(butyl cyanoacrylate) nanoparticles and measured the topical ocular delivery of progesterone using these particles. Kreuter *et al* (1988) discovered that a

significant increase in the pharmacodynamic effect of pilocarpine could be attained by incorporation into poly(butyl cyanoacrylate) nanoparticles. Recently park *et al* (1994) have studied the release of proteins such as bovine serum albumin, horseradish peroxide and tetanus toxoid from poly(isobutyl cyanoacrylate) particles coated with poly(lactic acid) and demonstrated that with tetanus toxoid-loaded particles, the time course of immunisation can be controlled by the type of polymer matrices used.

### 1.5.2 Polyanhydride Microspheres

Polyanhydrides are a novel class of biodegradable polymers under development as vehicles for the release of bioactive molecules including peptides and proteins (Chasin *et al.*, 1990). They are prepared from dibasic acid by melt polycondensation. For example, poly[bis(*p*-carboxyphenoxy)propane] anhydride is prepared by mixing 1,3-bis(*p*-carboxyphenoxy)propane with acetic anhydride to form mixed anhydride. On heating, it undergoes polycondensation to form anhydride.

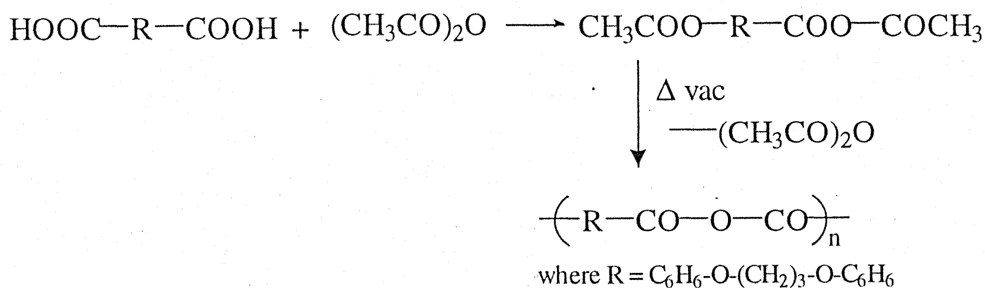



Fig. 1.5. Scheme for the preparation of polyanhydride

A series of biocompatibility studies reported on several polyanhydrides have shown them to be non-mutagenic and non-toxic (Leong *et al.*, 1986a). Several different techniques have been developed for the preparation of microspheres from polyanhydrides. One is hot melt encapsulation process where the drug to be encapsulated is mixed with melt polymer and mixture is suspended in a non-miscible solvent that heated 5°C above the melting point of the polymer and stirred continously. Once the emulsion is stabilized, it is cooled until microspheres are formed. Another method is by solvent removal and different solvent removal techniques have been developed (Mathiowitz *et al.*, 1988; Bindschaedler *et al.*, 1988). Mathiowitz *et al* (1990) studied the morphology of polyanhydride microspheres prepared by different fabrication methods. Due to their highly hydrophobic nature, polyanhydrides degrade by surface erosion. Polyanhydrides in general degrade more rapidly in basic media than in acidic media (Leong *et al.*, 1985). Leong *et al* (1986b) in another study found that poy(carboxyphenoxypropane anhydride) degrades in over 3 years. Degradation rate was found to change with the length of the alkyl group in the polymer backbone. By changing the composition of the copolymer, a wide range of degradation rate can be obtained. Insulin has been incorporated into polyanhydride microspheres prepared from bis(*p*-carboxyphenoxy propane) and sebacic acid and *in vitro* studies showed that much of insulin is released over the first 1-2 days but significant amount continues to be released for 4-5 days (Mathiowitz and Langer, 1987; Mathiowitz *et al.*, 1985). Low molecular weight drugs such as acid orange 63, acid red 8 and *p*-nitroaniline were incorporated in polyanhydride microspheres and microsphere degradation as well as drug release was studied (Tabata and Langer,

1993). Tabata and coworkers in their studies found that different proteins like lysozyme, trypsin, heparinase, ovalbumin, albumin etc., when encapsulated in polyanhydride microspheres retained their biological activity (Tabata *et al.*, 1993a). They observed that all the proteins irrespective of molecular weights and loading released at a near constant rate without large initial burst, and erosion of the microspheres also showed a near zero order kinetics.

### 1.5.3 Other Biodegradable Polymers

Other biodegradable polymers which find application in controlled drug delivery include poly(orthoester)s, poly(acetal)s, polycarbonates, poly(hydroxybutyrate)s, and poly(α-malic acid). Structures of these polymers are shown in Fig. 1.6. Poly(orthoester)s are stable in alkaline medium, but undergo degradation in acidic medium (Heller *et al.*, 1981, 1992). Therefore, addition of basic salts stabilises the interior of the matrix limiting degradation to the surface. Since the surface eroding device tend to release the drug incorporated at a constant rate, poly(orthoester)s appear to be particularly useful for the controlled release of drugs (Heller *et al.*, 1983, 1985, 1987; Heller, 1988). Application of this material in topical delivery have been investigated (Heller *et al.*, 1990). Release of norethintrone from poly(orthoester) and polyacetal sheets were attempted (Heller *et al.*, 1981).

Polyethylene carbonate and polypropylene carbonate have been tested as biodegradable carriers for the delivery of 5-fluorouracil (Kawaguchi *et al.*, 1982). Ouchi *et al* (1990) have prepared poly(α-malic acid)/5-FU conjugates and its antitumour activity has been evaluated. poly(hydroxy butyrate) and poly(hydroxy valerate) have been investigated as biodegradable polymers as homopolymers,

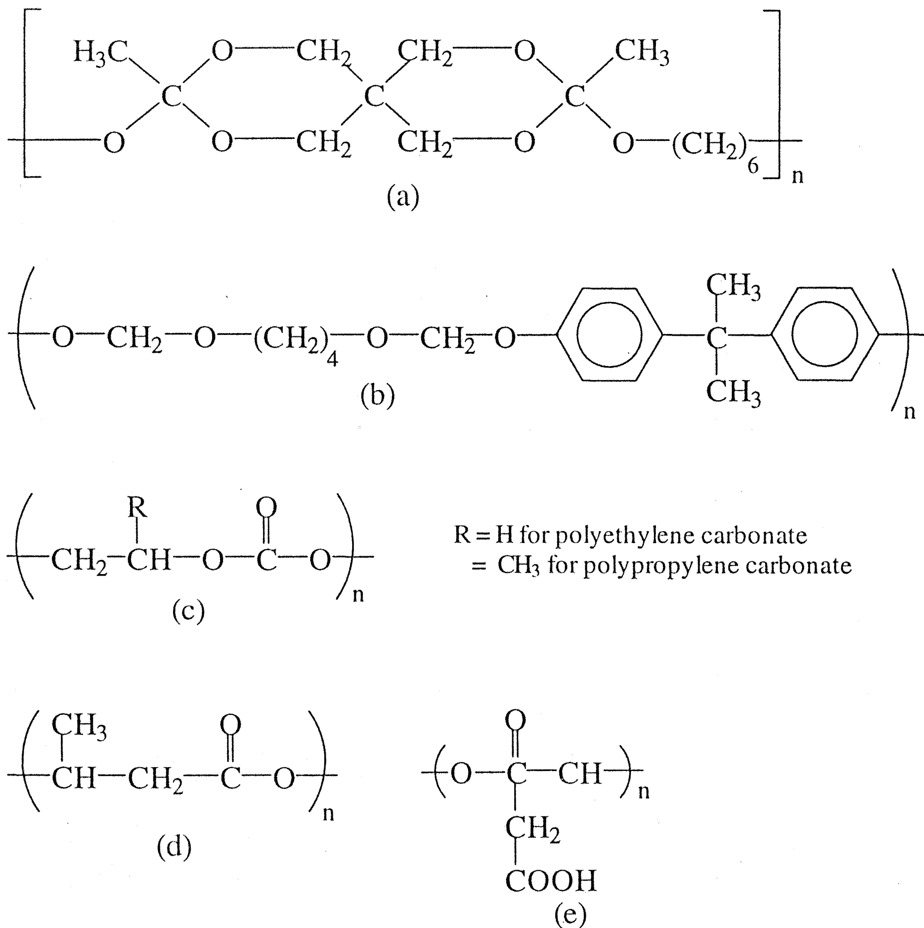



Fig. 1.6. Structures of some other biodegradable polymers

(a) Polyester formed from 3,9-bis(methylene)-2,4,8,10-tetraoxaspiro[5,5]-undecane and 1,6,hexanediol. (b) Polyacetal formed from bis-phenol A and divinyl oxybutane (c) Polycarbonate (d) β-hydroxybutyrate and (e) α-malic acid

copolymers, and as blends with polysaccharides and poly(ε-caprolactone) (Holland *et al.*, 1990; Yasin and Tighe 1992).

## 1.6 Microspheres Based on Natural Polymers

Generally, microspheres from natural polymers are prepared by a crosslinking process. A water-oil emulsion is prepared, where the water phase is a solution of the polymer which contains the drug to be incorporated. The oil phase is a suitable vegitable oil or oil-organic solvent mixture containing an oil-soluble emulsifier. Once the desired W/O emulsion is formed, the polymer is solidified by some kind of a crosslinking process. This may involve thermal treatment or the addition of a chemical crosslinking agent such as glutaraldehyde or photocrosslinking

Thermal denaturation has been used to crosslink proteins. Albumin, gelatin and collagen have been crosslinked by thermal treatment (Yannas and Tobolsky, 1966; Yannas and Tobolsky, 1967; Wetzel *et al.*, 1980; Burgess and Carless, 1987; Burgess *et al.*, 1987; Heller, 1987). Proteins become insoluble by the formation of interchain amide linkages (Yannas and Tobolsky, 1966, 1967).

Among different chemical crosslinking agents, glutaraldehyde has been used more frequently since it is less expensive, is readily available and is highly soluble in aqueous solution. In a recent review, we have examined and evaluated the merits and drawbacks of using this reagent as a fixative in bioprostheses and drug delivery matrices (Jayakrishnan and Jameela, 1996). It crosslinks biological polymers like proteins and polysaccharides, by reacting primarily with their amino groups to form imino bond (Schiff base).

Ionising radiations crosslink many synthetic and natural polymers. Beneficial changes due to curing are thus attainable without the addition of chemical reactant. Photocrosslinking may be induced by non-particulate radiations (UV, X ray, $\gamma$-ray

etc) or particulate radiations ($\alpha, \beta$, high energy electrons, protons, deutrons etc.) (Shultz, 1966). On irradiation, bond cleavage of polymer occurs and reactive intermediates are formed, and these undergo various reactions which result in crosslinking of the polymer. Photocrosslinkable polymers are classified as follows,

1. Those bearing functional groups which crosslink by themselves under photoirradiation. A sensitizer may be added but other crosslinking agents or polymerisable monomers are not necessary. The functional group is either a photopolymerisable group or a one-to one coupling group.
2. Those mixed with a crosslinker where the functional groups of the polymer are activated when contact is made with a photoexcited crosslinker or the photodecomposition products of a crosslinker. A sensitiser may be added.
3. Those polymerised in the presence of polyfunctional monomers. Here sensitiser is indispensible.

Applicability of the three processes depends on the required quality of the photocrosslinked product and the workability. The problems of designing photocrosslinkable polymer have been extensively reviewed (Tazuke, 1982).

### 1.6.1 Protein Microspheres

#### 1.6.1.1 Albumin Microspheres

During the last 30 years, extensive efforts have been made towards the design and development of albumin microspheres for the purpose of drug delivery. The exploitable features of albumin include its reported biodegradation into

natural products (Zolle *et al.*, 1970; Bernard *et al.*, 1980), its lack of toxicity, non-antigenicity (Taplin *et al.*, 1964; Rhodes *et al.*, 1969) and its ready availability. The use of albumin microspheres in drug delivery was first suggested by Kramer (1974). After that, a large number of publications and patents appeared on the synthesis and evaluation of albumin microspheres for drug delivery.

Albumin microspheres can be prepared by heat denaturation and cure temperature is a key factor in determining the aqueous swelling behaviour and biological life of protein microspheres. Sugibayashi *et al* (1979) reported that 5-fluorouracil release from albumin microspheres containing 3.3 wt% drug into pH 7.4 buffer is a strong function of the treatment temperature. The *in vitro* release behaviour of doxorubicin hydrochloride from albumin microspheres is also determined by the treatment temperature. Effect of crosslinking time on the *in vitro* release of dexamethasone from heat-crosslinked microspheres has been studied (Dilova and Shishkova, 1993). Using chemical crosslinking agents such as glutaraldehyde, formaldehyde, terephthaloyl chloride etc., albumin microspheres can be prepared at room temperature. Heat denatured or chemically crosslinked albumin has been found to be least immunogenic (Yapel, 1985; Kim *et al.*, 1985).

Lee *et al* (1981) described the preparation of glutaraldehyde crosslinked bovine serum albumin and rabbit serum albumin microspheres. Tomlinson (1983) reported that over 90 drugs have been incorporated into various microsphere systems, while bovine serum albumin microspheres have been used to deliver more than 40 drugs (Yapel, 1979). The delivery of drugs from albumin microspheres was reviewed extensively by Davis *et al* (1984) and Tomlinson and Burger (1987). Fate of albumin microspheres *in vivo* has been studied by radioimaging

techniques. Albumin microspheres tagged with $^{99}Tc$ injected intravenously into rats showed a rapid accumulation in the liver (Oppenheim, 1980). In intramuscular administration, about 5% and in intraperitoneal about 3% of radioactivity was observed in the liver by the same authors. Albumin microspheres have been labelled with $^{99}Tc$ using the surface OH functions using a reduction procedure with Sn(II) (Oppenheim *et al.*, 1978). Willmott *et al* (1989) studied the biodegradation of albumin microspheres by radiolabelled techniques.

It has been shown that albumin microspheres can be degraded by collagenase, papain, protease and trypsin (Morimoto and Fujimoto, 1983; Willmott *et al.*, 1989; Mahato *et al.*, 1991). Lee *et al* (1981) studied the enzyme degradable properties of glutaraldehyde-crosslinked microspheres loaded with progesterone and found that intra-muscular life of microspheres crosslinked using 1% glutaraldehyde was about 2 months. Recently Cumming *et al* (1994) studied pharmacokinetics and metabolism of mitomycin C encapsulated in albumin microspheres in mice bearing MAC 16 colon adenocarconoma. Epirubicin was loaded into albumin microspheres and its *in vitro* release and biological activity on human breast carcinoma cells implanted into Nu/Nu mice were carried out (Novotn and Zinek, 1994). Antitumour effect of adriamycin-loaded albumin microspheres was evaluated in rats by monitoring change in body weight, tumour size and survival over a period of 3 weeks (Gupta *et al.*, 1990). Morimoto and Fujimoto (1983), Gupta and Hung (1989a, 1989b) and Arshady (1990) have reviewed the use of albumin microspheres as drug carriers. Glutaraldehyde crosslinked albumin microbeads containing insulin was prepared by Goosen *et al* (1982) and its therapeutic efficacy was measured in diabetic rats. They reported that the blood insulin level was between 10-67 $\mu$g over a

60 day period and found that microspheres were not completely degraded even after 5 months. This study showed that insulin encapsulated in albumin microspheres by glutaraldehyde crosslinking was bioactive. Royer *et al* (1983) conducted a similar experiment, protecting amino groups of insulin by citraconylation at pH 8-10 before crosslinking . They found a rapid *in vitro* release of 60% of the insulin in the first few minutes followed by a second release phase which lasted for nine hours. Dextran was encapsulated in glutaraldehyde crosslinked albumin-heparin microspheres as a model drug by two methods, by *in situ* loading and by post loading (Kwon *et al.*, 1992). In both cases *in vitro* release of dextran showed an initial burst followed by a slower release.

### 1.6.1.2 Gelatin Microspheres

Gelatin is a non-toxic and biodegradable natural polymer with low antigenicity and it has been investigated as a drug carrier in microsphere form (Jayanthi and Panduranga Rao, 1987; Oner and Groves, 1993). Gelatin microspheres are usually prepared by crosslinking with formaldehyde or glutaraldehyde (Yoshioka *et al.*, 1981, Yan *et al.*, 1991). Studies showed that release profile of drugs from gelatin microspheres depends on the amount of gelatin employed in making microspheres and on the amount of cosslinking agent added (Nixon and Walker, 1971; Yoshioka *et al.*, 1981; Goto *et al.*, 1983). Release of some drugs from gelatin microspheres showed first order kinetics (Goto *et al.*, 1983). Glutaraldehyde crosslinked gelatin microspheres containing methotrexate was prepared and the release of the drug was reported to be zero order in simulated gastric and intestinal fluid (Narayani and Panduranga Rao, 1994). Forni *et al* (1992) studied the effect

of loading and dynamic swelling on drug release of aminophylline-loaded and clonidine hydrochloride-loaded gelatin microspheres. Oppenheim and Stewart (1979) studied the uptake of fluorescein-labelled albumin and gelatin nanoparticles by certain tumour cells and found that gelatin particles were taken up but not albumin. Interferon encapsulated glutaraldehyde crosslinked gelatin microspheres were prepared by Tabata and Ikada (1989) and they found that release of interferon was dependent on the extent of crosslinking. *In vitro* degradation studies without collagenase showed that no degradation of microspheres or release of interferon occur within two days in phosphate buffered saline. The study showed that interferon release occur only by the degradation of the gelatin backbone. When the amount of glutaraldehyde was increased from 0.03 to 1.33 mg/mg gelatin, the percentage of degraded microspheres decreased from 98 to 60% and interferon release into phosphate buffer containing collagenase decreased from 100% to 20% in 24 hours. $I^{125}$ labelled interferon was encapsulated and *in vitro* studies on macrophage uptake was done in suspension of mouse peritoneal macrophages. It was found that microspheres were phagocytosed and degraded gradually in the interior of the macrophages and resulted in the slower release of the encapsulated interferon in the cells compared to the *in vitro* release.

### 1.6.1.3 Casein Microspheres

The bovine milk protein, casein has also been investigated as a microsphere matrix for controlled drug delivery applications (Chen *et al.*, 1987). Controlled release formulations of different antitumour drugs have been designed using casein microspheres as matrix. Casein microspheres of 1-5 and 10-20 $\mu$m

size were prepared by glutaraldehyde crosslinking. The avid phagocytic uptake of casein microspheres was demonstrated with fluorescein-labelled microspheres using a macrophage-like mouse myelomonocytic leukaemia cell line (Knepp *et al.*, 1993). Post synthesis loading of 25% mitoxantrone was achieved for casein microspheres containing 20% poly(glutamic acid). Preliminary intratumoural chemotherapy experiments with a mouse Lewis lung carcinoma indicated that mitoxantrone-loaded casein-poly(glutamic acid) microspheres exhibited lower toxicity when administered intratumourally. Methotrexate was incorporated into casein microspheres to the extent of 15 wt% loading and *in vitro* release was examined in phosphate buffer at 37°C (Jayakrishnan *et al.*, 1994). Drug release of 35% was observed in 24 h, suggesting that most of the incorporated drug was strongly associated with microspheres. Enzymatic degradation studies with the enzyme protease suggested that the casein microsphere with lower crosslink density degraded faster than those with higher crosslink density.

Glutaraldehyde crosslinked casein microspheres were loaded with 5-fluorouracil (5-FU) from aqueous solution of the drug after the microspheres were synthesised (Latha *et al.*, 1994). *In vitro* release of the drug was examined in phosphate buffer in the absence and in the presence of protease at 37°C. Drug release data showed that only about 20% of the drug is released in the absence of protease even after 5 days, while digestion of the matrix with protease released the entrapped drug completely in about 24 h. A casein-drug conjugate was synthesised via carbamoyl linkage using 6-(5-FU-1-yl)hexyl isocyanate and the drug release was examined in phosphate buffer at 37°C. Release from the conjugate was slower compared with the release from microspheres in the presence of protease. Glutaraldehyde crosslinked

casein microspheres have also been evaluated for sustained delivery of an oral drug theophylline. Glutaraldehyde crosslinked microspheres were not succeptible to proteolytic enzymes present in the GI tract and sustained theophylline release was shown *in vivo* in a rabbit model. (Latha and Jayakrishnan, 1994; Latha *et al.*, 1995).

### 1.6.2 Polysaccharide Microspheres

#### 1.6.2.1 Starch Microspheres

Among polysaccharides, starch and its derivatives have been investigated most widely as drug delivery vehicles. Starch microspheres are biocompatible and readily degradable in biological fluids by amyloglycosidase or α-amylase, if the extent of derivatization is low (Artursson *et al.*, 1984a; Laakso *et al.*, 1986; Laakso and Sjohlm, 1987). The potential of injectable starch microspheres for drug targeting to the reticuloendothelial system (RES) has been investigated (Atrursson *et al.*, 1984a; Artursson *et al.*, 1984b; Artursson *et al.*, 1987; Starnkvist *et al.*, 1989, 1991a, 1991b; Degling *et al.*, 1991). When injected intravenously into mice, these microspheres were found to be phagocytosed by macrophages of the RES. It is reported that the degradation rate of starch microspheres, and thus the release of the drug could be controlled by adjusting the degree of starch modification. (Laakso *et al.*, 1986; Stjarnkvist *et al.*, 1989). Targeted delivery of macromolecules encapsulated in starch microspheres to the lysosomes of RES was reported using lysozyme (Artursson *et al.*, 1984a), human serum albumin (Kost and Shefer, 1990; Artursson *et al.*, 1984a), carbonic anhydrase (Artursson *et al.*, 1984a) and immunoglobulin G (Artursson *et al.*, 1984a).

### 1.6.2.2 Dextran Microspheres

Another polysaccharide, dextran has been investigated extensively as a drug carrier. Dextran and its derivatives are degraded by dextranase which are present in the liver, intestinal mucosa, colon, spleen and kidney of humans (Serry and Hehre, 1956; Fisher and Stein, 1960; Edman *et al.*, 1980; Larsen, 1989; Vercauteren *et al.*, 1990; Crepon *et al.*, 1991). Polyacryl dextran microspheres prepared by crosslinking with N,N′-methylene bisacrylamide (BIS) were injected in mice and it was found that 60-80% of the particles were taken up by the cells in the spleen and liver and stored in the lysosomal vacuoles (Edman and Sjoholm, 1982; Edman *et al.*, 1983; Edman and Sjoholm, 1983). Studies with radiolabelled BIS particles showed that the particles were eliminated by first order kinetics with a half-life of about 12 to 30 weeks (Edman and Sjoholm, 1983). For macromolecular release also, dextran has been investigated as a matrix. Different proteins such as human serum albumin (HSA), Immunoglobulin G, bovine carbonic anhydrase and catalase etc. were encapsulated in polyacryl dextran microspheres by physical entrapment (Edman *et al.*, 1980). Crosslinking was done with BIS. The *in vitro* release studies showed that the amount of protein released after 80 days as 65% for carbonic anhydrase (MW 31,000), 33% for HSA (MW 65,500) 44% for immunoglobulin G (MW 150,000) and 30% for catalase (MW 240,000).

## 1.7 Chitin and Chitosan

Chitin which is chemically (1-4)-2-acetamido-2-deoxy-β-D-glucan is a natural polysaccharide second in abundance to cellulose in nature. Chitosan is prepared

by N-deacetylation of N-acetyl glucosamine unit of chitin by strong alkali and it is chemically (1-4)-2-amino-2-deoxy-β-D-glucan.

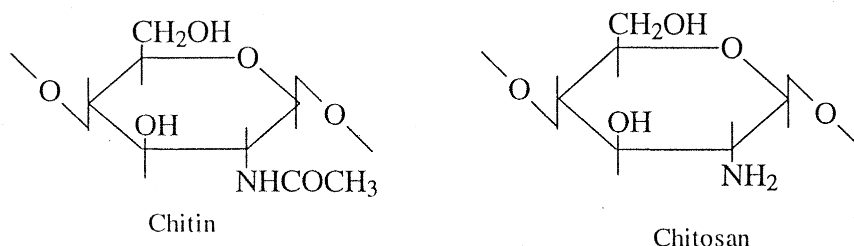



Fig. 1.7. Structure of chitin and chitosan

Even though chitin has got an unusual combination of properties such as toughness, biodegradability, and relative inertness which makes it an attractive special material, its insolubility in water and other ordinary solvents has restricted its use. On deacetylation, hydrophilicity of chitin increases and maximally deacetylated chitin, called chitosan, is readily soluble in dilute acidic solutions. Chitosan in solution exhibits the polyelectrolyte effect; in the absence of salt, there is an increase in the viscosity of the more dilute solution because of an enlarged effective volume due to charge repulsion and stretching out of the molecule. Viscosity can be decreased by the addition of salts. The degree of deacetylation is an important characteristic of chitosan and chitosan with different degree of N-deacetylation can be prepared. Degree of deacetylation can be determined by various spectroscopic methods (Miya *et al.*, 1980; Hirano *et al.*, 1981; Aiba, 1986) or by titration method (Terayama, 1952; Broussignac, 1968; Muzzarelli, 1977).

### 1.7.1 Isolation and Purification of Chitin and Chitosan

Chitin is mostly present in crab shells, and also in insects, mushrooms and in the cell walls of infectious pathogens such as bacteria and yeast. Raw chitinous material is often associated with proteins and minerals like $CaCO_3$, and removal of these materials requires harsh chemical treatments. Several procedures have been developed to isolate chitin and chitosan (Muzzarelli, 1977). According to the method of Hackman, chitin is isolated as follows. Crab shells are cleaned by washing and scrapping under running water, and dried in an air oven at 100°C. The shells are then digested with 2N HCl at room temperature, washed, dried and ground to a fine powder. The finely ground material is extracted with 2N HCl at 0°C under vigorous agitation. The collected material is washed and extracted with 1N NaOH at 100°C under occasional stirring. The alkali treatment is repeated four more times to yield chitin.

Pigments melanine and carotenoids can be eliminated with 0.02% potassium permanganate at 60°C. Purification is done by extracting with boiling water, ethanol and ether.

Chitosan is produced by the deacetylation of chitin. The procedure reported by Rigby (1936) and Wolfrom et al (1958) is as follows. Chitin is treated with a 40% aqueous solution of sodium hydroxide at 115°C for 6 h under $N_2$. After cooling, the mixture is filtered and washed with water. Upto 82% deacetylation can be achieved.

### 1.7.2 Biomedical Applications of Chitosan

Chitosan is reported to be digested by lysozyme and other related enzymes, such as N-acetyl'β-D-glucosamine occuring in the human body, and its degradation

product is aminosugars which do not impart any hazards to body (Bourbouze *et al.*, 1991). Therefore, a large number of biomedical applications of this material has been reported in the excellent reviews of Muzzarelli (1977) and Allan *et al* (1984).

Chitin is an attractive candidate for wound healing treatment because it forms a tough, water-absorbing, biocompatible film with sufficient oxygen permeability. Chitosan solution in very dilute acid provides a cool and pleasant soothing effect when applied to open wounds. Chitosan will be slowly degraded by lysozyme, which is transported to the wounded area by the inflammatory cells (polymorpho nuclear leucocytes) and hence need not be periodically removed from the wound at all. Acetic acid solution of chitosan is also found to be effective for the treatment of dermatitis in monkeys.

Chitosan membranes have been proposed as dialysis membranes in artificial kidneys since it possesses high mechanical strength and is permeable to urea and creatine. The membrane is impermeable to serum proteins and prevents mixing of toxic metals into the blood streams. Membranes with high tensile strength and urea permeability can be prepared from blends of chitosan with a synthetic polymer such as poly(vinyl alcohol).

Chitosan is found to be a suitable starting material for the production of heparin-like blood anticoagulants. N-and O-sulphated chitosan have been tested and found to possess 15-45 % of the activity of heparin *in vitro.*

Bacteriostatic action of chitosan has been demonstrated against different bacteria and fungi. The growth of Staphylococcus epidermis and Candida tropicals is found to be completely inhibited by 1% solution of chitosan in 1% acetic acid. It

also showed growth inhibitory effect against *S. aureus* and *P. aeruginosa*. It slightly impaired the growth of *S. pyrogenes* and *E. coli*.

Soft contact lens has been prepared from chitosan by casting of the acidic solution or by moulding of film. In moulding procedure, chitosan acetate film was plasticised by the addition of the polymeric structure breaker like lithium chloride. The moulded form afforded a soft, tough, water absorbant chitosan lens with high oxygen permeability. With chitosan lens, colour modification can be easily carried out by the covalent attachment of colour moiety with its functional groups. Lenses with wide range of tints can be prepared.

Chitosan is also reported to have application as drug carriers which will be discussed in detail in the section 1.7.4.

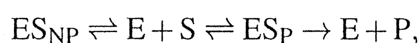
### 1.7.3 Biodegradation of Chitosan

The most important properties of chitosan which makes it attractive as a drug carrier are its biodegradability and biocompatibility. Chitin and chitosan are reported to be digested by lysozyme. Its degradation product is a common aminosugar in the body . It enters the innate metabolic pathway and may finally be incorporated into glycoproteins or be excreted as $CO_2$. Pangburn *et al* (1982) conducted degradation studies of lysozyme on partially deacetylated chitin solutions, partially deacetylated chitin film and glutaraldehyde crosslinked hydrogels. Action of lysozyme on soluble partially deacetylated chitin was studied for different concentrations of enzyme and found that rate of reaction was linear over two hour experiment for the lowest concentration but became progressively non-linear at higher concentration. The influence of substrate concentration on the

reaction was also studied. It is seen that the rate of degradation as a function of substrate concentration was culvilinear following first order kinetics at low concentration and zero order kinetics at higher concentrations. Experiments with film and glutaraldehyde crosslinked hydrogels showed that they are also lysozyme digestible. The enzymatic reaction can be formulated as,

$$E + S \rightleftharpoons ES \rightarrow E + P$$

where E is the enzyme, S is the Substrate, P is the product. In view of the probable formation of non-productive complex this equation can be rewritten as,



$$ES_{NP} \rightleftharpoons E + S \rightleftharpoons ES_{P} \rightarrow E + P,$$

*P* and *NP* denote productive and non-productive complex.

Nakajima *et al* (1986) reported that complete resorption of chitin sutures takes place within 2 months. Chitosan ascorbate used for the reconstruction of paradondal tissue has also been observed to degrade in the oral cavity within 2 months (Muzzarelli *et al.*, 1989). Hirano *et al* (1989) showed that degradation of chitosan by lysozyme depended on the degree of acetylation. The lysozyme degradability of chitosan having different degree of substitution (0.2, 0.4, 0.6, and 0.8 acetylated) was examined. They found that partially N-acetylated derivative (d.s. 0.4-0.8 for N-acetyl) were 1.5 to 4 times more digestible than N-acetyl chitosan (d.s. 1 for N-acetyl).

### 1.7.4 Chitosan as a Drug Carrier

Both chitin and chitosan have received some attention as possible carriers for sustained delivery of drugs. Miyazaki *et al* (1981) used indomethacin and

papaverin hydrochloride as model drugs and found that drugs dispersed in the chitosan gels were released at a constant rate. The applicability of compressed tablets of chitosan as a vehicle for sustained release of water soluble drug was examined by Sawayanagi *et al* (1982). They selected propanolol hydrochloride as model drug and found that chitosan could be used as a vehicle for the controlled release of water soluble drugs. Preparation of controlled release theophylline granules coated with a polyelectrolyte complex of sodium polyphosphate chitosan was done by Kawashima *et al* (1985). They found that the drug release pattern of the coated granules followed zero-order kinetics and the release rates were significantly reduced compared with that of the original granule. Indomethacin-loaded chitosan granules were prepared and the release rate was compared with a conventional commercial indomethacin capsule and a sustained release capsule by Miyazaki and coworkers (1988). In contrast with the rapid release seen from a commercial conventional capsule form, sustained release from the chitosan granules was observed. In a bioavailability study conducted in rabbits, they found chitosan granules were superior to conventional commercial capsule in maintaining the plasma concentration. 5-fluorouracil (5-FU)-chitosan conjugate was prepared by Ouchi *et al* (1989). Four kinds of chitosan derivatives were prepared attaching 5-FU through spacers such as ether, amide, ester or carbomyl bond. The release behaviour of 5-FU and 5-FU derivatives was studied *in vitro* at 37°C . The antitumour activity was tested against p-388 lymphocytic leukemia in female CDF1 mice *in vivo* by intraperitoneal injection of the leukemia cells. Chitosan fixing 5-FU at 2-position through hexamethylene spacer and carbomoyl linkage exhibited a high effect with

respect to prolongation of life than free 5-FU. The chitosan-5-FU conjugates obtained did not display accute toxicity in the high dosage range.

Inouye *et al* (1989) prepared sustained release intragastric floating granules of chitosan of different degree of deacetylation in granular or in laminated preparations. Using prednisolone as model drug, they studied the *in vitro* release, and absorption studies in beagle dogs showed a sustained drug absorption. Theophylline tablets using chitosan as a sustained release base were evaluated by Nagalaye *et al* (1990). When chitosan was used at a concentration of more than 50% of tablet weight, an insoluble non-erosion type matrix was found. Tablets prepared with chitosan concentrations of less than 33% were fast releasing, chitosan used at a concentration of 10% acted as a disintegrant and the drug was dissolved within an hour. Citric acid and carbomer-934P were used as coadjuvants which gelled the chitosan and imparted sustained release properties. Citric acid slowed down the release rates of chitosan-based theophylline tablets. Watanabe *et al* (1990) prepared gels from 6-O-carboxymethylchitin (CM-Chitin) by the addition of Fe(III) chloride. They incorporated bovine serum albumin and doxorubicin in the gel and found increasing release rate by lysozyme digestion of the matrix in a time-dependent manner.

The potential of chitosan film containing diazepam as an oral drug delivery system was investigated in rabbits (Miyazaki *et al.*, 1990). *In vitro* release of cisplatin from chitosan microspheres was examined. Effect of incorporating chitin in the system on the drug content and release profile was examined (Nishioka *et al.*, 1990). Song *et al* (1992) synthesised conjugates of mitomycin C with N-succinyl chitosan and carboxymethyl chitosan and drug release was studied.

*In vitro* release of nifedipine from chitosan beads and granules was examined by Chandy and Sharma (1992). Ohya *et al* (1993) prepared glutaraldehyde crosslinked chitosan gel microspheres immobilising 5-FU derivatives and their release behaviour was studied. Chitosan gel beads containing indomethacin were prepared by a polyelectrolyte complexation of sodium triphosphate and chitosan (Shiraishi *et al.*, 1993). The effect of the molecular weights of chitosan hydrolysates on the release and absorption rates from gel beads were examined. The release rates of indomethacin decreased with increasing molecular weight and indomethacin concentration. A negative correlation was observed between the molecular weight and release rate constant. The release of indomethacin depended upon the dispersion of the indomethacin solid particles in the beads, as well as the porosity, tortuosity and surface area of the matrix. The plasma concentration of indomethacin after oral administration of chitosan gel beads to beagle dogs exhibited the sustained release pattern. Chandy and Sharma (1993) reported *in vitro* release of ampicillin from chitosan beads and microgranules. Polk *et al* (1994) examined controlled release of albumin from chitosan-alginate microcapsules. The effect of variables such as concentration of alginate, molecular weight of chitosan and pH on the release behaviour was studied.

## 1.8 Aim and Scope of the Study

Although chitin and chitosan have received some attention as drug carrier, most of the work reported is based on chitosan as granules and as a tablet binder for sustained delivey of oral drugs (Kawashima *et al.*, 1985; Miyazaki *et al.*, 1988; Inouye *et al.*, 1989; Nagalaye *et al.*, 1990). Chitosan is known to degrade by the

action of lysozyme and its degradation products are non-toxic (Muzzarelli, 1977). The *in vivo* life of chitosan and chitosan ascorbate in the oral cavity is reported to be about 2 months (Muzzarelli, 1989). Pangburn *et al* (1982) have reported that glutaraldehyde crosslinked chitosan is also succeptible to lysozyme although slowly. It is well known that the biological life of protein and polysaccharide microspheres can be altered by chemical crosslinking. Thus, depending on the crosslinking density, albumin microspheres crosslinked using glutaraldehyde are reported to have an *in vivo* life of 2-6 months when implanted intramuscularly (Lee *et al.*, 1981). Matrix crosslinking also influences the rate of release of drugs incorporated into protein and polysaccharide microspheres (Goto *et al.*, 1983; Kim and Lee, 1986; Thanoo *et al.*, 1992). Thus matrix crosslinking offers an elegant way of controlling the drug release profile from microspheres.

The possibility of preparing glutaraldehyde crosslinked chitosan microspheres containing various oral drugs such as theophylline, griseofulvin, and aspirin for sustained delivery was reported in a preliminary communication by Thanoo *et al* (1992). They prepared glutaraldehyde crosslinked chitosan microspheres from aqueous acetic acid solution of chitosan. Microspheres having different degree of swelling were made by varying the crosslinking density. Theophylline, aspirin and griseofulvin-loaded microspheres were prepared and *in vitro* release studies of the drugs were carried out in simulated gastric and intestinal fluids. They found that drug release rates were influenced by the crosslinking density, particle size and initial drug loading in the microspheres.

Hence, the aim of this study is to prepare and evaluate glutaraldehyde crosslinked chitosan microspheres with different crosslinking densities for the

controlled release of various micro- and macromolecular drugs.

Oral administration of conventional dosage form often leads to rapid elimination and occasionally destruction of drug activity by gastrointestinal enzymes. Hence, controlled drug delivery assumes much importance for oral drugs. Here, an oral drug such as diclofenac sodium will be incorporated in chitosan microspheres and *in vitro* release will be examined from microspheres with different crosslinking densities. Bioavailabiliy of the drug incorporated in the microspheres will be examined in a rabbit model.

Owing to the importance of controlled/targeted delivery in cancer chemotherapy, another aim is to prepare an anticancer drug, mitoxantrone-loaded chitosan microspheres and evaluate its antitumour activity. *In vitro* release of the drug will be examined in phosphate buffer at room temperature as well as at 37°C. Antitumour activity of the incorporated drug will be evaluated in a mice model against Ehrlich ascites carcimoma.

Steroids are another important class of bioactive agents which are having very short half life and controlled release formulations can enhance their therapeutic efficacy. Hence, another objective of this work is to prepare progesterone-loaded chitosan microspheres and examine the *in vitro* release in phosphate buffer at 37°C. The bioavailability studies of the progesterone-loaded chitosan microspheres will be carried out in a rabbit model.

In recent years, major advances in genetic engineering have been made. Polypeptides and proteins having interesting and useful pharmacological activity can now be produced. Currently, clinical administration of these agents require repeated intramuscular or subcutaneous injection because oral administration is

not suitable due to degradation caused by proteolytic enzymes present in G. I tract. Vaccines are another important class of macromolecular drugs on which there has been a considerable amount of research activity to prepare controlled release dosage forms because of the need to immunise large populations particularly in the developing countries. In order to be effective, most vaccines reqiure two or three primary immunisations followed by booster doses every few months or years. Patient compliance for such multiple shot immunisation is rather poor in developing countries. Hence, in order to minimise dosing frequency and improve patient compliance, controlled release vaccine formulations have been investigated. Therefore, another aim of this study is to prepare, crosslinked chitosan microspheres containing proteins such as bovine serum albumin and diphtheria toxoid and to evaluate them for sustained delivery of these macromolecules. Since chitosan is a hydrophilic polymer that swells in aqueous medium, these macromolecules will be incorporated into preformed microspheres by passive loading techniques thereby limiting the possibility of loosing the antigenicity of the protein. Immunogenicity studies of the diphtheria toxoid-loaded chitosan microspheres will be carried out in Wistar rats.

The biological life of the carrier matrix is a very important parameter for the controlled release of drugs for prolonged periods. Hence, *in vitro* and *in vivo* degradation studies of chitosan microspheres prepared by glutaraldehyde crosslinking will be carried out. In vitro degradation will be studied at different concentrations of lysozyme at 37°C. *In vivo* degradation will be studied by implanting the microspheres intramuscularly in rats for various periods of time, explanting them and examining the extent of degradation microscopically. .

Chitosan possesses different functional groups such as -OH and $NH_2$. Making use of the reactivity of these functional groups, chemical modification of chitosan is possible. Therefore, another aim of this study is the preparation of photocrosslinkable chitosan by the reaction of $NH_2$ group of chitosan with azidated epiclorohydrin. The work is finally aimed at studing the the possibility of using this photocrosslinkable matrix for the controlled release of drugs. Here, as a preliminary study, theophylline will be incorporated into photocrosslinkable chitosan films and release kinetics will be examined *in vitro*.

# CHAPTER 2
# MATERIALS AND METHODS

# MATERIALS AND METHODS

## 2.1 Materials

Chitosan (purified, viscosity grade 50) obtained as a gift from Central Institute of Fisheries and Technology, Cochin, India was used without further purification. Glutaraldehyde (biological grade, 25% aqueous solution), sorbitan sesquioleate, dioctyl sulphosuccinate, progesterone (4-pregnene-3,20-dione), bovine serum albumin (BSA, fraction V), lysozyme, Tween 20, Tween 80, o-phenylene diamine and theophylline were purchased from Sigma Chemical Company, USA, and were used as such. Liquid paraffin (heavy, viscosity 90 cP at 30°C and light, viscosity 18 cP at 30°C), petroleum ether (60–80°C), perchloric acid, acetonitrile (HPLC grade), o-phosphoric acid, sodium carbonate, sodium hydroxide, sodium potassium tartrate, phenol reagent (Folin and Ciocalteu) and epichlorohydrin (1-chloro-2,3-epoxy propane) were from S.D Fine Chemicals, Bombay, India. Diclofenac sodium was a grift from EROS Pharma Pvt. Ltd., Bangalore, India. Mitoxantrone (1,4-Dihydroxy-5,8-bis {[2 – [(2–hydroxyethyl) amino] ethyl]amino}-9,10- anthracenedione dihydrochloride) manufactured by American Cyanamid Company, New York was a gift from Prof. E. P. Goldberg of the

University of Florida, USA. Diphtheria Toxoid (DT), Molecular weight 62,000, (3,500 Lf units/mL) was from Serum Institute, Pune, India. Poly(lactic acid) (PLA, Molecular weight 100,000) was purchased from Polysciences, USA, and sodium azide and tetrabutyl ammonium bromide were from Spectrochem, Ltd., Bombay, India. Progesterone Ovucheck ELISA kit was from Cambridge Veterinary Sciences, Cambridge, England. All other reagents and chemicals were of analytical or equivalent grade and were obtained from locally available sources.

## 2.2 Methods

### 2.2.1 Determination of Molecular Weight of Chitosan

The molecular weight of chitosan was determined using an Ubbelohde viscometer at 25°C. Solvent used was 0.1 M acetic acid containing 0.2 M NaCl. Limiting viscosity number for chitosan solutions of different concentrations was determined. Molecular weight was calculated using the equation of Maghami and Roberts (1988).

$$[\eta] = KM^a$$

$$\text{where } K = 1.81 \times 10^{-3}\ \text{cm}^3\ \text{g}^{-1} \text{ and}$$

$$a = 0.93$$

### 2.2.2 Determination of Degree of Deacetylation of Chitosan

The degree of deacetylation of chitosan was determined by the method of Muzzarelli (Muzzarelli, 1977). Chitosan, 0.5 g was dissolved in 20 mL 0.3N HCl and the solution was titrated potentiometrically against 0.1N NaOH. A blank

experiment was done with 20 mL 0.3N HCl. The difference in volume of NaOH used is equivalent to $NH_2$ groups on chitosan. From this value, weight of glucosamine unit and the degree of deacetylation was calculated.

### 2.2.3 Preparation of Chitosan with Low Degree of Deacetylation

Chitosan was acetylated further according to the method of Roberts and Taylor (Roberts and Taylor, 1989). Chitosan 1.2 g was dissolved in 100 mL 0.5% aqueous acetic acid. 100 mL methanol was added and the solution mixed. Then, 58 mL of 0.5% methanol solution of acetic anhydride was poured in whilst the solution was well stirred. After standing at room temperature for 2 h, the solution was poured into methanolic ammonia and the precipitate was filtered off, washed well with aqueous methanol, then with methanol and dried under vacuum.

### 2.2.4 Preparation of Glutaraldehyde Saturated Toluene (GST)

GST was prepared according to the method of Longo *et al* (1982). Aqueous glutaraldehyde, (25% solution), 25 mL was added to 200 mL toluene..The mixture was sonicated using a probe type sonicator (Model Soniprep 150, England) for 1 min. The glutaraldehyde saturated toluene layer was separated using a separating funnel and dried over anhydrous sodium sulphate.

### 2.2.5 Determination of Glutaraldehyde Content in Saturated Toluene

Glutaraldehyde in GST was estimated by the ε-aminocaproic acid method (Lynn *et al.*, 1990). GST 1 mL was taken in a screw capped test tube. It was then mixed

with 5 mL of 0.01M phosphate buffer and rotated in a haemetology mixer (Fisher Scientific, USA) overnight to extract the glutaraldehyde to the aqueous medium. This solution was mixed with 0.5 mL of 0.006 mol(%) ε-aminocaproic acid in 0.01M phosphate buffer and kept at 90°C in a water bath for 90 min. It was chilled and the absorbance was noted at 253 nm and 300 nm. Concentration and hence amount of glutaraldehyde was calculated from the calibration curve.

### 2.2.6 Preparation of Placebo Chitosan Microspheres by Glutaraldehyde Crosslinking

A 4% solution of chitosan in 5% aqueous acetic acid containing 2% NaCl was dispersed in a mixture of 35 mL of light paraffin oil and 25 mL of petroleum ether containing 0.85 g of sorbitan sesquioleate in a 100 mL round-bottomed flask at room temperature (27°C). The dispersion was stirred using a stainless steel half–moon paddle stirrer having a diameter of 3 cm for 5 min at 2000 rev $min^{-1}$. The required amount of GST was added (depending on the crosslinking density desired) and the stirring continued for 1.5h. The hardened microspheres were separated by centrifugation, washed several times with petroleum ether, methanol, 5% solution of sodium metabisulphite, water and finally with acetone. The microspheres were dried overnight in an air oven at 60°C. Microspheres having different crosslinking densities were prepared by employing various amounts of GST for crosslinking. Highly crosslinked microspheres were prepared by adding aqueous glutaraldehyde in addition to GST. Microspheres with different sizes were prepared by changing the speed of rotation of the stirrer.

Microspheres of smaller size were prepared by sonicating the dispersion using a probe type sonicator (Cole–Parmer 4710, USA) for 5 min., following which the dispersion was stirred using a stainless steel half–moon paddle stirrer having a diameter of 3 cm at 2000 rev min$^{-1}$. GST was added while stirring the system with paddle stirrer. Stirring was continued for 1.5 h as before. After that, the system was stirred magnetically overnight. The hardened microspheres were separated by centrifugation, washed and dried as before.

### 2.2.7 Preparation of Diclofenac Sodium–Loaded Chitosan Microspheres

A 4% solution of chitosan in 5% acetic acid containing 2% NaCl was prepared and 6 g of this solution was mixed with diclofenac sodium (20, 50 and 200% of the weight of chitosan) and dispersed in a mixture of 35 mL light liquid paraffin and 25 mL petroleum ether containing 0.85 g of sorbitan sesquioleate in a 100 mL round-bottomed flask at room temperature (27°C). The dispersion was stirred using a stainless steel half–moon paddle stirrer having a diameter of 3 cm at 200 rev min$^{-1}$ for 5 min. Crosslinking was carried out by the addition of 10 mL GST while the system was kept stirred. At the end of 30 min, 1 mL of 25% aqueous glutaraldehyde was added and the stirring continued for 1.5 h. Microspheres were also prepared by adding 4 mL and 10 mL GST alone. The hardened microspheres were separated by centrifugation and washed several times with petroleum ether and once with acetone. It was again washed once with a 5% solution of sodium metabisulphite to remove residual glutaraldehyde, followed by copious amounts of cold water and finally with acetone and centrifuged. Microspheres thus obtained were vacuum dried and kept in a desiccator at room temperature.

### 2.2.8 Preparation of Mitoxantrone-Loaded Chitosan Microspheres

The method of preparation of mitoxantrone–loaded microspheres was the same as described in section 2.2.7, the only difference being the dispersion was stirred at 2000 rev $min^{-1}$. Here, the drug was incorporated at an initial loading of 5 and 10% of the weight of chitosan. Microspheres were prepared with 3.2 mL GST and 0.8 mL aqueous glutaraldehyde, 4 mL GST alone and 10 mL GST alone. Hardened microspheres were separated, washed several times with petroleum ether, once with a 5% solution of sodium metabisulphite, water and finally with acetone and centrifuged. Microspheres were vacuum dried and stored in a desiccator at room temperature.

Microspheres were also prepared by sonicating the dispersion to obtain smaller sized particles. For this, the dispersion was sonicated using a probe type sonicator (Cole-Parmer 4710, USA) for 5 min following which the dispersion was stirred using a stainless steel half-moon paddle stirrer having a diameter of 3 cm at 2000 rev $min^{-1}$. 10 mL GST and 1 mL aqueous gultaraldehyde was added while stirring the system with paddle stirrer. Stirring was continued for 1.5 h. After that the system was stirred magnetically overnight. The hardened microspheres were separated and washed as before.

### 2.2.9 Preparation of Progesterone-Loaded Chitosan Microspheres

A 4% solution of chitosan in 5% acetic acid containing 2% sodium acetate was prepared and 6 g of this solution was mixed with definite amount of progesterone (20% of the weight of chitosan) and dispersed in a mixture of 35 mL of light liquid

paraffin and 25 mL of petroleum ether containing 0.85 g of sorbitan sesquioleate in a 100 mL round–bottomed flask at room temperature. The dispersion was stirred using a stainless steel half–moon paddle stirrer at 2000 rev $min^{-1}$ for 5 min and 10 mL GST was introduced into the flask and the stirring continued. At the end of 30 min, 1 mL aqueous glutaraldehyde was added. Stirring was continued for a total duration of 1.5 h, at the end of which the hardened microspheres were separated. Microspheres were then washed four times with petroleum ether, once with acetone, once with 5% solution of sodium metabisulphite, three times with water, centrifuged and finally vacuum dried and kept in a desiccator at room temperature. Microspheres were also prepared by adding 4 mL and 10 mL GST alone.

### 2.2.10 Preparation of Bovine Serum Albumin (BSA)-Loaded Chitosan Microspheres

A 4% solution of chitosan was prepared in 5% acetic acid solution containing 2% NaCl. This solution 7.5 g was dispersed in a mixture of 25 mL light and 50 mL heavy paraffin oil containing 1 mL of 8% solution of dioctylsulphosuccinate (DOS) in n-heptane in a 150 mL round–bottomed flask at room temperature. The dispersion was stirred at 500 rev $min^{-1}$ with a stainless steel half–moon paddle stirrer, and after 5 min, 2 mL GST was added and the stirring continued. After 30 min, 2 mL GST was again added and at the end of 1 h, 1 mL of 25% aqueous glutaraldehyde was added and the stirring continued for a total duration of 3 h. The hardened microspheres were filtered and washed several times with petroleum

ether, methanol, water and finally acetone. Microspheres thus obtained were dried in a vacuum desiccator at room temperature.

Glycine Quenched Microspheres: Glycine-quenced chitosan microspheres were prepared in the following manner. The glutaraldehyde crosslinked microspheres were treated with a 2% solution of glycine in distilled water (100 mg microspheres in 5 mL glycine solution for 4 h at room temperature) to end-cap the residual aldehyde handles. Microspheres were then centrifuged, washed several times with water followed by acetone, centrifuged and dried in a desiccator. The quenched microspheres, (500 mg) were swollen in 25 mL distilled water for 24 h at room temperature. The swollen microspheres were filtered through a fritted disc (IG3) and then introduced into 5 mL of a 20% solution of BSA in water in screw-capped vials and rotated in a haematology mixer (Fisher, model 346, USA) for 24 h at room temperature (27°C). The excess BSA solution was removed using a Pasteur pipette with a narrow orifice through which the microspheres did not pass through. The microspheres were then washed once with distilled water followed by acetone, centrifuged and dried in a vacuum desiccator at room temperature.

### 2.2.11 Preparation of Diphtheria Toxoid (DT)–Loaded Chitosan Microspheres

Glycine-quenched placebo Chitosan microspheres were prepared as described in section 2.2.10. Sixty four mg of microspheres having particle size below 75 $\mu$m were aseptically transferred into a screw-capped glass vial and 245 $\mu$L of 1:10 DT(equivalent to 0.1 mg) was introduced and incubated at 4°C overnight.

The volume was then made upto 1 mL using a 1% solution (sterile) of sodium carboxymethyl cellulose in saline and was used for immunogenicity studies.

### 2.2.12 Preparation of Photocrosslinkable Chitosan

#### 2.2.12.1 Preparation of Azidated Epichlorohydrin

Sodium azide 16 g was dissolved in 40 mL water. Epichlorohydrin 20 mL was added to this and stirred well in the presence of a phase transfer catalyst, tetrabutylammonium bromide at a concentration of 0.01 mole $L^{-1}$. The contents were stirred overnight magnetically in the dark at room temperature. The non–aqueous layer was separated using a separating funnel, dried over calcium chloride and the crude sample of azidated epichlorohydrin was used for further reaction.

#### 2.2.12.2 Incorporation of Azide Group into Chitosan

Chitosan 1.2 g was dissolved in 30 mL 5% acetic acid. To this was added 5 mL 5% HCl and 5 mL azidated epichlorohydrin and stirred at room temperature magnetically for 24 h. Azidated chitosan was precipitated in methanol, filtered, washed several times with methanol, once with water and finally dried in vacuum and kept in the dark in a desiccator until use. (Yield 90%).

#### 2.2.12.3 Preparation of Chitosan Film

Modified chitosan 1 g was dissolved in 25 mL 5% acetic acid. The solution was then cast on a glass plate, protected from light and allowed to dry for 4 days at

room temperature to obtain a film of thickness 0.1 mm. Theophylline loaded film was prepared in the same way by mixing definite amount of the drug (10% of the weight of chitosan) with the chitosan solution and casting the film.

#### 2.2.12.4 Photocrosslinking

Crosslinking of the film was carried out by UV irradiation. A piece of film having dimensions 1 cm× 1 cm × 0.1 mm was taken in a quartz tube and exposed to UV radiation from a Phillips HPL-N 125 W UV lamp for 2 h at a distance of 15 cm from the lamp.

### 2.2.13 Characterisation of Microspheres

#### 2.2.13.1 Particle Size Analysis

Particle size distribution of microspheres was determined using either one of the following methods.

**Using Sieves:** Large microspheres were sieved through standard test sieves (Filterwel, Bombay, India) and the fractions which passed through one sieve, but retained on the other sieve were collected and weighed in an analytical balance and the distribution was calculated.

**Using Laser Diffraction:** Microspheres were dispersed in water with sonication in the presence of trace amounts of Tween 20 and particle size analysis was carried out in a particle size analyser (Galai, Model CIS–1, Israel). The distribution was plotted using a computer programme supplied by the manufacturer.

#### 2.2.13.2 Determination of Swelling Kinetics

Swelling kinetics of the microspheres was examined in distilled water at room temperature (27°C). A known amount of dry microspheres was swollen in distilled water for different periods of time. Excess water was removed using a Pasteur pipette having a narrow orifice through which the swollen beads would not pass. The weight of swollen beads was determined after blotting them using a filter paper in an analytical balance and the equilibrium water content (EWC) was calculated using the following relation.

$$EWC(\%) = 100 \times \frac{\text{wt. of swollen beads–wt. of dry beads}}{\text{wt. of swollen beads}}$$

#### 2.2.13.3 Scanning Electron Microscopy (SEM)

SEM was performed as follows. Microspheres were sprinkled into double–sided adhesive tape fixed on aluminium stubs, sputter–coated with gold in an ion sputter (Hitachi, Model E101) and examined in the microscope (Hitachi, Model S 2400).

#### 2.2.13.4 Internal Structure of Microspheres

The internal structure of microspheres was examined using optical microscopy. Microspheres were stained with 2% aqueous osmium tetroxide solution for 2 weeks, washed with distilled water and vacuum dried. It was embedded in Spurr Resin and polymerised at 70°C for 8 h. Sections (1$\mu$m) were cut in an ultramicrotone (LKB - V, 2088, BROMMA, Sweden) using glass knives and viewed under light microscope (Nikon Binocular, Optiphot, Japan).

phosphate buffer (pH 7.4, 0.1 M) in a screw–capped vial by rotating the tubes head–to–tail in the haematology mixer for 24 h. (Complete extraction was ensured by extracting the powdered microspheres repeatedly with the buffer). Total protein content in the extract was determined by Lowry's method of protein assay which is discussed in section 2.2.14.

Theophylline loaded chitosan films were prepared at a drug content of 10% and hence the total drug content was taken as 10%.

### 2.2.14 Lowry's Method of Protein Assay

A 2% solution of $Na_2CO_3$ was prepared in 0.1 N NaOH (Reagent A). A 0.5% solution of $CuSO_4.5H_2O$ was prepared in 1% sodium potassium tartrate (Reagent B). Reagent B 1 mL was added to 50 mL of Reagent A and mixed well (Reagent C). One part of phenol reagent (Folin and Ciocalteu) was mixed with 2 parts of water (Reagent D). BSA solution 0.3 mL was taken in a test tube and 3 mL Reagent C was added and mixed well. After 10 min 0.3 mL Reagent D was added and shaken well. After 30 min absorbance was taken at 750 nm (Chaykin, 1966).

### 2.2.15 Infrared Spectra

The infrared spectra of chitosan films were recorded using a FTIR spectrophotometer (Nocolet, Model 410, USA) using baseline horizontal ATR accessory (Nicolet).

### 2.2.16 Preparation of Simulated Gastric Fluid (GF) and Simulated Intestinal Fluid (IF)

Simulated gastric fluid and intestinal fluid were prepared according to the US Pharmacopeia. For simulated gastric fluid, sodium chloride 2 g was dissolved in 7 mL concentrated HCl and sufficient quantity of water was added to make up to 1000 mL (pH 1.2).

For simulated intestinal fluid, monobasic potassium phosphate 6.8 g was dissolved in 250 mL water. To this was added 190 mL 0.2 N NaOH and 400 mL water. The pH of the resulting solution was adjusted to 7.4 with 0.2 N NaOH and diluted with water to 1000 mL.

### 2.2.17 *In Vitro* Drug Release from Microspheres

#### 2.2.17.1 Diclofenac Sodium Release

*In vitro* release of diclofenac sodium from chitosan microspheres was carried out in simulated IF at 37°C in a paddle type dissolution assembly according to US Pharmacopeia. Into a 1000 mL Erlenmeyer flask containing 500 mL medium was introduced 50 mg of drug–loaded microspheres and stirred at a speed of 125 rev $min^{-1}$ using a Teflon paddle stirrer. Aliquots of 3 mL were removed at various time intervals and assayed spectrophotometrically at 276 nm (Chowdary and Vijaya Ratna, 1992). An equal volume of the aliquot medium was immediately added to the dissolution medium. Each point on the release curves represents average of at least 3 determinations.

#### 2.2.17.2 Mitoxantrone Release

*In vitro* release of mitoxantrone was carried out in phosphate buffer (pH 7.4, 0.1 M) at room temperature (27°C). Drug loaded microspheres 50 mg were introduced into 50 mL of the dissolution medium. Release study was done under constant stirring conditions as described in section 2.2.17.1 and also under occasional shaking conditions, where the dissolution medium was hand-shaken 8–10 times a day. Aliquots of 0.5 mL were withdrawn at various time intervals and assayed for the drug spectrophotometrically at 610 nm (Windholz *et al.*, 1976). The medium was replenished with an equal volume of buffer after the withdrawal of each aliquot.

#### 2.2.17.3 Progesterone Release

*In vitro* release of progesterone was done in phosphate buffer (pH 7.4, 0.1 M) at 37°C. Progesterone–loaded microspheres 10 mg was taken in 100 mL of the medium and stirring was given as described in section 2.2.17.1. Aliquots of 0.5 mL were withdrawn at various time intervals and assayed for the drug spectrophotometrically at 247 nm (Dunn *et al.*, 1981).

#### 2.2.17.4 BSA Release

*In vitro* release of BSA was examined in phosphate buffer (pH 7.4, 0.1 M) at 37°C. BSA–loaded microspheres 50 mg was introduced into 50 mL of phosphate buffer and stirred at 125 rev $min^{-1}$ using the same apparatus described in section 2.2.17.1. Aliquots of 0.3 mL were withdrawn at various time intervals and BSA content was estimated by Lowry's method of protein assay as described in section 2.2.14.

In order to modulate the drug release, BSA–loaded microspheres were coated with paraffin oil and poly(lactic acid) (PLA). For coating with oil, 100 mg of the BSA–loaded microspheres was added to 1 mL of liquid paraffin in a test tube and mixed using a vortex mixer for 5 min. The excess oil was removed using a Pasteur pipette. For coating with PLA, 100 mg of the microspheres was introduced into 5 mL of 10% polymer in dichloromethane and mixed for 2 min and the spheres were separated by vacuum filtration and dried.

*In Vitro* release of BSA from the oil coated and PLA coated microspheres was done as described above.

### 2.2.18 *In Vitro* Release of Theophylline from Chitosan Film

Crosslinked chitosan film, 20 mg (1 cm × 1 cm × 0.1 mm) was taken in 100 mL simulated gastric fluid at 37°C using the same apparatus and stirring conditions discussed in section 2.2.17.1. Aliquots of 0. 5 mL were withdrawn at various time intervals and the medium was replenished with equal amount of gastric fluid. The drug released at various time intervals was assayed spectrophotometrically at 274 nm (Thanoo *et al.*, 1992). This experiment was repeated in simulated intestinal fluid also.

### 2.2.19 Bioavailability Studies

#### 2.2.19.1 Bioavailability of Diclofenac Sodium from Chitosan Microspheres

Rabbits (New Zealand white) weighing 1.6–2.2 kg were used for the study. Animals were fasted for 15 h prior to the administration of the drug. Each rabbit

was given a dose of 25 mg/kg body weight of diclofenac sodium powder or an equivalent dose in microsphere form with 10 mL of water orally through a catheter (n=3). Microspheres having a drug loading of 30% in the size range of 600–850 $\mu$m crosslinked using 10 mL GST followed by 1 mL 25% aqueous glutaraldehyde were employed for *in vivo* evaluation. The pharmacokinetic parameters considered were $C_{max}$ (maximum diclofenac plasma concentration); $t_{max}$ (the time taken to achieve $C_{max}$ and $AUC_{0-24h}$ (the area under the plasma concentration - time curve between zero and the final sample time as calculated by the trapezoidal rule). Water was provided ad libitum during fasting and throughout the experiment. Blood samples were collected at 0, 1, 2, 3, 4, 6, 8, 10, 12 and 24 h from the ear vein after administration.

Analysis of the drug in the blood plasma was done by High Performance Liquid Chromatography (HPLC) (Waters, USA, model 244, equipped with 486 tunable wavelength detector) (Garcia–Encina *et al.*, 1993). Blood samples were centrifuged at 3000 rev $min^{-1}$ for 10 min and plasma was separated. To 200 $\mu$l of plasma in a 1.5 mL centrifugal tube, 20 $\mu$L of 30% perchloric acid and 400 $\mu$L of acetonitrile were added. The tube was shaken for 2 min and centrifuged (Remi Research–Centrifuge, R–24) for 5 min at 10000 rev. $min^{-1}$. The resultant supernatant was injected into the HPLC system and analysed at 276 nm using 95:5 v/v acetonitrile and 0.03% phosphoric acid (pH 3.5) as the mobile phase at a flow rate 1 mL $min^{-1}$.

#### 2.2.19.2 Antitumour Activity of Mitoxantrone–Loaded Chitosan Microspheres

Swiss albino mice weighing 25–35 g were used for this study. Animals were inoculated intraperitoneally with $2 \times 10^6$ Ehrlich ascites carcinoma cells. On the second day after inoculation, one group of animals (n=8) was administered microspheres (average size $37 \pm 13$ $\mu$m) equivalent to 2 mg of the drug dispersed in 2 mL phosphate buffered saline containing 0.05% Tween 80 using a 23 G needle intraperitoneally. The second group (n=5) was injected with equivalent amount of placebo chitosan microspheres in the same vehicle, the third and fourth groups (n=8) were given 2 and 1 mg of the drug in the same vehicle and the control group (n=8) was left untreated. Therapeutic efficacy was determined by monitoring the change in body weight and survival time of each animal for 60 days. Statistical significance was evaluated using the student's t-test.

#### 2.2.19.3 Bioavailability of Progesterone from Chitosan Microspheres

Rabbits (New Zealand white, male) weighing 1.7–1.75 kg were used for this study (n=3). Each rabbit was injected with a dose of 5 mg/kg body weight of progesterone powder or equivalent dose in microsphere form with 2 mL sterile saline containing 0.1% Tween 80 intramuscularly into the gluteal muscle. Blood samples (1 mL each) were collected immediately before injection, and at different time intervals after injection up to 6 months. Blood was centrifuged at 3000 rev min$^{-1}$ for 10 min and plasma was separated and plasma progesterone conçentration was determined using Enzyme–Linked Immunosorbent

Assay (ELISA) (Bio–tek, USA, Model EL–311, Microplate autoreader) using the kit obtained from Cambridge Veterinary Sciences, Cambridge, England.

The 96–well ELISA plates were exposed, emptied the contents and tap dried on an absorbant paper. Test samples 10 $\mu$L each were loaded into the wells of plate in duplicate. Then 200 $\mu$L of conjugate was added to each well and covered with a sheet of paper and left at room temperature for 30 min. Wells were then emptied and washed thrice with cold water and tap dried on absorbant paper. Substrate reagent (Prepared by dissolving three substrate tablets in the substrate buffer) 200 $\mu$L were loaded into each empty well. Wells were then covered with a sheet of paper and left at room temperature (27°C) for 30 min. Stopping solution (100 $\mu$L) was then added to all wells and absorbance was read at 405 nm. Standards given in the kit was used for the preparation of calibration curve. The standard curve was run on each plate along with the test samples every time in each assay, in order to overcome plate to plate assay variation.

### 2.2.20 Immunogenicity of Diphtheria Toxoid (DT)–Loaded Chitosan Microspheres

DT–loaded microsphere–suspension (see section 2.2.11), equivalent to a dose of 0.01 mg DT was injected intramuscularly into Wistar rats (n=8). The control group received the same dose of DT on alum. Animals were bled through retro orbital plexus at 0, 15, 30, 45, 60, 90, 120 and 150 days, the serum was separated and checked for DT antibodies using ELISA, standardised in the laboratory of National Institute of Immunology, New Delhi.

The 96–well–ELISA plates (Nunc Maxisorp, Denmark) was coated with 100 $\mu$L/well of DT (3500 Lf/mL) solutions (1 $\mu$g/well) in 0.05 M phosphate buffer of pH 7.4. The plate was incubated for 60 min at 37°C and washed once with phosphate buffered saline (50 mM, pH 7.4) with 0.2% Tween 20 (PBS–T). Then 100 $\mu$L of 1% BSA solution in 0.05 M phosphate buffered saline was added to each well and incubated for 60 min at 37°C and the plate was washed thrice with 0.2% PBS–T. Different dilutions of serum samples 1: 300 to 1: 3000 were made with 0.2% PBS–T and 100 $\mu$L final volume of each samples was loaded into the wells of plate. Each test sample was loaded in duplicate. The plate was then incubated for 60 min at 37°C and washed 4 times with 0.2% PBS–T. Then 100 $\mu$L of the conjugate, Goat anti–rat HRPO (horse raddish peroxidase) (dilution 1: 7500) was added to each well and incubated at 37°C for 1h. After incubation, the plate was again washed four times with 0.2% PBS–T and 50 $\mu$L of substrate (prepared by dissolving 6 mg of o–phenylene diamine in 12 mL citrate phosphate buffer of pH 5.5 and mixed with 12 $\mu$L of 30% $H_2O_2$) was loaded in the plate and incubated for 20 min in dark. Then the reaction in each well was stopped with the addition of 5 $\mu$L per well of 5 M $H_2SO_4$. Absorbance was read at 492 nm. Rat anti–DT antibodies polyclonal were used in various dilutions ranging from 1:10 to 1:3000 in 0.2% PBS–T for preparation of the standard curve. The standard curve was run on each plate along with the test samples every times in each assay, in order to overcome plate to plate assay variation.

### 2.2.21 Degradation Studies

#### 2.2.21.1 *In Vitro* Degradation

Chitosan microspheres 5 mg was incubated in 1 mL phosphate buffer (pH 7.4, 0.1 M) containing 5 and 10 mg of lysozyme, at 37°C. Microspheres were filtered and washed at various time intervals and examined by SEM.

#### 2.2.21.2 *In Vivo* Degradation

*In vivo* degradation of the microspheres was examined up to 12 months period by implanting the microspheres in the skeletal muscle of Wistar rats. Microspheres (150–300 $\mu$m), 5 mg were suspended in 1 mL of physiological saline and injected into the skeletal muscle. Each animal received two injections on either side of the muscle. After definite time intervals, the tissue at the injection site was cut and fixed in formalin. 5 $\mu$m sections were cut, embedded in paraffin, stained with hematoxylin and eosin and examined microscopically for the extent of degradation. Tissue containing the microspheres were dehydrated in increasing concentrations of ethanol, dried, coated with gold, and were also examined by SEM.

### 2.2.13.5 Determination of Drug Payload in Microspheres

The amount of drug incorporated into chitosan microspheres was determined by extracting the drug–containing microspheres using a suitable solvent and estimating spectrophotometrically at respective $\lambda_{max}$ of the drug.

For diclofenac sodium, powdered microspheres 5 mg were added to 15 mL of methanol in a 50 mL stoppered Erlenmeyer flask and stirred magnetically for 24 h. The supernatant solution was filtered through a 0.45 $\mu$m filter (Sigma, USA) and analysed spectrophotometrically (Hitachi UV 220, Japan) at 276 nm (Chowdary and Vijaya Ratna, 1992). From the calibration curve prepared in the concentration range 2–20 $\mu$g/mL, the drug content was determined.

For mitoxantrone, powdered microspheres 5 mg was added to 15 mL methanol in a 50 mL stoppered Erlenmeyer flask and stirred magnetically for 24 h. The supernatant solution was filtered through a 0.45 $\mu$m filter and assayed spectrophotometrically at 610 nm according to Windolz *et al* (1976). Calibration curve was prepared in the concentration range 2–40 $\mu$g/mL and from this the drug content was determined.

Progesterone content was determined by extracting 5 mg of the powdered progesterone–loaded microspheres with 15 mL ethanol and the supernatant solution was filtered and assayed spectrophotometrically at 247 nm (Dunn *et al.*, 1981). Progesterone content was estimated from the calibration curve prepared in the concentration range of 2–20 $\mu$g/mL.

BSA content in the microspheres was estimated as follows. BSA–loaded microspheres, 5 mg were crushed in an agate mortar and extracted with 3 mL of

# CHAPTER 3
# RESULTS AND DISCUSSION

# RESULTS AND DISCUSSION

## 3.1 Preparation and Characterisation of Glutaraldehyde Cross-linked Placebo Chitosan Microspheres

Chitosan is a deacetylated derivative of the natural polysaccharide chitin, which is second in abundance to cellulose. The viscosity of chitosan in 0.1 M acetic acid containing 0.2 M sodium chloride at various concentrations was determined using an Ubbelohde viscometer at 25°C. Fig. 3.1.1 shows the plot of $\eta_{sp}/C$ versus different concentrations. Limiting viscosity number was obtained on extrapolating the graph to infinite dilution. The viscosity average molecular weight was calculated using the equation of Maghami and Roberts as described in section 2.2.1. The viscosity average molecular weight was found to be $3.11 \times 10^5$ Da. The degree of deacetylation was determined by potentiometric titration. For this, chitosan was dissolved in 0.3 N HCl and the dissolved product was titrated potentiometrically against NaOH. A blank experiment was conducted with HCl alone. From the volume of NaOH used which is the inflection point in the graph (Fig. 3.1.2), total milliequivalents of amino group in the sample was obtained and from this degree of deacetylation was calculated on molar basis. It was found to

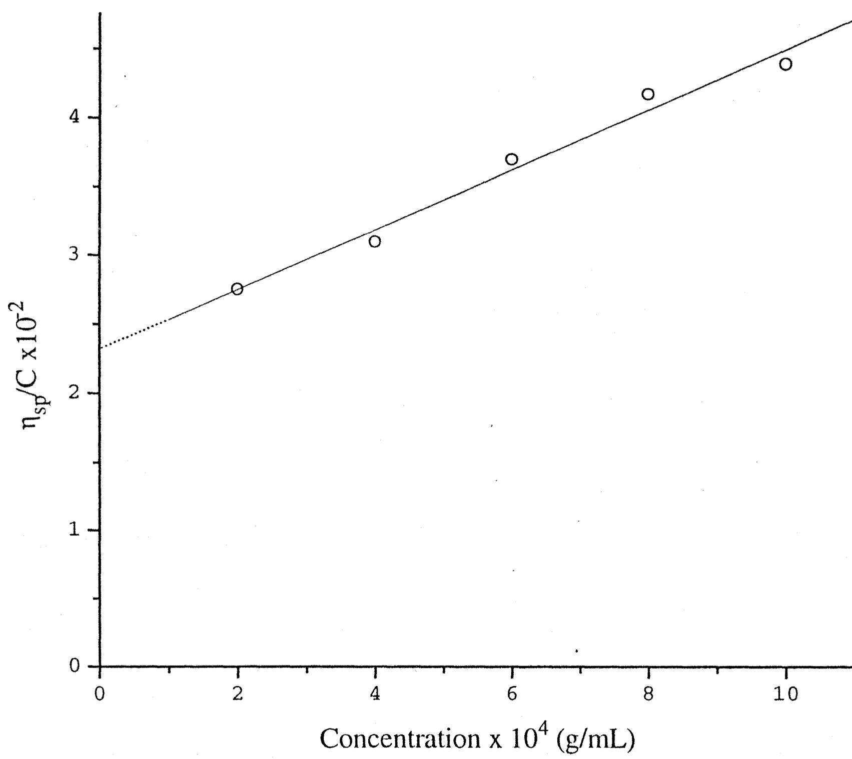



Fig. 3.1.1 Plot of $\eta_{sp}/C$ versus concentration of chitosan solution

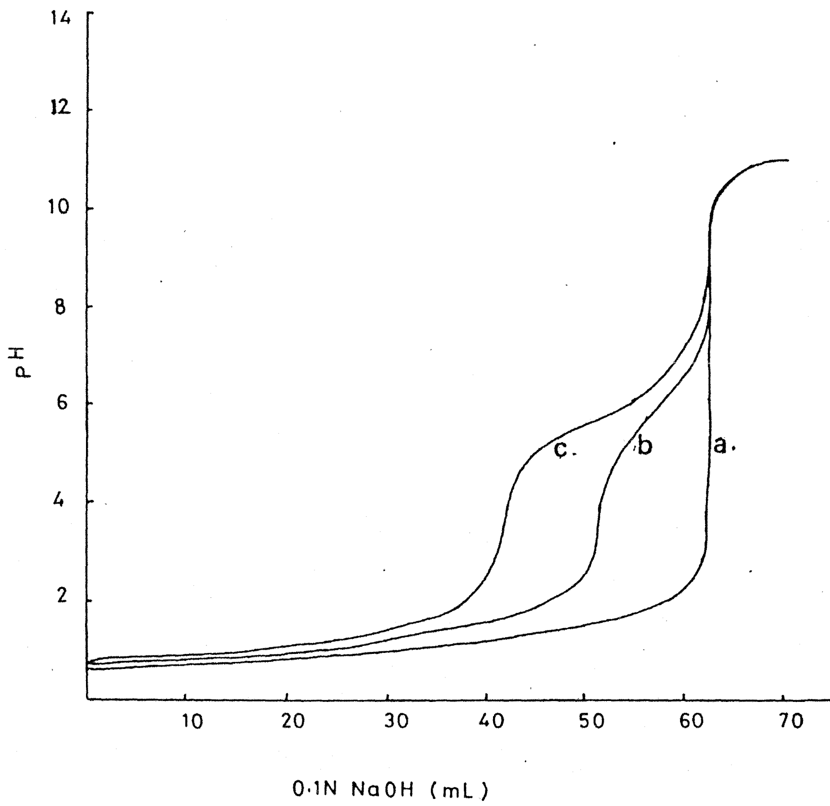



Fig. 3.1.2 Potentiometric titrations of chitosan dissolved in 20 mL of 0.3 N HCl. Reference curve for 0.3 N HCl (a), 0.25 g chitosan (b), 0.5 g chitosan (c)

be 74%. Chitosan sample acetylated further according to Robert and Taylor (1989) was found to have a degree of deacetylation 30%.

Chitosan microspheres were prepared from an aqueous acetic acid solution of chitosan containing salts such as sodium chloride or sodium acetate. Addition of electrolytes to chitosan solutions is essential in order to reduce the otherwise highly viscous nature of chitosan solutions due to its polyelectrolyte effect. That is, in the absence of salt, there is an abnormal increase in the viscosity of the more dilute solutions because of the enlarged effective volume due to charge repulsion and stretching out of the molecules. When sufficient salt is added to neutralise this charge effects the viscosity behaviour is normal (Muzzarelli, 1977). For crosslinking the microspheres, glutaraldehyde saturated toluene (GST) was employed instead of aqueous glutaraldehyde solution. Determination of glutaraldehyde content in saturated toluene solution by ε-aminocaproic acid method showed that the concentration was about 0.02 mole%. Because of the oil solubility of toluene saturated with glutaraldehyde in the dispersion medium, uniform surface crosslinking of the microspheres was expected to take place confining the crosslinking reaction mostly to the surface of the microspheres providing a surface net that would act as a barrier for diffusion of entrapped pharmacologically active agents. Highly crosslinked microspheres were prepared by adding aqueous glutaraldehyde in addition to GST. Here, in addition to surface crosslinking, extensive crosslinking of the microsphere matrix was expected to take place. During crosslinking, condensation reaction between aldehyde group and amino group present in chitosan takes place to form a Schiff base linkage. In order to remove the residual glutaraldehyde, the beads were washed with sodium bisulphite

followed by copious amounts of water (Thanoo *et al.*, 1993). Residual aldehyde handles present on the microspheres were also end-capped using aqueous solutions of glycine in certain preparations (Longo *et al.*, 1982). Fig. 3.1.3 shows the SEM of microspheres prepared by paddle stirring. The microspheres are large in size with good spherical geometry and a smooth surface. Fig. 3.1.4 shows the particle size distribution of microspheres prepared by this technique. Fig. 3.1.5 shows the SEM of microspheres prepared by sonication using a probe type sonicator. These smaller size particles having diameter $<10\ \mu m$ were also highly spherical and possessed smooth surface morphology. The particle size distribution of these microspheres is given in Fig. 3.1.6. Thus, smooth, highly spherical microspheres crosslinked using glutaraldehyde could be prepared in the desired size range depending on the reaction conditions employed.

Examination of the internal structure of placebo microspheres by optical microscopy showed that they possessed a solid internal structure without any pores or perforations. Fig. 3.1.7 shows the optical photograph of 1 $\mu m$ thick section of microspheres prepared by the paddle stirring method and crosslinked using only 4 mL of GST. Even at such low crosslinking densities, the internal structure was solid in appearance. Examination of microspheres crosslinked using higher amount of GST or aqueous glutaraldehyde also showed similar internal structure.

Although SEM examination of microspheres showed a very smooth non-porous outer surface and optical microscopy showed a solid internal structure devoid of pores and perforations inside the microspheres, these examinations were conducted on spheres that were dry. Since the polysaccharide will have affinity for water, swelling microspheres in aqueous solution would expand the crosslinked network

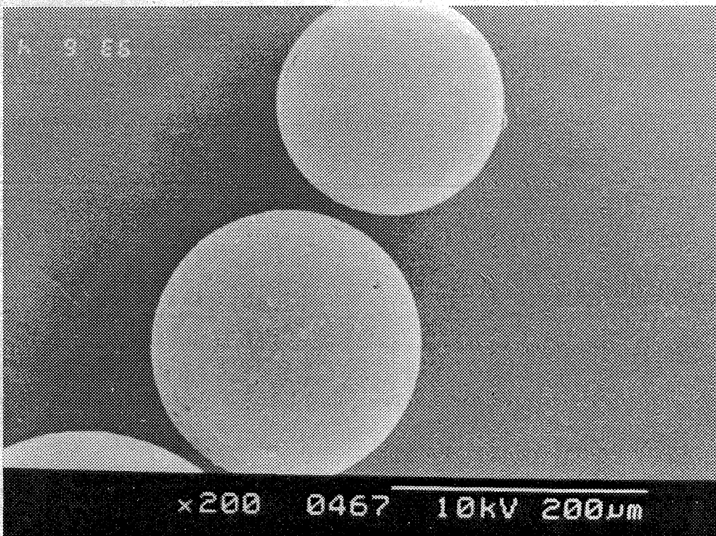



Fig. 3.1.3 SEM of chitosan microspheres prepared using paddle stirrer

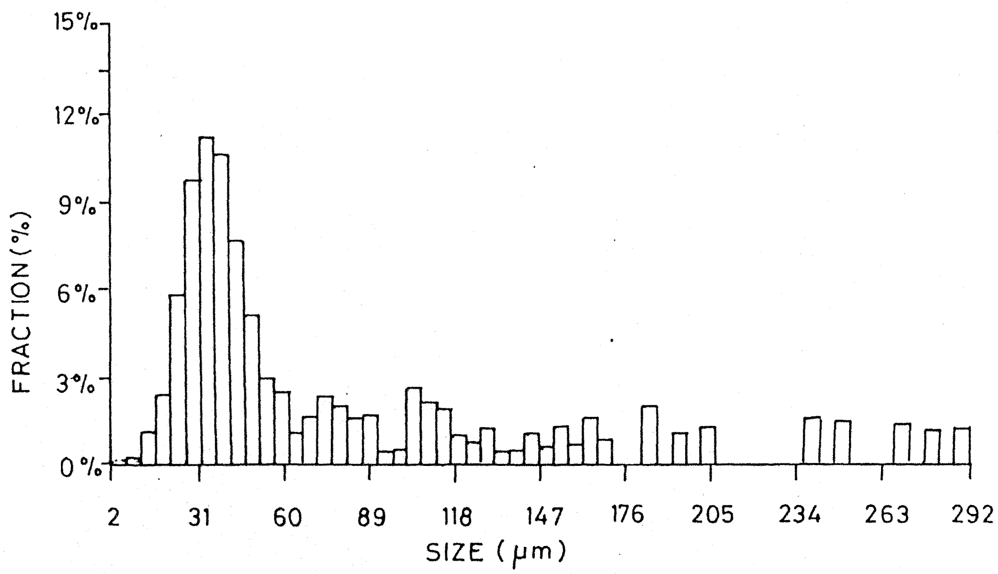



Fig. 3.1.4 Probability volume density distribution of chitosan microspheres prepared using paddle stirrer

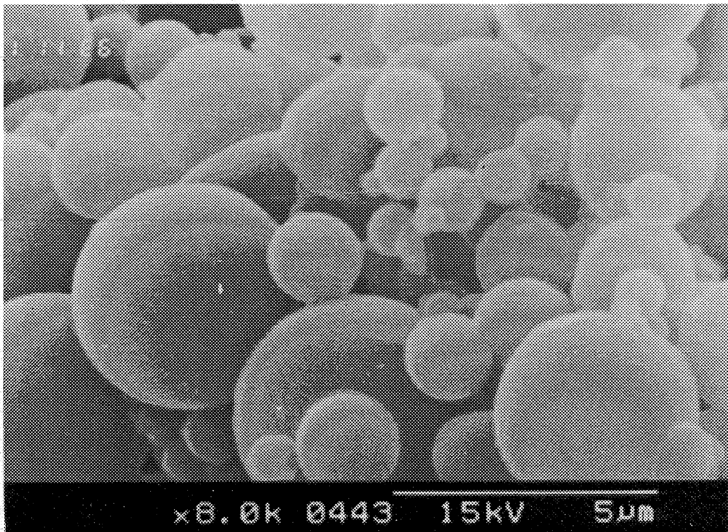



Fig. 3.1.5 SEM of chitosan microspheres prepared using probe type sonicator

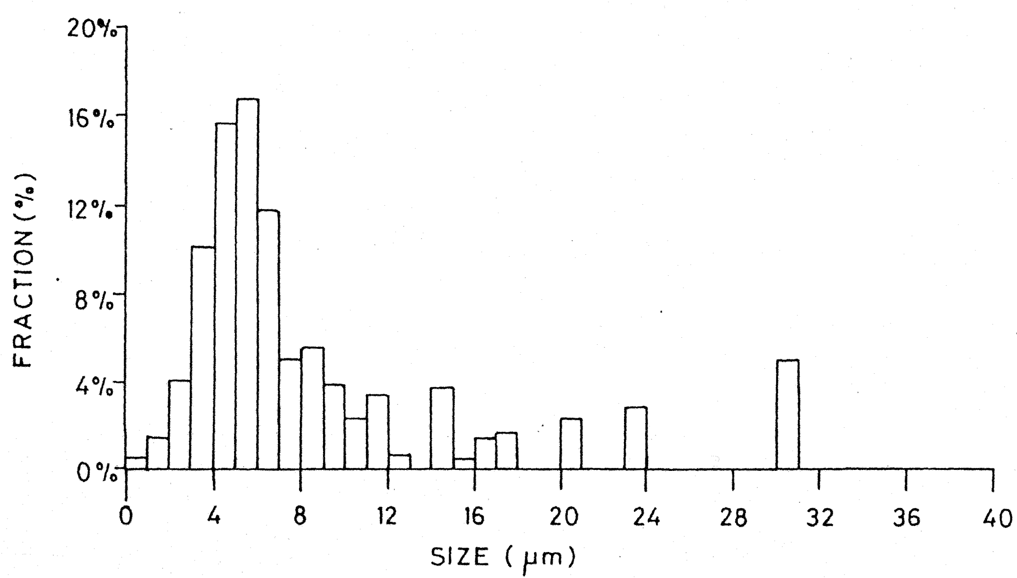



Fig. 3.1.6 Probability volume density distribution of chitosan microspheres prepared using probe type sonicator

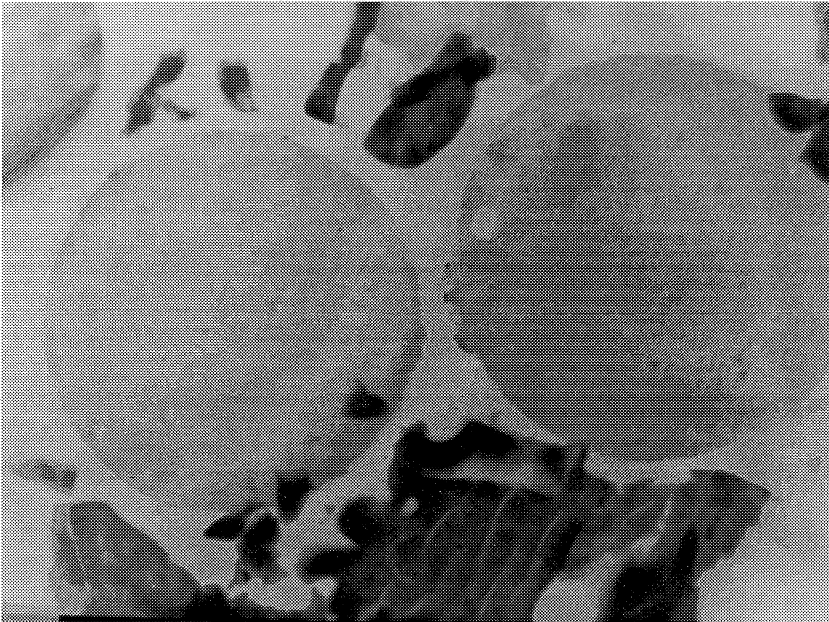


Fig. 3.1.7 Optical photograph of 1 μm thick section of chitosan microspheres prepared using paddle stirrer (Original magnification x 400)

depending on the degree of crosslinking. Therefore, microspheres having different crosslinking densities were swollen to equilibrium in distilled water at 27°C and the equilibrium uptake of water was estimated as described in section 2.2.13.2. Fig. 3.1.8 shows the swelling kinetics of these microspheres. Microspheres least crosslinked were found to take up more amount of water at equilibrium compared to microspheres which were extensively crosslinked. Therefore, although the microspheres in the dry form do not exhibit pores and perforations, once swollen they will be sufficiently porous in order to take up pharmacologically active agents from their aqueous solutions. This property of the microspheres would be advantageous in loading drugs in their most native form into pre-formed microspheres. Glutaraldehyde crosslinking of microspheres in the presence of drugs which possess functionalities such as amino groups that can enter into reaction with aldehyde affect the activity of such drugs. For example, aldehyde crosslinking affects the activities of drugs such as methotrexate, epinephrine and salbutamol (Gupta and Hung, 1989a). Therefore, unlike hydrophobic particles which exhibit little affinity for water, chitosan microspheres by virtue of their swelling ability would be suitable for loading water soluble drugs in their most native form after the microspheres are prepared and cleaned thoroughly for any impurities that may become incorporated during their preparations.

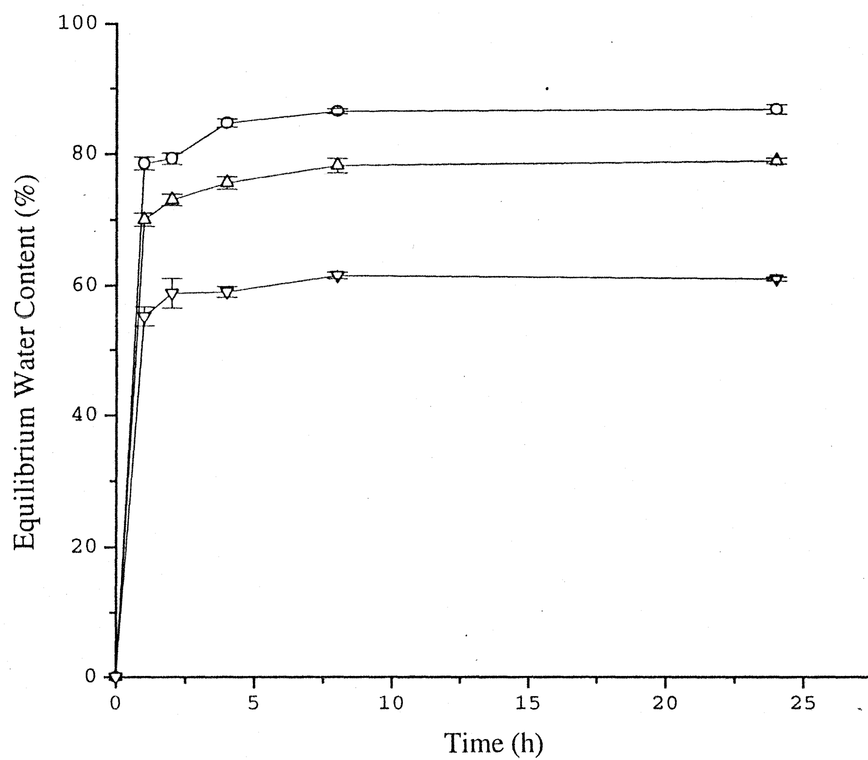



Fig. 3.1.8. Swelling kinetics of chitosan microspheres crosslinked with different amounts of glutaraldehyde in distilled water at room temperature (27°C): 2 mL GST (—○—) 4 mL GST (—△—) and 10 mL GST (—▽—).

## 3.2 Degradation Studies of Chitosan Microspheres

### 3.2.1 *In Vitro* Degradation

The biological life of carrier matrix is important from the point of view of determining the duration of chemical delivery that can be effected using a particular matrix. Hence degradation studies of chitosan microspheres assumes importance.

Partially deacetylated chitin and gels prepared from it by glutaraldehyde crosslinking are reported to be degraded *in vitro* by the enzyme lysozyme (Pangburn *et al.*, 1982). Chitin sutures are reported to be resorbed in the body in about 2 months (Nakajima *et al.*, 1986). Muzzarelli *et al* (1989) reported that Chitosan ascorbate used for the reconstruction of paradontal tissue was resorbed in the oral cavity in about the same period. The rate of lysozyme-catalysed soluble partially deacetylated chitin is linear at low enzyme concentrations ($<10$ $\mu$g/mL) but non-linear at higher enzyme concentrations (Pangburn *et al.*, 1982).

In order for the enzyme catalysed hydrolysis to occur, the substrate should bind to the active site of the enzyme as shown in a model developed by Pangburn *et al* (1982). With increased degree of deacetylation, the rate of enzymatic hydrolysis is expected to be low, as the fully deacetylated material was not found to be digested with lysozyme. Glutaraldehyde crosslinking of chitosan is likely to produce unfavourable conformations of the polysaccharide for productive enzyme binding. Therefore unlike soluble chitin or chitosan, the crosslinked material may be less susceptible to lysozyme. Microspheres least crosslinked were therefore exposed to high lysozyme concentration *in vitro*. Any visible degradation to the matrix was monitored by SEM examination. Fig. 3.2.1 shows the SEM of

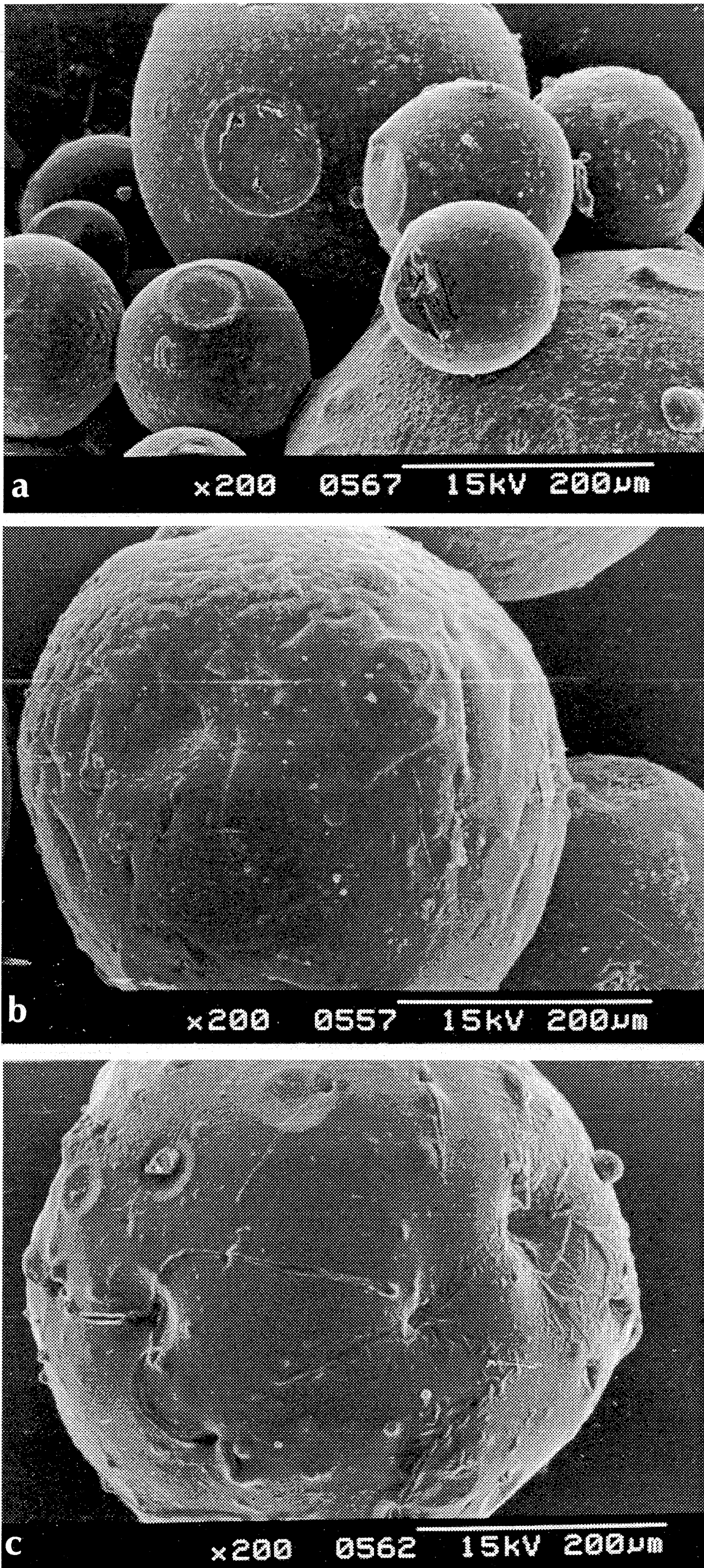



Fig. 3.2.1 SEM of chitosan microspheres (from 74% deacetylated chitin) after incubation in lysozyme solution for 1 month at 37°C. Microspheres incubated in phosphate buffer (a), buffer containing 5 mg lysozyme (b), buffer containing 10 mg lysozyme (c).

microspheres incubated in phosphate buffer (a), in buffer containing 5 mg lysozyme (b), and buffer containing 10 mg lysozyme (c) for one month at 37°C. Although there appeared to be changes in the surface morphology of the microspheres incubated in lysozyme solution, there was no significant degradation. Even after 3 months and 6 months incubation in lysozyme solution there appeared to be very little changes in the surface morphology of the microspheres. Determination of the weight of microspheres after incubation in lysozyme solution did not show any change from their original weight before incubation within the limits of experimental error. Therefore the susceptibility of these crosslinked microspheres to lysozyme degradation *in vitro* at concentrations examined was rather poor. Pangburn *et al* (1982) reported that glutaraldehyde crosslinked gels prepared from 31% deacetylated chitin was completely dissolved on treatment with lysozyme *in vitro*. In their work 3 mg of crosslinked gel on treatment with 3 mg of lysozyme was found to dissolve in 1.5 h at 37°C. Thus the degree of deacetylation appears to be a critical factor in determining the enzymatic degradation of the material *in vitro*. Increased deacetylation thus provides an unfavourable conformational environment for productive enzyme binding to occur rapidly. Nevertheless, the low susceptibility of the crosslinked microspheres to lysosomal degradation could be advantageous in drug delivery applications, where prolonged release of the active agent by diffusion from the matrix assumes importance. Furthermore, often there exists very little correlation between *in vitro* and *in vivo* degradation profiles of many biodegradable polymers. It was therefore necessary to determine the actual *in vivo* degradation of these microspheres in a suitable animal model.

### 3.2.2 *In Vivo* Degradation

Although chitin sutures and chitosan ascorbate employed in the reconstruction of paradontal tissue in the oral cavity are reported to be degraded *in vivo* in about 2 months (Muzzarelli, 1989), little published information exists on the *in vivo* degradation of glutaraldehyde crosslinked gels or microspheres prepared from chitin or chitosan. Therefore this study was undertaken in order to evaluate the degradation characteristics of glutaraldehyde crosslinked chitosan microspheres *in vivo*. Since the polysaccharide with high degree of deacetylation was not found to be digested *in vitro* by lysozyme, microspheres were also prepared from chitosan acetylated further as described in section 2.2.3. Thus microspheres prepared from 74% deacetylated chitin and 30% deacetylated chitin crosslinked using 4 mL GST were employed in the study. Microspheres injected into the gluteal muscle of rats were explanted at various periods of time and examined histologically for tissue reaction and extent of degradation under the microscope.

Fig. 3.2.2 shows the histological section of the rat muscle containing chitosan microspheres from 74% deacetylated chitin 7 days post implantation. Numerous beads present evoked the initial inflammatory response with lymphocytes, macrophages and fibrocytes appeared around the implant.

At 6 weeks post implantation (Fig. 3.2.3) microspheres from both 74% and 30% deacetylated chitin could be seen in the muscle. Histological pictures showed the presence of fibrocytes, macrophages and giant cells around the implant. Lot of fibrocytes and fibroblasts were observed. Histologically there was no adverse tissue reaction to the implant and the cellular response was moderate. Spheres from 30%

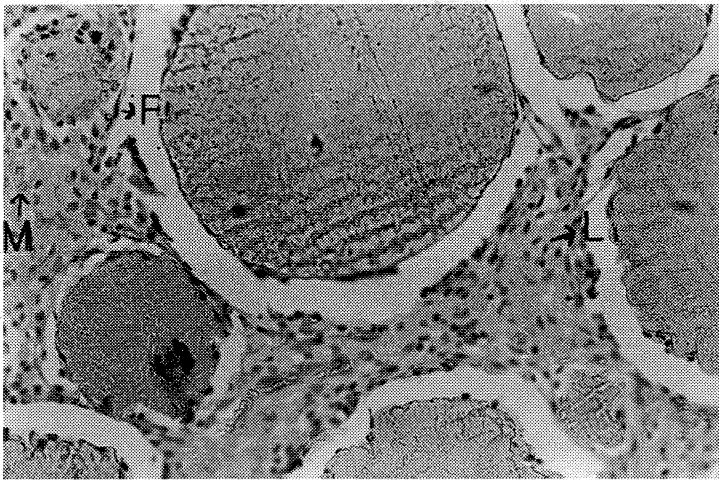



Fig. 3.2.2 Histological section of rat skeletal muscle containing chitosan microspheres (from 74% deacetylated chitin) 7 days post implantation showing initial inflammatory response with lymphocytes (L), macrophages (M) and fibrocytes (F) around the implant (Original magnification x 100)

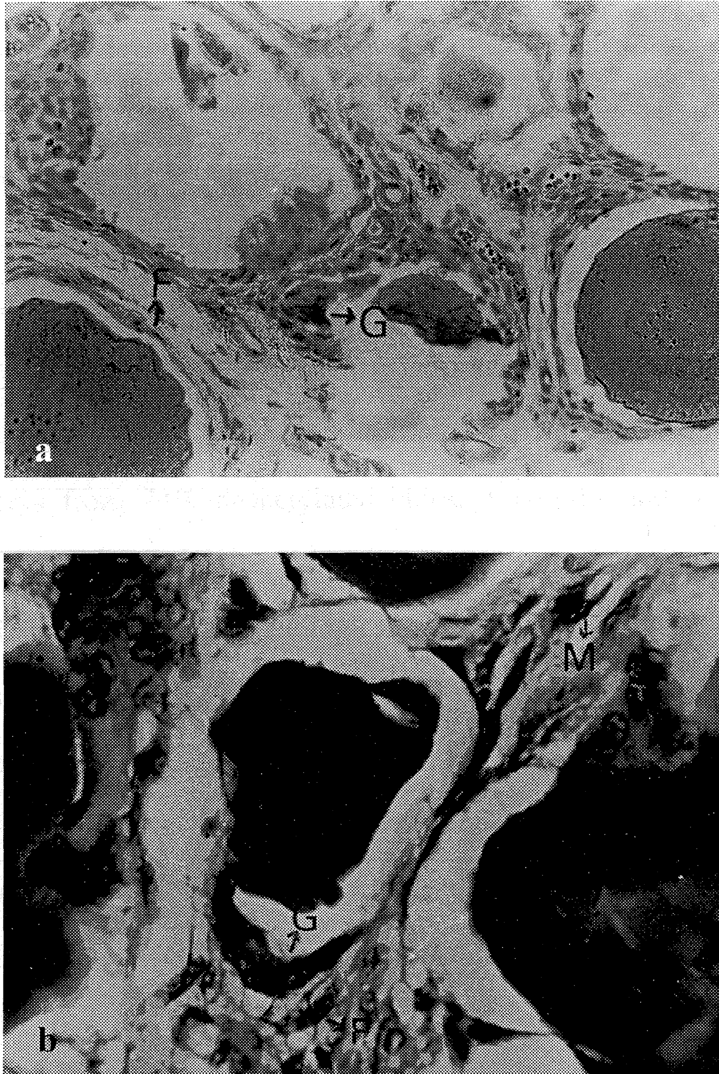


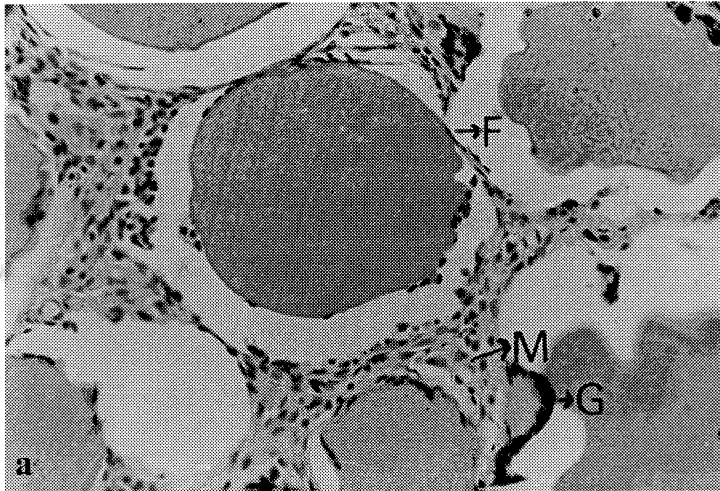

Fig. 3.2.3 Histological section of rat skeletal muscle containing chitosan microspheres from 74% deacetylated chitin (a) and 30% deacetylated chitin (b) 6 weeks post implantation showing the presence of fibrocytes (F), macrophages (M) and giant cells (G) around the implant (Original magnification x 100)

deacetylated polysaccharide appeared to be more deformed than those from 74% deacetylated material.

At 3 months and 6 months post implantation, microspheres from both 74% and 30% deacetylated polysaccharide could be still seen histologically although there appeared to be degradation of the material (Fig. 3.2.4). Tissue reaction was similar to the 6 week implant with fibrocytes, macrophages and giant cells still present around the microspheres.

Further evidence to the degradation of the material was obtained by SEM examination of the tissue containing microspheres. Fig. 3.2.5 shows the SEM of microspheres from 74% deacetylated chitin 3 months and 6 months after implantation. The degradation of the microspheres was evident from the photograph. Examination of the histological section revealed that the surface of the microspheres have undergone degradation rather than the bulk. As can be seen from the photomicrographs the edges of the spheres have become irregular in appearance. SEM examinations confirm this observation. It can be seen from the SEM pictures, that the surface of the microspheres have become rough owing to biodegradation (Compare with surface morphology of microsphere in Fig. 3.1.3).

At the end of 1 year, microspheres from both 74% and 30% deacetylated polysaccharide still persisted in the tissue. The histological section of the tissue containing microspheres are shown in Fig. 3.2.6. Apparently spheres from 30% deacetylated material have undergone more degradation compared to spheres from 74% deacetylated material. However both microspheres could still be visualised in the tissue even after 1 year of intramuscular implantation. Fibroblasts with occasional macrophages were observed. That means cellular response was



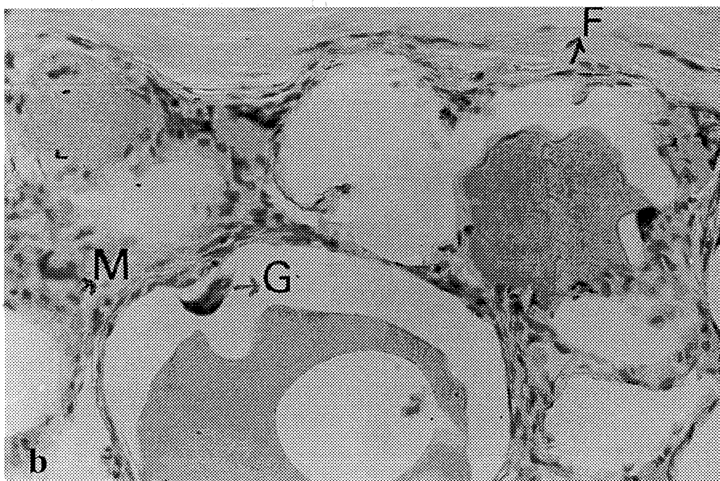




Fig. 3.2.4 Histological section of rat skeletal muscle containing chitosan microspheres 3 months and 6 months post implantation again showing the presence of fibrocytes (F), macrophages (M) and giant cells (G) around the microspheres. Microspheres from 74% deacetylated chitin, 3 months post implantation (a), 6 months post implantation (b) (contd...).

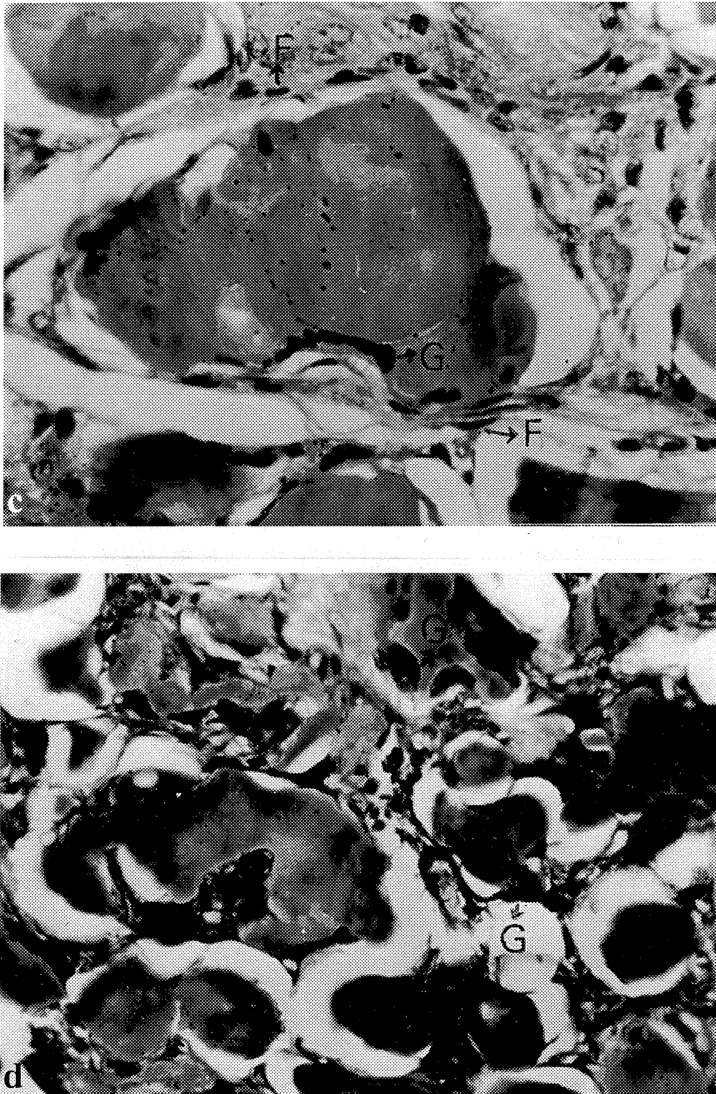
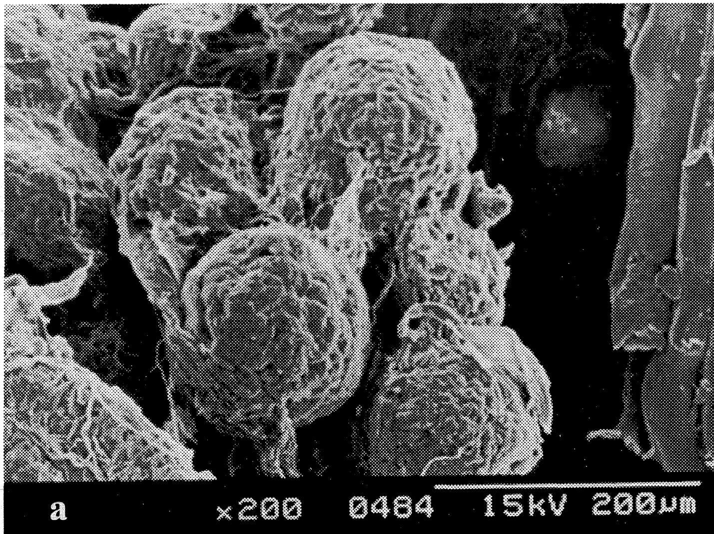



Fig. 3.2.4 Microspheres from 30% deacetylated chitin
3 months post implantation (c), 6 months post implantation (d).
(Original magnification x 100).



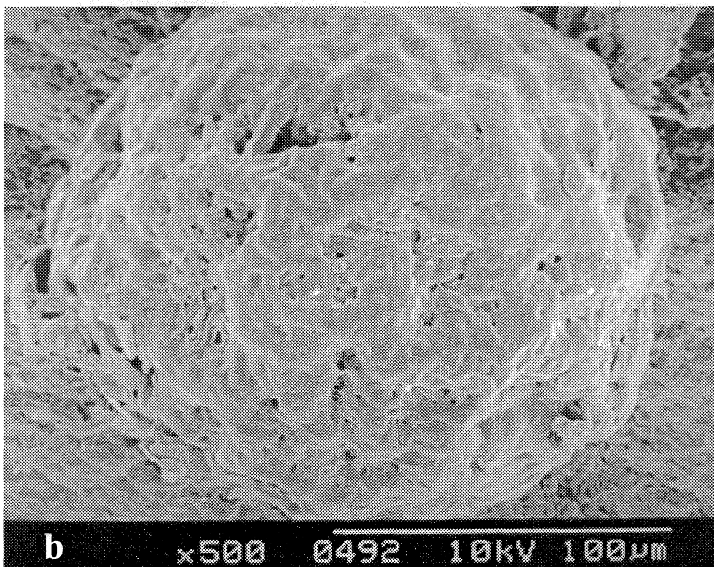
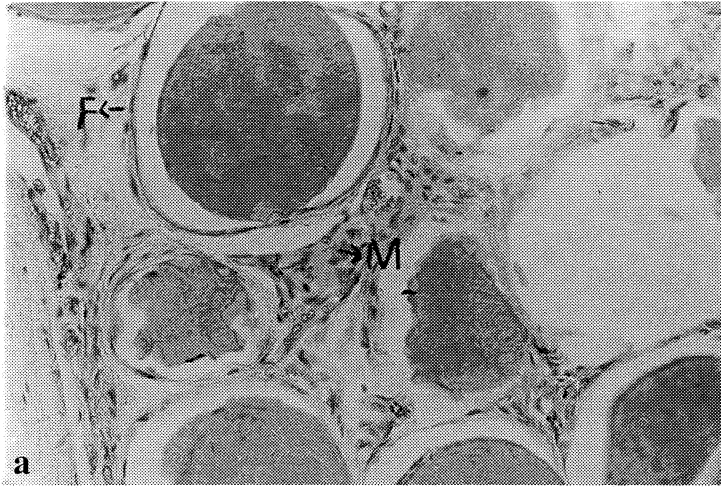




Fig. 3.2.5 SEM of chitosan microspheres explanted from the rat muscle 3 months after implantation (a), 6 months after implantation (b) showing surface degradation.



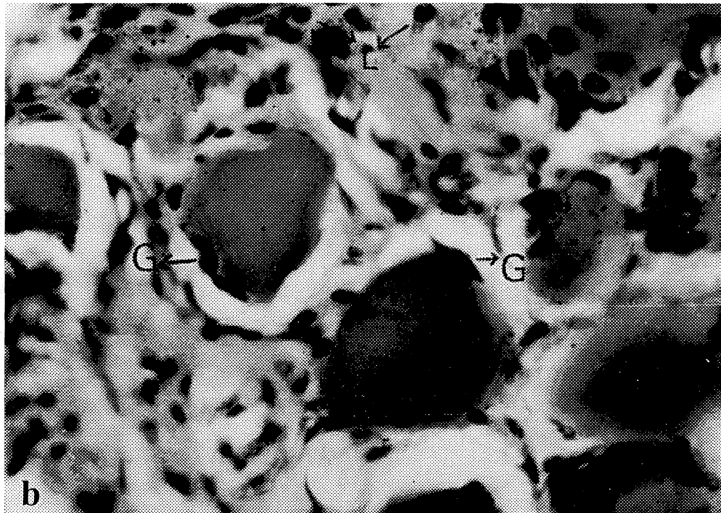




Fig. 3.2.6 Histological section of rat skeletal muscle containing chitosan microspheres 1 year after implantation. Microspheres from 74% deacetylated chitin (a), 30% deacetylated chitin (b) (Original magnification x 100)

moderate in both cases and it reduced with time. At 1 year post implantation more number of fibroblasts with very little inflammatory reaction was observed.

Although Pangburn *et al* (1982) have shown that glutaraldehyde crosslinked gels from 31% deacetylated chitin are degraded and dissolved completely by lysozyme *in vitro*, the observations of the *in vivo* degradation of microspheres in the skeletal muscle of rats in this study assumes importance. The amount of lysozyme concentration in the human blood is reported to be 500 $\mu$L/mL (Watanabe *et al.*, 1990). At such low concentration lysozyme present *in vivo*, the biodegradation of microspheres even from 30% deacetylated chitin was not complete after a whole year. Microspheres from the polysaccharide deacetylated further (74%) may thus be present for larger period of time in the tissue. Thus, the extent of acetylation of chitin appears to be a critical factor in determining the extent of biodegradation that would take place *in vivo*. The slow degradation of glutaraldehyde crosslinked microspheres could therefore be exploited for effecting prolonged release of pharmacologically active agent *in vivo*.

# 3.3 Chitosan Microspheres as a Carrier for the Controlled Delivery of Mitoxantrone

## 3.3.1 Background

Anticancer drugs are often limited in their use by high systemic toxicity, poor stability and short biological half life (Balis *et al.*, 1983). Hence, selective efficient and targeted delivery of drugs to the desired site of action assumes importance in cancer chemotherapy (McLaughlin and Goldberg, 1983). Many protein and polysaccharide–based microspheres have been evaluated for sustained and targeted delivery of cytotoxic drugs (Guiot and Couvreur, 1986; Puisieux *et al.*, 1994).

Mitoxantrone, an anthracene dione is a novel anticancer agent with a wide spectrum of antitumour activity. Its chemical structure is shown in Fig. 3.3.1. Anticancer activity of mitoxantrone is comparable to that of doxorubicin, methotrexate, cyclophosphamide and cytosine arabinoside against P388 and L1210 leukemias and B16 melanoma and colon tumour in mice. Compared with doxorubicin, it has much reduced cardiotoxicity (Cheng *et al.*, 1979). The pharmacokinetics of mitoxantrone in man and laboratory animals has been reviewed by Batra *et al* (1986).

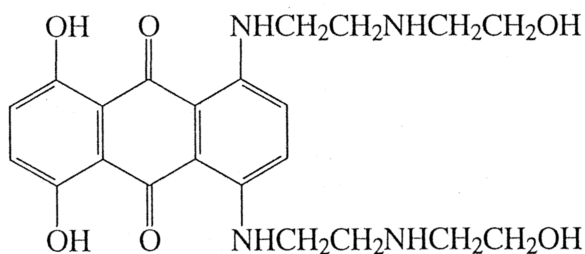



Fig. 3.3.1 Chemical structure of mitoxantrone

Anticancer drugs such as 5–fluorouracil and methotrexate were encapsulated in protein microspheres and their *in vitro* release profiles were examined by Jayakrishnan *et al* (1994) and Latha *et al* (1994). Knepp *et al* (1993) prepared mitoxantrone–loaded bovine casein microspheres and tested their antitumour activity against mouse Lewis lung carcinoma. Mitoxantrone–loaded casein microspheres exhibited lower toxicity and better therapeutic efficacy compared the free drug when administered intratumourally.

There are very few reports in the literature on the antitumour effect of sustained polymeric formulations of antineoplastic agents against Ehrlich ascites carcinoma (EAC). Miyazaki *et al* (1985 and 1986) investigated the antitumour effect of 5-FU-loaded ethylene-vinyl alcohol (EVA) copolymer discs and doxorubicin-loaded fibrinogen microspheres against EAC intraperitoneally in mice. Sugibayashi *et al* (1979) and Morimoto *et al* (1980) investigated the antitumour effect of 5-FU entrapped albumin microspheres on EAC. This chapter describes the preparation of mitoxantrone-loaded chitosan microspheres, the *in vitro* release profiles of mitoxantrone from microspheres of varying crosslinking densities, size ranges and drug payloads and their antitumour activity against EAC in a mice model.

### 3.3.2 Mitoxantrone–Loaded Chitosan Microspheres

Mitoxantrone-loaded microspheres obtained were spherical in shape as evidenced by SEM analysis (Fig. 3.3.2). The morphology appears to be a little rough, presumably due to the aqueous washing removing the drug crystals present on the surface of the microspheres. The incorporation efficiency of mitoxantrone

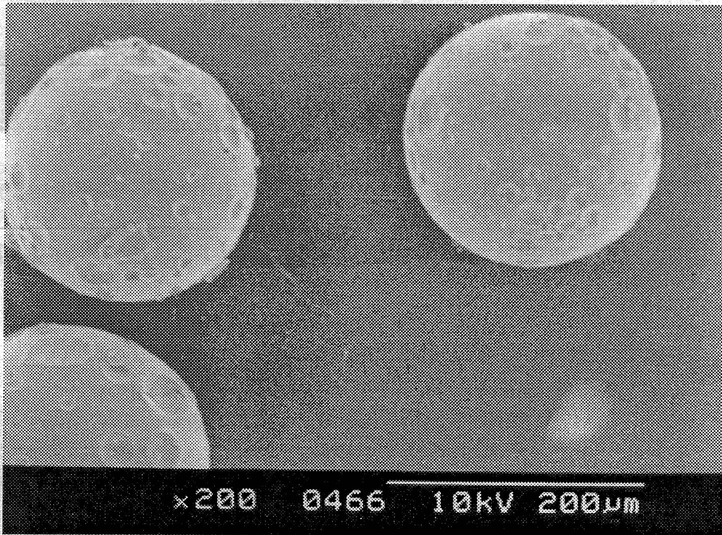



Fig. 3.3.2 SEM of mitoxantrone-loaded chitosan microspheres

in microspheres of different crosslinking densities at 5 and 10% initial loadings is shown in Table I. The incorporation efficiency of drug appears to be low at all crosslinking densities. Mitoxantrone is soluble to the extent of 12% in water (American Cyanamid Co., 1991). During the crosslinking and hardening process, water is extruded from the microspheres along with the dissolved drug and this appears to be responsible for the rather low incorporation efficiency. Sieve analysis (Fig. 3.3.3) of the particles showed that 48% were below 75 μm, while 32% had particle size from 75 to 150 μm and 20% were in the range of 150 to 300 μm. Microspheres for *in vivo* experiments were prepared by sonication of the dispersion. Particle size analysis showed a volume average particle size of 37± 13 μm (Fig. 3.3.4) and drug content analysis showed a loading of Ca. 4%.

Table I
Incorporation efficiency of mitoxantrone (MTX) in chitosan microspheres crosslinked with different amounts of glutaraldehyde saturated toluene (GST)

| Initial MTX Content (wt%) | Final MTX content (wt%) | GST used (ML) | Incorporation efficiency (%) |
|---|---|---|---|
| 5 | 1.4 | 2 | 28.0 |
| 5 | 1.1 | 4 | 22.0 |
| 5 | 0.9 | 10 | 18.0 |
| 5 | 0.8 | 3.2* | 16.0 |
| 10 | 4 | 10 | 40.0 |

* In addition, 0.8 mL of 25% aqueous glutaraldehyde was used for crosslinking this preparation.

### 3.3.3 *In Vitro* Release of Mitoxantrone

The *in vitro* release studies of mitoxantrone from chitosan microspheres were conducted at 27°C instead of 37°C since the release from the matrix spanning

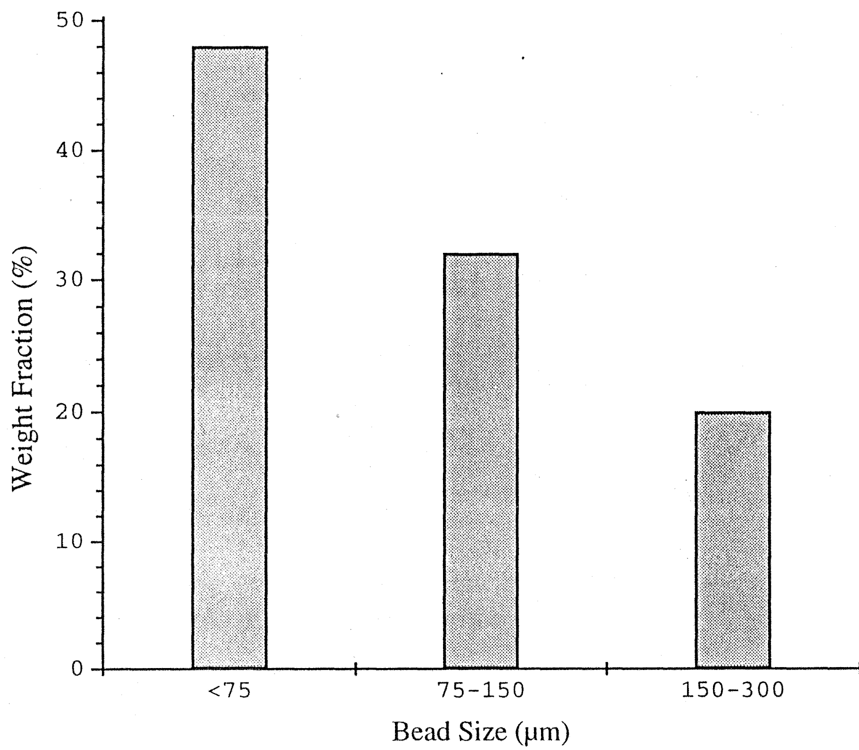



Fig. 3.3.3 Particle size distribution of mitoxantrone loaded chitosan microspheres prepared at a stirring speed of 2000 rpm

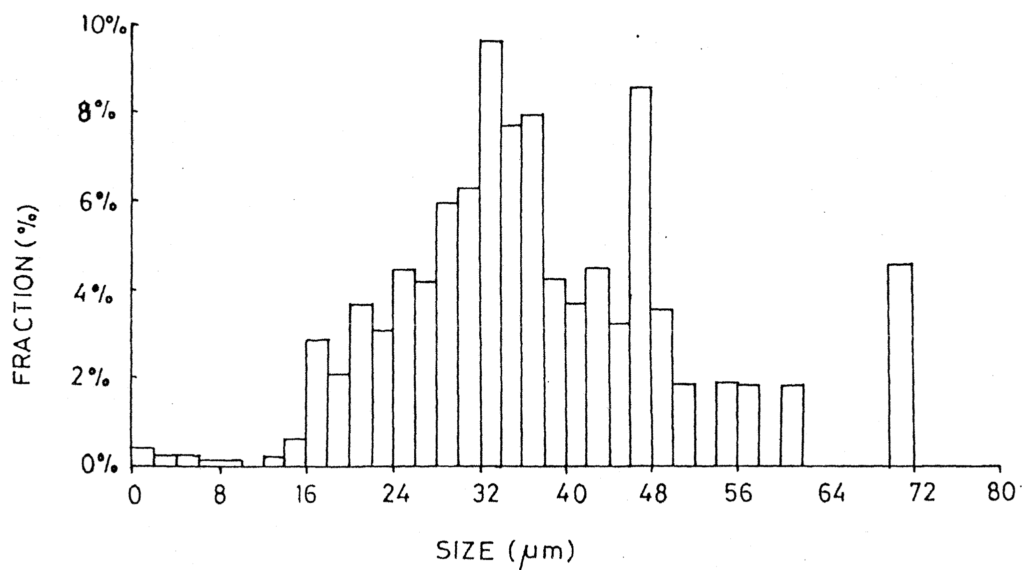



Fig. 3.3.4 Probability volume density distribution of mitoxantrone-loaded chitosan microspheres prepared using probe type sonicator

several weeks was examined and the recommended storage temperature of the drug was between 15 and 30°C according to the data sheet from American Cyanamid Company (American Cyanamid Co., 1991). Moreover, serum stability studies and degradation half-lives of mitoxantrone have been reported at 25°C, possibly because of the increased instability of the drug at higher temperatures (Batra *et al.*, 1986).

The release of mitoxantrone from the microspheres into the dissolution medium under constant stirring and occasional shaking conditions was first examined. Fig. 3.3.5 shows the release profiles under these conditions. This experiment was conducted in order to examine whether release rates could vary considerably if the medium was kept stirred constantly or only occasionally. Since release spanning several weeks was to be determined, if no significant difference in the release rate was observed, the occasional shaking condition would be more suitable for such a study. Interestingly there was virtually no difference in the extent of release of the drug whether the dissolution medium was kept constantly stirred or not. Since the aqueous solubility of the drug was about 12% (American Cyanamid Co., 1991), and the amount of drug present in the microspheres was only in the range of 1–2 mg, sink conditions would be maintained even after complete dissolution of the drug in the medium. All further release experiments were therefore conducted under occasional stirring conditions.

The *in vitro* release of the drug into phosphate buffer from particles having different crosslinking densities is shown in Fig. 3.3.6. As can be seen, the release from the least crosslinked spheres was most rapid; almost all the incorporated drug was released in 24 h. From microspheres having the highest crosslinking

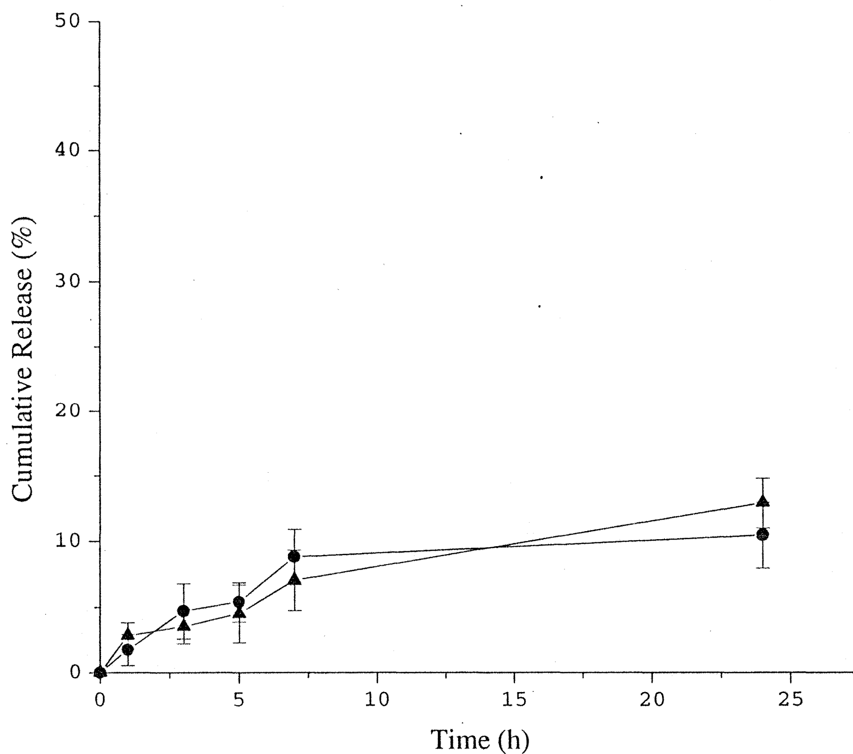



Fig. 3.3.5 In vitro release profiles of mitoxantrone from chitosan spheres (150-300 μm) having a drug payload of 1% crosslinked with 10 mL of glutaraldehyde saturated toluene into phosphate buffer at 27°C under constant stirring (—●—) and occasional shaking (—▲—) conditions.

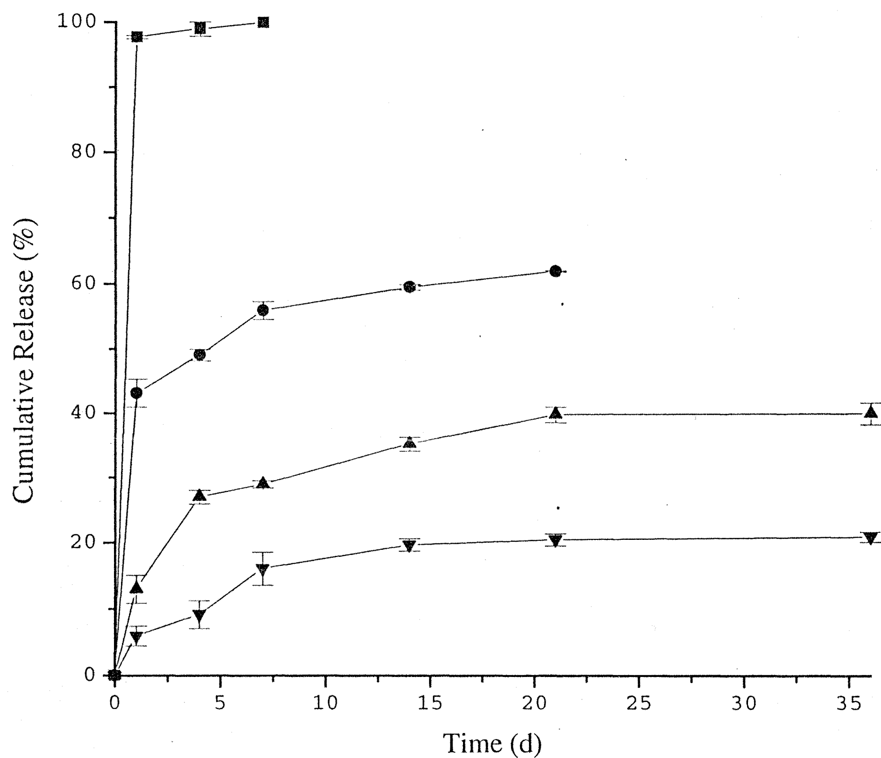



Fig. 3.3.6. Effect of crosslinking density on the release profile of mitoxantrone from chitosan microspheres of size 150-300 μm and drug content 1% into phosphate buffer at 27°C. Microspheres were crosslinked with 2 mL (—■—), 4 mL (—●—), 10 mL (—▲—) GST and 3.2 mL GST followed by 0.8 mL aqueous glutaraldehyde (—▼—).

density, only about 25% of the drug was released even after 36 days. The data clearly demonstrated that the drug release could effectively be controlled by varying the crosslinking density of the microspheres. The effect of crosslinking on drug release from chitosan matrix appeared to be more remarkable than from a protein matrix (Latha and Jayakrishnan, 1994). Possibly the poor affinity of chitosan towards water could be held responsible for the effective control of drug diffusion as opposed to a protein matrix, which is more hydrophilic and swelling in nature. The fact that only about 25–60% of the drug was released from microspheres having high crosslinking densities even after 1 month in the dissolution medium was very intriguing in view of the good aqueous solubility of the drug. Therefore it was felt necessary to determine whether the unreleased portion of the drug still remained within the microsphere matrix. Microspheres were separated from the release medium and subjected to methanol extraction. The amount of the drug extracted into methanol was estimated and found to tally with the theoretical amount that should be found in the microsphere matrix within experimental error.

The release of mitoxantrone from chitosan microspheres of different size ranges is shown in Fig. 3.3.7. The ease of release of drug was dependent on the microsphere size as expected. Drug release was faster from spheres of smaller size owing to the decreased diffusional path length and increased surface area in contact with the dissolution medium. With increased drug payloads in the microsphere matrix, there was increased release as shown by the data in Fig. 3.3.8. At higher loading, drug diffusion from the matrix produces more pores and channels through which release occurred at a faster rate.

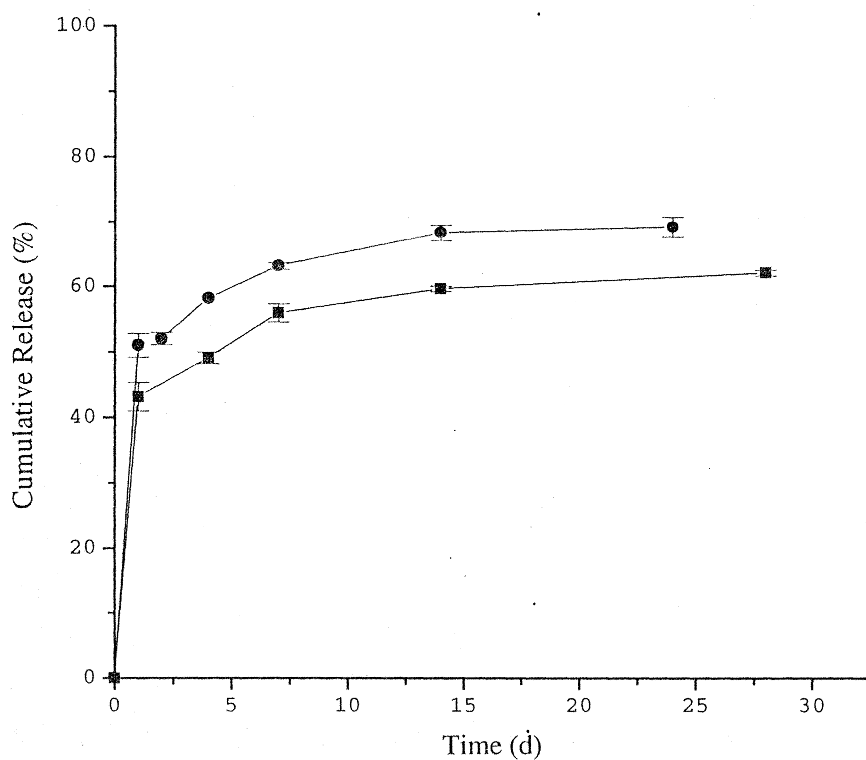



Fig. 3.3.7. Rate of release of mitoxantrone from 1% loaded chitosan microspheres crosslinked with 4 mL GST into phosphate buffer at 27°C as a function of microsphere size. 150-300 μm (—■—), 75-150 μm (—●—).

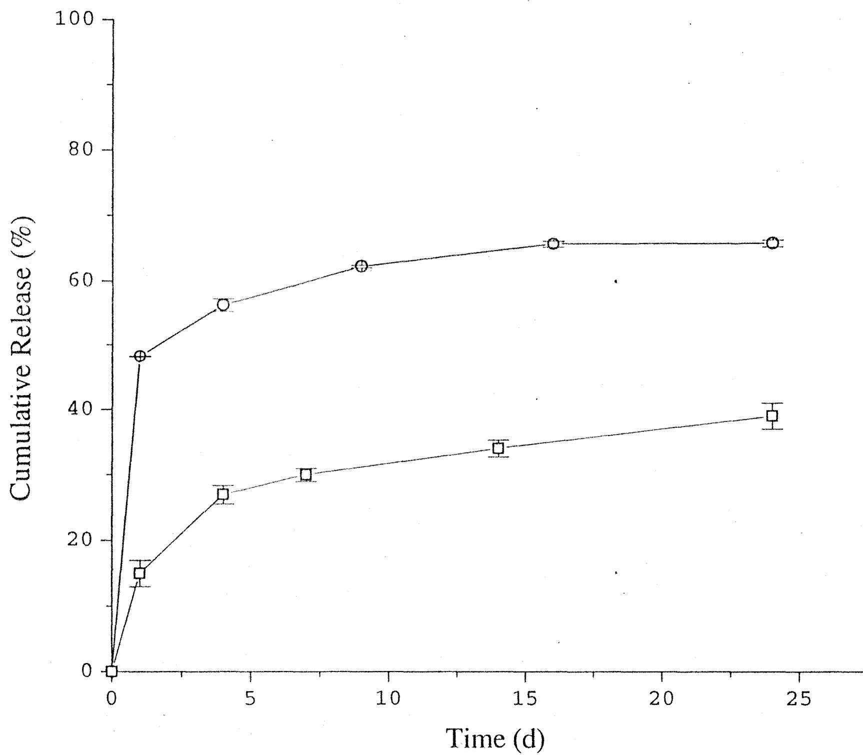



Fig. 3.3.8 Rate of release of mitoxantrone from chitosan spheres (Size 150-300 μm) crosslinked with 10 mL GST into phosphate buffer at 27°C as a function of drug loading. 4% loaded (—o—) and 1% (—□—) loaded.

Fig. 3.3.9 shows plots of the cumulative release of mitoxantrone versus the square root of time for microspheres having two different crosslinking densities in phosphate buffer. For both crosslinking densities the plots were linear, having correlation coefficients 0.98 and 0.93 demonstrating that the release of mitoxantrone from the microsphere matrix was diffusion-controlled and conforms to the Higuchi model (Higuchi, 1963).

Thus, in spite of the good aqueous solubility of the drug, release from the polysaccharide matrix could be effectively controlled by changing the degree of crosslinking. Even at very low glutaraldehyde concentrations employed for crosslinking the microspheres, its effect on the drug release profiles was highly significant. Estimation of the glutaraldehyde content in toluene saturated with glutaraldehyde by the ε-aminocaproic acid method (Lynn *et al.*, 1990) showed that the aldehyde concentration was 0.02 mole%. Thus the concentrations of glutaraldehyde in the reaction medium at 2 mL, 4 mL and 10 mL GST were $5.6\times10^{-4}$, $1.05\times10^{-3}$ and $2.63\times10^{-3}$%. It can be seen that at 2 mL GST, complete release of mitoxantrone occurred in less than 2 days, but when the concentration was doubled only around 50% of the drug was released within the same period and even after 3 weeks the release reached only around 60%. With 10 mL GST and with 0.8 mL aqueous glutaraldehyde, the release rates could be brought down still further. Although particle size and the drug payload also influence the drug release profiles as shown in Fig. 3.3.7 and Fig. 3.3.8. their influence was not as remarkable as the degree of crosslinking of the microsphere matrix. Therefore, the method provides a simple and logical way to control the release of the entrapped

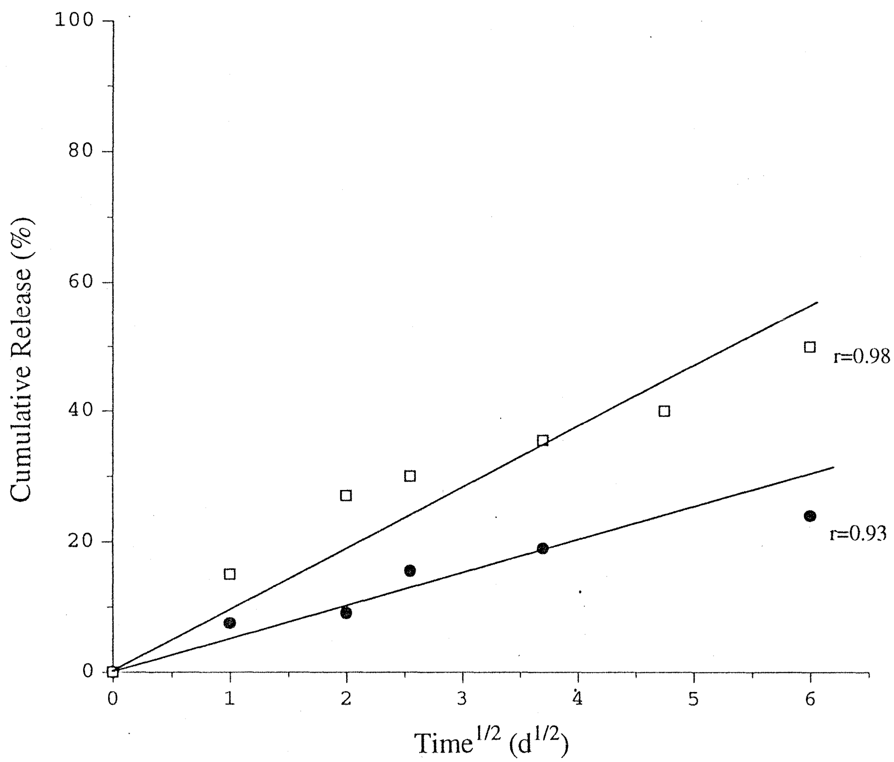



Fig. 3.3.9 Plot of mitoxantrone released into phosphate buffer at 27°C. versus square root of time. Spheres crosslinked with 10 mL GST ( □ ) and 3.2 mL GST followed by 0.8 mL aqueous glutaraldehyde ( • )

drug from the microsphere matrix from a few days to few weeks depending on the final application.

### 3.3.4 Evaluation of Antitumour Activity of Mitoxantrone–Loaded Chitosan Microspheres

For evaluating the antitumour activity of mitoxantrone–loaded chitosan microspheres against EAC, drug-loaded microspheres in phosphate buffered saline containing 0.05% Tween 20 were intraperitoneally injected in mice. Antitumour effect was evaluated by following the animal survival and change in their body weight. The percentage mice survived is plotted against time in Fig. 3.3.10. Animals which received EAC cells but no therapy showed a survival time of 17.2±1.13 (Mean±S.E) days. Fig. 3.3.11 shows the mice two weeks after inoculation with EAC cells. All animals died within 25 days. The mean survival time of animals (n=5) which received placebo chitosan spheres was 19.5± 1.1 days which was not significantly different from the value for the untreated group ($P$ <0.05). All animals in this group died within 22 days. The mean survival time of animals which received therapy via mitoxantrone-loaded chitosan microspheres was 50 ±4.6 days which was significantly different from the value of 2.1±0.67 days for those which received 2 mg of free drug ($P$ <0.001) or 4.6±0.67 days which received 1 mg of free drug ($P$ <0.001). Five out of eight animals which received mitoxantrone-loaded chitosan microspheres were still alive at 60 days. Fig. 3.3.12 shows mice inoculated with EAC cells after receiving mitoxantrone therapy via microsphere modality after 60 days. In the case of animals which received 2 mg of free drug, 7 out of 8 animals died within 5 days and remaining one died on the

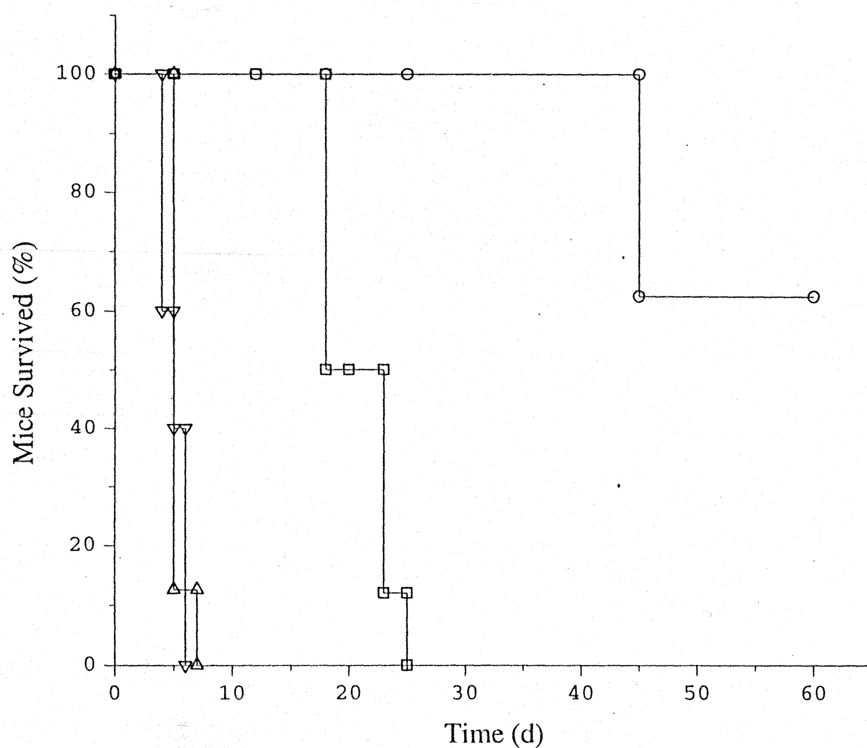



Fig. 3.3.10 Percentage survival of mice receiving mitoxantrone therapy plotted against time. Mice receiving 2 mg mitoxantrone encapsulated in chitosan microspheres (—o—), mice receiving no therapy (—□—), mice receiving 2 mg mitoxantrone as free drug (—△—) and mice receiving 1 mg mitoxantrone as free drug (—▽—)

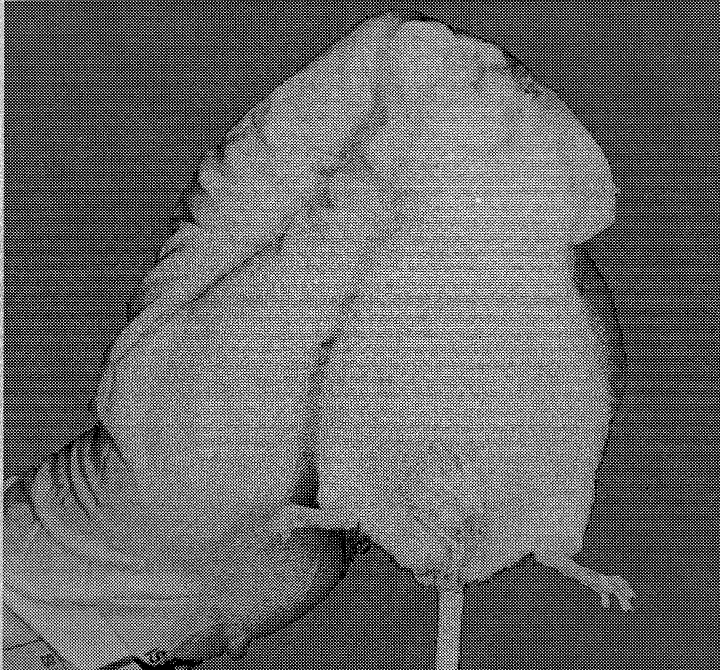


Fig. 3.3.11 Mice two weeks after inoculation with EAC cells

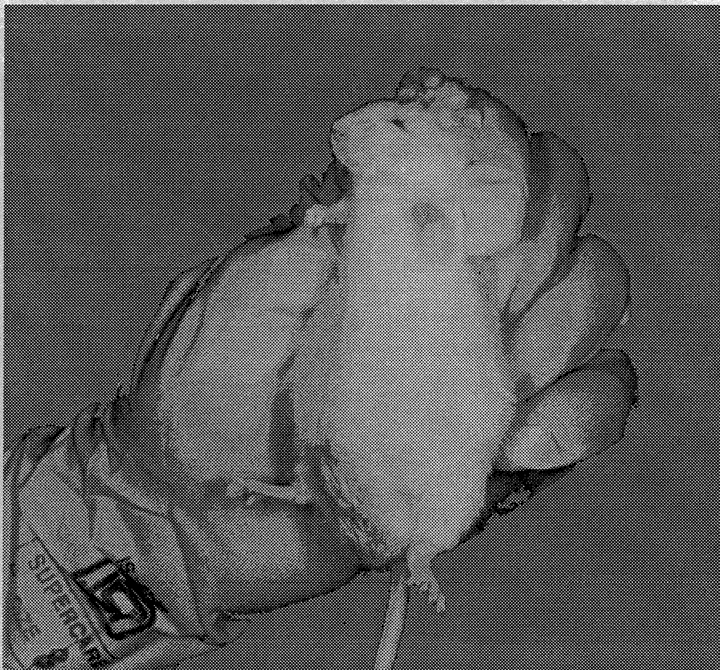


Fig. 3.3.12 Mice inoculated with EAC cells after receiving mitoxantrone therapy via microsphere modality after 60 days.

seventh day. In the case of animals which were given 1 mg of free drug, all animals died within 6 days. Drug toxicity was apparent in the group which received free mitoxantrone. The percent T/C ratio (mean survival time of treated group divided by the mean survival time of untreated control in percentage) for the group treated via microsphere modality was 290 as opposed to 27 for those received 1 mg of free drug or 12.2 for those received 2 mg of free drug (Table II). The $LD_{50}$ of mitoxantrone administered intraperitoneally in mice and rats ranges from 8.0 to 19.7 mg per kg body weight (American Cyanamid Co., 1991). It is remarkable that even at a dose of 4 to 8 times the $LD_{50}$ of the drug, the therapeutic efficacy of the microsphere preparation is very significant. Thus as seen in the *in vitro* studies, the very slow diffusion of the drug from the microsphere matrix is believed to be responsible for the excellent therapeutic effect seen *in vivo*.

Table II
Effect of single doses of chitosan microspheres containing mitoxantrone or free drug in solution on survival time of mice inoculated intraperitoneally with Ehrlich ascites carcinoma

| Compound | Dose mg $kg^{-1}$ | Survival time (Mean±S.E) | T/C (%) | Survivors at 60 days |
|---|---|---|---|---|
| Control | - | 17.2±1.13 | 100 | 0/8 |
| Free drug | 1 | 2.1±0.67 | 27 | 0/8 |
| | 2 | 4.6±0.67 | 12.2 | 0/8 |
| Chitosan microspheres containing drug | 2 | 50±4.6 | 290 | 5/8 |

The antitumour effect was also evaluated by following the change in body weight of animals with time. Fig. 3.3.13 shows the average body weight of animals treated via different modalities against time. In the case of animals untreated as well as those received placebo microspheres, progressive growth of tumour is

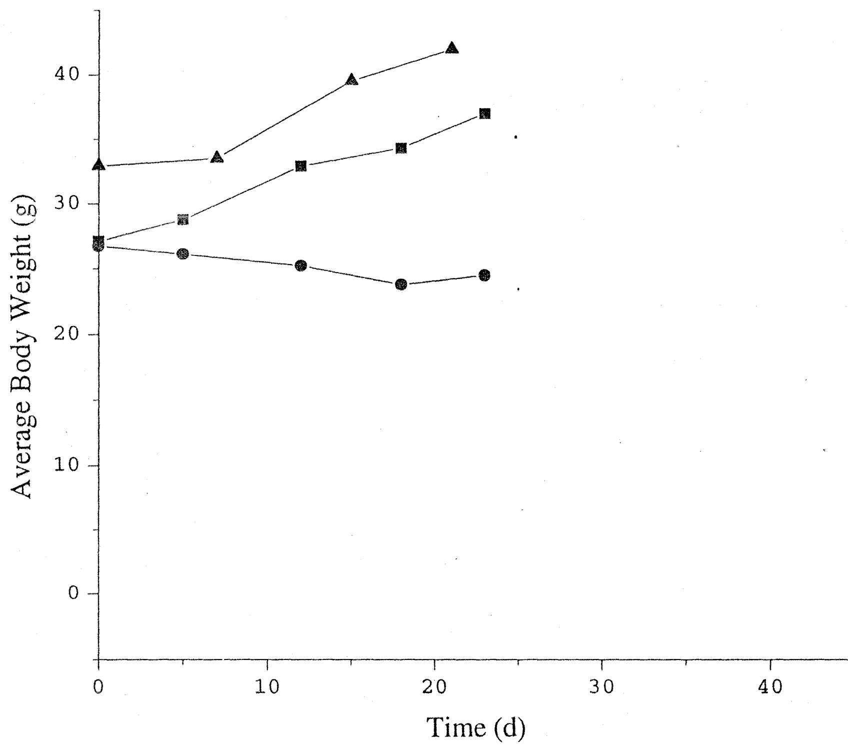



Fig. 3.3.13 Average body weight of mice bearing Ehrlich ascites carcinoma and receiving mitoxantrone therapy. Mice receiving placebo chitosan microspheres (—▲—), mice receiving no therapy (—■—) and mice receiving chitosan microspheres containing 2 mg mitoxantrone (—●—)

observed as evidenced by the increase in their body weight. Animals which received mitoxantrone therapy via microsphere modality showed a more or less constant body weight with respect to time. Therefore, it was evident that mitoxantrone loaded microspheres were not imparting any toxicity during the entire period of time. Drug toxicity was apparent in the case of animals which received 1 or 2 mg of the free drug.

The results obtained using mitoxantrone-loaded chitosan microspheres in the chemotherapy of EAC appears to be very significant. Using doxorubicin which has similar therapeutic efficacy like mitoxantrone against many neoplastic diseases, Miyazaki *et al* (1986) evaluated the antitumour effect of drug loaded fibrinogen microspheres against EAC. The percent T/C ratio observed by these authors was approximately three times that of free doxorubicin administered at a dose corresponding to 2–5 times the $LD_{50}$ of the drug. In the present study, it can be seen that the percent T/C ratio of chitosan microspheres containing 2 mg of mitoxantrone is approximately 23 times that of the free drug at the same dose. Also noteworthy is the fact that the mean survival time of mice treated via microsphere modality was 50 days and survival at 60 days was 62.5%. On the other hand, with doxorubicin containing fibrinogen microspheres administered intraperitoneally against EAC, Miyazaki *et al* (1986) found a mean survival time of 40 days with only one out of 6 mice surviving at the end of 60 days. The much reduced cardiotoxicity of mitoxantrone coupled with its slow sustained release from the microsphere matrix is believed to be responsible for the very high survival rate seen in the present investigation.

The data obtained thus conclusively demonstrate that intraperitoneal administration of mitoxantrone–loaded chitosan microspheres is an effective means of therapy against EAC. The formulation minimises drug toxicity to a significant extent and enhances therapeutic efficacy. Chitosan microspheres loaded with anti-neoplastic agents therefore appears to be very promising in the treatment of neoplastic diseases.

## 3.4 Chitosan Microspheres as a Carrier for the Controlled Delivery of Progesterone

### 3.4.1 Background

The need to control population is very critical in today's world. The pharmacological approach to control fertility has developed primarily through orally administered steroids. Disadvantages of oral contraceptives are the requirement of daily ingestion and the subsequent daily variations in blood steroid concentration. If anti-fertility steroids could be delivered in therapeutic concentrations over a prolonged period using drug delivery devices, the major drawbacks of the oral formulations viz., the necessity for daily ingestion could be overcome. Sustained release doses obviate the problem of cyclic overdosing and underdosing associated with the conventional administration of steroids; the technology in principle affords a means of effecting an optimum pharmacological responses with a minimum dose of steroid.

Considerable attention has been focussed over the past 20 years to develop steroid delivery systems using biodegradable polymers. Implantable rods and injectable microspheres have been prepared from a large number of biodegradable non-toxic polymers over the period. Jackanicz *et al* (1973) incorporated d-norgestrel in poly(lactic acid) (PLA) and studied the release rates *in vitro* and *in vivo* and showed that films containing 33% steroid released the steroid at a rate of 3 $\mu$g/day/sq.cm for over 80 days. In another study, norethisterone was incorporated into PLA and a fairly linear and sustained release demonstrated for 3 months (Anderson *et al.*, 1976). Copolymers of lactic and glycolic acids have

been used in a variety of configurations including solid rods for the release of levonorgestrel (Benagiano, 1979). After an initial unstable period, a reasonably steady rate of release was obtained for a period of 100 weeks. Beck and Cowsar (1980) prepared PLA microspheres containing 20% norethisterone and showed intramuscular injection in baboons produced sustained release for 6 months.

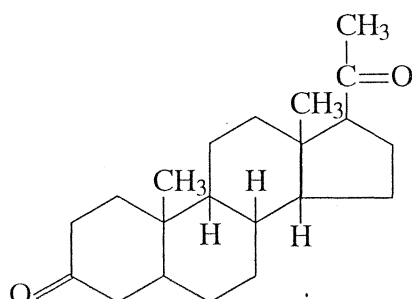



Fig. 3.4.1 Chemical structure of progesterone

Among contraceptive steroids, progesterone is attractive because it occurs in high concentration under natural conditions without known side effects. Its chemical structure is shown in Fig. 3.4.1. Clinical use of this steroid however is hampered because it is not orally active except in high doses and it has a short biological half life. Progestasert[R], a medicated IUD, a non-biodegradable sustained release device which releases progesterone through a rate-controlling ethylene-vinyl acetate copolymer membrane has reached commercialisation. It delivers 65 $\mu$g/day of progesterone directly to the uterus which is reported to inhibit ovulation in healthy women (Kulkarni *et al.*, 1973; Martinez-Maunautou, 1975). Pitt *et al* (1976) showed that progesterone incorporated into PLA film and implanted in rabbit released the steroid in a suitable sustained fashion for up to 14 weeks.

Injectable biodegradable drug reservoirs from glutamic acid/leucine copolymers in the form of tubes and solid rods were developed by Sidman *et al* (1977) to provide controlled release of progesterone for 6–12 months. Beck *et al* (1979) designed a long acting injectable microcapsule system for the release of progesterone using PLA and found that the steroid was released at a rate of 1.3 $\mu$g/mg/day for 30 days. In a recent study, Gangrade and Price (1991) investigated poly(hydroxy butyrate) and poly(hydroxy valerate) as matrix for sustained progesterone delivery.

Most of the investigations reported are based on synthetic polymers as carriers and very little is reported on natural polymers as carriers for steroid delivery. Lee *et al* (1981) entrapped progesterone in crosslinked serum albumin microbeads. Injection of these microbeads into rabbits recorded a sustained steroid release up to 20 days. Progesterone-loaded albumin microparticles were prepared and effect of crosslinking method on the *in vitro* release was studied recently by Orienti and Zecchi (1993). Steroids such as testosterone, progesterone, and β-oestradiol were entrapped in chitosan films and microbeads and *in vitro* release profiles were examined (Chandy and Sharma, 1991). Chitosan beads containing steroids were prepared by injecting an acetic acid solution of chitosan into NaOH-methanol solution. No *in vivo* studies have been reported by these authors. The literature on contraceptive drug delivery systems has been reviewed (Duncan and Kalkwarf, 1975; Benajiano, 1976; Nash, 1984; Zatuchni *et al.*, 1984; Chasin and Langer, 1990).

In the earlier study using mitoxantrone, it was demonstrated that it would be possible to prepare a microsphere matrix from chitosan using glutaraldehyde crosslinking to deliver the drugs from days to weeks by adjusting the crosslinking

density of the microspheres. The aim of this study was therefore to prepare glutaraldehyde crosslinked chitosan microspheres containing progesterone and to examine the *in vitro* release behaviour of the drug from the microspheres with respect to crosslinking density and particle size, to select the most promising preparation for prolonged delivery and then to examine the bioavailability of the steroid in a suitable animal model by intramuscular injection.

### 3.4.2 Progesterone-Loaded Chitosan Microspheres

Progesterone-loaded microspheres were prepared from chitosan solution containing 20% progesterone by weight of chitosan. Fig. 3.4.2 shows the SEM of progesterone-loaded chitosan microspheres. The particles were highly spherical in appearance. The surface morphology of the particles at a higher magnification is shown in Fig. 3.4.3. Drug crystals could be seen on the surface of the microspheres. The particle size distribution of the drug-loaded microspheres is shown in Fig. 3.4.4. All the microspheres were below 300 $\mu$m in diameter. Out of these, 67% of the microspheres was between 45–90 $\mu$m, 20% between 90–150 $\mu$m and 13% between 150–300 $\mu$m. The theoretical loading of the drug is 20%. But actual drug content obtained was 7.3% (Table III).

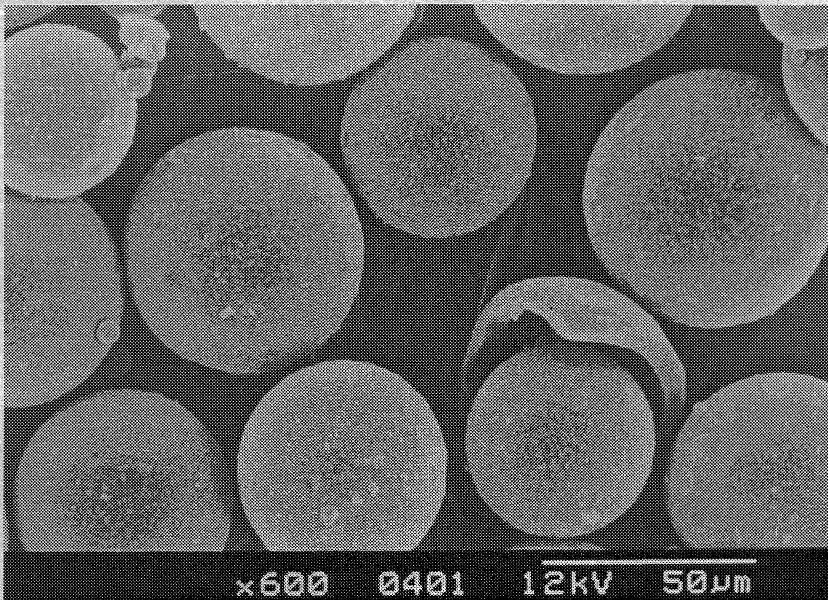



Fig. 3.4.2 SEM of progesterone-loaded chitosan microspheres

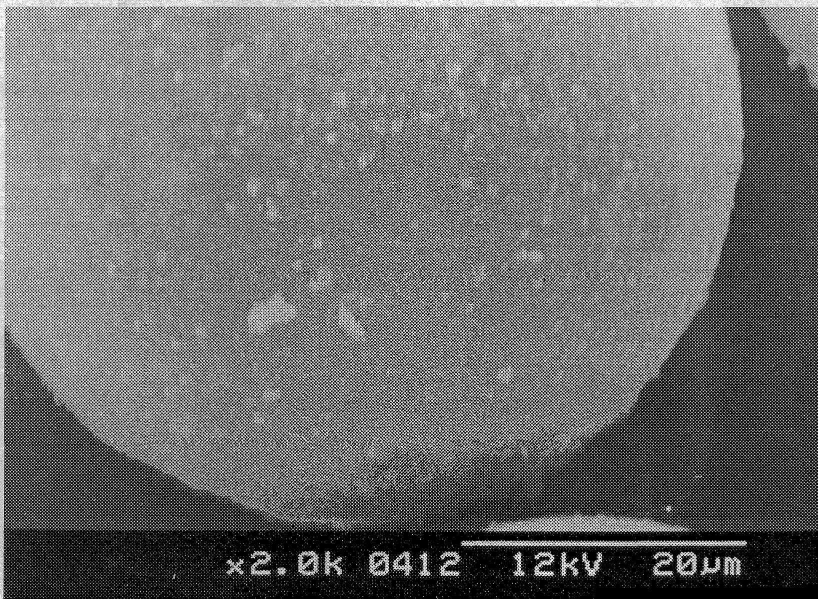



Fig. 3.4.3 SEM showing the surface morphology of progesterone-loaded chitosan microspheres

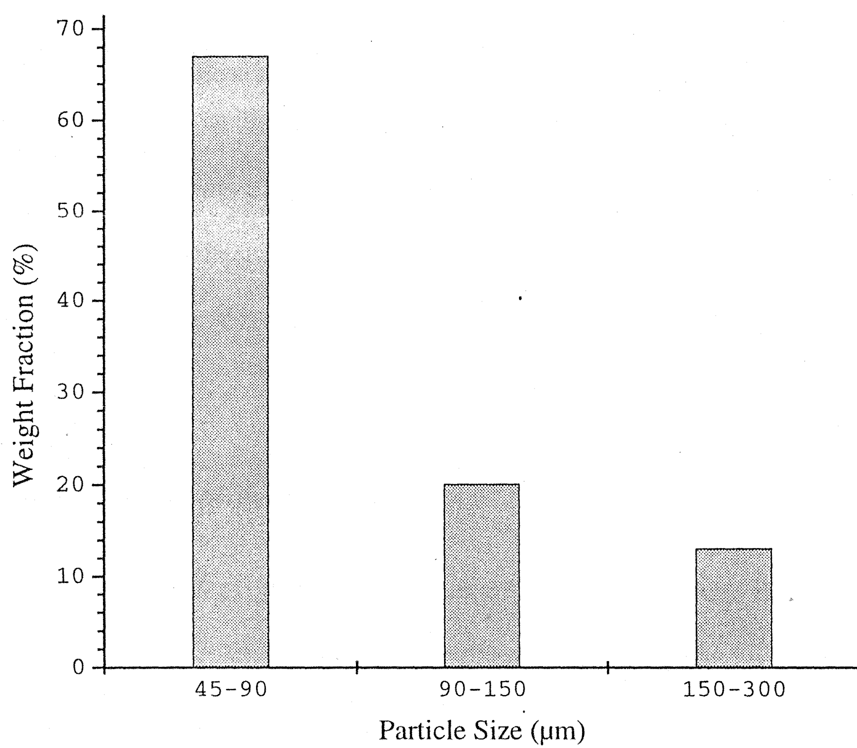



Fig. 3.4.4 Particle size distribution of progesterone-loaded chitosan microspheres prepared at a stirring speed of 2000 rpm

Table III
Amount of Progesterone loaded into chitosan microspheres

| Expt No. | Progesterone loaded (wt%) |
|---|---|
| 1 | 6.6 |
| 2 | 8.5 |
| 3 | 6.8 |
| Mean ± SD | 7.3±0.85 |

Chitosan microspheres is prepared from its acetic acid solutions. The lower incorporation efficiency of the drug could be due to its lipophilic character with less affinity for the aqueous dispersed phase and more affinity for the non-aqueous dispersion medium. Furthermore, washing the microspheres with petroleum ether and acetone also could have removed the drug to some extent resulting in rather low loading.

### 3.4.3 *In Vitro* Release of Progesterone

The *in vitro* release of progesterone was examined in phosphate buffer (0.1 M, pH 7.4) at 37°C. Drug release from microspheres having different crosslinking densities is shown in Fig. 3.4.5. The release from the microspheres of higher crosslinking density was slower compared to release from microspheres having lower crosslinking density. Within 40 days, about 70% of the drug was released from least crosslinked microspheres, whereas it was only around 36% from highly crosslinked microspheres. All the formulations showed a steady steroid release with time without peaks and valleys. It is noteworthy that there was no significant

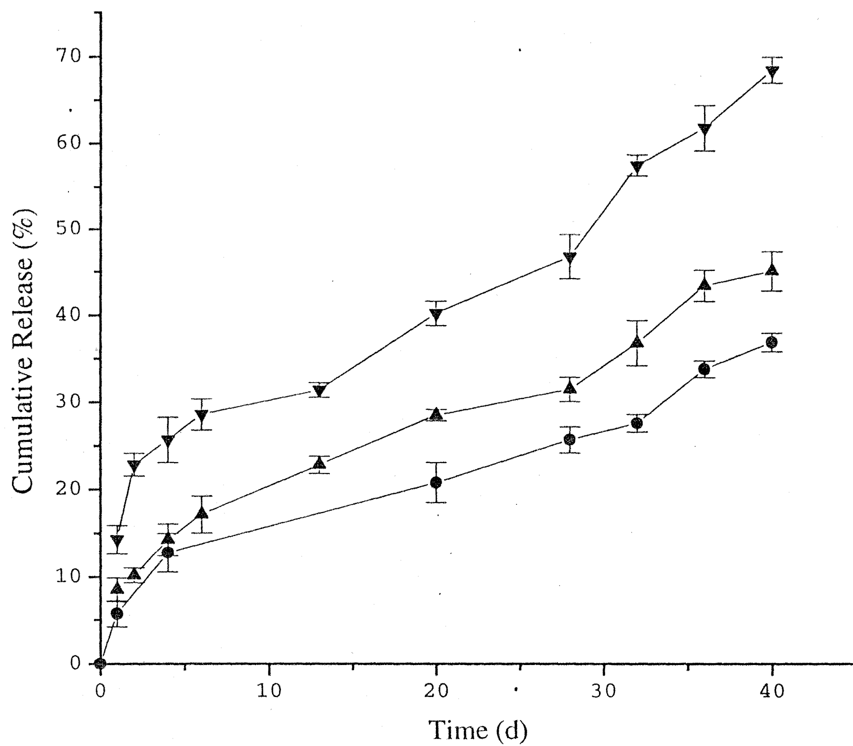



Fig. 3.4.5 In vitro release of progesterone from chitosan microspheres of size 45-90 μm having a drug payload of 7.3% into phosphate buffer at 37°C crosslinked with 4 mL GST (—▼—), 10 mL GST (—▲—) and 10 mL GST + 1 mL aqueous glutaraldehyde (—●—)

burst effect from any of the preparations. The low aqueous solubility of the drug is considered to be responsible for this behaviour in spite of the fact that drug crystals could be seen on the surface of the microspheres by SEM examinations.

The rate of release was also dependent on the size of the microspheres employed (Fig. 3.4.6). Release from smaller microspheres was faster compared to release from larger spheres, because of the larger area of contact of smaller microspheres with the dissolution medium. Within 32 days, drug release was 36.8% from microspheres of 45–90 $\mu$m in size. It was 34% and 28% respectively from microspheres of size 90–150 $\mu$m and 150–300 $\mu$m.

The rates of *in vitro* release of progesterone from glutaraldehyde crosslinked chitosan microspheres was relatively much slower compared to the release rates observed from a protein matrix such as crosslinked albumin microspheres (Orienti and Zecchi, 1993). Albumin microspheres having an average particle size of 5 $\mu$m and having similar progesterone payloads ($\sim$ 6%) was reported to release the entire drug into phosphate buffer at 37°C in less than a month. Possibly, the porous nature of the protein matrix coupled with its highly swelling nature could be attributed to the rapid diffusion of the drug into the dissolution medium. In the case of the polysaccharide chitosan, it was demonstrated that the internal structure of glutaraldehyde crosslinked microspheres was non-porous. This coupled with the low affinity of chitosan towards water could be held responsible for the slow release of progesterone from the microspheres.

The plot of cumulative release versus square root of time is shown in Fig. 3.4.7 for three different crosslinking densities. The plots were linear with correlation coefficients of 0.978, 0.994, 0.972 demonstrating that the drug diffusion from

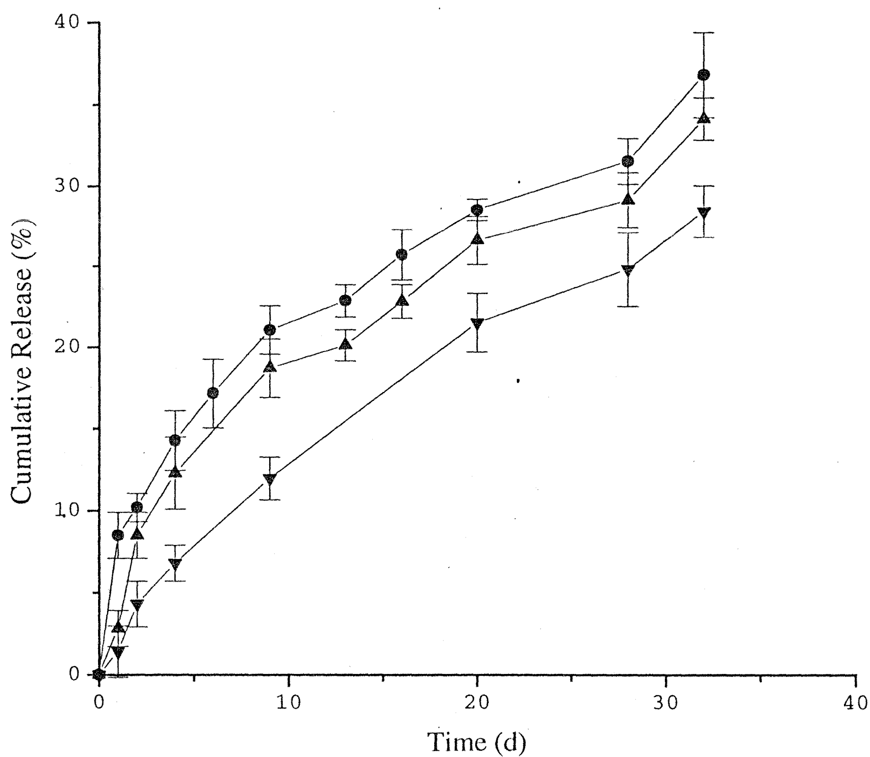



Fig. 3.4.6 Release of progesterone from chitosan microspheres having a drug payload of 7.3% into phosphate buffer at 37°C crosslinked with 10 mL GST, having sizes 45-90 μm (—●—), 90-150 μm (—▲—) and 150-300 μm (—▼—).

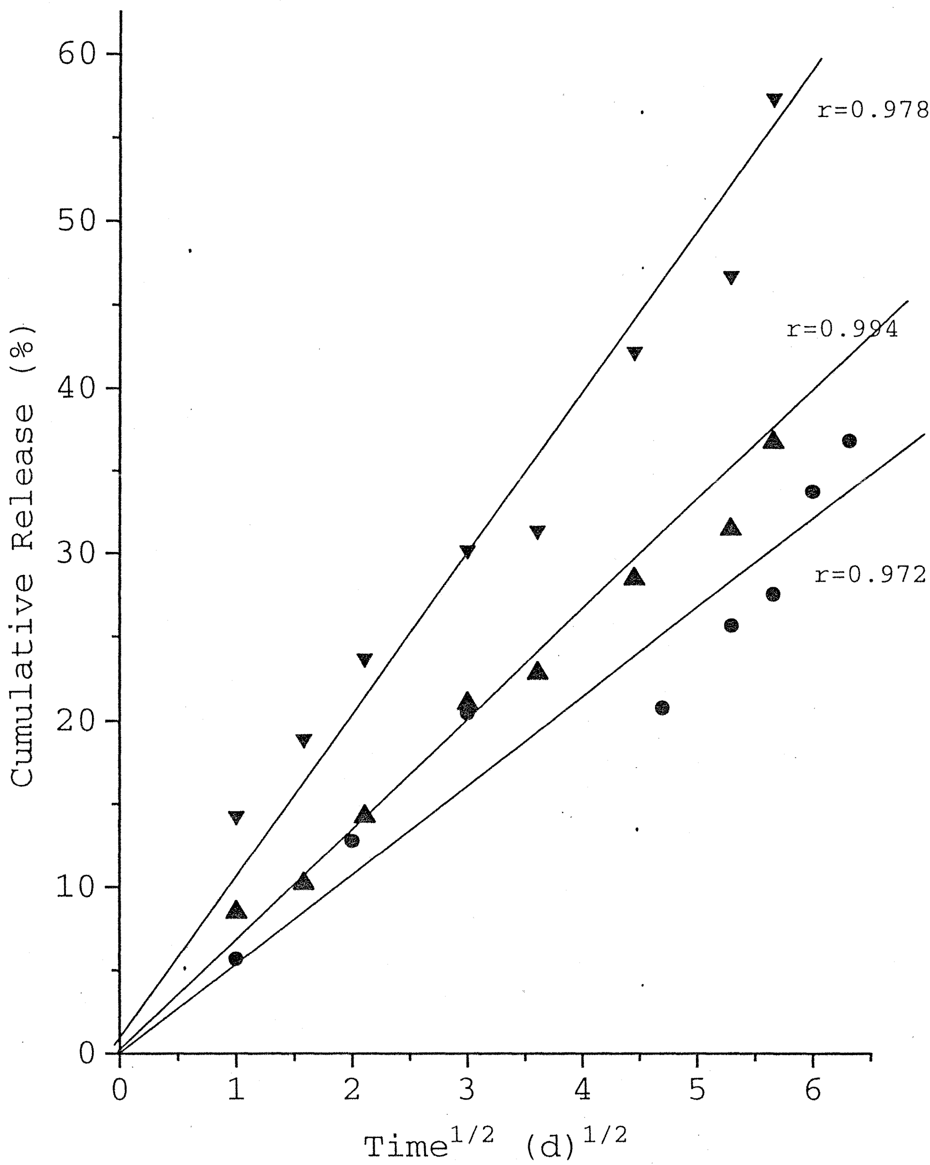



Fig. 3.4.7 Plot of progesterone released versus square root of time from chitosan microspheres crosslinked with 4 mL GST ( ▼ ) 10 mL GST ( ▲ ) and 10 mL GST and 1 mL aqueous glutaraledhyde ( ● ) having a drug payload of 7.3% into phosphate buffer at 37°C.

the matrix follows the Higuchi model (Higuchi, 1963) as shown in the case of mitoxantrone-loaded chitosan microspheres.

### 3.4.4 Bioavailability of Progesterone

The bioavailability of progesterone examined was by intramuscular injection of free as well as encapsulated progesterone in rabbits at a dose of 5 mg/kg body weight. Plasma-progesterone concentrations were determined using ELISA. Fig. 3.4.8 shows the plasma concentration of progesterone in rabbits from microspheres as well as from free drug plotted against time. The restive progesterone concentration in rabbits was around 0.7 $\mu$g/mL. Estimation of progesterone concentration was done till the plasma concentration of the animals injected with free or encapsulated drug reached this level. Animals which has received progesterone through microspheres showed a sustained steroid concentration of 1–2 ng/mL for about 5 months. On the other hand, the control animals which received free progesterone did not show a sustained steroid concentrations beyond 4 days. There was a large 'burst effect' reaching over 20 ng/mL in the case of free drug which is very toxic (DeLuca *et al.*, 1993).

There was very little 'burst effect' from microspheres containing progesterone. In 6 h, the amount of progesterone detected in the plasma was 6.5 ng/mL. The only *in vivo* study using a naturally occurring polymer as a carrier matrix for sustained delivery of progesterone is that of Lee *et al* (1981) who used glutaraldehyde crosslinked albumin microspheres in rabbits. Serum concentration of intramuscularly injected albumin beads containing progesterone showed a peak value of 16 ng/mL which dropped to approximately 2 ng/mL in 5 days. Drug

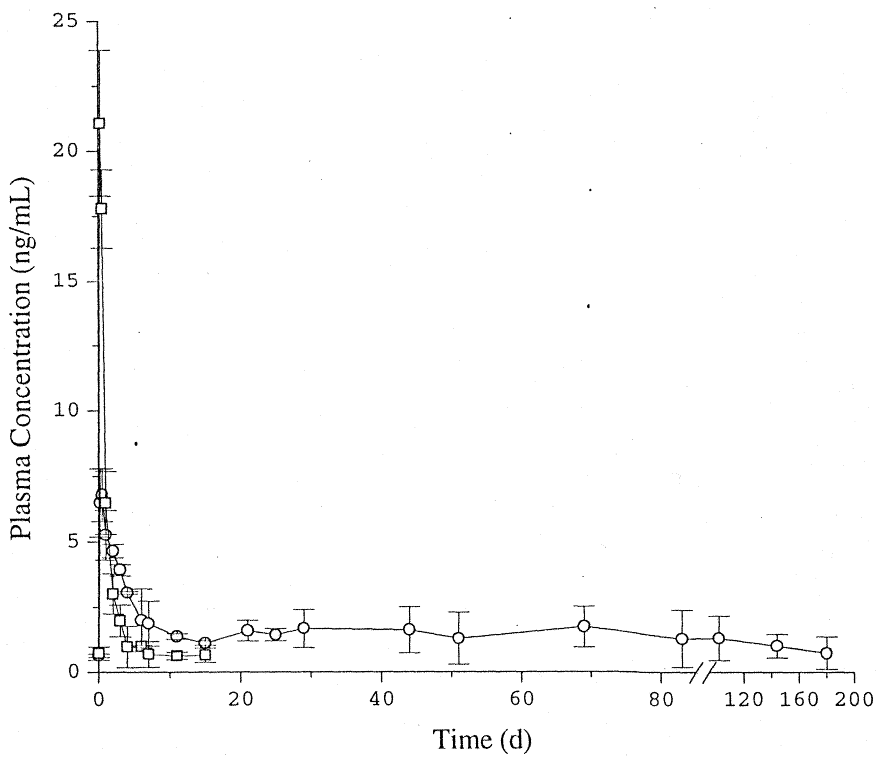



Fig. 3.4.8 Progesterone concentration in rabbit plasma after intramuscular administration of progesterone powder (—□—) and progesterone-loaded chitosan microspheres (—○—).

concentration of approximately 1 ng/mL was sustained for only 20 days. Therefore, compared to a protein matrix, such as albumin, the polysaccharide chitosan appears to offer significant advantage for prolonged delivery of steroids. Glutaraldehyde crosslinked albumin microspheres are reported to biodegrade completely in the muscle in about 2 months (Lee *et al.*, 1981). Therefore, the drug release is governed both by diffusion and degradation of the matrix. On the other hand, we have seen that biodegradation of glutaraldehyde crosslinked chitosan microspheres is a rather slow process. Thus, the mechanism of drug release is rather purely by diffusion and the extent of crosslinking imparted to the matrix can effectively control the diffusion of the encapsulated drug.

In a recent study (El-Nokaly *et al.*, 1993) using poly(lactic acid) microspheres loaded with progesterone, it was shown that at a dose of 1.4 g progesterone administered in mares in the microsphere form, sustained release was observed only for a period of 12 days in the concentration range of 2–4 ng/mL of serum. Therapeutically effective concentration of progesterone is 2–5 ng/mL of plasma. Serum levels greater than 6 ng/mL do not provide any additional benefit and may be toxic. Although, the delivery system in the present work is not optimised to deliver the ideal concentrations of progesterone for prolonged periods, the *in vivo* data clearly points out that it should be possible to manipulate the system to optimise progesterone delivery.

Progesterone has been employed for the treatment of a variety of disorders, including dysmenorrhoea, amenorrhoea, disfunctional uterine bleeding, endometriosis, cancer of the endometrium, habitual abortion and infertility due to an inadequate luteal phase production of endogenous progesterone (Israel, 1967).

In most cases, synthetic progestins are employed in the place of progesterone because of its short biological half-life. The growing concern over the side effects of synthetic progestins will eventually restrict their wide spread use. The possibility of using a biodegradable, injectable sustained delivery system delivering therapeutically desirable concentrations of progesterone to produce optimum pharmacological response therefore assumes importance. The data obtained with chitosan microspheres in the *in vivo* study reported in this investigation point out to the possibility of developing such a delivery system.

# 3.5 Chitosan Microspheres as a Carrier for the Controlled Delivery of Diclofenac Sodium

## 3.5.1 Background

Oral route is the most commonly employed route of drug administration because it is the most convenient route for access to the systemic circulation. The major limitation of oral route is the low bioavailability. This is because of a large number of reasons such as brief transit time in the gastro-intestinal tract, variability in stomach emptying time, severe chemical conditions in the stomach, and variable absorbing surface of the gastro-intestinal tract (Ballard, 1978). Encapsulating the oral drug in a suitable polymer matrix can reduce the gastro-intestinal adverse effects.

Diclofenac sodium is an effective nonsteroidal anti-inflammatory agent used in degenerative joint diseases, rheumatoid arthritis and allied conditions (Brodgen *et al* 1980). The chemical structure of the drug is shown in Fig. 3.5.1.

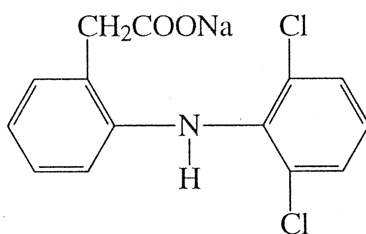



Fig. 3.5.1. Chemical structure of diclofenac sodium

Compared with other non-steroidal anti-inflammatory drugs such as acetyl salicylic acid, phenyl butazone, indomethacin, ibuprofen and naproxen, this drug is reported to have high therapeutic efficacy and improved tolerability and is available

in various formulations (Zucker, 1986; Todd and Sorkin, 1988; Small, 1989). Usual dosage of this drug is 25 mg three or four times a day (Budavari, 1989) and it has a short biological life of 2 h. It is reported that the drug causes gastro-intestinal disturbances and peptic ulcer if present in large concentrations in gastro-intestinal tract (Martindale, 1982). Different enteric coated products of this drug have been developed and commercialised (Lin and Kao, 1991; Verbruggan and Moll, 1992). Preparation and evaluation of ethyl acetate microspheres of diclofenac sodium has been reported (Chowdary and Vijaya Ratna, 1992). Nylon coated ion exchange resins containing sodium diclofenac were prepared and *in vivo* evaluation was done in rabbits (Garcia Encina *et al.*, 1993). Jain and Vyas (1994) prepared diclofenac sodium bearing magnetic erythrocytes and *in vitro* characterisation was carried out. Diclofenac-wax microspheres were prepared and *in vitro* release of the drug was studied (Vilivalam and Adeyeya, 1994). Bhatnagar *et al* (1995) prepared Polaxamer[R] coated three-ply walled microcapsules containing diclofenac sodium and its *in vivo* performance was evaluated in albino rats. This chapter describes *in vitro* and *in vivo* evaluation of diclofenac sodium encapsulated in chitosan microspheres.

### 3.5.2 Diclofenac Sodium-Loaded Chitosan Microspheres

Diclofenac sodium-loaded chitosan microspheres were prepared from an aqueous acetic acid solution of chitosan containing the drug. Fig. 3.5.2 shows the SEM of diclofenac sodium-loaded chitosan microspheres. The drug-loaded spheres have good spherical geometry, but the incorporation of the drug has roughened their surface. Particle size analysis of the microspheres prepared is shown in Fig. 3.5.3.

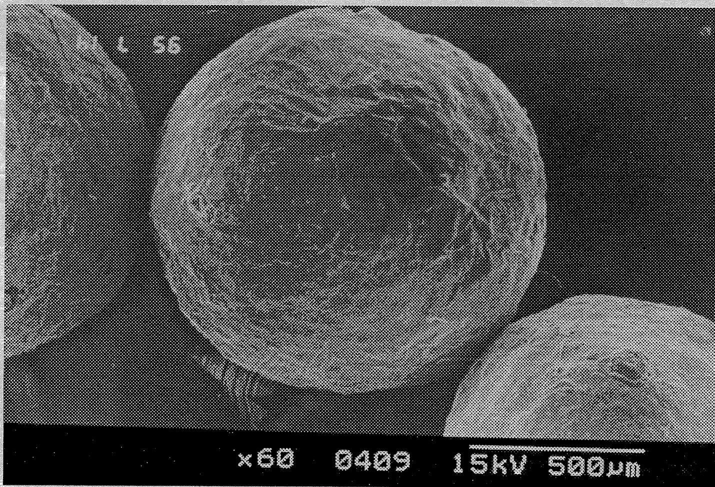



Fig. 3.5.2 SEM of diclofenac sodium-loaded chitosan microspheres

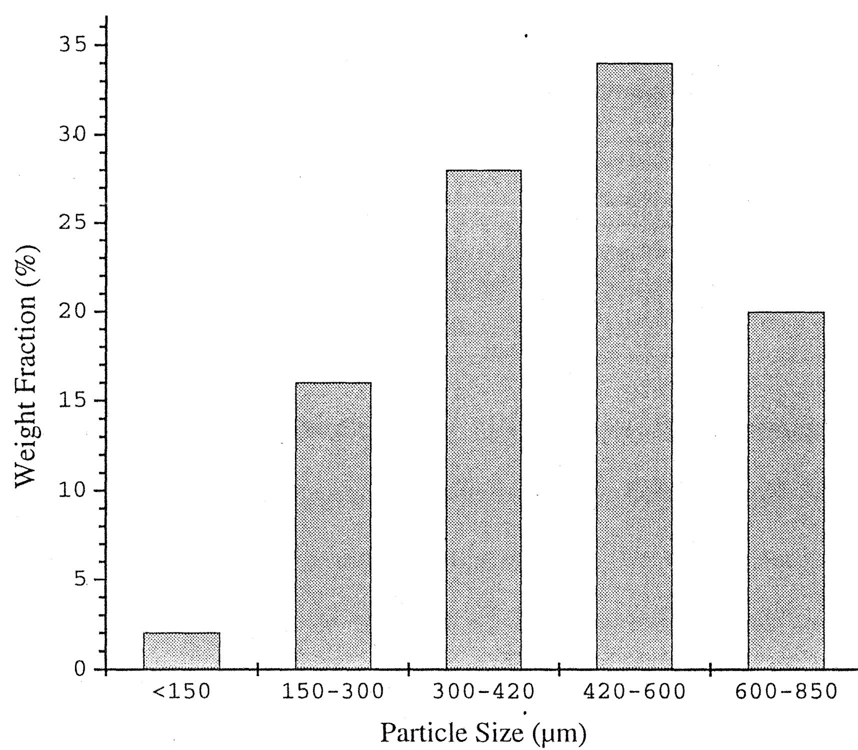



Fig. 3.5.3 Particle size distribution of diclofenac sodium-loaded chitosan microspheres prepared at a stirring speed of 200 rpm

Sieve analysis showed that 98% of the microspheres were above 150 $\mu$m in diameter. The major fraction was within the size range of 420–600 $\mu$m and the entire batch of microspheres was below 850 $\mu$m in diameter. Drug incorporation efficiency at various initial drug loadings is given in Table IV. Incorporation efficiencies were rather poor, the loading obtained being 5.75, 10.5 and 30% for initial loadings of 20, 50 and 200% respectively. The incorporation efficiency was found to decrease with increasing concentrations of the drug in the dispersed phase.

Table IV
Incorporation efficiency of diclofenac sodium-loaded chitosan microspheres prepared with different initial drug contents

| Initial loading (%) | Actual loading (%) | Incorporation efficiency (%) |
|---|---|---|
| 20 | 5.75 | 28.75 |
| 50 | 10.05 | 21.00 |
| 200 | 30.00 | 15.00 |

In an earlier work by Thanoo *et al* (1992) from this laboratory, it was demonstrated that high incorporation efficiencies (>80%) could be obtained in glutaraldehyde crosslinked chitosan microspheres for drugs such as aspirin griseofulvin and theophylline. In the case of drugs which are less water soluble such as griseofulvin and aspirin, incorporation efficiencies in the range of 90–95% could be achieved in comparison with 80% for a more water soluble drug such as theophylline. With diclofenac sodium it was found that mixing the drug with chitosan solution did not give rise to a homogeneous solution particularly at high initial drug loadings. During the microencapsulation process, it was seen that a large amount of the drug migrated into the dispersion medium thereby loading to rather poor incorporation efficiency. Moreover, diclofenac sodium is

soluble in water to the extent of 0.5%. Therefore, washing the microspheres after encapsulation using aqueous solutions of sodium metabisulphite followed by copious amounts of cold water to remove the unreacted glutaraldehyde, acetic acid etc. would also have removed considerable amount of the incorporated drug leading to poor incorporation efficiency.

### 3.5.3 *In Vitro* Release of Diclofenac Sodium

*In vitro* release of diclofenac sodium was examined in stimulated intestinal fluid at 37°C. The effect of crosslinking density of microspheres on the release profile is shown in Fig. 3.5.4. Crosslinking was done with 4 mL GST, 10 mL GST and 10 mL GST followed by 1 ml aqueous glutaraldehyde to prepare microspheres of different crosslinking densities. The drug release was faster from microspheres having lower crosslinking density. Thus, about 100% release was observed in 8 h from microspheres crosslinked with 4 mL GST. Release rate decreased as the crosslinking was increased. When 10 mL GST was used for crosslinking, cumulative release of the drug at 8 h was about 85% whereas from microspheres crosslinked with 10 mL GST followed by 1 mL aqueous glutaraldehyde it was about 60%. Thus, the extent of crosslinking plays a very important role in determining the rate of diclofenac release from crosslinked chitosan microspheres.

The effect of drug loading on the release rate from microspheres is shown in Fig. 3.5.5. Release rate increased with increase in drug loading. Rapid release was seen from 30% loaded microspheres. Within 2 h, these microspheres released 100% of the drug incorporated. In the same period, microspheres with 10.5% payload showed only about 54% release. It again decreased to about 19% for the

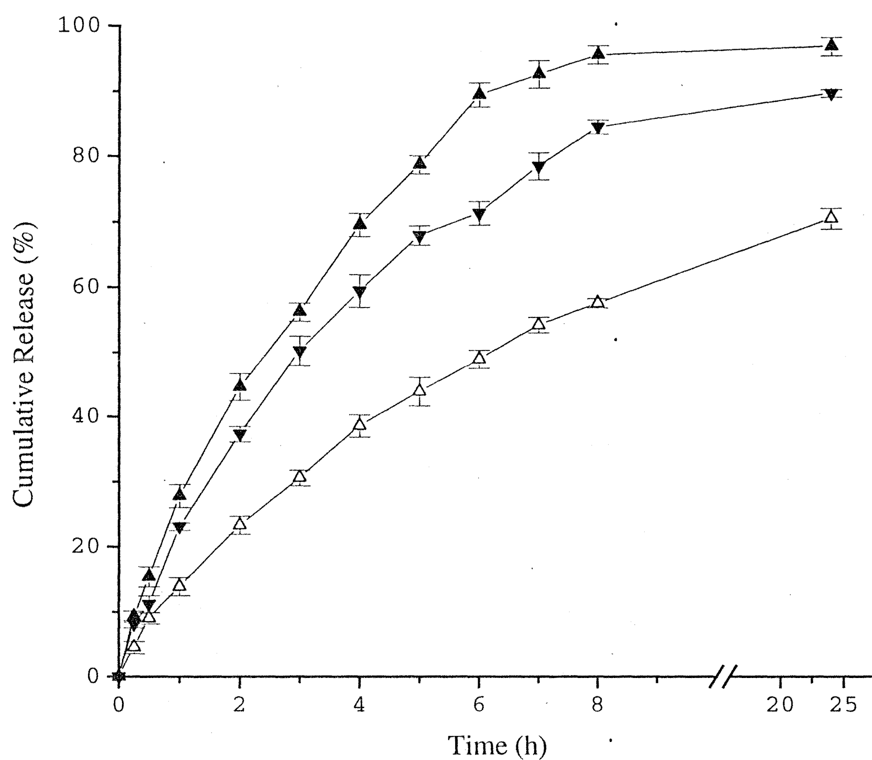



Fig. 3.5.4. Effect of crosslinking density on the in vitro release profile of diclofenac sodium from 30% loaded chitosan microspheres of size 600-850 μm into simulated intestinal fluid at 37° C. Microspheres were crosslinked with 4 mL GST (—▲—), 10 mL GST (—▼—) and 10 mL GST followed by 1 mL aqueous glutaraldehyde (—△—)

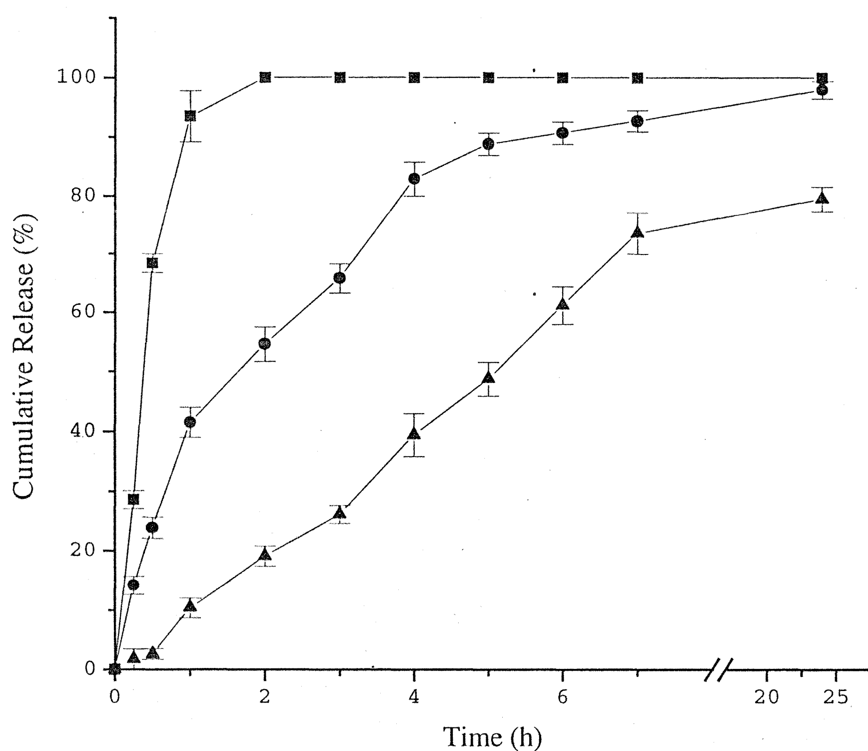



Fig. 3.5.5 In vitro release of diclofenac sodium from chitosan microspheres of size 300-420 μm crosslinked with 10 mL GST into simulated intestinal fluid at 37° C having drug contents 30% (—■—), 10.25% (—●—) and 5.75% (—▲—)

microspheres having 5.75% payload. Fig. 3.5.6 show the release profile of drug from microspheres having different size ranges. Release of the drug was found to be 100% from microspheres having size range 300–420 $\mu$m at 8 h. When the size range was increased to 420–600 $\mu$m, the cumulative release at 8h was about 75% and it was only 57% from the microspheres in the size range of 600–850 $\mu$m. Thus, the particle size of microspheres exert a very significant influence on the rate of release of diclofenac sodium from chitosan microspheres. Greater surface area of small sized particles result in a greater area of contact with the dissolution medium resulting in a rapid rate of release. The plots of cumulative release versus square root of time is shown in Fig. 3.5.7. The plots are all linear with correlation coefficients above 0.99. Thus the release of drug from the microspheres is diffusion-controlled as it obeys Higuchi relation $Q = kt^{1/2}$ where, $Q$ is the amount released at time t and k is a constant which depends on surface area and diffusion coefficient (Higuchi, 1963).

### 3.5.4 Bioavailability of Diclofenac Sodium

*In vivo* release studies of diclofenac sodium was done by oral administration of the microspheres containing the drug at a dose of 25 mg/kg body weight in rabbits. Only one microsphere preparation was tested *in vivo*. Since microspheres with low crosslinking densities such as those crosslinked with 4 and 10 mL GST were found to release the drug more rapidly in *in vitro* studies, particles having the highest crosslinking density, viz., those crosslinked with 10 mL GST followed by 1 mL 25% aqueous glutaraldehyde was employed in the study. Also, the other preparation with low drug loading (5.75% and 10.25%) were not thought to be

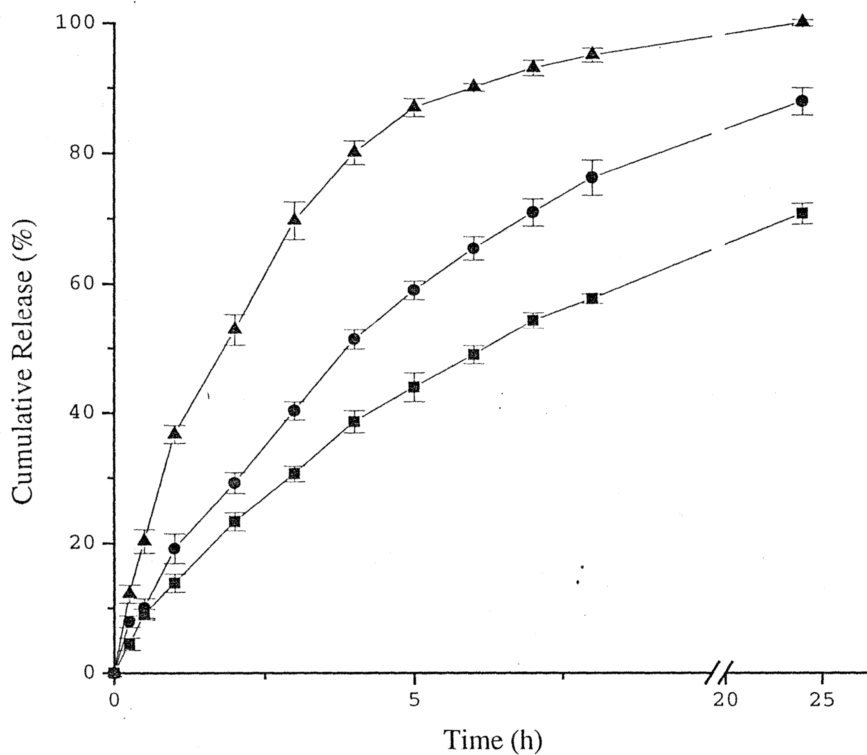



Fig. 3.5.6 Rate of release of diclofenac sodium from 30% loaded chitosan microspheres crosslinked with 10 mL GST and 1 mL aqueous glutaraldehyde as a function of microsphere size into simulated intestinal fluid at 37° C. 600-850 μm (—■—), 420-600 μm (—●—) and 300-420 μm (—▲—)

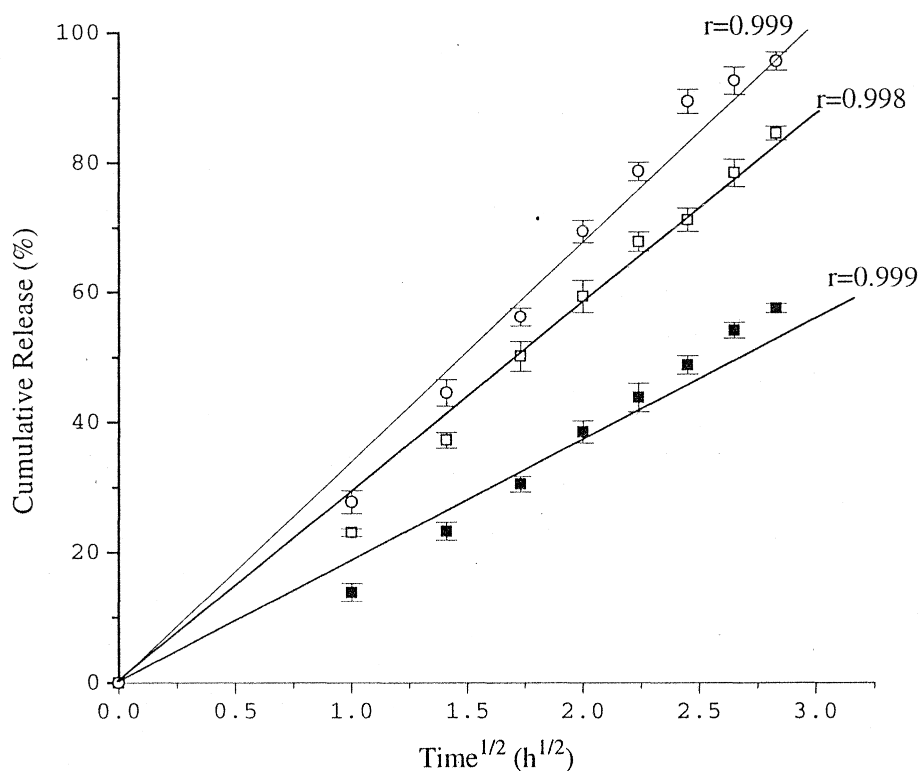



Fig. 3.5.7 Plot of diclofenac sodium released versus square root of time from chitosan microspheres crosslinked with different amounts of glutaraldehyde into simulated intestinal fluid at 37° C. 4 mL GST ( ○ ), 10 mL GST ( □ ) 10 mL GST and 1 mL glutaraldehyde ( ■ )

suitable for *in vivo* applications. Therefore only particles having a diclofenac content of 30% was used. Fig. 3.5.8 shows plasma concentration versus time profile of free drug and the drug encapsulated in chitosan microspheres. The peak plasma concentration attained ($C_{max}$ in the case of free drug was 4.65 $\mu$g/mL, whereas for the encapsulated drug it was 2.45 $\mu$g/mL. The time required to reach the peak level ($t_{max}$ for the free drug was 4 h and it took 6 h in the case of microsphere formulation. Thus compared to the free drug group, a sustained plasma level of the drug was observed up to 12 h in animals which received the drug encapsulated in chitosan microspheres. The pharmacokinetic parameters are listed in Table V.

Table V
Pharmacokinetic parameters* of diclofenac sodium formulations after oral administration in rabbits

| Property | Microspheres | Free drug |
|---|---|---|
| **$AUC_{O-24h}$ ($\mu$g.mL/h) | 25.21±5.25 | 26.43±1.18 |
| ***$C_{max}$ ($\mu$g/mL) | 2.44±0.18 | 4.62±0.31 |
| $t_{max}$ (h) | 6.0 | 4.0 |

* Mean ± S.D
** P> 0.5, not significantly different
***P< 0.01 significantly different

There was no significant difference between the free drug and the encapsulated drug as regards the relative extent of absorption calculated from the $AUC_{0-24h}$ values. Very similar observations have been reported by Garcia–Encina *et al* (Garcia–Encina *et al.*, 1993) when they tested Dolotren Retard$^R$, a commercial sustained release formulation and several ion-exchange resin formulations containing diclofenac. *In vitro* release from commercial sustained- release preparations such as Dolotren Retard$^R$ has been reported to be very rapid, almost

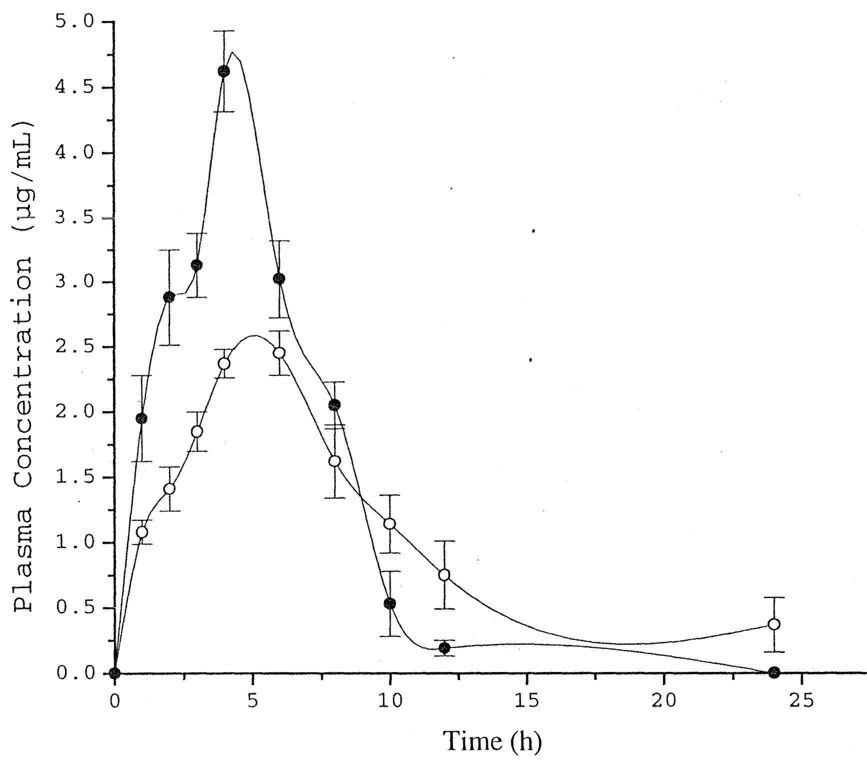



Fig. 3.5.8 Diclofenac sodium concentration in rabbit plasma after oral administration of diclofenac sodium powder (—●—) and the drug loaded microspheres (—○—).

the entire drug was found to be released in less than 30 min. However, the $t_{max}$ for this formulation *in vivo* was 7.5 h in rabbits and the $C_{max}$ was 1.83±0.45 μg/mL. (Garcia Encina *et al.*, 1993). In the present study, it can be seen that the $C_{max}$ was 2.44±0.18 μg/mL and the $t_{max}$ was attained in 6 h. Thus, compared to the free drug, the encapsulated form maintained a sustained release pattern comparable to one of the commercially available preparations to a great extent.

## 3.6 Chitosan Microspheres as a Carrier for the Controlled Delivery of Macromolecules

### 3.6.1 Background

In controlled release technology, biodegradable polymeric carriers offer potential advantages for the prolonged release of macromolecular drugs (Heller, 1984; Wise *et al.*, 1987; Pitt, 1990b). Drugs in this class include polypeptides, hormones, polysaccharides, antigens, antibodies and other biologically active agents. A number of bioerodible polymeric carriers have been investigated for the sustained release of macromolecular drugs. These include poly(lactic acid), poly(lactic acid- co-glycolic acid), poly(orthoesters), polyanhydrides, and poly(ε-caprolactone). Very little is reported on the use of natural polymers for the sustained delivery of macromolecular drugs. One possible reason may be the fact that such materials are degraded *in vivo* fairly rapidly thereby limiting the delivery for short period. For example, glutaraldehyde crosslinked albumin microspheres are reported to be completely degraded in about two months (Lee *et al.*, 1981). Crosslinked gelatin spheres also have similar life *in vivo*. Many macromolecular drugs require delivery for prolonged periods in order to be effective.

Vaccines are another important class of macromolecular drugs which require special attention in this area because of the need to immunise large populations particularly in the developing countries (Asano *et al.*, 1989; O'Hagan *et al.*, 1991; Eldridge *et al.*, 1991; Singh *et al.*, 1992; O'Hagan *et al.*, 1993b). Vaccines require administration over a 2–3 month schedule involving a primary immunisation followed by one or more boosters to generate the desired antibody response. Patient

compliance for such multiple shot immunisation therapy is rather poor in the developing countries.

Most biological macromolecular drugs are very sensitive to organic solvents, surfactants, pH, and temperature. Incorporation of such molecules in a polymer matrix is usually carried out by dissolving the polymer in a suitable organic solvent, mixing the drug, and evaporating the solvent (Asano *et al.*, 1989; Spenlehauer *et al.*, 1989; O'Hagan *et al.*, 1991; Eldridge *et al.*, 1991; Singh *et al.*, 1992; Tabata *et al.*, 1993b; O'Hagan *et al.*, 1993b). Microspheres, films, rods etc., containing the protein are essentially prepared in this fashion. The biological activity or antigenicity of such macromolecules is often compromised by this technique, because the material is in contact with undesirable organic solvents, surfactants, sometimes crosslinking agents, etc. If macromolecular drugs could be incorporated into matrices after the matrices are prepared, many of these problems could be overcome. However, many of the biodegradable polymers that are investigated for the sustained delivery of macromolecules are unsuitable for this purpose since they are not hydrophilic in character. They do not exhibit reasonable degree of swelling in aqueous solutions to permit drug incorporation from aqueous solution of the drug at right pH and temperature. Furthermore, there is also the possibility that molecules incorporated in such a fashion into the matrix would be released very rapidly from the matrix and a sustained release would not be observed. In this chapter, the release of a model protein bovine serum albumin (BSA), passively absorbed into crosslinked chitosan microspheres was examined. A preliminary evaluation of immunogenic response produced by Diphtheria Toxoid (DT)–loaded microspheres was also carried out in a rat model.

### 3.6.2 BSA–Loaded Chitosan Microspheres

The chitosan microspheres used in this study was prepared as described in section 2.2.10. The equilibrium water content of microspheres was around 50% as can be seen from Fig. 3.6.1. Equilibrium swelling was attained in about 8 h in distilled water at room temperature. The particle size distribution of microspheres is shown in Fig. 3.6.2. Table VI shows the amount of BSA passively loaded into pre-formed glycine-quenched chitosan microspheres. Since BSA was loaded from highly concentrated solution, a fairly high loading was seen as expected. The swelling nature of the microspheres was responsible for the loading of macromolecules into the matrix. The surface morphology of microspheres before and after loading with macromolecules was not different as evidenced by SEM (Fig. 3.6.3.a& b).

Table VI
Amount of BSA loaded into glycine-quenched, glutaraldehyde crosslinked chitosan microspheres by passive absorption from 20% aqueous solution of BSA at room temperature (27°)C

| Expt No. | BSA loaded (wt%) |
|---|---|
| 1 | 4.26 |
| 2 | 4.80 |
| 3 | 5.60 |
| 4 | 4.98 |
| Mean ± S.D | 4.91±0.48 |

### 3.6.3 *In Vitro* Release of BSA

The release of BSA from chitosan microspheres into phosphate buffer at 37°C is shown in Fig. 3.6.4. As expected, there was an initial burst in which about 65%

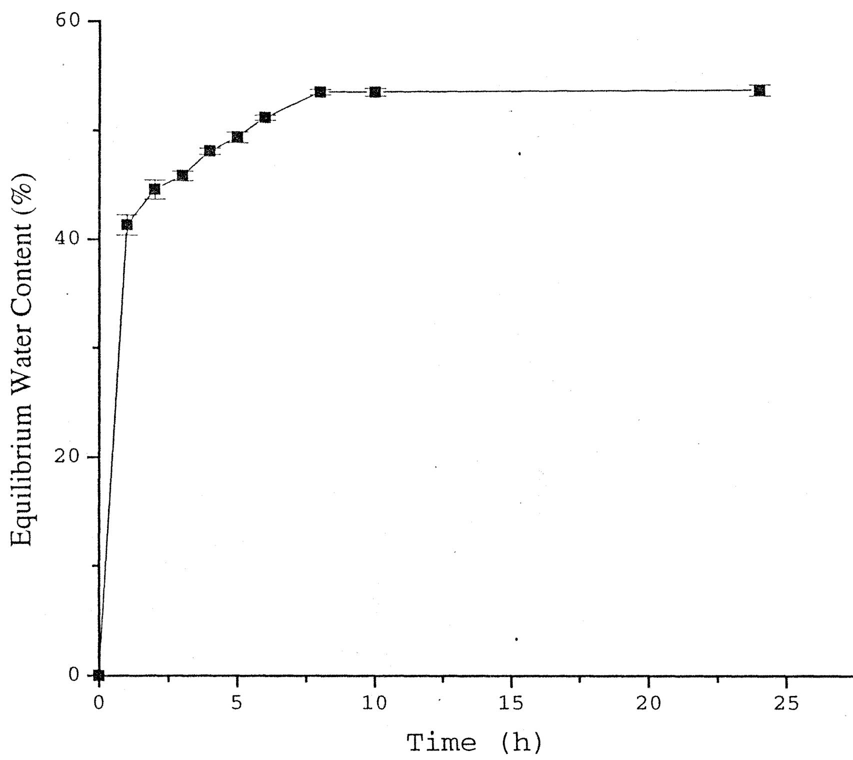



Fig. 3.6.1 Equilibrium water content of chitosan microspheres at room temperature (27° C)

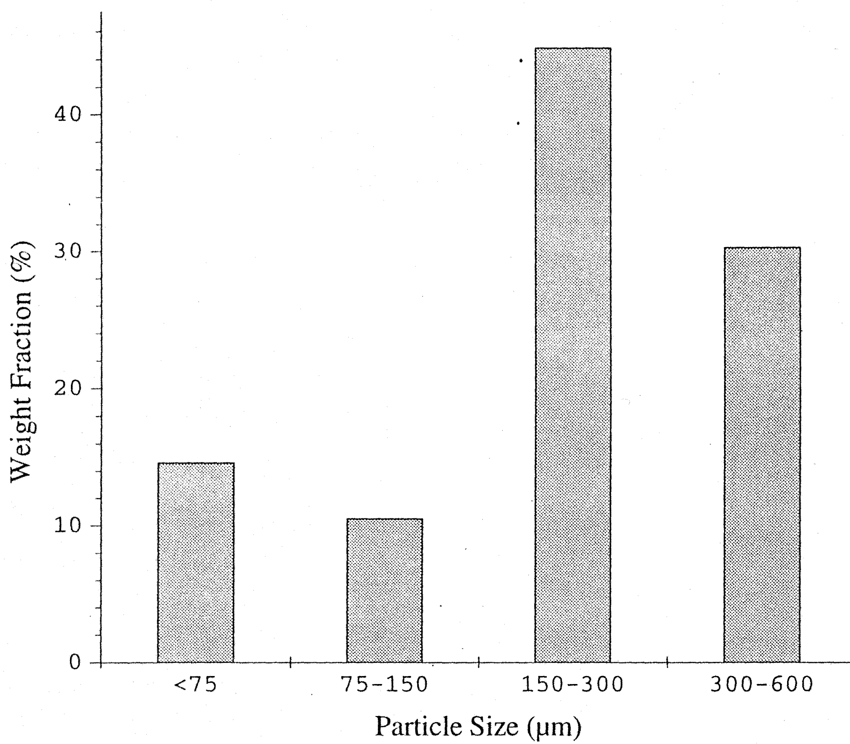



Fig. 3.6.2 Particle size distribution of BSA-loaded chitosan microspheres prepared at a stirring speed of 500 rpm

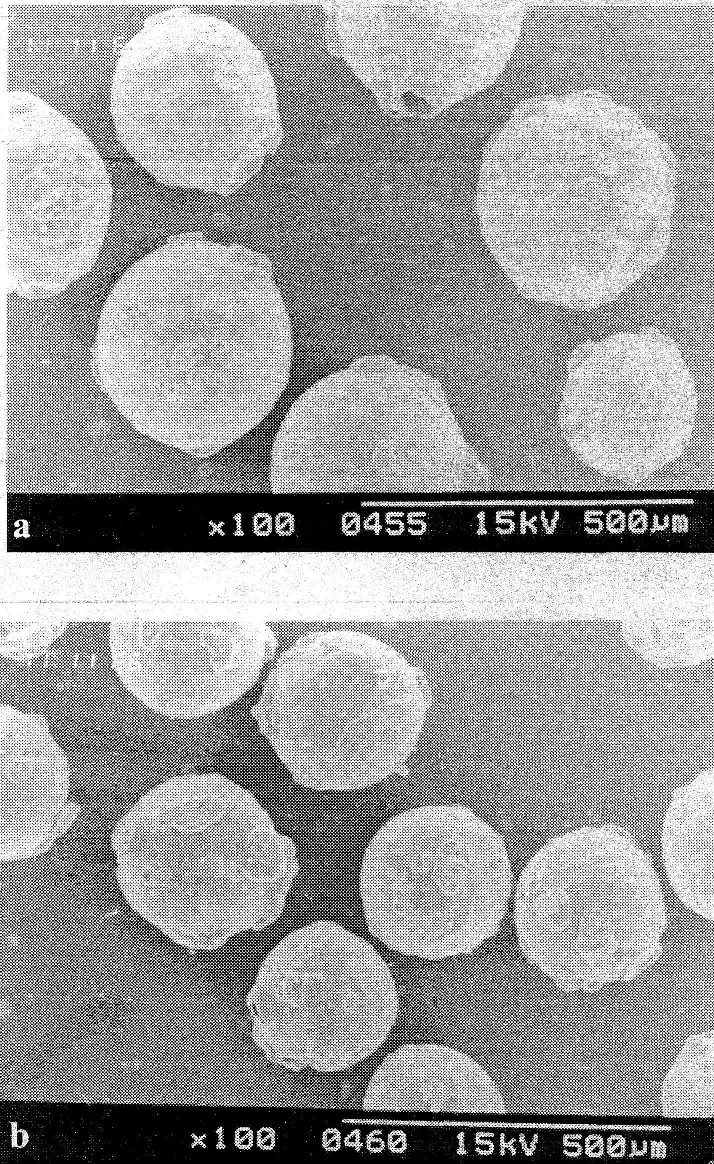



Fig. 3.6.3 SEM of placebo chitosan microspheres (a), microspheres loaded with BSA (b).

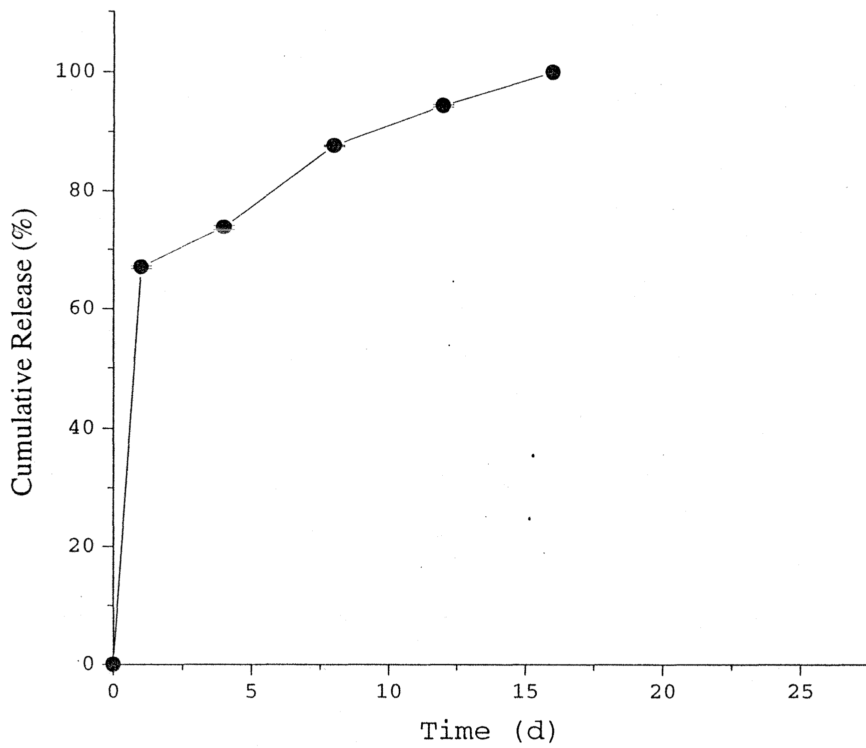



Fig. 3.6.4 BSA release from glycine-quenched chitosan microspheres (150-300 μm) at 37° C into phosphate buffer

of the incorporated BSA was released from the microspheres. This is followed by a steady increase in the amount released with time and almost 100% release was observed in about 16 days. This is indeed noteworthy because of the fact that BSA was incorporated into the microspheres by passive absorption. The mechanism of release can only be diffusion, but the diffusion of the BSA molecules from the matrix was not rapid as expected. From Fig. 3.6.1 it is seen that equilibrium swelling of the particles is attained in about 8 h, but is tooks nearly 16 days for 100% of the incorporated BSA to be released after the initial 'burst'. This suggests that once loaded into a water swollen matrix and dried, reswelling the matrix does not allow the macromolecules to suddenly escape. Possibly, the pores and channels that initially permitted the macromolecules to diffuse in are not reformed in an identical fashion when the matrix is reswollen to allow rapid diffusion and dissolution of the molecules. Once loaded and dried, the macromolecules may get thoroughly entangled inside the carrier matrix. Moreover chitosan has different affinity for various proteins (Caprisi *et al.*, 1993; Homma *et al.*, 1993). This affinity may also account for the slow release of BSA from microspheres.

Subtracting the burst release seen at day 1 and analysing the data rigorously, the amount of BSA released per milligram of the matrix per millilitre of the dissolution medium is plotted against time in Fig. 3.6.5. A steady increase in the amount released with respect to time can be seen.

Modulation of the release of BSA from microspheres was attempted by coating the loaded microspheres with poly(lactic acid) (PLA) and paraffin oil. When the particles were coated; with PLA (Fig. 3.6.6, curve A), the release of BSA was found to be prolonged and there was a fairly steady increase in the amount released

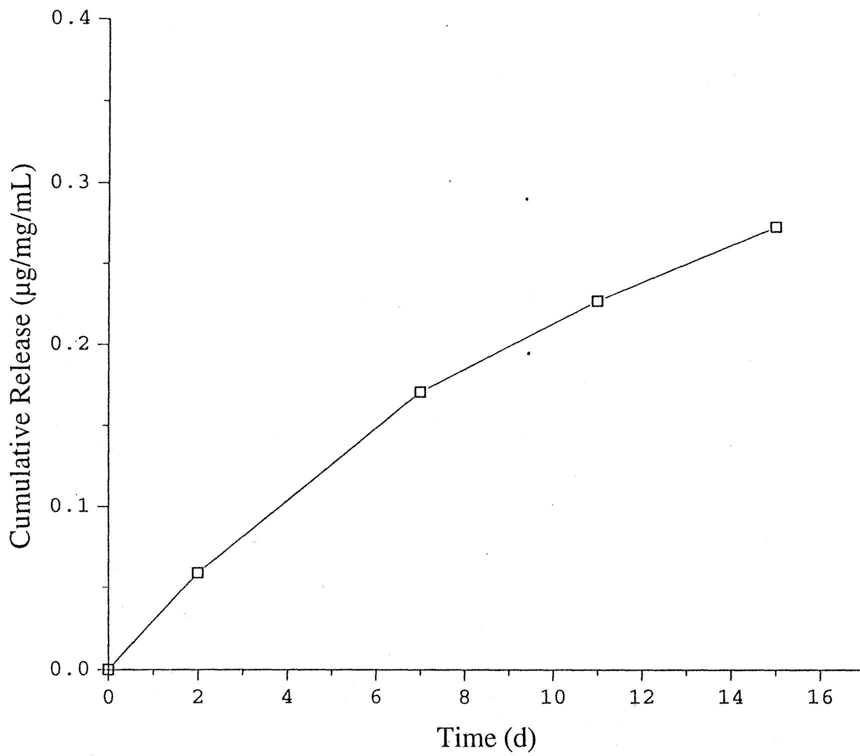



Fig. 3.6.5 Rate of release of BSA into phosphate buffer at 37° C from glycine-quenched microspheres (150-300 µm) after correcting for the initial burst on day 1

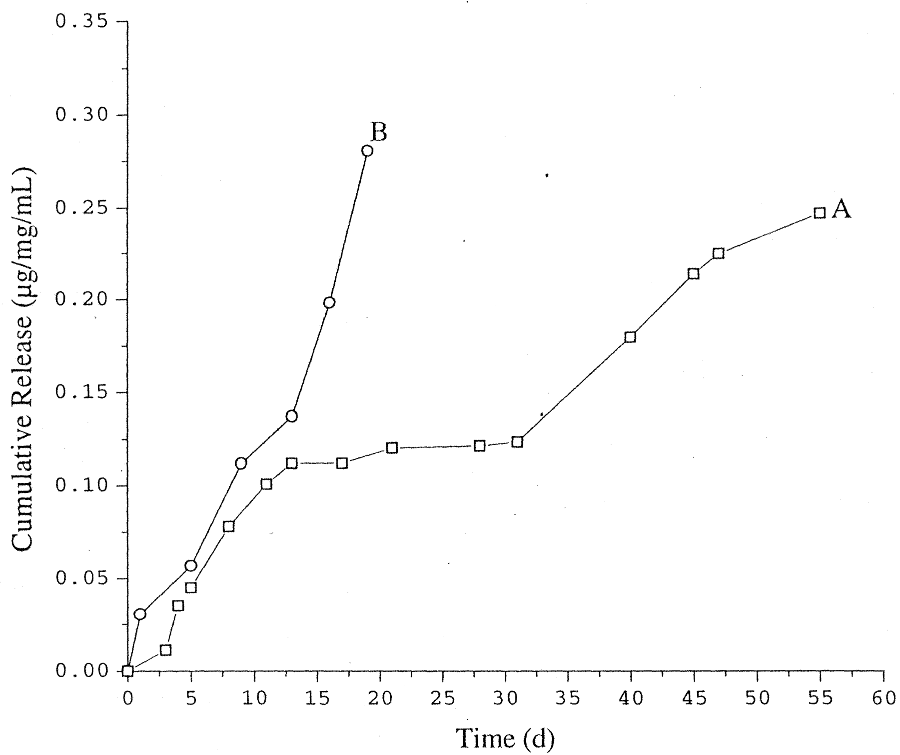



Fig. 3.6.6 BSA release from chitosan microspheres into phosphate buffer at 37° C coated with paraffin oil and PLA after correcting for the initial burst at day 1. Paraffin oil coated (—○—), PLA coated (—□—)

with time for nearly 2 months. The Fig. 3.6.6 curve B shows release of BSA from microspheres coated with paraffin oil after correcting for the initial 'burst' on day 1. These data point to the possibility of modulating the release of passively absorbed macromolecules from microspheres to obtain a near zero order release after an initial burst.

### 3.6.4 Immunogenicity of DT–Loaded Chitosan Microspheres

The preliminary data on the antibody response to chitosan microspheres passively loaded with DT is given in Fig. 3.6.7. Even though the titres were rather low compared with the control, the response was rather significant. The antibody response was fairly uniform for the period tested without peaks and troughs. Within-group variation was also considerably less, indicating better control over antigen release as compared to the alum absorbed modality. Incubation of the microspheres in a very small volume of DT (245 $\mu$L) was not found to absorb the entire amount. Therefore, all the DT added may not diffuse into microspheres. It was thus possible that a good amount of DT was not within the spheres, but outside it and was thus made available as "soluble antigen" which is definitely less immunogenic. It is also possible that the DT incorporated is released only in very minute quantities and the response is likely to be sustained for prolonged periods.

In summary, the method of passive absorption of macromolecular drugs into a crosslinked biopolymer matrix appeared to have some significant advantages and disadvantages. As seen in this study, the amount of protein that could be incorporated into such matrices by taking advantage of their swelling behaviour was rather low. However, for most immunisation purposes, such low concentrations

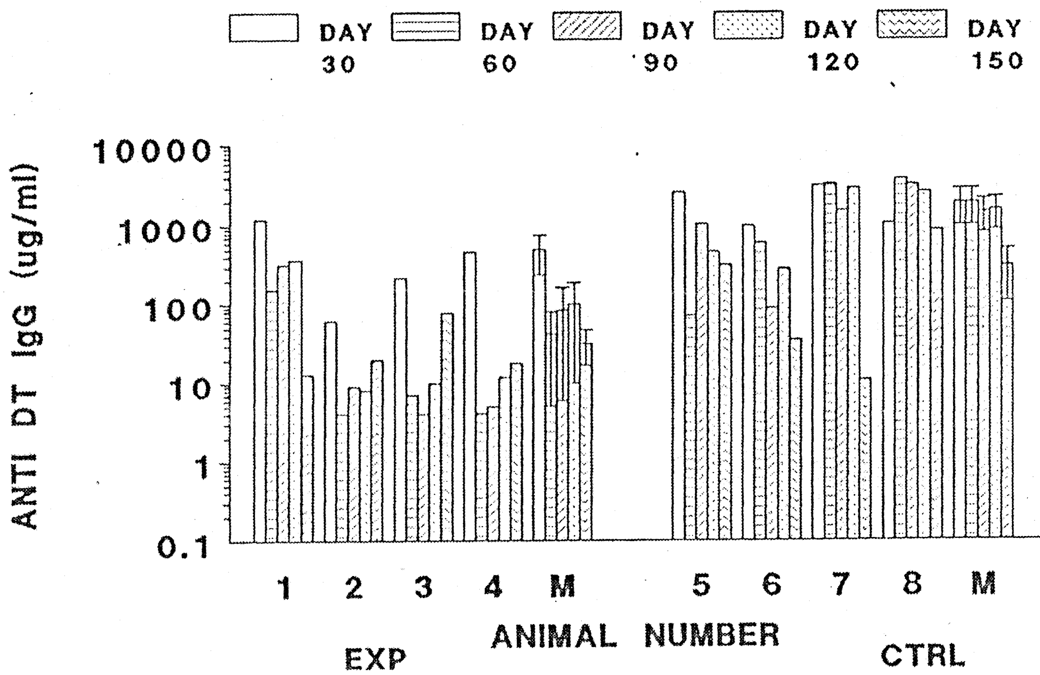



Fig. 3.6.7 Anti-DT antibody response to chitosan microspheres of < 75 μm size containing DT (1 to 4) and to DT on alum control (5 to 8) in rats

are more than sufficient. The surface bound and loosely incorporated protein was released from these matrices in the initial 'burst' to a large extent, although it was possible to control the 'burst' effect by coating the spheres after drug loading with oil or a suitable biodegradable polymer. After the initial 'burst', which may be expected to provide the primary immunisation dose *in vivo*, a fairly linear relationship was observed between the amount released and time. This was encouraging. Most of the biological macromolecules are denatured and degraded during the encapsulation process in the presence of organic solvents to various extents depending on the protein (Eppstein *et al.*, 1986; Hora *et al.*, 1990; Cohen *et al.*, 1991; Tabata *et al.*, 1993b). Protein degradation also take place under the effect of ultrasound, usually employed for emulsification thereby affecting the biological activity (Macleod and Dunn, 1968). In addition, polymers such as PLA are also reported to catalyse the degradation of proteins such as porcine somatotropin (Wyse *et al.*, 1989) and atriopeptin (Johnson *et al.*, 1991). Determination of biological activity of many proteins after emulsification using organic solvents and ultrasound has demonstrated that they lose their activity to varying degrees but significantly during the process (Tabata *et al.*, 1993b). The passive loading technique reported here therefore would be potentially advantageous in preserving the biological integrity of protein molecules. Once loaded into a matrix by passive absorption technique, one could think of crosslinking the surface using photosensitive groups incorporated into such matrices. This may reduce the 'burst' effect and control the release of the incorporated macromolecules in a more desirable fashion.

## 3.7 Preparation and Evaluation of Photocrosslinkable Chitosan as a Drug Delivery Matrix

### 3.7.1 Background

Studies described in earlier chapters have shown that glutaraldehyde crosslinked chitosan microspheres could be potential carriers for prolonged delivery of many micro- and macromolecular drugs. It was also shown that the biodegradability of chitosan could be altered by glutaraldehyde crosslinking and the extent of drug release is a function of the crosslinking density of the matrix.

The present study, although preliminary in nature, was undertaken in order to evaluate the potential of a photocrosslinkable form of chitosan as a matrix for sustained delivery of drugs. Drugs which possess active functions that enter into reaction with aldehyde can not be incorporated into chitosan matrix with glutaraldehyde crosslinking *in situ*. Thus, for eg., activities of drugs such as methotrexate, epinephrine and salbutamol etc. are affected by aldehyde crosslinking (Gupta and Hung, 1989a). If a photocrosslinkable matrix could be prepared, then the matrix could be crosslinked to varying extent by irradiation with light after the drugs are loaded into the matrix. The extent of drug release, in principle can therefore be controlled by the irradiation dose.

An attempt was therefore made to prepare a derivative of chitosan that would crosslink on irradiation with UV light. The azide group is a photosensitive one and undergoes photodecomposition to highly reactive nitrenes (Fig. 3.7.1).

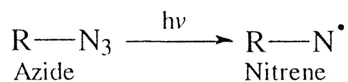



Fig. 3.7.1 Photodecomposition of azide into nitrene

The photogenerated nitrene can react via several non-selective reactions, including cycloaddition to double bonds, insertion into C – H bond or by hydrogen atom abstraction on the polymer (Turner and Daly, 1989). All these reactions can lead to crosslinking.

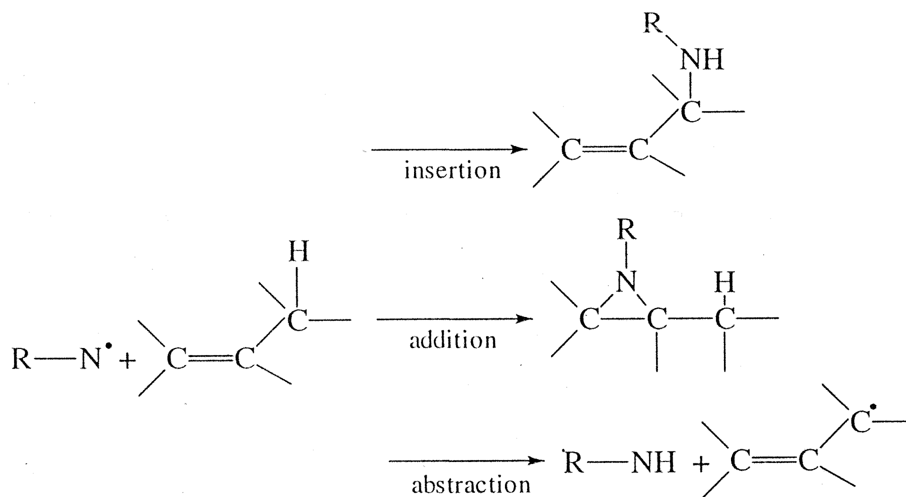



Fig. 3.7.2 Crosslinking reactions of nitrene

Thus, in principle azidated chitosan should crosslink on photoirradiation.

### 3.7.2 Azidated Chitosan

Attempts were first made to azidate chitosan via diazotisation reaction. Chitosan was first treated with sodium nitrite in the presence of hydrochloric acid at temperatures below 5°C to diazotise the amino function present on the glucosamine ring. After 5 min reaction, the excess acid in the solution was neutralised with 1N NaOH. Azidation was attempted by the addition of sodium azide in 2 mole excess as shown in Fig. 3.7.3.

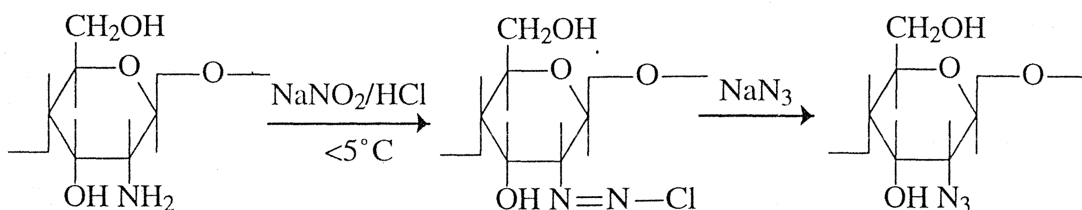



Fig. 3.7.3 Reaction Scheme for azidation of chitosan via diazotisation.

Efforts to precipitate the azidated compound by the addition of large volumes of methanol were not successful. It was seen that during treatment with nitrous acid, there was considerable reduction in the viscosity of the chitosan solution possibly due to rapid degradation of the polysaccharide. The degradative nature of chitosan in the presence of nitrous acid has been reported recently by other workers (Allan and Peyron, 1989).

Therefore, another approach was attempted for the preparation of chitosan bearing azide functions. Epichlorohydrin was azidated as described in section 2.2.12.1.

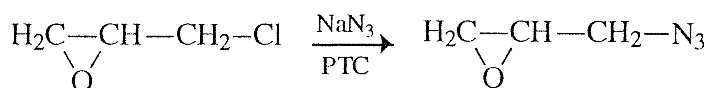


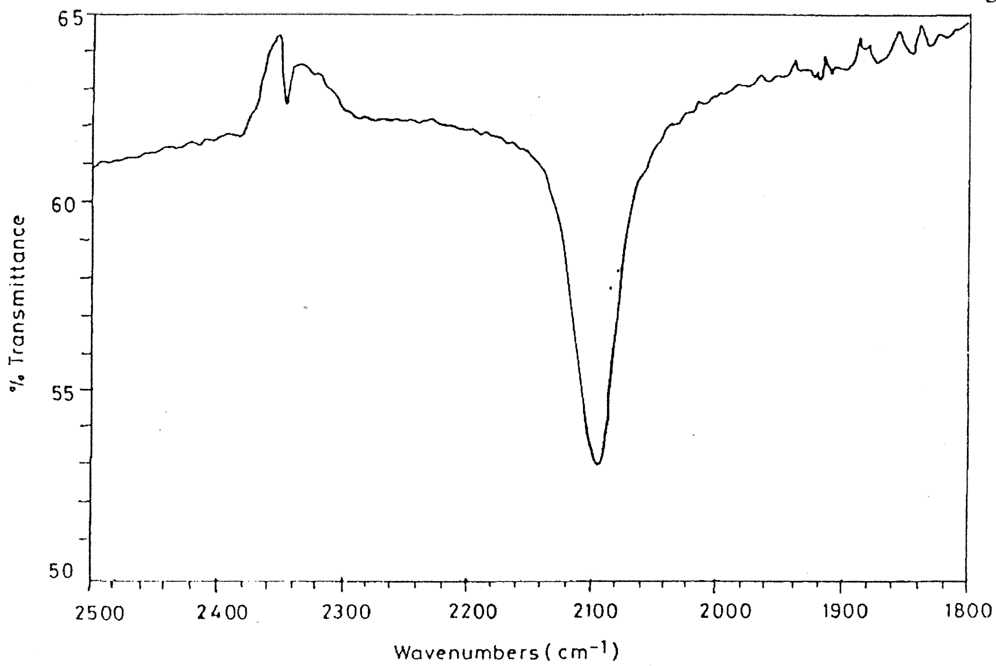

Fig. 3.7.4 Reaction scheme for the preparation of azido epoxypropane from epichlorohydrin

The azidated epichlorohydrin was then reacted with chitosan in the presence of a mineral acid such as HCl as described in section 2.2.12.2. The epoxy group of azidated epichlorohydrin react with the OH group of chitosan to form azidated chitosan

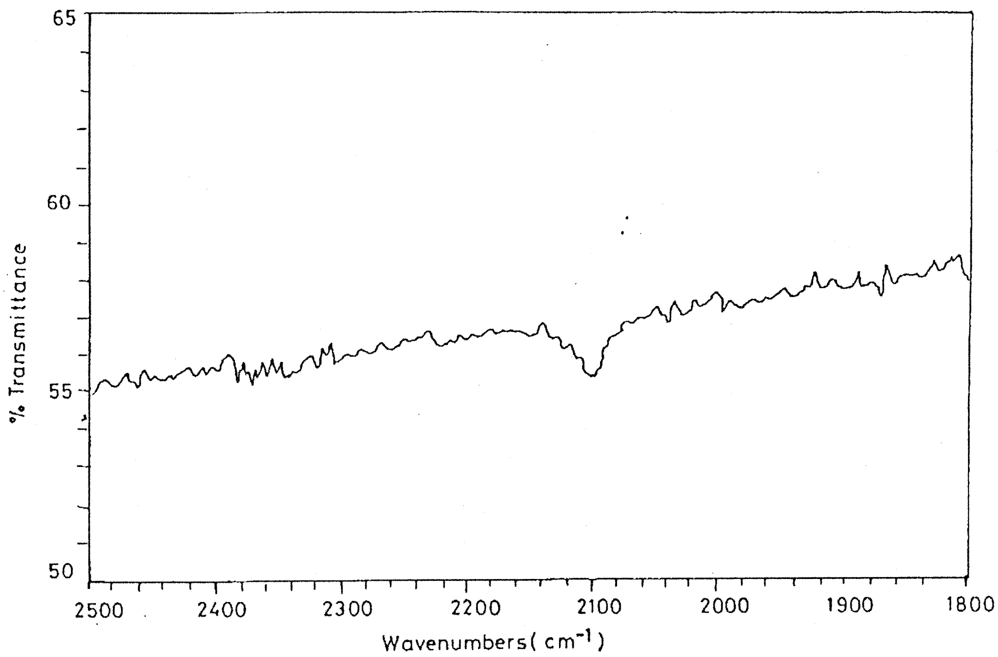
Films were cast from an acetic acid solution of azidated chitosan. Fig. 3.7.5 shows the IR spectra of azidated chitosan film with a strong azide peak at 2100 $cm^{-1}$. This peak disappears almost completely after 2 h irradiation demonstrating complete surface crosslinking (Fig. 3.7.5).

### 3.7.3 *In Vitro* Release of Theophylline

Fig. 3.7.6 shows the *in vitro* release of theophylline from azidated chitosan films before and after irradiation into simulated gastric fluid. Unirradiated film was found to dissolve in the gastric fluid leading to 100% of the release of the incorporated drug within 2 min. From the film irradiated for 2 h, it can be seen that only around 70% is released in about 10 min and thereafter there is a steady increase in the release rate with time. It took nearly two hours for about 90% of the incorporated drug to be released.




(a)




(b)

Fig. 3.7.5. ATR-IR spectrum of azidated chitosan film before UV irradiation showing the strong azide peak at 2100 $cm^{-1}$(a) and after 2 h irradiation with a 125 W UV lamp showing almost complete disappearance of the peak at 2100$cm^{-1}$ (b).

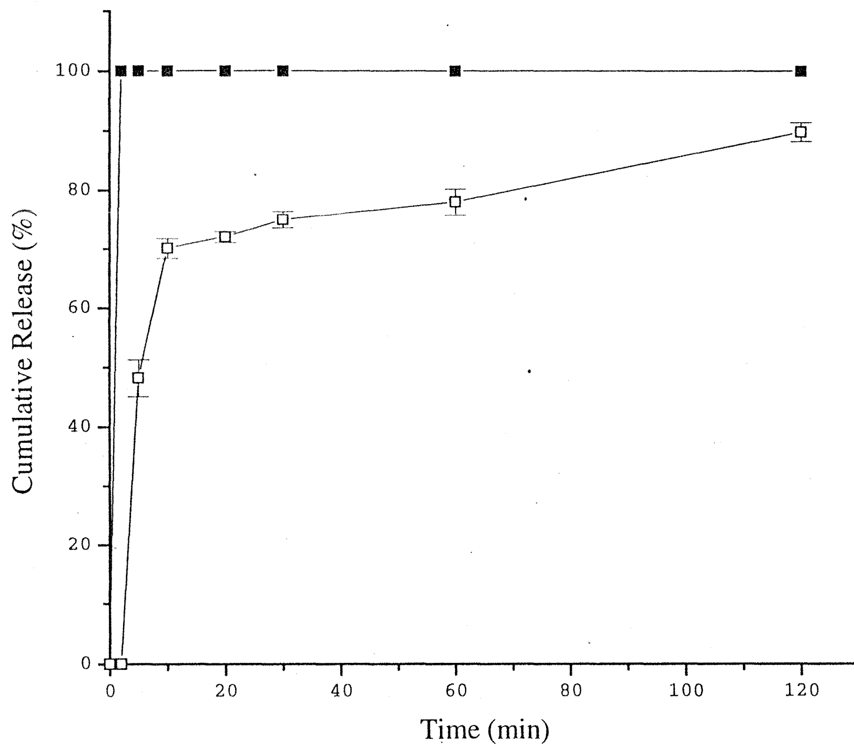



Fig. 3.7.6 Release of theophylline from azidated chitosan films at 37° C into simulated gastric fluid before and after photocrosslinking. Before crosslinking (—■—), after crosslinking (—□—).

In simulated intestinal fluid (Fig. 3.7.7), however, the release from unirradiated film was not so rapid as in simulated gastric fluid. Complete release was seen in about 20 min. The pH of the medium being slightly alkaline (pH 7.4), the film was not found to undergo dissolution in intestinal fluid unlike in gastric fluid. The less rapid release seen in intestinal fluid compared to the release seen in gastric fluid is because of the lower solubility of the film in the former. From the irradiated film, the release was less rapid, 80% of the drug diffused out in about 20 min and thereafter there was a steady increase in the rate with time. Complete release was seen in about 2 h.

The preliminary data reported in this study showed that photocrosslinking will be an interesting possibility for controlling drug diffusion from the chitosan matrix. Although, this system is not optimised to deliver a drug for prolonged periods of time, the possibility of modifying chitosan with other photosensitive functions may give rise to a photocrosslinkable matrix from which drug diffusion could be more effectively controlled.

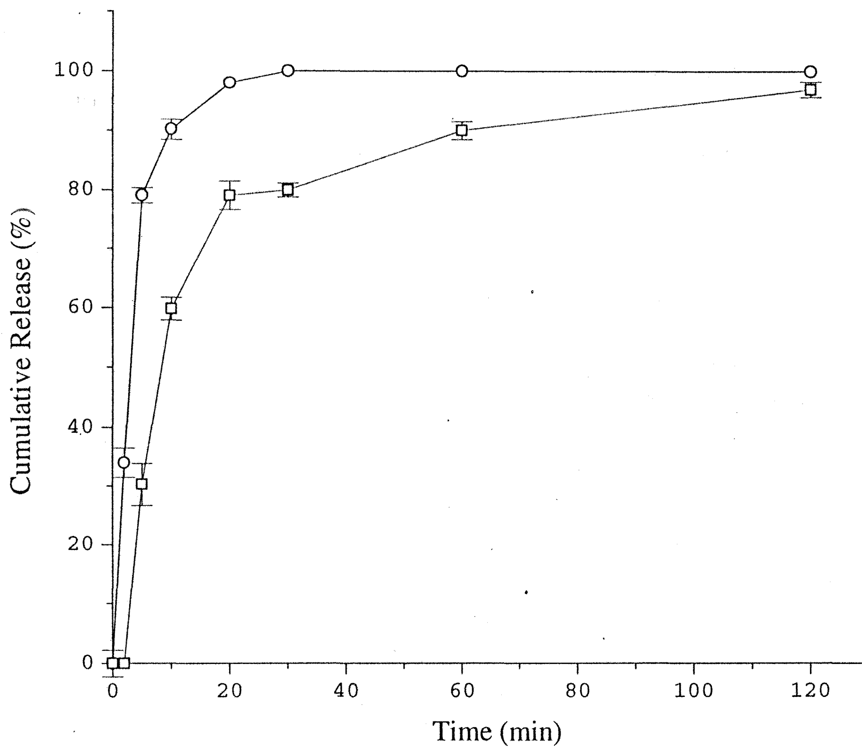



Fig. 3.7.7 Release of theophylline from azidated chitosan films at 37° C into simulated intestinal fluid before and after photo-crosslinking. Before crosslinking (—o—) and after cross-linking (—□—).

# CHAPTER 4

# SUMMARY, CONCLUSIONS AND FUTURE PROSPECTS

# SUMMARY, CONCLUSIONS AND FUTURE PROSPECTS

## 4.1 Summary and Conclusions

Development of a viable drug delivery system with controlled release characteristics requires a fundamental understanding and optimisation of pharmaceutics, pharmacokinetics and pharmacodynamics. Most highly developed form of drug delivery system involves various devices composed of synthetic polymers. Significance of polymeric microspheres as matrix for the controlled release of active agents has been well explored. This work has been devoted to studying the possibility of using a biodegradable natural polymer, chitosan as a matrix for controlled release. Glutaraldehyde crosslinked chitosan microspheres were prepared and their *in vitro* and *in vivo* degradation were examined. These microspheres were used as a matrix for the controlled release of *in situ* loaded drugs like mitoxantrone, an anticancer drug, progesterone, a steroid and diclofenac sodium, an oral drug. Also examined was the possibility of delivering macromolecules such as BSA and DT which were incorporated into pre-formed microspheres by the passive absorption method. Attempts to prepare

a photocrosslinkable chitosan as a drug delivery matrix are also included in this study.

Chitosan microspheres prepared from an aqueous acetic acid solution by glutaraldehyde crosslinking was found to be smooth and spherical. Microspheres of large size were prepared by paddle stirring and microspheres of smaller size (<10 $\mu$m) were prepared by ultrasonication. Microspheres were found to have an internal structure without any pores and perforations. It was also found that microspheres with different swelling characteristics could be prepared by employing different amounts of glutaraldehyde for crosslinking.

*In vitro* degradation studies of these microspheres were done by incubating in lysozyme solution at 37°C. No significant degradation was observed over a period of 6 months. *In vivo* degradation studies of glutaraldehyde crosslinked chitosan microspheres were carried out by implantation in rat muscle. Microsphere degradation was found to be slow irrespective of the degree of deacetylation. Although it was reported that chitin sutures and chitosan ascorbate are degraded *in vivo* in about 2-3 months, the glutaraldehyde crosslinked microspheres were found to be less susceptible to biodegradation. SEM and histological analysis showed that microspheres were degraded *in vivo* mostly by surface erosion and they persisted in the rat muscle even after a year. Spheres were found to be well tolerated by the living tissue without any adverse tissue reaction.

Mitoxantrone-loaded chitosan microspheres of different crosslinking densities were prepared. *In vitro* release studies showed that the drug release can be effectively controlled by changing the crosslinking density. Release rate was also found to be influenced by the drug payload and size of the microspheres. This

study demonstrated that chitosan microspheres can be used as matrix for sustained delivery of chemotherapeutic agents which are highly toxic.

Mitoxantrone-loaded chitosan microspheres were found to be therapeutically many times more effective than the free drug against EAC in mice when administered intraperitoneally. The microsphere formulation minimised drug toxicity and enhanced the therapeutic efficacy. Even at a dose corresponding to 4 to 8 times the $LD_{50}$ of mitoxantrone, the microsphere formulation was found to be non-toxic and therapeutically very effective. Thus glutaraldehyde crosslinked chitosan microspheres loaded with antineoplastic agents appeared to have good potential as a sustained drug delivery system in the treatment of cancer.

Another study conducted was on steroid delivery. Progesterone was incorporated in chitosan microspheres and the *in vitro* release was examined in phosphate buffer at 37°C as a function of crosslinking density and particle size. Progesterone release from the microspheres was found to be influenced by the extent of crosslinking and size of the microspheres. Bioavailability studies in a rabbit model by intramuscular injection showed that a plasma concentration of 1-2 ng/mL was maintained for about 5 months. While progesterone delivered as the free steroid peaked to toxic levels *in vivo*, the release from microspheres preparation was well below toxic limits. Progesterone has a poor biological half life and with the free steroid the serum concentration was not maintained beyond 15 days whereas the microsphere formulations sustained the serum progesterone levels without peaks and troughs for about 5 months.

Diclofenac sodium-loaded chitosan microspheres were prepared and found that the drug loading retained the spherical geometry of microspheres. *In vitro* release

of the drug was carried out in simulated intestinal fluid. This study showed that the extent of crosslinking, particle size and drug content of microspheres played an important role in determining the rate of release of the drug. Bioavailability studies done in rabbits by single oral administration showed that compared to the free drug group, a sustained plasma level of the drug was maintained upto 12 h in animals which received the drug encapsulated in chitosan microspheres. There was no significant difference between the free drug and the encapsulated drug as regards the relative extent of absorption calculated from the $AUC_{0-24h}$ values. It was comparable to a commercial sustained release formulation Dolotren Retard[R]. The study showed a $C_{max}$ of 2.44±0.18 $\mu$g/mL and a $T_{max}$ of 6h. Thus compared to the free drug, the encapsulated form maintained a sustained release pattern comparable to one of the commercially available preparations to a great extent.

Studies with macromolecules showed that incorporating BSA and DT by passive absorption method from their aqueous solutions into pre-formed chitosan microspheres can give rise to reasonable protein loadings. BSA incorporated in microsphere matrix was not found to be diffusing out completely in the initial 'burst' even under sink conditions. After the initial 'burst', a gradual increase in the amount released was observed and it lasted for several days. The duration of release could be prolonged by coating the drug-loaded spheres with paraffin oil or poly(lactic acid) (PLA). Such passive loading of biological macromolecules which are sensitive to organic solvents, surfactants, pH and temperature into hydrophilic matrices might offer an opportunity to preserve the biological activity of such molecules. Preliminary immunisation studies of DT-loaded chitosan microspheres

carried out in rats showed that though titres were low compared with the control, the response was uniform without peaks and troughs for the period tested.

Attempts to prepare photocrosslinkable chitosan was carried out by different methods. First was to azidate chitosan via diazotisation reaction of the amino group present on the glucosamine ring. But in the presence of nitrous acid degradation of chitosan was observed. Another approach attempted was the reaction with azido epoxypropane to prepare chitosan with azide functional group. Azido epoxypropane was prepared by the reaction between epichlorohydrin and sodium azide in presence of a phase transfer catalyst. Films prepared from the modified chitosan was found to have an azide functional group as evidenced by ATR-IR spectrum. Theophylline release from the film was examined in simulated gastric fluid and intestinal fluid at 37°C. Difference in release was observed from uncrosslinked and photocrosslinked films. This data showed that photocrosslinking would be an interesting method to control drug diffusion from a polymer matrix. Theophylline is a small drug molecule and therefore the diffusion was found to be more facile. With large drug molecules such as steroids or macromolecules, the diffusivity through crosslinks may be retarded and a prolonged release may be possible.

## 4.2 Future Prospects

The study reported in this thesis demonstrated that glutaraldehyde crosslinked chitosan microspheres are effective carriers for the sustained delivery of many drugs. The results obtained using mitoxantrone and progesterone were particularly striking. While proteins passively absorbed into pre-formed chitosan microspheres could evoke an immune response although lower in magnitude compared to the alum modality, this method has many advantages. This is an area that could be further investigated. The amount of antigen passively loaded into preformed microspheres should be quantified before they are injected. This would make sure that the same amount of antigen is administered to the animal by the microsphere modality and the control modality. Microspheres need to be incubated in radiolabelled antigens and the amount absorbed should be quantified by scintillation methods. Since microspheres *per se* in saline are known not to generate an inflammatory response to attract macrophages to generate a potent primary response, the microsphere modality should be investigated using immunostimulatory vehicles such as alum. The immune response generated by microspheres delivered in alum and in saline would be worth comparing. Antigens covalently bound to polymeric carriers have recently been shown to evoke immune response without the need for additional adjuvants. Glutaraldehyde crosslinked microspheres of chitosan appear to be promising in this regard. Antigens could be covalently bound to residual aldehyde handles of unquenched chitosan microspheres. The immune response evoked by such microspheres would be an interesting problem to investigate.

Since glutaraldehyde crosslinked chitosan microspheres have been found to have an *in vivo* life of more than a year in the muscle, it may be worth investigating the effect of other crosslinking agents on the rate of biodegradation of microspheres. Formaldehyde and 2,3- butanedione are potential crosslinking agents that could be investigated. Also, it would be worth examining the effect of molecular weight of chitosan on the biodegradation after crosslinking with different crosslinking agents. It may thus be possible to tailor a crosslinked chitosan matrix with the desired biodegradation characteristics.

It is demonstrated that a photocrosslinkable matrix of chitosan could be prepared by appropriate chemistry. There is hardly any published literature on this type of matrices for drug delivery. The photocrosslinking method will have many advantages for drug delivery applications although the data obtained using the model system in this investigation are very preliminary in nature. Large molecules such as steroids and macromolecules such as proteins are important candidates to investigate the efficacy of this matrix further. The diffusivity of large molecules from photocrosslinked matrix may be slower and a sustained release may be possible. Other photocrosslinkable matrices of chitosan could be prepared and investigated for sustained delivery.

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# Appendix A: List of Abbrevations

| | | |
|---|---|---|
| ATR | - | Attenuated total reflection |
| BIS | - | N-N' Methylene bisacrylamide |
| BSA | - | Bovine Serum albumin |
| DOS | - | Dioctyl Sulphosuccinate |
| DT | - | Diphtheria toxoid |
| EAC | - | Ehrlich ascites carcinoma |
| ELISA | - | Enzyme linked immuno sorbant assay |
| EVA | - | Ethylene vinyl alcohol |
| EWC | - | Equilibrium water content |
| FTIR | - | Fourier transform infrared |
| 5-FU | - | 5-fluorouracil |
| GF | - | Gastric fluid |
| GI | - | Gastro intestinal |
| GST | - | Glutaraldehyde saturated toluene |
| HPLC | - | High performance liquid chromatography |
| HRPO | - | Horse raddish peroxidase |
| HSA | - | Human serum albumin |
| IF | - | Intestinal fluid |
| LF | - | Lime flocculations |
| MTX | - | Mitoxantrone |
| MW | - | Molecular weight |
| PBS-T | - | Phosphate buffered saline with Tween 20 |
| PCL | - | Poly(ε-caprolactone) |
| PGA | - | Poly(glycolic acid) |
| PLA | - | Poly(lactic acid) |
| RES | - | Reticulo endothelial system |
| SEM | - | Scanning electron microscopy |
| Tg | - | Glass transition temperature |

# Appendix B: List of Publications

**Publications from the thesis work**

1. Crosslinked chitosan microspheres as carriers for prolonged delivery of macromolecular drugs, Jameela S. R., Misra A, Jayakrishnan A, J. Biomat. Sci. Polym. Edn., Vol. 6, 621-623, 1994.
2. Glutaraldehyde crosslinked chitosan microspheres as a long acting biodegradable drug delivery vehicle: Studies on the *in vitro* release of mitoxantrone from microspheres, Jameela S. R, Jayakrishnan A., Biomaterials, Vol. 16, 769-775, 1995.
3. Glutaraldehyde as fixative in bioprostheses and drug delivery matrices, a review, Jayakrishnan A, Jameela S. R., Biomaterials, Vol 17, 471-484. 1995.
4. Antitumour activity of mitoxantrone-loaded chitosan microspheres against Ehrlich ascites carcinoma, Jameela S. R, Latha P. G, Subramonium A, Jayakrishnan A. J. Pharm. Pharmcol, Vol. 48, 685-688, 1996.
5. Glutaraldehyde crosslinked chitosan as a long acting biodegradable drug delivery vehicle, A. Jayakrishnan S. R., Jameela, Proc. Eur. Conf. Biomat. p. 33, 1994.

6. Progesterone-loaded chitosan microspheres: a long acting biodegradable sustained delivery system. Jameela S. R., Kumari T. V., Lal A. V, Jayakrishnan A, J. Control. Rel. (Communicated-under revision).
7. Preparation and evaluation of photocrosslinkable chitosan as a drug delivery matrix, Jameela S. R., Lakshmi S., Jayakrishnan A, Biomaterials (Communicated-under revision)

**Author's other Publications related to controlled drug delivery**

1. Poly(ε-caprolactone) microspheres as carrier for vaccine delivery: Immune response to bovine serum albumin loaded microspheres in rats, Jameela S. R., Suma N, Misra A, Raghuvanshi R, Ganga S, Jayakrishnan A, Current Science, 70, 1996.
2. Protein release from Poly(ε-caprolactone) microsphere prepared by melt encapsulations and solvent evaporation techniques; A comparative study Jameela S. R, Suma N, Jayakrishnan A, J. Biomat. Sci., Polym. Ed. Vol. 8, 457-466, 1997.