

PREPARATION AND EVALUATION OF CHITOSAN-BASED CONTROLLED
DRUG DELIVERY SYSTEMS

A dissertation submitted in partial fulfillment of the requirement for the award of the degree of

Masters of Science

In

Biotechnology



Submitted By

Ravleen Kaur

(Reg No. 301201018)

Under the supervision of

Mrs. M. Vasundhara

Assistant Professor

Department of Biotechnology

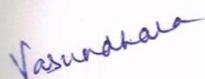
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CERTIFICATE

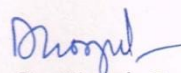
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Mrs. M. Vasundhara

Assistant professor

Department of Biotechnology



Dr. Dinesh Goyal

Head of Department

Department of Biotechnology



Dr. S.K. Mahapatra

Dean (Academic Affairs)

Thapar University

Patiala

CANDIDATE'S DECLARATION

I hereby declare that the work presented in the dissertation entitled "**Preparation and Evaluation of Chitosan-Based Controlled Drug Delivery Systems**" in partial fulfillment of the requirement for the award of the Degree of Masters of Science in Biotechnology, is an authentic record of my own work during the academic year 2012-2014, under the guidance of Mrs. M. Vasundhara, Assistant Professor, Department of Biotechnology, Thapar University, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other university.

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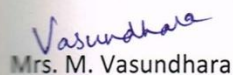


RAVLEEN KAUR

Date: 18 July 2014

301201018

This is to certify that the above statements made by the student are correct and true to the best of our knowledge and belief.



Mrs. M. Vasundhara

Assistant professor

Department of Biotechnology



Dr. Dinesh Goyal

Head of Department

Department of Biotechnology

DEDICATED

TO

MY FAMILY AND FRIENDS

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Date: 18 july 2014

RAVLEEN KAUR (301201018)

ABSTRACT

Controlled drug release enhances the safety, efficacy and reliability of the drug therapy. Regulation of the drug release rate results in the reduction in the frequency of drug administration and should encourage patients to comply with dosing instructions. These controlled release methods are more desirable patterns for drug administration, as the new and most powerful drugs continue to be developed. Many approaches were explored for controlled release of drug to the site of action, thus microspheres and hydrogels have received considered attention.

In the present study, attempts have been made to prepare microspheres by emulsion cross-linking and hydrogels by physical cross-linking methods. The number of polymers suitable for controlled release of viable therapeutics is quite limited because of inherent toxicity or lack of certain properties such as biodegradability. In this thesis, chitosan is chosen as a base polymer, as it is non-toxic and biodegradable natural polymer. The interesting characteristics of chitosan make it ideal candidate for use in controlled drug release. Glutaraldehyde is used as cross-linking agent due to its commercial availability and low cost.

Ampicillin loaded-chitosan microspheres were prepared by dropping the chitosan-drug solution in light liquid paraffin and span 80 emulsion, followed by adding the glutaraldehyde. The droplets instantaneously formed gelled spheres by emulsion cross-linking method. The effect of varying chitosan concentration on the preparation and properties of microspheres were studied. Microspheres were characterized by their percentage yield, particle size, surface morphology, drug encapsulation efficiency and in vitro drug release rate. Similarly hydrogels were prepared by preparing the chitosan-drug solution and then adding the glutaraldehyde, and stirring the solution for 15 minutes on the magnetic stirrer. Gelly type liquid forms by chemical cross-linking method. The effect of varying glutaraldehyde concentration and volume of glutaraldehyde was studied. They were characterized by their percentage yield, swelling ratio, surface morphology, drug encapsulation efficiency and in vitro drug release rate. In vitro drug release studies of microspheres and hydrogels both were done in buffer (pH 1.2) and subsequently in buffer (pH 6.8). The release of ampicillin from microspheres were affected by

chitosan concentration i.e., increase in chitosan concentration decreases the release of drug. It was observed that highest efficiency and highest percentage release was achieved in the batch 1, having 1% (w/v) chitosan conc., 1% w/v drug concentration, 20 ml volume of chitosan-drug concentration and 2.5 hrs stirring time. In hydrogels, highest efficiency and highest percentage release was observed in batch 10, having 1% (w/v) chitosan concentration, 1% (w/v) drug concentration, 20 ml chitosan- drug solution, and with 15 minutes Stirring time.

Chitosan microspheres crosslinked with the glutaraldehyde had good shape and good pH-responsive drug release properties. High drug incorporation in chitosan microspheres were obtained by emulsion cross-linking method, as by this method size of particles can be controlled by controlling the size of aqueous droplets. The chitosan hydrogels also show good pH-responsive properties. Both emulsion cross-linking and chemical cross-linking method can be carried out under very mild conditions using simple equipments. The control of various manufacturing parameters play a very important role in obtaining microspheres and hydrogels of high yield and high drug encapsulation, followed by high percentage release.

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LIST OF ABBREVIATIONS

Abbreviations	Word(s)
AIDS	Acquired immunodeficiency syndrome
CCHP	Cationic cholesteryl group bearing pullulan group
CMC	Carboxymethyl cellulose
CNS	Central nervous system
GIT	Gastrointestinal tract
HAMC	Hyaluronan methyl cellulose
hMSC	Human mesenchymal stem cells
LHRH	Luteinizing-hormone-releasing-hormone
mESCs	Mouse embryonic stem cells
MLTH	MRI-monitored long term therapeutic hydrogels
PBCCP	Poly (bis (carboxylate-phenoxy) phosphazene
PEG	Poly (ethylene glycol)
PEO	Poly (ethylene oxide)
PHB	Polyhydroxybutyrate
PMMA	Poly (methyl methacrylate)
PsPA	Pneumococcal surface protein A
PVA	Poly (vinyl alcohol)
r-IL-2	Recombinant Interleukin-2

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C **HAPTER 1**

INTRODUCTION

INTRODUCTION

In the last few decades, the way of administering drug has gained increasing attention, with the concomitant recognition of therapeutic advantages of controlled drug delivery (Park 1997). Conventional drug dosage forms, including pills, tablets, capsules, injections, ointments, creams, and aerosols, provide an instantaneous release of drug in a bolus form. So multiple dosing system is required for achieving and maintaining the drug concentration within the therapeutically effective range. With the conventional dosing formulations, the concentration of the released drug is initially high peaks and then declines sharply below the minimum therapeutic level, which cause fluctuation in the drug levels, followed by unacceptable side effects at the peaks, and insufficient therapy in the troughs (Ravi Kumar 2000)(Figure 1a). Regulating the rates of drug release with the aid of controlled drug release formulations offers numerous advantages over conventional dosage forms (Figure 1b).

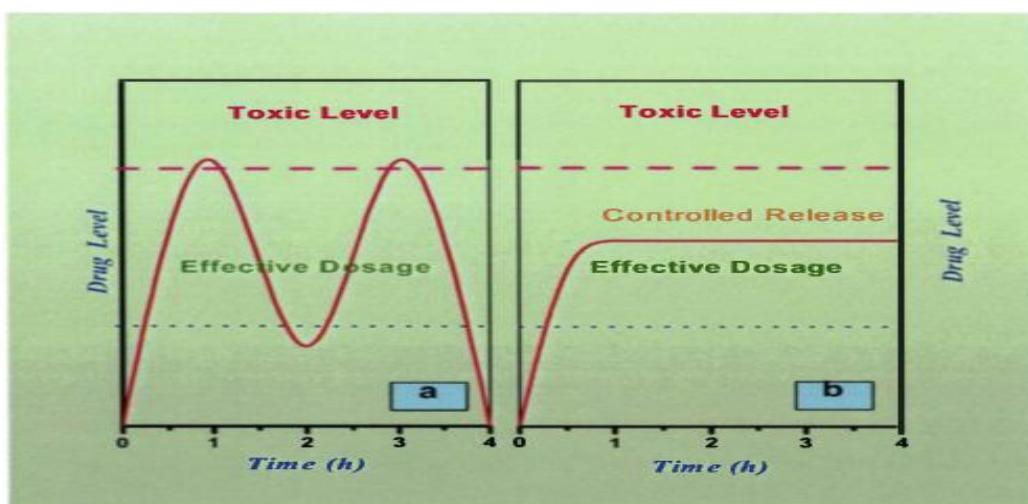


Figure 1: Graphical representation showing pharmacokinetic profiles in case of
(a) Conventional and (b) Controlled drug release formulations (Ravi Kumar 2000)

1.1 Controlled Release Drug Delivery System

Controlled release drug delivery system means any drug delivery system that maintains adequate and desired release of drug over an extended period of time and is designed to deliver the drug at specific release rates. Targeted delivery systems are also considered as a

controlled delivery system, since they provide spatial control of drug release to a specific site of the body (Brahmankar and Jaiswal 2009). Hydrophilic polymer matrix is widely used for formulating a controlled dosage form (Wani et al. 2008).

Main advantages of controlled release forms (Kumar et al. 2003)

- Controlled release of drug results in a reduction in frequency of the drug administration and should encourage patients to comply with the dosing instructions.
- The drug is released in a controlled fashion, which could be in terms of onset of release, the duration of release, and the release profile itself.
- The drug is released in targeted region, which can be achieved by timed release of the drug and it can minimize the drug efficacy.
- The controlled release formulations enable the drug to be protected from the physiological environment for longer durations, thus extending the residence time of drug.
- Systemic exposure of drug could be reduced upon its targeting to desired environment, which decreases the side effects of drug.

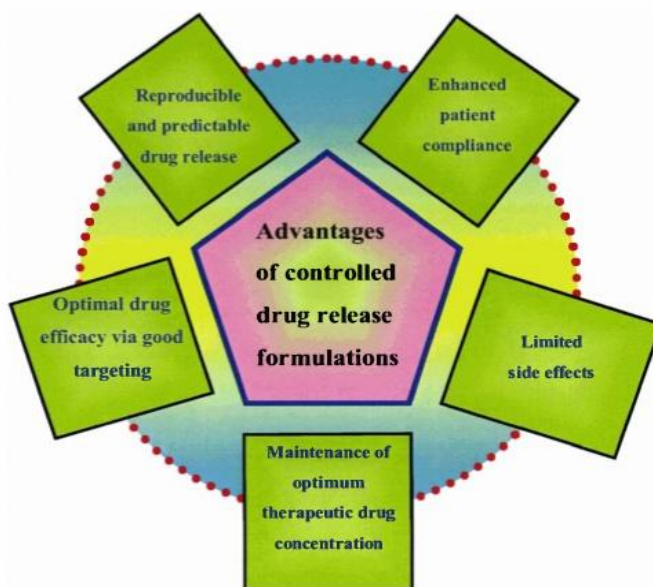


Figure 2: Advantages of controlled drug release formulations (Kumar et al. 2003)

However, the cost of controlled drug release formulations may be considerably higher than the conventional forms. Controlled drug release formulations also exhibit some limitations such as (Ritschel 1989):

- 1) Delayed onset
- 2) Increased potential for first-pass metabolism
- 3) Less accurate dose adjustment
- 4) Dependence on residence time in gastrointestinal (GI) tract

1.2 Hydrogels in controlled drug delivery system

Hydrogels have been used extensively in the development of the smart drug delivery systems. In 1960, Wichterle and Lim prepared and investigated a hydrogels of poly(2-hydroxyethyl methacrylate) for contact lens application (Wichterle and Lim 1960), thus hydrogels have been of great interest to biomedical scientist and widely involved in development of drug delivery system (Shiva Kumar et al. 2006; Hejcl et al. 2008).

Hydrogels are hydrophilic, polymeric networks of three dimensional cross linked structures that absorb substantial amount of water, while maintaining the structure. The network is composed of homopolymers or co-polymers. Cross linking facilitates insolubility in water because of ionic interaction and hydrogen bonding. It also provides required mechanical strength and physical integrity to the hydrogels. The high hydrophilicity of the hydrogels is mainly due to presence of a number of hydrophilic moieties such as amino, carboxyl, amide and hydroxyl groups distributed along the backbone of the polymeric strand (Peppas et al., 2000; Park et al. 2001). Hydrogels can imbibe water nearly 10-20 times its molecular weight and hence becomes swollen. In the swollen state, hydrogels are soft and have a rubbery structure, resembling the living tissues to a great extent (Kim et al. 1992).

1.2.1 Classification of hydrogels

- ❖ According to the hydrogel response to the environment factor

These hydrogels are known as “intelligent” or “smart” hydrogels. The main reason for using smart hydrogels in drug delivery is that, a good drug delivery system responds to physiological

requirements, sense the changes and accordingly amend the drug release pattern (Gupta et al. 2002).

Table 1: Environment sensitive hydrogels and their polymer system (Gupta et al. 2002)

Sensitivity type	Polymer system	Uploaded drug
Ph	Poly(2-hydroxyethyl methacrylate)	Salicylic acid
	Chitosan-poly ethylene oxide	Amoxicillin, metronidazole
	Poly (acrylic-acid)- poly-ethylene oxide	Nicotinamide, salicylamide
Temperature	Poly(N-isopropylacrylamide)-poly (ethylene glycol)diacrylate	Heparin
Temperature and pH	poly-N-isopropylacrylamide	Calcitonin
Electric field	Poly(2-hydroxyethyl methacrylate)	Propanolol, hydrochloride

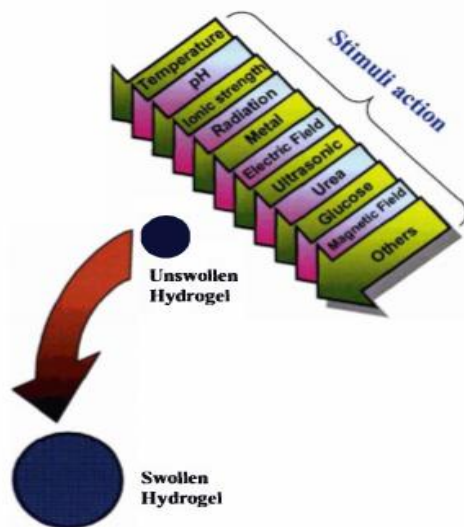


Figure 3: Stimuli-responsive swelling of hydrogels (Gutowaska 1992)

❖ According to the method of preparation

Hydrogels can be classified as homopolymers (one type of hydrophilic monomer unit), copolymer (cross-linking of two comonomer units) and multi-polymer (cross-linking of three or more comonomer units).

❖ According to the origin

Hydrogels can be classified into natural, synthetic and semi-synthetic hydrogels.

Table 2: Examples of polymers for preparation of hydrogels (Hoffman 2001)

Polymer category		Example
Natural polymers	Neutral	Agarose, dextran
	Cationic	Chitosan
	Anionic	Pectin, Carrageenan, Alginic acid
	Amphipathic	Collagen, carboxymethylchitin, gelatin
Synthetic polymer	Polyesters	PEG-fumarate, PHB
	Others	PBCPP, PVA, PEO
Semi- synthetic polymer		Collagen- acrylate

1.2.2 Applications of hydrogels (Kashyap 2005)

- The hydrogels of gelatin and polyvinyl alcohol together with blood coagulants are formulated for wound healing (Azad et al. 2004).
- Silicon hydrogels have been used for development of soft contact lenses.
- In industries, hydrogels have been used as absorbent such as methylene blue dyes. E.g. adsorption of dioxins by hydrogel beads.
- In tissue engineering, micronized hydrogels are used to deliver macromolecules in cytoplasm of antigen-presenting cells (Lee et al. 2001)
- In GI tract delivery, drug is loaded with colon specific hydrogels and is liberated due to change in pH or enzymatic action.
- Other: Hydrogels are used for rectal, ocular and transdermal delivery.

1.3 Microspheres in controlled drug delivery system

Microspheres are characteristically free flowing powders consisting of protein or synthetic polymers, which are biodegradable in nature ideally having size less than 200 μm (Vyas and Khar 2001). They facilitate accurate delivery of small quantities of potent drug and reduce the concentration of drug at site other than target organ or tissue. They provide protection for unstable drugs before and after administration, prior to availability at the site of action. They provide the availability to manipulate the in vivo action of the drug, pharmacokinetic profile, tissue distribution and cellular interaction of drug. They enable controlled release of drug. E.g. Narcotic, Antagonist, Steroid hormones (Omkar et al. 2009).

Table 3: Examples of polymers used for microsphere preparation (Vyas and Khar 2001)

Polymer category		Examples
Synthetic polymers	Non-biodegradable polymers	Polymethyl methacrylate (PMMA), Acrolein, Epoxy polymers
	Biodegradable polymers	Lactides, Polyalkyl Cyano Acrylate, Polyanhydrides
Natural Polymers	Proteins	Albumin, Gelatin, Collagen
	Carbohydrates	Agarose, Chitosan, Starch
	Chemically-modified carbohydrate	Poly (acryl) dextrane, Poly (acryl) starch

1.3.1 Types of microspheres and their application

Table 4: Types and application of microspheres (Prasanth et. al. 2011; Patel et al. 2011)

Types of microspheres	Applications
-----------------------	--------------

Bioadhesive microspheres	Buccal, oral, ocular, colonic drug delivery Nasal- Gentamicin, Insulin, GI- Glipizide Colonic- Insulin Ocular- Methyl prednisolone
Magnetic microspheres	Used in DNA analysis, cell isolation, protein purification and targeting drugs to tumour sites (Doxorubicin)
Floating microspheres	Carrier for drugs for antiviral, antifungal and antibiotic agents (so called absorption windows), non-steroidal anti-inflammatory drugs, Prednisolone, Lansoprazole.
Radioactive microspheres	For diagnostic purpose- Diagnostic radioembolization: ^{99m}Tc -macro-aggregated human serum albumin(MAA), Thrombus imaging in deep vein thrombosis: ^{99m}Tc -sulfur colloid For therapeutic purpose- Radioembolization of liver and spleen tumours: ^{90}Y -microspheres, Local radiotherapy: ^{212}Pb -sulfur colloid.
Polymeric microspheres	Vaccine delivery: Hepatitis, influenza, Pertusis, Diphtheria toxoid Oral drug delivery of easily degraded drugs; gene therapy with DNA plasmids; delivery of insulin, LHRH Controlled drug delivery after local application: release of proteins, hormones and peptides over extended times

To conclude ideally, controlled release drug delivery systems aim to maintain plasma concentration of drugs within the therapeutic window for longer period of time, thereby to ensure sustained therapeutic action. With the localization of drug action one may achieve specificity and control which could improve the quality of treatment and at the same time

minimize adverse side effects, due to the unnecessary drug exposure of non targeted tissues (Muzzarelli et. al, 1989). Hydrogels have played a significant role in biomedical applications. They can swell and contract in response to external stimuli, thus have potential applications in the site-specific delivery of drugs to specific regions of GIT whereas microspheres is a short term but it is having wide applications in drug delivery system, such as, it is used for targeting tumors using anticancer drugs. It is important carrier for safe and effective *in vivo* drug delivery.

For controlled release system, there are many formulations in market for various diseases such as nicotine patches, hormonal patches, controlled release tablets of metformin, iron, cisplatin, doxorubicin, nystatin, etc. Research is going on towards the development of controlled delivery systems for diabetes, hypertension and cancer drugs and some are under different stages of clinical trials. After FDA approval, they may be expected to come in market in near future.

Objective:

- To prepare microspheres and hydrogels by varying formulation parameters.
- To evaluate drug loaded microspheres and hydrogels for percentage yield, equilibrium swelling, average particle size, encapsulation efficiency and *in vitro* drug release
- To study *in vitro* drug release profiles of microspheres and hydrogels.

C **HAPTER 2**

REVIEW OF LITERATURE

REVIEW OF LITERATURE

- ★ Sawayangi *et al.* (1982) reported that chitosan had excellent properties as an excipient for direct compression of tablets where the addition of 50% chitosan resulted in rapid disintegration. The degree of deacetylation determines the extent of moisture absorption.
- ★ Thanoo *et al.* (1992) prepared the chitosan microspheres by emulsion technique, where cross linking of chitosan solution in paraffin oil as an external medium with glutaraldehyde was done using dioctyl sulfosuccinate as the stabilizing agent. Addition of stabilizing agent during formation produces microspheres with spherical geometry and smooth surface. Microspheres formed have encapsulation efficiency up to 80% for theophyllin, aspirin or griseofulvin.
- ★ Jameela *et al.* (1998) prepared smooth, highly spherical, cross-linked chitosan microspheres in the size range of 45-300 μ m for the controlled release of progesterone. An aqueous acetic acid dispersion of chitosan containing progesterone was emulsified in the dispersion medium consisting of liquid paraffin and petroleum ether stabilized by using sorbitan sesquioleate and the droplets were hardened by glutaraldehyde cross-linking. Encapsulation efficiency of these microspheres is up to 35-70% for steroids.
- ★ Al-Helw *et al.* (1998) used glutaraldehyde extracted in toluene as a cross-linking agent to prepare chitosan microspheres encapsulated with phenobarbitone, which produced uniform and spherical microspheres with loading efficiency up to 57.2%. In the first hour 20-30% of the drug was released and release was faster from small sized microspheres i.e., 75-95% of drug was released within 3 hours.
- ★ Genta *et al.* (1998) described a very simple coacervation method for the preparation of chitosan beads. They prepared chitosan solution in aqueous acetic acid and then solution was blown into NaOH or NaOH-methanol using a compressed air nozzle. Thus tiny droplets or coacervates were obtained, which were finally filtered and washed with hot and cold water successively. Chitosan beads formed possess porous surfaces and are applicable in various fields such as immobilization of protease, carriers for the cancer drug adriamycin, oral sustained delivery of nifedipine, ampicillin and various steroids.

- ★ Lorenzo-Lamosa *et al.* (1998) prepared the micro-encapsulated chitosan microspheres for colonic delivery of sodium diclofenac. Sodium diclofenac was entrapped into chitosan microcores by spray-drying and then micro-encapsulation into Eudragit R -100 and Eudragit R S-100 using an oil-in-oil solvent evaporation method. Chitosan microspheres of 1.8-2.9 μm sizes were prepared.
- ★ B.Conti *et al.* (2000) prepared microparticles composed of chitosan and designed as powders for topical wound-healing properties. Both blank and ampicillin loaded microspheres were prepared by spray-drying method, which show good wound healing properties in albino rats.
- ★ Singla and Chawla (2001) reported that chitosan is an excellent viscosity enhancing agent in an acidic environment and acts as a pseudo plastic material, exhibiting a decrease in viscosity with increasing rates of shear.
- ★ S, Ozbas-Turan *et al.* (2002) prepared chitosan microspheres loaded with recombinant human interleukin-2 (rIL-2) by dropping recombinant interleukin-2 with sodium sulfate solution in acidic chitosan solution. The recombinant human interleukin-2 was released from microspheres in a sustained manner for up to 3 months.
- ★ Huang *et al.* (2002) prepared chitosan microspheres by spray-drying method using type-A gelation and ethylene oxide-propylene oxide block copolymer as modifier. Betamethasone disodium phosphate-loaded microspheres demonstrated good drug stability, 95% entrapment efficiency and 37.5 mV surface charge.
- ★ Radi Hejazi *et al.* (2003) investigated the chitosan based gastrointestinal delivery system. They concluded that the physical and chemical properties of chitosan, such as inter and intramolecular hydrogen bonding and the cationic charge in acidic medium makes the polymer attractive for the development of conventional and novel pharmaceutical product. Being a bioadhesive polymer and having antibacterial activity, chitosan is a good candidate for oral cavity drug delivery.
- ★ Agnihotri and Aminabhavi (2004) developed a simple yet novel method to produce chitosan microparticles. Microparticles were prepared by cross-linking chitosan to obtain non-sticky glassy hydrogels followed by passing through a sieve; the microparticles were washed with 0.1N NaOH solution to remove un-reacted excess glutaraldehyde and dried overnight in the oven at

40°C. Clozapine was incorporated into chitosan before cross linking with the entrapment efficiency up to 98.9%. Average particle size was in the range 543-698 μm and in vitro release was extended up to 12 hours, while in vivo release of Clozapine was slow.

- ★ Said *et al.* (2004) reported the preparation of hydrogels of CMC by grafting CMC with acrylic acid in presence of electron beam irradiation, in aqueous solution. Electron beam is used to initiate the free radical polymerization of acrylic acid on the backbone of CMC. Irradiation of CMC and acrylic acid produces free radicals that can combine to produce hydrogels. They proposed the application for the recovery of metal ions such as copper, nickel, cobalt and lead and in dressing for temporary skin covers.
- ★ Cai *et al.* (2005) reported the preparation of thermo- and pH- sensitive hydrogels by graft copolymerization of chitosan and N-isopropylacrylamide. The results showed that graft efficiency and graft percentage increased with the increase of monomer concentration and total irradiation dose. These hydrogels showed good thermo- and pH-sensitivity and swelling property.
- ★ Spinelli *et al.* (2008) reported the preparation of hydrogels by chemical grafting, in which macromolecular backbones are activated by the action of a chemical reagent. Starch grafted with acrylic acid by using N-vinyl-2-pyrrolidone is used to produce hydrogels, which show excellent pH-dependent swelling behavior and possess ideal characteristic to be used as drug and vitamin delivery devices in the small intestine.
- ★ James Blanchette *et al.* (2010) showed that bleomycin, a chemotherapeutic agent, glycopeptide antibiotic used for the last 30 years in chemotherapy when loaded in a hydrogel nano spheres was able to enhance the permeability of an in vitro model of the intestinal epithelium. The enhancement of permeability of the intestinal epithelium aided the transport of bleomycin from the lumen of small intestine out into the blood stream.
- ★ Y. Zhao *et al.* (2010) used collagen hydrogels as a matrix material to generate the engineered hepatic units to reconstitute 3D, vascularized hepatic tissue *in vivo*. Thus significant cell engraftment was found upon stacking the hepatic units (hepatocyte/collagen hydrogels constructs) under the subcutaneous space. They have been successfully used for cell encapsulation in neural TE.

- ★ Wu *et al.* (2010) reported a novel class of hybrid core shell nano hydrogels, as a multitasking single nanoparticle system for simultaneous optical temperature sensing, targeted tumor cell imaging and combined chemophotothermal treatment. These nano hydrogels were composed of a Ag-Au bimetallic nanoparticle core coated with PEG based hydrogel as a shell and semi IPN of HA chains as the targeting ligands into the network of gel shell. Due to the small size of the Ag-Au nanoparticles the deep penetration into cells was achieved.
- ★ Kim *et al.* (2012) developed MRI-monitored long term therapeutic hydrogel (MLTH) system using a thermosensitive/magnetic poly (organophosphazene) hydrogels. This magnetic hydrogel showed its potential as a biodegradable imaging platform for anticancer drugs. The surgical reaction needed for the implantable Gliadel wafers containing carmustin was rectified by an injectable MLTH system, which provides an easy route of administration and distinguished it from surgical implantation.
- ★ Wang *et al.* (2012) pursued “epicortical delivery” by using Hyaluron/HAMC hydrogel for release of erythropoietin to induce endogenous neural stem cells and progenitor cells, which effectively repair after stroke injury in mouse brains. These promising results gave a potential invasive mode of delivery of erythropoietin to the brain. Thus, hydrogels based drug delivery vehicles can provide sustained, site specific delivery to CNS.
- ★ Kong *et al.* (2013) used nano hydrogels based on pneumococcal surface protein A (PsPA) intranasal vaccines, which prevents invasive disease and colonization of *streptococcus pneumonia*. This hydrogel was developed using a cationic cholesteryl group bearing pullulan group (CCHP). A reduced colonization and invasion by bacteria in the upper and lower respiratory tract was observed. Pneumococcal surface protein A (PsPA) was not delivered to either the olfactory bulbs or central nervous system during the nasal delivery.
- ★ L. Weng *et al.* (2013) prepared the hydrogels of carboxymethyl chitosan. These hydrogels were utilized by vascular intervention in kidney that involves embolization and drug delivery.
- ★ B.Chen *et al.* (2013) demonstrated the use of semi-permeable alginate based hydrogels for the encapsulation, storage and release of stem cells. Human mesenchymal stem cells (hMSC) and mouse embryonic stem cells (mESCs) were successfully stored inside these gels in an air tight sealed ceyovial. The survival rate of these cells was favorable as compared to cryopreservation.

C **HAPTER 3**

RESEARCH ENVISAGED

Controlled release drug delivery is most preferred and convenient option as controlled release route provides maximum active surface area among all drug delivery system for administration of various drugs. This allows the drug to be released carefully to the specific treatment site through the choice and formulation of various drug-polymer combinations. Using innovative microencapsulation technologies, and by varying the copolymer ratio, molecular weight of polymer etc., hydrogels and microspheres can be developed into an optimal drug delivery system which will provide the desired release profile. Being small in size, they have large surface to volume ratio and can be used for controlled release of insoluble drugs (Sinha et al. 2004).

3.1 Selection of drug

The efficacy of many drugs is often limited by their potential to reach the site of therapeutic action. Design of controlled release product is normally a very difficult task because of the interplay of physicochemical and biological properties of the drug, the patient's disease state and the technologies limitation in the fabrication of the final dosage form.

Properties of drug

- Molecular size: Large molecular size may be unsuitable for a sustained release system because they show small diffusion coefficient.
- Dose size: The therapeutic dose of drug should be low.
- Drug stability: Drug used for sustained drug delivery should be stable over the entire length of gastrointestinal tract.
- Partition coefficient: Extremes in partition coefficient of drug are undesirable.
- Biological half-life: A drug with short half-life requires frequent dosing and this makes it a desirable candidate for sustained release formulations.
- Absorption: Drugs that are slowly absorbed or absorbed with variable absorption rate are poor candidates for sustained release formulations.
- Distribution: Drugs with high apparent volumes of distribution are poor candidates.

- Therapeutic index: Drugs with narrow therapeutic index require precise control over the blood levels of drug placing a constraint on controlled release dosage form.

3.2 Ampicillin

Ampicillin is an antimicrobial agent with a wide spectrum of activity. It is free flowing, white crystalline powder. It has an odor characteristic of penicillin.

3.2.1 Mechanism of action

Ampicillin belongs to the penicillin group of beta-lactum antibiotics, which is able to penetrate gram positive and gram negative bacteria. Ampicillin acts as an irreversible inhibitor of the enzyme transpeptidase, which is needed by bacteria to make their cell walls. It inhibits the third and final stage of bacterial cell wall synthesis in binary fission, which ultimately leads to cell lysis (Petri 2011).

3.2.2 Pharmacokinetics

Ampicillin is well absorbed in gastro-intestinal tract. C_{max} is approximately $3\mu\text{g/ml}$ (500mg/capsules) and $3.4\mu\text{g/ml}$ (500mg oral suspension). Oral absorption of ampicillin is found to be $35\%\pm 5$. Volume of distribution is found to be 0.38 L/kg and plasma protein binding is 15-25%. Presystemic metabolism is noted to be $5\%\pm 5$. Renal excretion accounts for 70-80% and plasma half-life is 1-2 hour. Orally its bioavailability is 40% and metabolism rate is noted to be 12-50 % (Hauser 2013).

3.2.3 Drug description

Ampicillin [6-[d-(-)- α -aminophenyl acetamido] penicillanic acid] is an antibiotic useful for the treatment of number of bacterial infections. It is beta-lactum antibiotic that is a part of the aminopenicillin family. The common brand name is principen. It is free flowing, white crystalline powder. It has molecular weight 349.41 g/mol . The empirical formula of ampicillin is $\text{C}_{16}\text{H}_{19}\text{N}_3\text{O}_4\text{S}$ and its chemical structure is as follows:

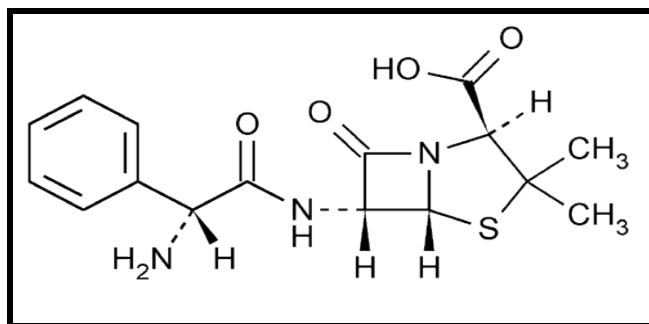


Figure 4: Chemical structure of Ampicillin (Akova, M., 2008)

Ampicillin is available in three forms, viz., ampicillin trihydrate, ampicillin anhydrous and ampicillin sodium. Ampicillin trihydrate is most widely used form, which is taken orally and is stable in acid environment. Ampicillin sodium is given as an injection (Rapson et al. 1968; Grant 1961). It comes as a capsule, liquid and pediatric drops to be taken by mouth. It is usually taken every 6 hours. Its preparation is in the form of capsule (250 and 500 mg), powder oral suspension (125 and 250 mg/5ml), and powder for injection (250 mg, 500 mg, 1g, 2g). The formulation, strength and duration of treatment depends on severity of infection and the causative microorganism.

3.2.4 Clinical use of ampicillin

Ampicillin is an antibiotic which is effective against gram positive bacteria (such as *Streptococcus pneumonia*, *Streptococcus pyogenes*, *Staphylococcus aureus* and some *Enterococci*) and gram negative bacteria (such as *Neisseria meningitides*, some *Haemophilus influenza*, and some *Enterobacteriaceae*). Spectrum of activity is enhanced by co-administration of sulbactam, a drug that inhibits beta lactamase, and enzyme produced by bacteria to inactivate ampicillin and related antibiotics (Hauser 2013)

It is used in gastrointestinal tract infection, genitourinary tract infection, meningitis, respiratory tract infection, septicemia and soft tissue infection. It is a drug of first choice against *streptococcus faecalis* (biliary tract and intestinal infection), *haemophilus influenza* (respiratory tract infection) and *proteus mirabilis* (urinary tract infection) (Finberg et al. 2012).

3.2.5 Adverse effects

Ampicillin is relatively non-toxic. The most common side effects include rash, diarrhea, nausea and vomiting. In very rare cases, it causes severe side effects such as anaphylaxis and *Clostridium difficile* diarrhea.

3.2.6 Precautions

Ampicillin is contraindicated in patients with infectious mononucleosis and hypersensitivity and also in infections caused by penicillinase producing organisms.

- Nursing Mothers: Ampicillin-class antibiotics are excreted in milk. If used by nursing mothers may lead to sensitization of infants; therefore, a decision should be made whether to discontinue nursing or to discontinue ampicillin, taking into account the importance of the drug to mother.
- Pediatric use: Penicillins are excreted primarily unchanged by the kidney; therefore, the incompletely developed renal functioning neonates and young infants will delay the excretion of penicillin. The administrations of ampicillin should be limited to neonates and young infants.
- Labor and delivery: Oral ampicillin-class antibiotics are poorly absorbed during labor. Intravenous administration slightly decreases the uterine tone and frequency of contraction, but moderately increases the height and duration of contractions. Thus forceps delivery of newborn will be necessary.

3.3 Selection of polymer

Polymers are becoming increasingly important in the field of drug delivery. An appropriate selection of the polymer matrix is necessary in order to develop a successful drug delivery system. The polymer could be non-biodegradable or biodegradable. Biodegradable polymers are highly desirable in long term drug delivery situation because they degrade in the body to biologically inert and compatible molecules. By incorporating drugs in biodegradable polymers, dosage forms that release the drug over a prolonged length of time can be prepared in variety

of shapes and sizes. As a result biodegradable polymers offer a novel approach for developing sustained release drug delivery system that are simple and convenient to patients.

3.3.1 Advantages of biodegradable polymer as drug delivery

Polymer is a large molecule composed of many smaller units called monomers that are bonded together. Advantages of polymeric drug delivery system are: localized delivery of drug, sustained delivery of drug, stabilization of the drug, release rate which is less dependent on drug properties and steady release rate with time (Jones David 2007).

3.3.2 Development of polymer

The development of appropriate carriers for drug delivery is a challenge for biomedical scientists. Peptides, proteins, oligonucleotides, and genes are unstable compounds that need to be protected from degradation in the biological environment. Moreover, their efficacy is highly limited by their ability to cross biological barriers and reach the target site. The future of these molecules as therapeutic agents clearly depends on the design of an appropriate carrier for their delivery to the body. Several studies have been reported so far in the development of these carriers, among which the design of biodegradable nanoparticles has drawn considerable interest. In particular, nanoparticles made of hydrophobic or amphiphilic polymers such as poly (lactide-co-glycolide) (PLGA) and poly (lactic) acid (PLA)-poly (ethylene) glycol (PEG) offer great promise (Blanco and Alonso 1997; Tobio et al. 2000). But these nanoparticles have a limitation in their preparation procedure, which requires the use of organic solvents and surfactants as well as sonication. Challenging alternatives to these hydrophobic particles are colloidal carriers made of hydrophilic polymers. For this application, chitosan has come to be a particularly interesting polymer for the association and delivery of labile macromolecular compounds (Prabaharan 2005). Among water-soluble polymers available, chitosan is one of the most extensively studied.

3.4 Chitosan

Chitosan is a natural, cationic aminopolysaccharide copolymer of glucosamine and N-acetylglucosamine obtained by the alkaline, partial deacetylation of chitin. It is second most abundant natural polysaccharide and originates from shells of crustaceans such as crabs, shrimps and lobsters. Chitosan is also found in some microorganisms, yeast and fungi (Illum

1998). Chitosan is biodegradable, biocompatible, positively charged non-toxic mucoadhesive biopolymer (Pillai et al. 2009). Chitosan is used because of its property to improve the solubility of poorly water soluble drugs (Sawayanki et al. 1982) as well as to control the release of drugs by slow erosion from hydrated matrix (Shu et al. 2000). Its natural mucoadhesive properties allow design of bioadhesive drug carrier systems that can bind to the intestinal mucosa, and thus improve the residence time of drugs in the intestinal lumen and consequently, their bioavailability (Staddon 1995). Chitosan is available in a wide range of molecular weight and degree of deacetylation. Molecular weight and degree of deacetylation are the main factors affecting the particle size, particle formation and aggregation. The gelation property of chitosan offers diverse use including microencapsulation and controlled release via microparticulate system (Agnihotri and Aminabhavi 2004).

3.4.1 Chitosan sources and chemical structure

Chitosan is found in the exoskeleton of some anthropods, insects and some fungi. Commercial source of chitin are the shell wastes of crab, shrimp, lobster, etc. chitosan is usually prepared by the deacetylation of chitin. Chitosan is linear copolymer polysaccharide consisting of β (1-4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) units (figure 3.2). The properties, biodegradability, and biological role of chitosan is frequently dependent on the relative proportions of N-acetyl-D-glucosamine and D-glucosamine residues (Rinaudo 2006).

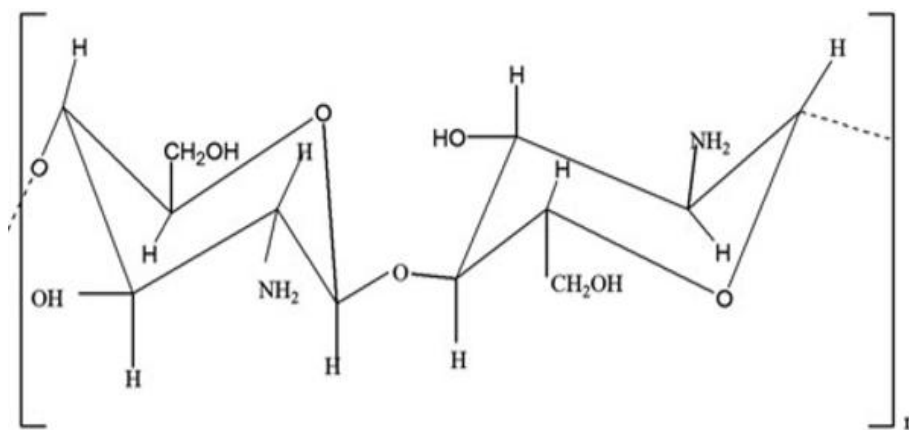


Figure 5: Chemical structure of chitosan (Rinaudo 2006)

3.4.2 Preparation of chitosan

Preparation of chitosan involves four steps: deproteinization, demineralization, decolorization and deacetylation. Deproteinization is carried out by alkaline treatment using 3-5% NaOH (w/v) aqueous solution at room temperature overnight. Remaining constituents are removed by treatment with 3-5% aqueous HCl (w/v) solution at room temperature for 5 hours. The product is again reacted with 40-45% NaOH solution at 120°C for 4-5 hours. This treatment gives the crude sample of chitosan. The crude sample is purified by precipitation of the chitosan from its aqueous acetic solution to NaOH and washing with distilled water until neutralized (Felt 1998).

3.4.3 Physiological and biological properties of chitosan

Chitosan is a semi-crystalline polymer that exhibits polymerization. Chitosan belongs to a series of polymers with different degree of deacetylation and molecular weight (Jayakumar 2010), which are two important properties of chitosan. Typical commercial chitosan has degree of deacetylation of 70-95% and molecular weight of 10-1000 kDa.

Chitosan appears as colorless, odorless flakes. It is readily soluble in aqueous acidic solution. The solubilization occurs through protonation of amino groups on the C-2 of D-glucosamine residues, whereby polysaccharide is converted into polycation in acidic media. Chitosan has low physiological pH of 7.4 as it is a weak base (pKa 6.2-7) (Jayakumar 2009). The viscosity of chitosan solution increases with an increase in chitosan concentration and degree of deacetylation. It possesses good complexing capacity, as it can complex with oppositely charged polymers such as poly(acrylic acid), alginate, sodium salt of poly(acrylic acid), carboxymethyl cellulose, pectin etc. The biological properties of chitosan are shown in figure 6.

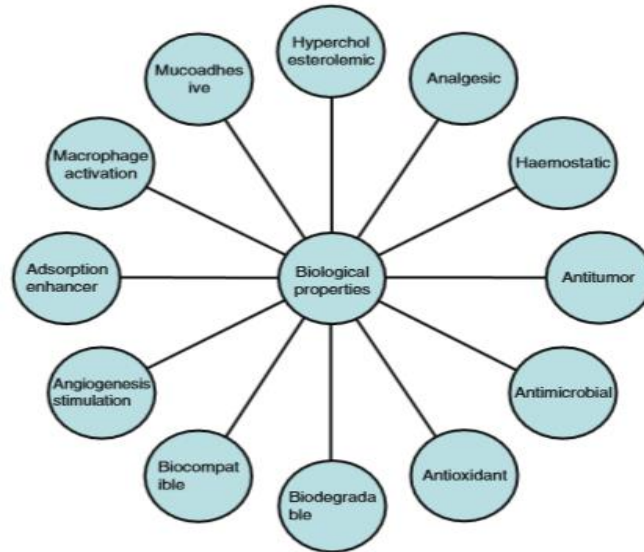


Figure 6: Biological properties of chitosan (Jayakumar 2009)

3.4.4 Usage of chitosan

- Agricultural: Reduce environmental stress due to drought and soil deficiencies, strengthen seed vitality, increase yields, and reduce fruit decay of vegetables, fruits and citrus crops (Linden and Stoner 2007).
- Horticulture: Increase blooms and extend the life of cut flowers and Christmas trees.
- Water filtration: Removes heavy minerals, phosphorous and oil from the water; precipitates caseins from bovine milk and cheese making (Alan Woodmansey 2002; Ausar et al. 2002); clarify wine, mead (alcoholic beverage created by fermenting honey with water, and frequently fruits, spices, grains or hops) and beer (Rayner Terry 2006).
- Winemaking and fungal source chitosan: Fining agent in winemaking (Quintela 2012); increase setting activity, reduction of oxidized polyphenolics in juice and wine, chelation and removal of copper (Escudero-Abarca et al. 2004).
- Biomedical use: Medical bandages and other hemostatic agents, contact lens and other equipments (Brown et al. 2009)
- Food industry: As preservatives and color stabilizer.
- Preliminary research: enzyme immobilization, cell recovery, chromatography.

3.5 Crosslinking agent-Glutaraldehyde

Glutaraldehyde, a linear 5-carbon dialdehyde is a clear, colorless to pale straw-colored, pungent oily liquid that is soluble in all proportions in water and alcohol, as well as organic solvents. It is mainly available as acidic aqueous solutions (pH 3.0-4.0), ranging in concentration from less than 2% to 70% (w/v). Glutaraldehyde has had great success because of its commercial availability and low cost in addition to its high reactivity. It reacts rapidly with amine groups at around neutral pH (Okuda et al. 1991). Other names of glutaraldehyde are 1,5-pentanedial, 1,3-diformylpropane and Glutaral. Chemical structure of glutaraldehyde is shown in figure 7.

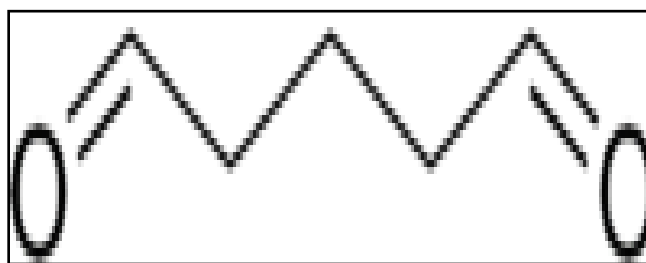


Figure 7: Chemical structure of glutaraldehyde (Christian Kohlpaintner et al. 2005)

The cross linking reaction is mainly influenced by the size and type of cross linker agent and the functional groups of chitosan (Ross-Murphy et al. 1994). The entrapment efficiency is slightly higher ($P < 0.05$). *In vitro* studies showed that chitosan particles crosslinked with glutaraldehyde showed slower release rate of drug.

3.5.1 Production of glutaraldehyde

It is produced industrially by the oxidation of cyclopentene and by the Diels-Alder reaction of acrolein and methyl vinyl ether followed by hydrolysis. It exists in the form of hydrates, which have several structures, shown in figure 8 (Eral et al. 1974).

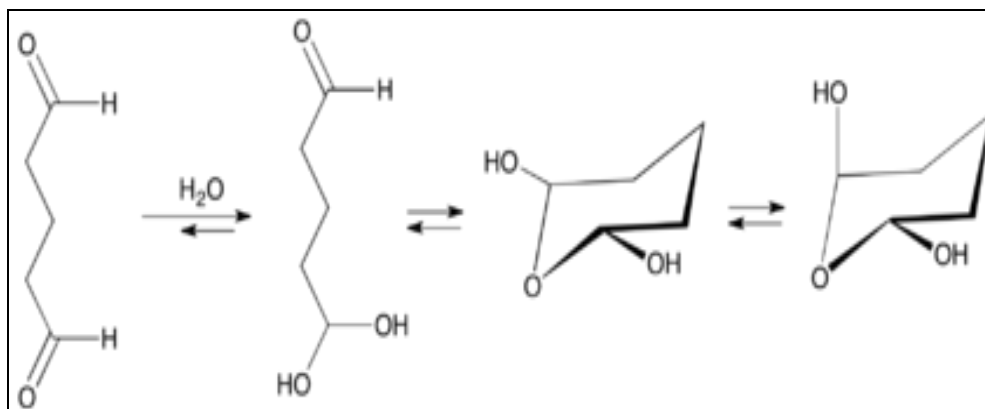


Figure 8: Structures of glutaraldehyde (Eral et al. 1974)

3.5.2 Usage

- Fixative: For system disinfection, preservation for long term storage, inactivation of bacterial toxins to create toxoid vaccines, tanning of leather and in biological electron microscopy (Karnovsky 1965).
- Biological reagent: Biochemistry applications such as an amine-reactive homobifunctional crosslinker to examine oligomeric state of proteins; SDS-PAGE to fix proteins and peptides.
- Wart treatment: “Diswart solution” to remove common and plantar warts.
- Hydraulic Fracturing Fluid

3.6 Sorbitan Monooleate (Span 80)

Sorbitan Monooleate is a light yellow viscous oily liquid, which is insoluble in water and soluble in organic solvents. It is primarily used as an emulsifier to keep water and oils mixed (water/oil type emulsion). The molecular formula of span 80 is $C_{24}H_{44}O_6$ and molecular weight is 428.6 g/mol. The chemical structure of span 80 is shown in figure 9.

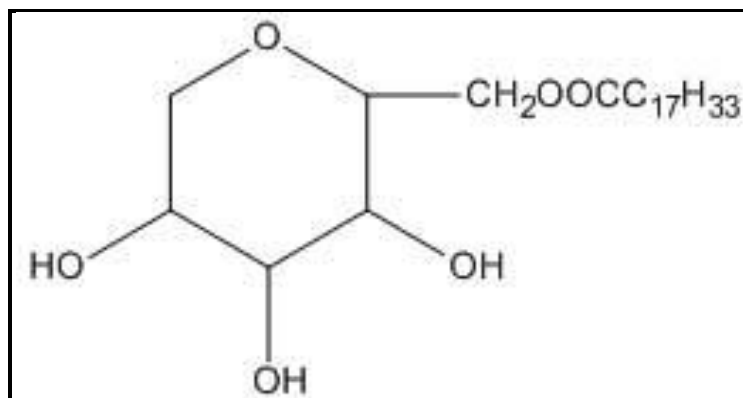


Figure 9: Chemical structure of span 80

3.6.1 Specification

Table 5: Specifications of Span 80

Item	Index
Acid value	$\leq 8\text{mgKOH/g}$
Saponification value	145-160mgKOH/g
Hydroxyl value	193-210mgKOH/g
Water	$\leq 2.0\%$

3.6.2 Uses

Span 80 is used as emulsifier, solubilizer, stabilizer, softener, anti-static agent etc. suitable for medicine, cosmetic, textile, paints etc.

3.6.3 Storage

Span 80 should be stored in cool, dry, and draughty place. Shelf life is 2 years.

3.7 Light liquid paraffin oil

Light liquid paraffin oil is highly purified mixture of liquid saturated hydrocarbons obtained from petroleum and is highly paraffin in nature. It is transparent and free from fluorescence in

daylight. It is colorless, tasteless and odorless when cold. It is highly refined hydro-treated oil which has excellent thermal and chemical stability, having high flash point and is soluble in chloroform and solvent ether.

3.7.1 Applications

It is mainly used as external medium in the preparation of chitosan based microspheres. It may also be used in the manufacture of pharmaceuticals and cosmetic products like creams, lotions, perfumery industry, bulk drugs and food industry etc.

C **HAPTER 4**

EXPERIMENTAL WORK

EXPERIMENTAL WORK

4.1 Experimental Material and Equipments

Various chemicals and instruments used for the preparation and evaluation of cross-linked hydrogels and microspheres are listed in table below:

Table 6: Experimental materials

Experimental materials	
❖ Chitosan (85% deacetylated, Sigma-Aldrich)	❖ Acetic acid
❖ Ampicillin Sodium	❖ Buffer (pH 1.2)
❖ Glutaraldehyde (25%)	❖ Buffer (pH 6.8)
❖ Light liquid paraffin oil	❖ Sodium hydroxide
❖ Sorbitan monooleate (Span 80)	❖ Conc. Hydrochloric acid
	❖ Distilled water

Table 7: Experimental equipments

Experimental equipments	
❖ Magnetic stirrer	❖ Disposable syringe
❖ Magnetic bead	❖ Glass Beakers (100ml, 150ml, 200ml, 250ml)
❖ Analytical balance	❖ Measuring Cylinders
❖ Optical microscope	(100ml, 500ml, 1000ml)
❖ UV-Visible Spectrophotometer	❖ Micropipette (200µl, 1 ml)
❖ Incubator	❖ Glass Flasks (100 ml)
❖ Shaking incubator	❖ Petriplate
❖ Nikon SMZ 800 microscope	

4.2 Preparation of standard curve of Ampicillin Sodium

The concentration of ampicillin sodium was estimated at 222.2nm by stable beam spectrophotometer. The standard curves were prepared in distilled water, pH 1.2 and pH 6.8 buffer.

4.2.1 Preparation of buffer (pH 1.2)

50 ml of potassium chloride solution (0.2 M) was mixed in 85 ml of 0.2 M of hydrochloric acid and then distilled water was added to make the volume to 200 ml.

4.2.2 Preparation of buffer (pH 6.8)

50 ml of potassium dihydrogen phosphate (0.2 M) was mixed in 22.4 ml of 0.2 M of sodium hydroxide and then the volume was made up to 200 ml with distilled water.

4.2.3 Preparation of standard stock solution of ampicillin sodium

Standard Stock Solution (1 mg/ml): 100 mg of accurately weighed drug was dissolved in distilled water and volume was made up to 100 ml with distilled water.

Working solution: Aliquots of 0.1 ml to 0.5 ml was pipetted out from the standard stock solution and volume was made up to 10 ml with distilled water to obtain solution containing desired concentration of ampicillin sodium ranging from 10µg/ml to 50µg/ml. The absorbance was measured at 222.2 nm against distilled water as blank, using stable beam spectrophotometer. Similarly, standard stock solution and working solution was prepared in buffer pH 1.2 and buffer pH 6.8 and standard curves were plotted based on the data.

4.2.4 Standard curves

Table 8: Standard curve data of ampicillin sodium in water

S. no.	Concentration (µg/ml)	Absorbance (222.2nm)
1	10	0.114
2	20	0.271
3	30	0.396
4	40	0.517
5	50	0.684

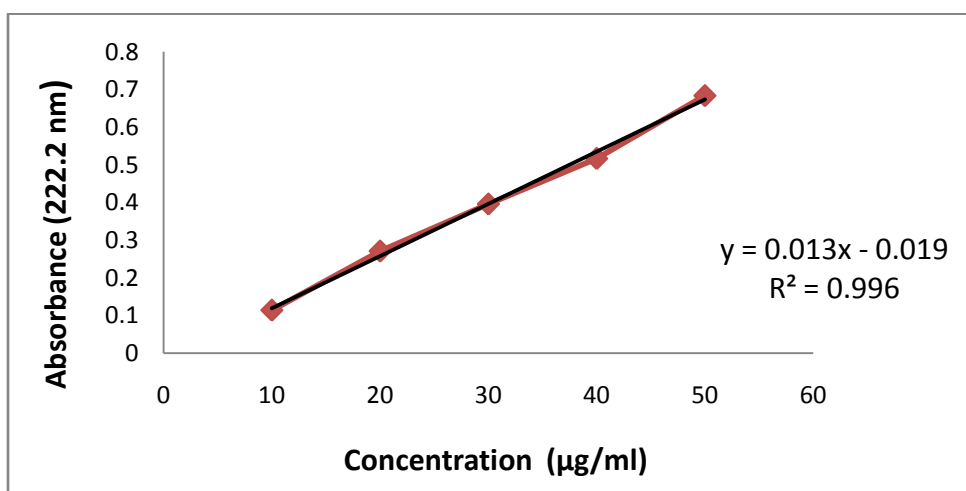


Figure 10: Standard curve of ampicillin sodium in water

Table 9: Standard curve data of ampicillin sodium in buffer pH 1.2

S. no.	Concentration (µg/ml)	Absorbance (222.2nm)
1	10	0.180
2	20	0.303
3	30	0.438
4	40	0.559
5	50	0.656

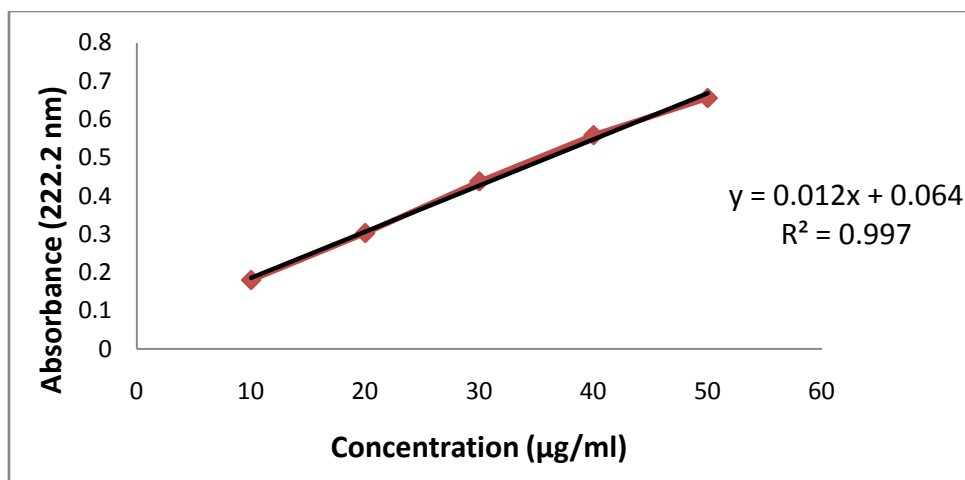


Figure 11: Standard curve of ampicillin sodium in buffer pH 1.2

Table 10: Standard curve data of ampicillin sodium in buffer pH 6.8

S. no.	Concentration (µg/ml)	Absorbance (222.2nm)
1	10	0.135
2	20	0.310
3	30	0.429
4	40	0.580
5	50	0.723

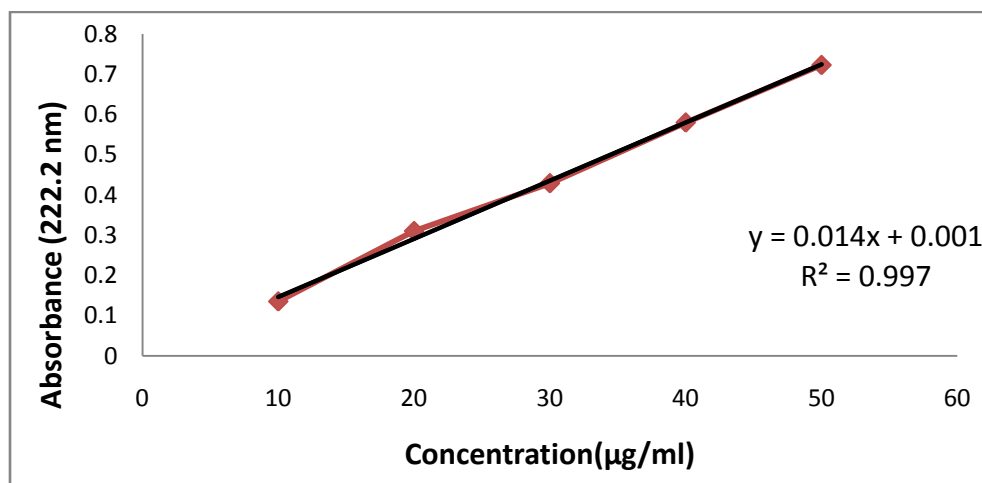
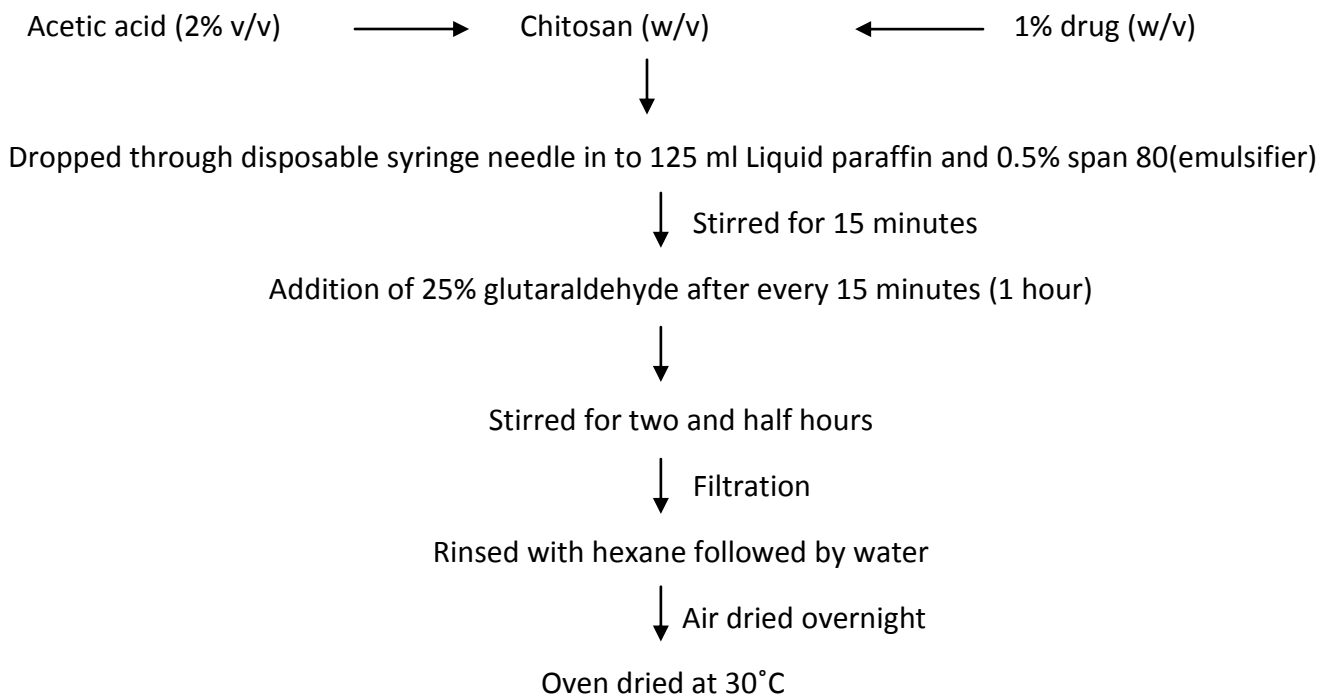


Figure 12: Standard curve of ampicillin sodium in buffer pH 6.8

4.3 Preparation of chitosan microspheres

Chitosan microspheres were prepared by Emulsion cross-linking method (Akbuga et al. 1994). This method utilizes the reactive functional amine group of chitosan to cross-link with aldehyde groups of the cross-linking agent.

In this method, chitosan solution (1%, 1.5%, 2%, 2.5% w/v) was prepared by dissolving chitosan in acetic acid (2% v/v) at room temperature. The drug ampicillin sodium (1% w/v) was dissolved directly into the above prepared chitosan solution. 20ml solution was dropped through a disposable syringe needle into gently agitating 125 ml light liquid paraffin containing 0.5% span 80 as an emulsifier. The dropping rate and falling distance were kept constant. The above water-in-oil emulsion was stirred for 15 minutes. Then glutaraldehyde (25% v/v) was added dropwise after every 15 minutes, for an hour. The total volume of glutaraldehyde added was 2.5ml. The solution was magnetically stirred for two and half hours, followed by filtration and rinsing with hexane and water. Microspheres obtained were air dried overnight for 24 hours followed by oven drying at 30°C.



Different batches of crosslinked chitosan microspheres were prepared by altering the following formulation variables:

Table 11: Formulations variables for preparation of different batches of microspheres

Concentration of chitosan	(1%, 1.5%, 2%, 2.5% w/v)
Drug concentration	(1% w/v)
Volume of 25% v/v Glutaraldehyde	2.5ml
Volume of chitosan-drug solution	20 ml
Total volume of solution	147.5ml
Stirring time	2.5 hours

Table 12: List of formulation of cross-linked chitosan microspheres

Batch no.	Conc. of Chitosan (% w/v)	Conc. of drug (% w/v)	volume of Glutaraldehyde (25% v/v) (ml)	Stirring time (hours)	Volume of Chitosan-Drug Solution (ml)
M1	1	1	2.5	2.5	20
M2	1.5	1	2.5	2.5	20
M3	2	1	2.5	2.5	20
M4	2.5	1	2.5	2.5	20

4.4 Preparation of chitosan based hydrogels

Hydrogels were prepared by chemical cross-linking method. In this chitosan stock solution (1%w/v) was prepared by dissolving chitosan in acetic acid (1% v/v) at room temperature. The drug ampicillin sodium (1% w/v) was dissolved directly into the above prepared chitosan solution. The solution was properly dissolved using magnetic stirrer. Glutaraldehyde (25%, 40%, 50%, 60% v/v) was added to the above chitosan solution. The solution was magnetically stirred

for 15 minutes to form a gelly type liquid. Then it was washed with distilled water to remove extra glutaraldehyde. The gel was poured in to petriplate and kept overnight for cross-linking at room temperature.

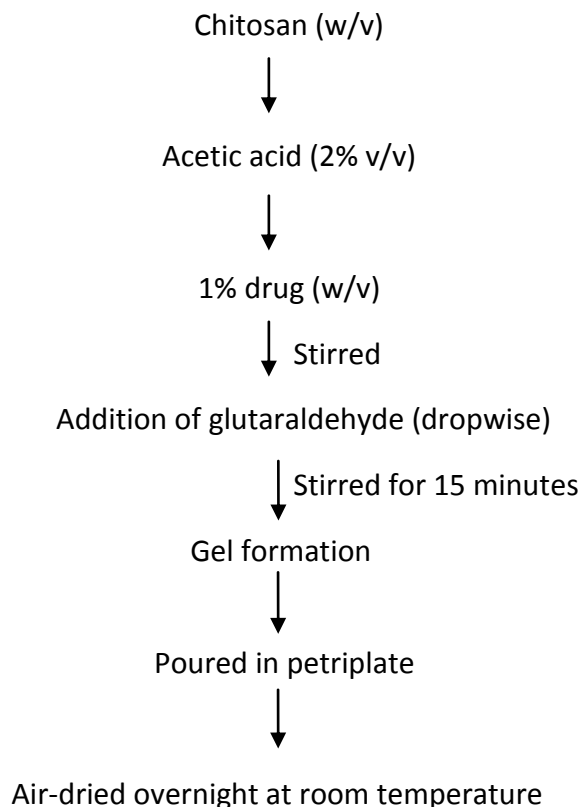


Table 13: Formulation variables for preparation of different batches of hydrogels

Concentration of chitosan	(1% w/v)
Drug concentration	(1% w/v)
Concentration of glutaraldehyde	(25%, 40%, 50%, 60% v/v)
Volume of chitosan-drug solution	(10-20ml)
Stirring time	(15 minutes)

Table 14: List of formulations of cross-linked chitosan based hydrogels:

Batch no.	Conc. of Chitosan (%w/v)	Conc. of Drug (% w/v)	Conc. of Glutaraldehyde (% v/v)	Volume of glutaraldehyde (μl)	Stirring Time (minutes)	Volume of Chitosan-Drug Solution (ml)
B1	1	1	25	500	15	10
B2	1	1	25	1000	15	10
B3	1	1	50	500	15	10
B4	1	1	50	1000	15	10
B5	1	1	25	1000	15	20
B6	1	1	50	1000	15	20
B7	1	1	50	2000	15	20
B8	1	1	50	200	15	20
B9	1	1	50	800	15	20
B10	1	1	40	1000	15	20
B11	1	1	40	2000	15	20
B12	1	1	60	1000	15	20
B13	1	1	60	2000	15	20

C **HAPTER 5**

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Microspheres and hydrogels are the most important approaches used for controlled release of drug to the site of action. Microspheres were prepared by emulsion crosslinking method, in which water-in-oil emulsion was prepared by emulsifying the chitosan aqueous solution in oil phase. Hydrogels were prepared by simple chemical crosslinking method, in which chitosan-drug solution was crosslinked with glutaraldehyde solution. Both methods are simple and can be carried out under mild conditions and with simple equipments.

The aim of our present investigation was to study the influence of varying chitosan concentration on the preparation and release of the drug from the microspheres and to study the influence of varying glutaraldehyde concentration on the preparation and release of the drug from the hydrogels.

Different batches of microspheres and hydrogels were prepared by altering the formulation variables, which include concentration of chitosan, concentration of glutaraldehyde and volume of chitosan-drug solution. All the batches of chitosan based microspheres and hydrogels were evaluated for the following properties:

- I. Percentage yield
- II. Average particle size
- III. Equilibrium Swelling studies
- IV. Drug loading
- V. Encapsulation efficiency
- VI. In vitro release studies

5.1 Evaluation of cross-linked chitosan based microspheres

5.1.1 Percentage yield

The prepared microspheres were collected, dried and weighed. The percentage yield was calculated as:

$$\% \text{ age yield (w/w)} = \frac{\text{Weight of dried microspheres recovered}}{\text{Weight of chitosan} + \text{Weight of glutaraldehyde}} \times 100$$

Table 15: Percentage yield of different batches of chitosan based microspheres

Batch no.	Yield (gm)	Percentage yield (%)
M1	0.343	12.03
M2	0.382	12.94
M3	1.038	34.03
M4	1.208	38.34

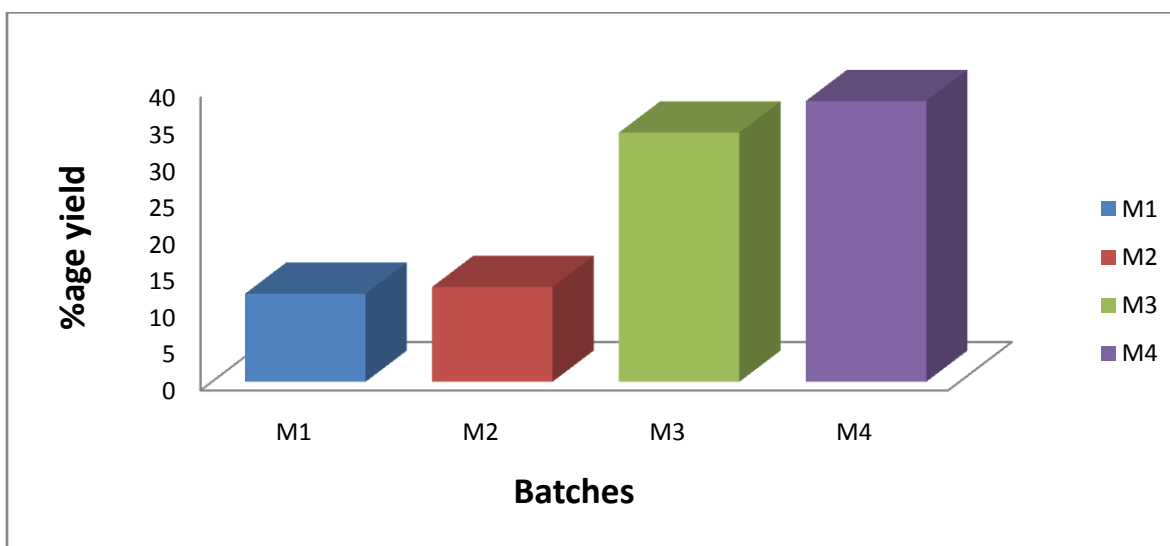


Figure 13: Percentage yield of different batches of microspheres

The percentage yield of microspheres varied from 12.03% to 38.34%. The percentage yield increased on increasing the concentration of chitosan at same glutaraldehyde concentration. As polymer-drug ratio increased, the percentage yield also increased, with maximum yield obtained from batch M4 having 2.5% chitosan concentration.

5.1.2 Average particle size

The particle size of the microspheres was measured directly by using “Nikon SMZ 800 light Microscope”. The chitosan microspheres were mounted on the stage of microscope and diameter of microspheres was measured.

Table 16: Average size of different batches of chitosan microspheres

Batch no.	Average size (μm)
M1	81.6
M2	84.8
M3	89.0
M4	94.1

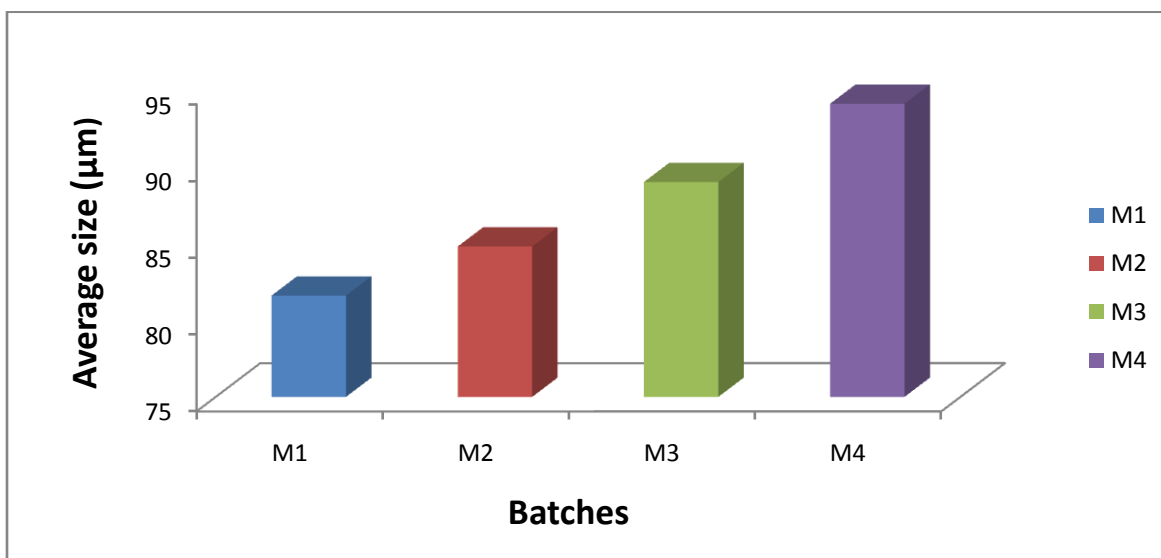


Figure 14: Average size of different batches of microspheres

The average particle size of various batches of cross-linked microspheres varies from 81.6 μm to 94.1 μm . The average particle size increases with increase in chitosan concentration from 1% to 2.5%. Maximum particle size was observed in batch M4.

Topographs of microspheres

The morphology and surface appearance of microspheres was examined by “Nikon SMZ 800 light Microscope”. Pictures are given below in figure 15 (a-d):

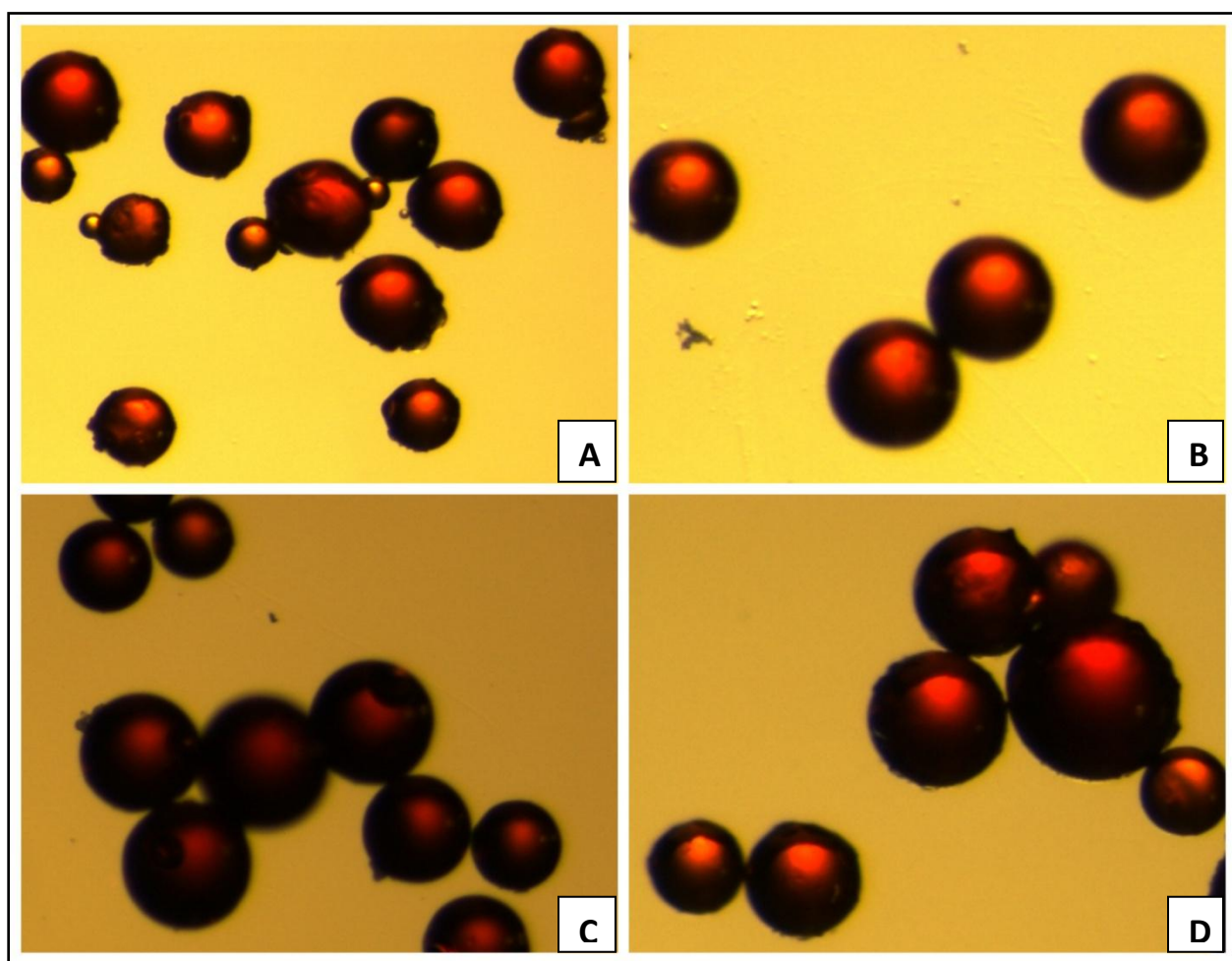


Fig. 15: Microscopic photographs of microspheres (A) Batch 1, (B) Batch 2, (C) Batch 3, (D) Batch 4.

5.1.3. Equilibrium swelling studies

Swelling is the most significant characteristic as it reflects the affinity of chemical structure of microspheres for water and other surrounding fluids. The swelling property of chitosan based microspheres was determined in water. 20 mg of chitosan microspheres were placed in 5ml of water at 37°C, overnight. The swollen microspheres were filtered, blotted (to remove excess moisture adhering to surface) and weighed immediately on weighing balance. The percentage swelling at equilibrium was calculated from formula given below:

$$\% E_{sw} = \frac{W_e - W_o}{W_o} \times 100$$

Where,

E_{sw} = percentage swelling of microspheres at equilibrium

W_o = initial weight of microspheres

W_e = weight of microspheres at equilibrium

Table 17: Percentage swelling of different batches of chitosan microspheres

Batches	% Esw in water
M1	161.05
M2	137
M3	104.55
M4	64.55

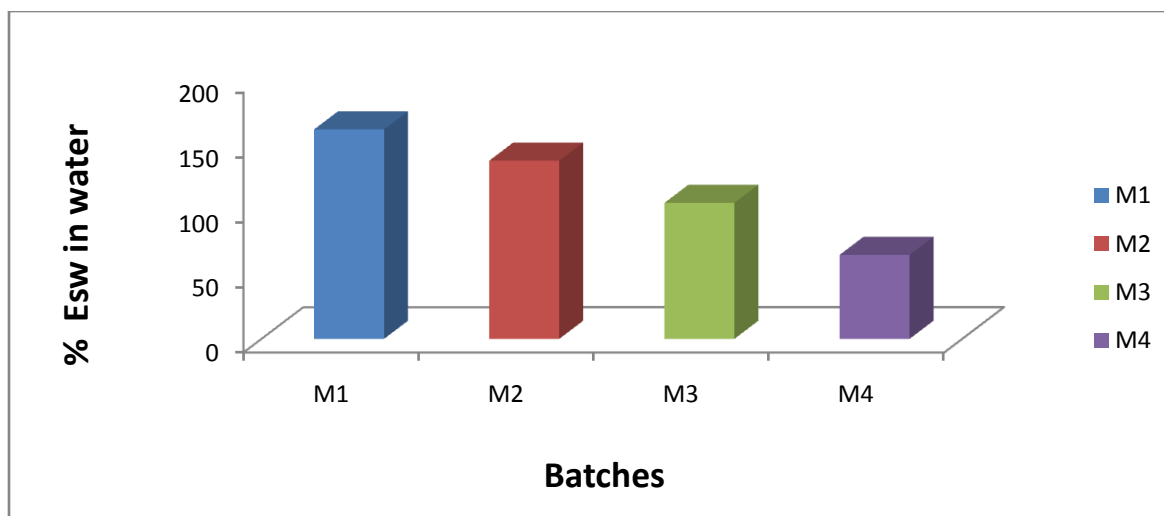


Figure 16: Percentage swelling of different batches of microspheres

Equilibrium swelling studies of various batches of microspheres were done in distilled water. Percentage equilibrium swelling decreases with increase in chitosan concentration from 1% to 2.5%. It is due to increase in polymer concentration, which reduces swelling of the microspheres. Maximum swelling was seen in batch M1 having 1% chitosan concentration and minimum swelling was seen in batch M4 having 2.5% chitosan concentration.

5.1.3 Drug loading into microspheres

Ampicillin was entrapped in microspheres by incubating 20 mg of glutaraldehyde cross-linked chitosan in 1% ampicillin solution in water for 24 hours in glass vials. After 24 hours, the microspheres were filtered and dried (air-drying followed by oven-drying).

5.1.4 Determination of encapsulation efficiency

20 mg of dried ampicillin loaded cross-linked chitosan microspheres were crushed and soaked in water for 24 hours. Then after filtration and subsequent dilution from this, the absorbance was determined spectrophotometrically at 222.2 nm against a suitable blank. The encapsulation efficiency was calculated by expressing the actual entrapment level divided by the theoretical entrapment level, as percentage.

$$\% \text{age Encapsulation efficiency} = \frac{\text{Actual entrapment level}}{\text{Theoretical entrapment level}} \times 100$$

Table 18: Encapsulation efficiency of different batches of chitosan based microspheres

Batch no.	Encapsulation efficiency (%)
M1	167.14
M2	83.57
M3	85.35
M4	69.57

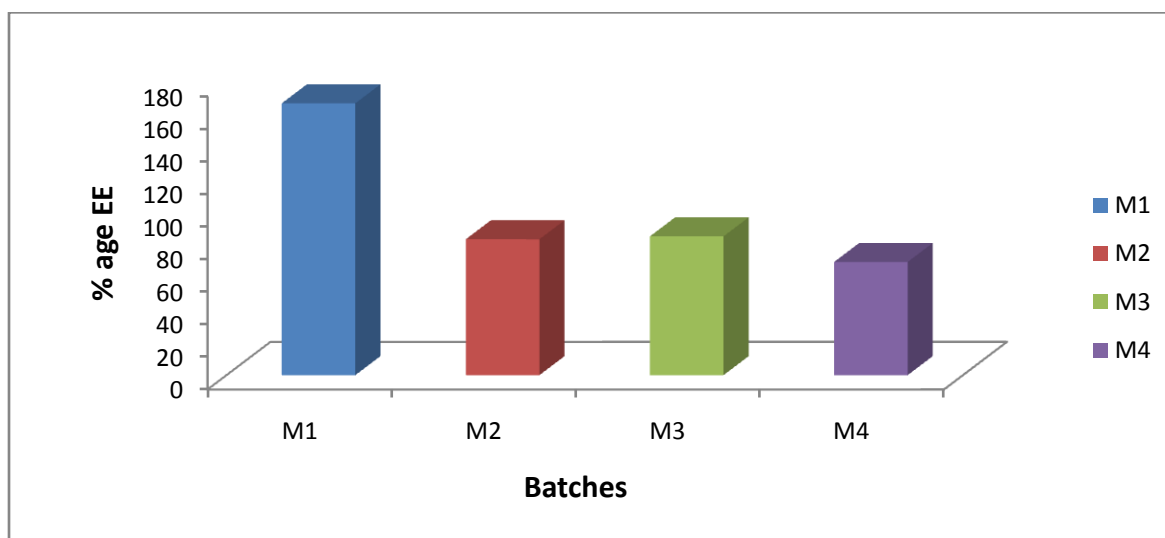


Figure 17: Encapsulation efficiency of different batches of microspheres

The encapsulation efficiency decreased from 167.14 to 69.57%, as the concentration of the polymer was increased. Encapsulation efficiency is highest in the batch M1, where chitosan concentration is 1 %. As the concentration of chitosan increased to 2.5%, the encapsulation efficiency decreased to 69.57%. This may be due to high viscosity of chitosan-drug solution, which forms large inner cavities thus not allowing the drug to diffuse inside the microspheres.

5.1.6 Evaluation of In vitro Drug Release Rate

Based upon the physiological gastrointestinal conditions, the in vitro release study was performed in buffer (pH 1.2) and buffer (pH 6.8). The dissolution behavior of chitosan-glutaraldehyde microspheres was dependent on pH. They swell in buffer (pH 1.2) but didn't

show much change in buffer (pH 6.8). An initial burst effect was observed in all formulations of microspheres, which can be attributed to fast dissolution of drug molecule, attached to the surface, and subsequently slow release phase, during which drug molecule present in the core of microspheres diffused out. The initial burst effect decreased with an increase in polymer concentration. In buffer (pH 1.2), protonation of phosphate ions breaks the hydrogen bonds, leading to weaker electrostatic interaction. This causes swelling of microspheres and higher release of drug in buffer (pH 1.2), while in buffer (pH 6.8), there is strong attractive force between phosphate and chitosan, which causes slower release of drug. Thus biphasic mode of release of drug is achieved due to change in pH of the medium i.e., pH 1.2 causes swelling and pH 6.8 causes deswelling.

100 mg of drug loaded chitosan microspheres were incubated in 100 ml buffer (pH1.2) in a 150 ml conical flask kept at 37°C shaking incubator at 100 strokes per minute. After 3 hours, they were filtered and transferred to 100 ml buffer (pH 6.8) incubated at 37°C in a shaking incubator at 100 strokes per minute. Starting from time 0 hour and at desired intervals of time, 4 ml sample was withdrawn and replaced with same amount of fresh medium. Drug in the release medium was measured spectrophotometrically. Release studies and release profiles of cross-linked chitosan microspheres of various batches are given below.

Table 19: Drug release data of cross linked chitosan microspheres

S. No.	Time (hrs)	Cumulative %age release			
		M1	M2	M3	M4
1	0.25	0.5	0.2	0.96	0.12
2	0.5	4.8	3.5	16.9	1.62
3	0.75	15.9	14.3	28.33	6.33
4	1	42.08	26.8	40.09	11.9
5	2	56.6	53.7	51.9	18.33
6	3	72.8	68.8	64.77	33.6
7	4	85.6	81.1	74.73	40.2
8	5	87	82.6	74.9	41.4

9	6	88.9	83.6	75.34	42.4
10	7	89.2	85	75.81	43.6
11	8	90.2	86.4	76.58	44.3
12	24	91.5	87.7	77.02	45.4

Figure 18 (a-d): The drug release curves of ampicillin sodium from cross-linked chitosan microspheres by varying chitosan conc. (M1-M4)

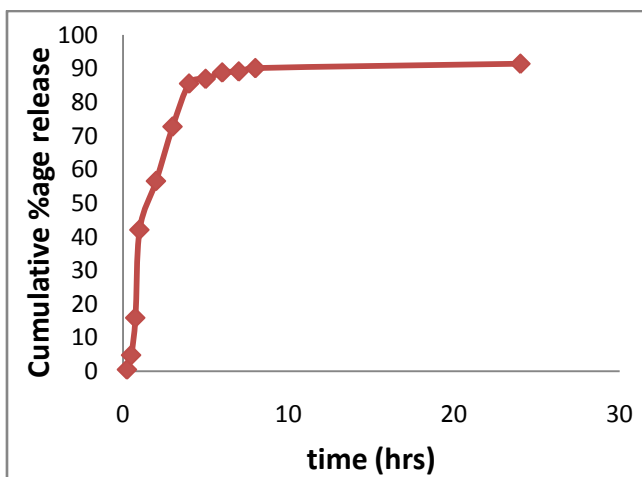


Fig 18(a): Drug release curve of M1

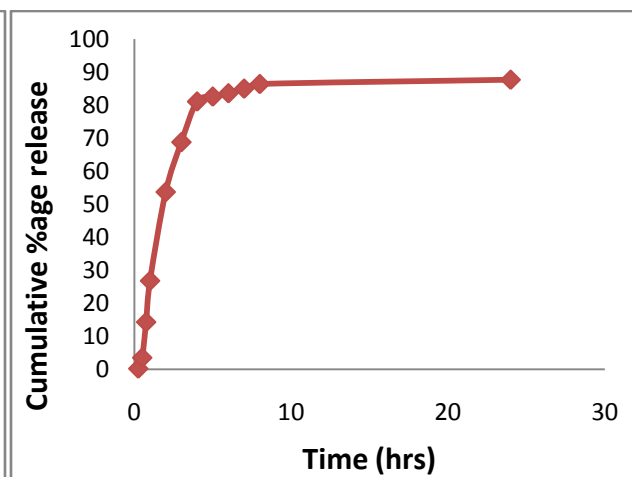


Fig 18(b): Drug release curve of M2

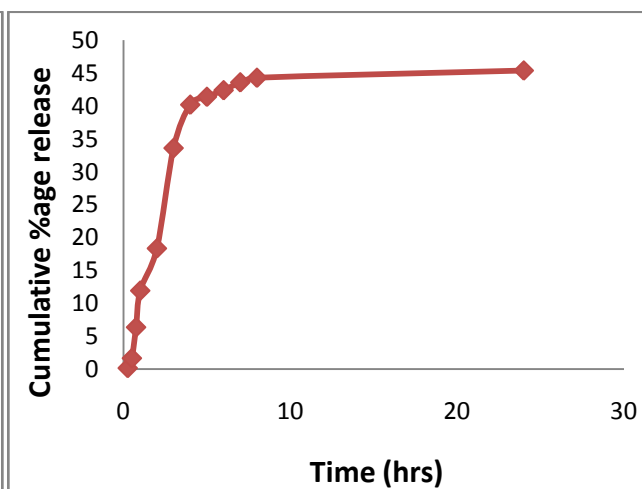
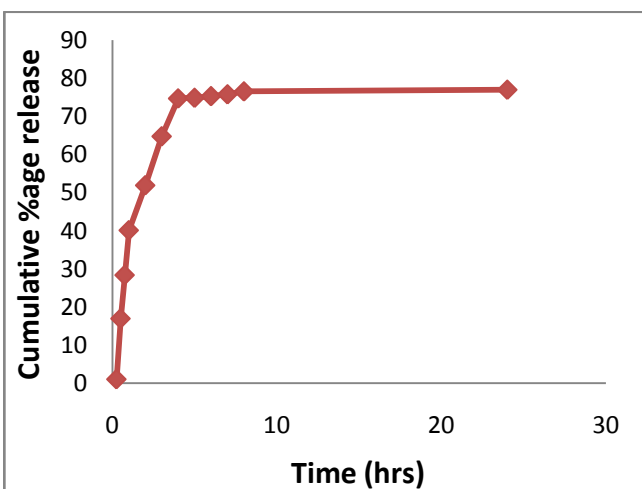


Fig 18(c): Drug release curve of M3

Fig 18(d): Drug release curve of M4

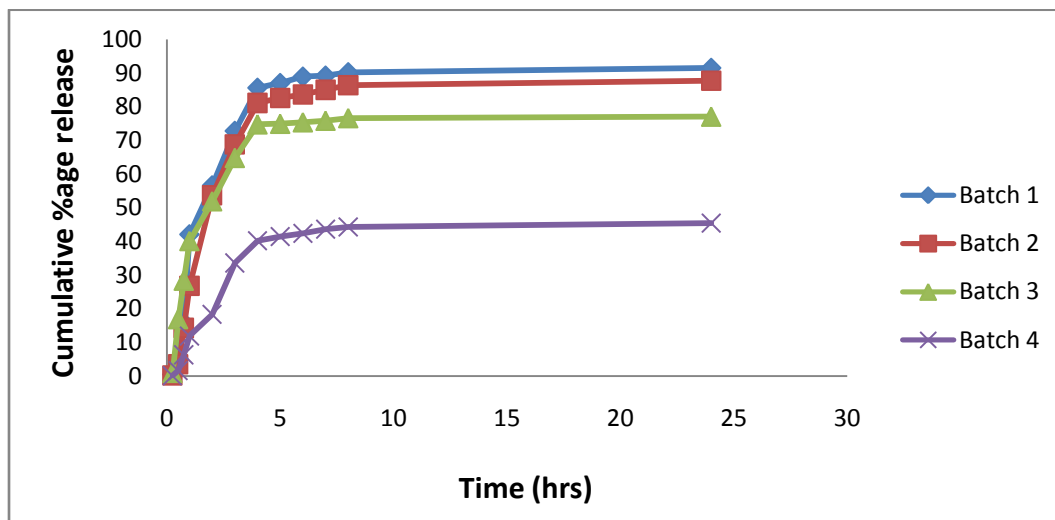


Figure 19: The drug release profiles of ampicillin sodium by varying chitosan conc.

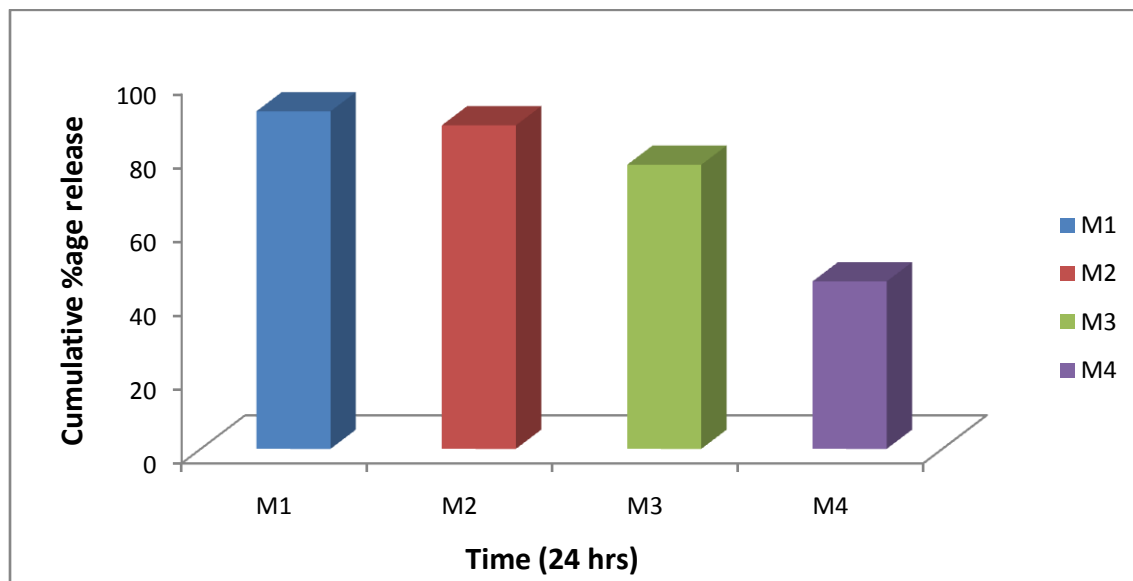


Figure 20: Cumulative %age release of drug from microspheres after 24 hours

The cumulative percentage release of drug decreased from 91.5% to 45.4%, with an increase in chitosan concentration. Maximum cumulative percentage release was observed in the batch 1,

with chitosan concentration of 1%. The increased chitosan concentration prolongs the diffusion route of drug by reducing the burst effect.

Chitosan microspheres with 2.5% could not be prepared. Due to increase in viscosity of the solution, it becomes difficult to drop the chitosan solution through the syringe needle. But by warming chitosan solution, its viscosity decreases and microspheres were formed. Chitosan microspheres with 0.5% w/v chitosan concentration were also prepared but they did not retain shape.

5.2 Evaluation of cross-linked chitosan based hydrogels

5.2.1 Percentage yield

The prepared hydrogels were collected, dried and weighed. The percentage yield was calculated as:

$$\% \text{age yield (w/w)} = \frac{\text{Weight of dried hydrogels}}{\text{Weight of chitosan} + \text{Weight of glutaraldehyde}} \times 100$$

Table 20: Percentage yield of different batches of chitosan based hydrogels

Batch no.	Yield (gm)	Percentage yield (%)
B1	0.2109	33.47
B2	0.4102	35.36
B3	0.283	44.92
B4	0.234	20.17
B5	0.461	36.58
B6	0.264	20.95
B7	0.122	5.25
B8	0.153	37.13
B9	0.138	13.16
B10	0.212	16.82
B11	0.266	11.46

B12	0.205	16.26
B13	0.201	8.66

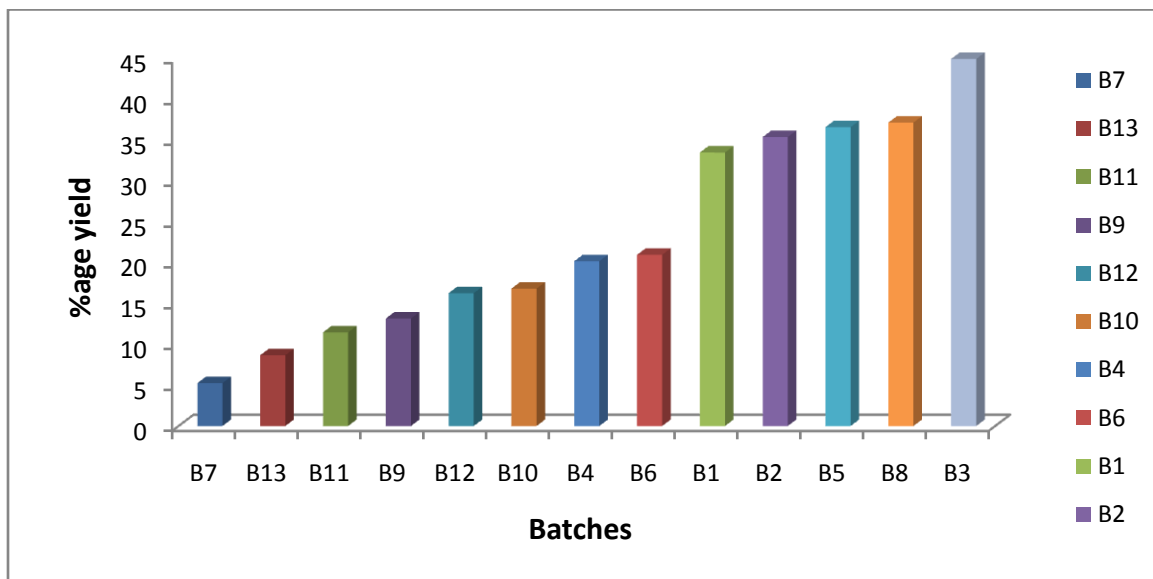


Figure21: Percentage yield of different batches of hydrogels

The percentage yield of chitosan based hydrogels varied from 5.25% to 44.92%. Maximum yield was obtained from batch 3, prepared by adding 500 µl of 50% v/v glutaraldehyde solution.

5.2.2 Equilibrium swelling studies

Swelling is the most significant characteristic as it reflects the affinity of chemical structure of hydrogels for water and other surrounding fluids. The swelling property of chitosan based hydrogels was determined in water. 20mg of chitosan based hydrogels were placed in 5ml of water at 37°C, overnight. The swollen hydrogels were filtered, blotted (to remove excess moisture adhering to surface) and weighed immediately on weighing balance.

$$\%E_{sw} = \frac{W_e - W_o}{W_o} \times 100$$

Where,

E_{sw} = percentage swelling of microspheres at equilibrium

W_0 = initial weight of microspheres

W_e = weight of microspheres at equilibrium

Table 21: Percentage swelling of different batches of chitosan hydrogels

Batch no.	% Esw in water
B1	119.88
B2	133.43
B3	249.80
B4	146.24
B5	121.02
B6	176.20
B7	132.09
B8	28.04
B9	30.26
B10	104.92
B11	31.34
B12	49.43
B13	35.26

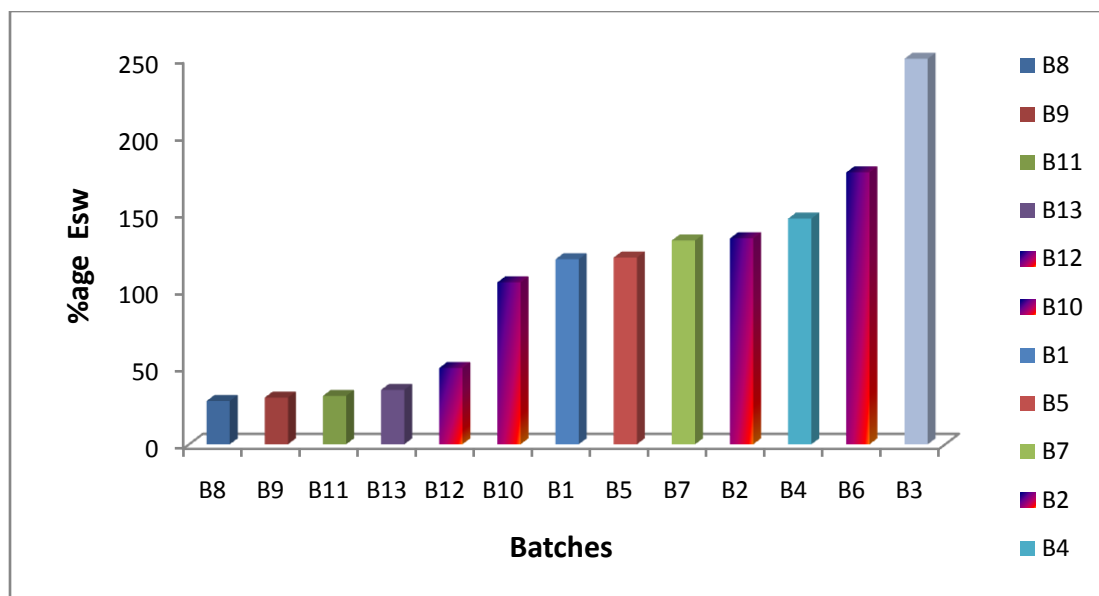


Figure 22: Percentage swelling of different batches of hydrogels

Percentage equilibrium swelling varied from 28.04 to 249.80%. Maximum percentage swelling was observed in batch 3, prepared by using 500 μ l of 50% glutaraldehyde. When glutaraldehyde concentration varies, the percentage equilibrium swelling also varies. When compared between batch 2, batch 6, batch 10 and batch 12, it was observed that when glutaraldehyde concentration increased from 25% to 40%, then the percentage swelling decreased from 133.43% to 104.92. Further, when glutaraldehyde concentration increased from 40% to 50%, the percent equilibrium swelling increased to 176.20% and when concentration of glutaraldehyde solution was further increased to 60%, the percentage equilibrium decreased to 49.43%. The decrease in percentage equilibrium is due to increase in cross-linking density, which reduces the swelling of hydrogels.

5.2.3 Drug loading

Ampicillin sodium was entrapped in hydrogels by incubating 20 mg of hydrogels in 1% ampicillin solution in water for 24 hours in a glass vials. After 24 hours, the hydrogels were filtered and dried (air drying followed by oven drying).

5.2.4 Determination of Encapsulation efficiency

20 mg of ampicillin loaded cross-linked hydrogels were crushed and soaked in distilled water for 24 hour. Then the hydrogels were filtered. Subsequent dilutions were made from this solution and the absorbance was determined spectrophotometrically at 222.2 nm against a suitable blank. The encapsulation efficiency was calculated by expressing the actual entrapment level divided by the theoretical entrapment level, as percentage.

$$\% \text{ Encapsulation efficiency} = \frac{\text{Actual entrapment level}}{\text{Theoretical entrapment level}} \times 100$$

Table 22: Encapsulation efficiency of different batches of chitosan based hydrogels

Batch no.	Encapsulation efficiency (%)
B1	136.61
B2	37.84
B3	106.61
B4	84.15
B5	122.92
B6	36.92
B7	37.22
B8	40
B9	34.61
B10	146.15
B11	123.52
B12	125.68
B13	101.06

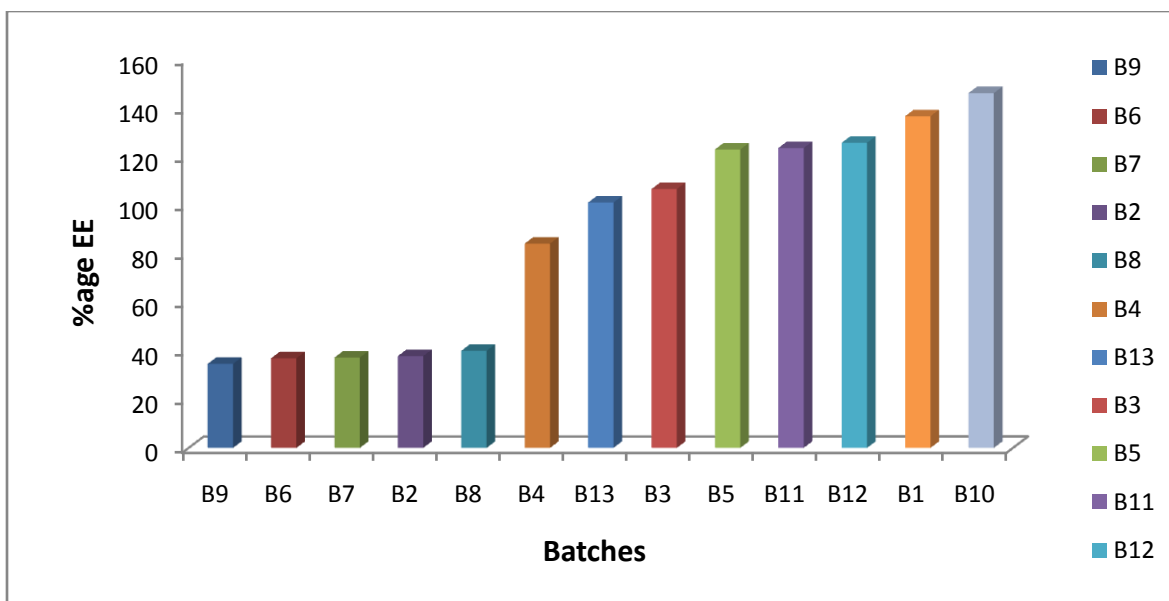


Figure 23: Encapsulation efficiency of different batches of hydrogels

The percentage encapsulation efficiency varied from 34.61% to 146.15%. Maximum percentage encapsulation efficiency was observed in batch 10, prepared from 1ml of 40% glutaraldehyde solution.

5.2.5 Evaluation of *in vitro* Drug Release Rate

Based upon the physiological gastrointestinal conditions, the *in vitro* release study was performed in buffer (pH 1.2) and buffer (pH 6.8). The dissolution behavior of chitosan-glutaraldehyde hydrogels was dependent on pH. They swell in buffer (pH 1.2) but didn't show much change in buffer (pH 6.8). An initial burst effect was observed in all formulations of hydrogels, which can be attributed to fast dissolution of drug molecule, attached to the surface, and subsequently slow release phase, during which drug molecule present in the core of hydrogel diffused out. The initial burst effect decreased with an increase in polymer concentration. In buffer (pH 1.2), protonation of phosphate ions breaks the hydrogen bonds, leading to weaker electrostatic interaction. This causes swelling of hydrogels and higher release of drug in buffer (pH 1.2), while in buffer (pH 6.8), there is strong attractive force between phosphate and chitosan, which causes slower release of drug. Thus biphasic mode of release of

drug is achieved due to change in pH of the medium i.e., pH 1.2 causes swelling and pH 6.8 causes deswelling.

100 mg of drug loaded chitosan hydrogels were incubated in 100 ml buffer (pH1.2) in a 150 ml conical flask kept at 37°C shaking incubator at 100 strokes per minute. After 3 hours, they were filtered and transferred to 100 ml buffer (pH 6.8) incubated at 37°C in a shaking incubator at 100 strokes per minute. Starting from time 0 hour and at desired intervals of time, 4 ml sample was withdrawn and replaced with same amount of fresh medium. Drug in the release medium was measured spectrophotometrically. Release studies and release profiles of cross-linked chitosan hydrogels of various batches are given below.

Table 23: Drug release data of cross-linked chitosan hydrogels at 25% glutaraldehyde conc.

S. no.	Time (hrs)	Cumulative %age release												
		B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13
1	0.25	0.05	0.56	0.29	0.92	0.01	0.01	0.04	0.06	0.3	0.06	0.01	0.01	0.17
2	0.5	0.46	1.24	0.7	1.95	1.8	0.3	0.39	0.5	1.36	0.56	0.21	0.23	0.82
3	0.75	1.8	2.15	1.37	3.34	2.81	1.3	1.27	1.63	2.03	1.84	0.86	1.09	1.33
4	1	2.4	3.21	1.9	4.76	3.91	2.51	2.28	3.07	2.88	3.33	1.56	2.22	1.96
5	2	3.2	4.74	2.63	6.25	5.16	3.78	3.38	4.72	3.79	4.97	2.36	3.53	2.69
6	3	4	5.73	3.26	7.66	6.13	5.11	4.59	6.49	4.75	6.72	3.23	5.08	3.51
7	4	4.5	6.25	3.51	7.83	6.77	6.57	5.88	8.51	5.81	8.62	4.26	6.61	4.52
8	5	4.9	6.94	3.9	8.27	6.93	7.67	6.93	10.04	6.61	10.08	5.17	7.79	5.31
9	6	5.3	7.7	4.35	8.78	7.1	7.9	7.1	10.61	6.86	10.52	5.49	7.95	5.68
10	7	5.6	8.2	4.6	9.42	7.33	8.63	7.34	10.76	7.5	11.01	5.86	8.59	5.99
11	8	5.8	8.6	4.88	9.81	7.82	9.44	7.5	10.92	7.89	11.45	6.23	9.45	6.3
12	24	6.1	9.05	5.44	10.27	8.07	9.75	7.91	11.07	8.15	11.7	6.44	9.7	6.52

Figure 24(a-m): The drug release curves of ampicillin sodium from chitosan hydrogels with varying glutaraldehyde conc.

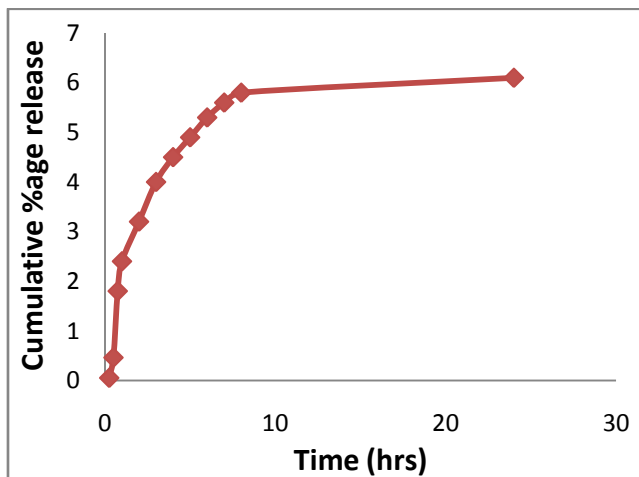


Fig. 24(a): Drug release curve of B1

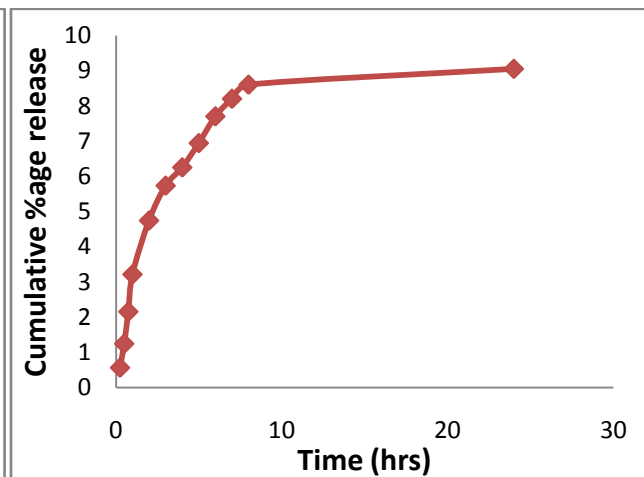


Fig. 24(b): Drug release curve of B2

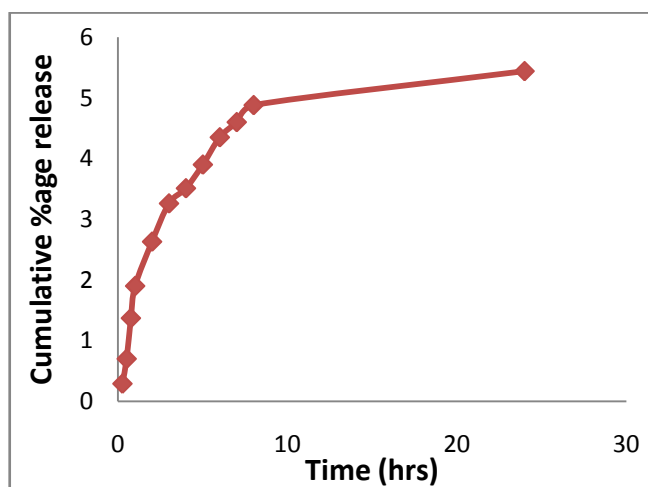


Fig. 24(c): Drug release curve of B3

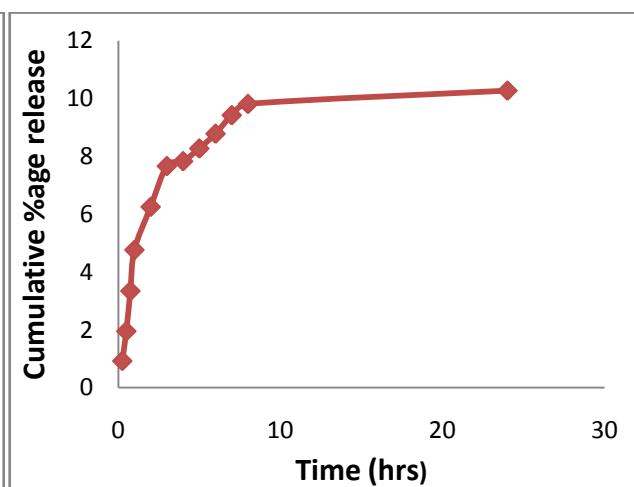


Fig. 24(d): Drug release curve of B4

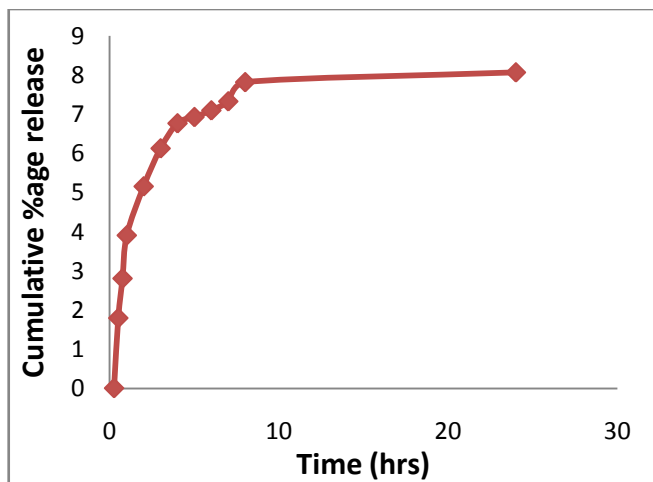


Fig. 24(e): Drug release curve of B5

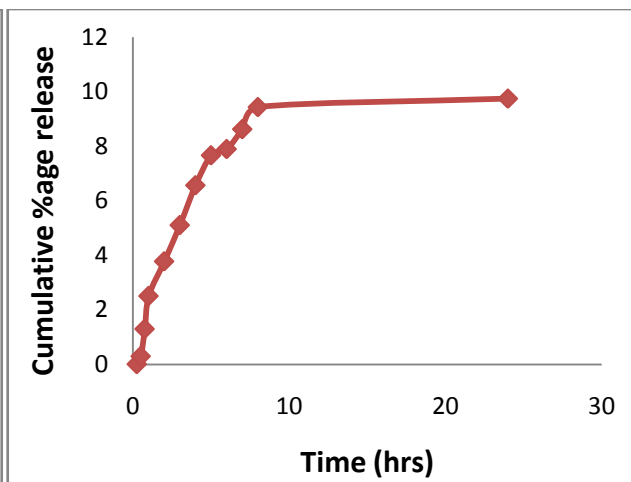


Fig. 24(f): Drug release curve of B6

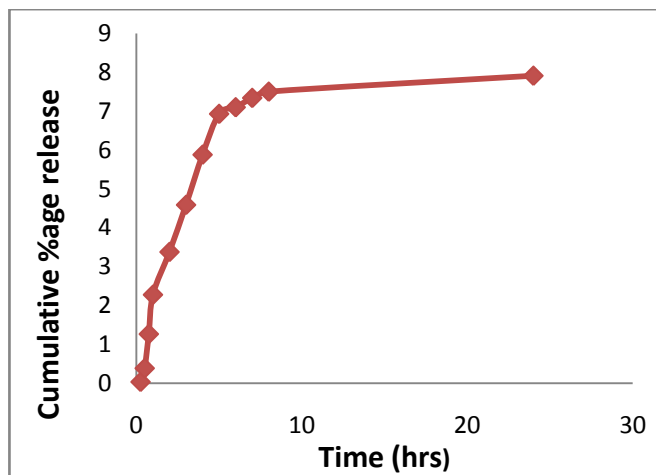


Fig. 24(g): Drug release curve of B7

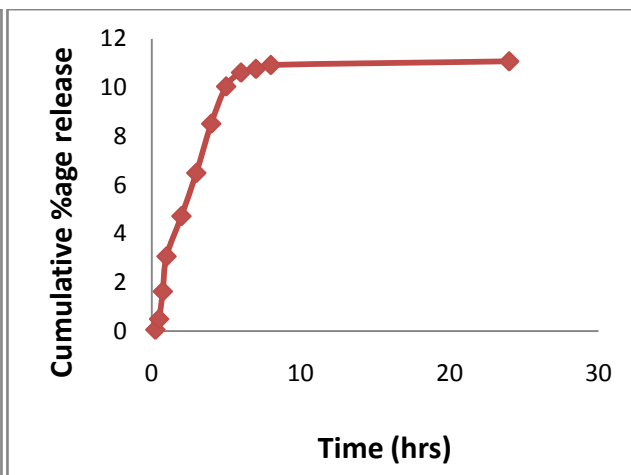


Fig. 24(h): Drug release curve of B8

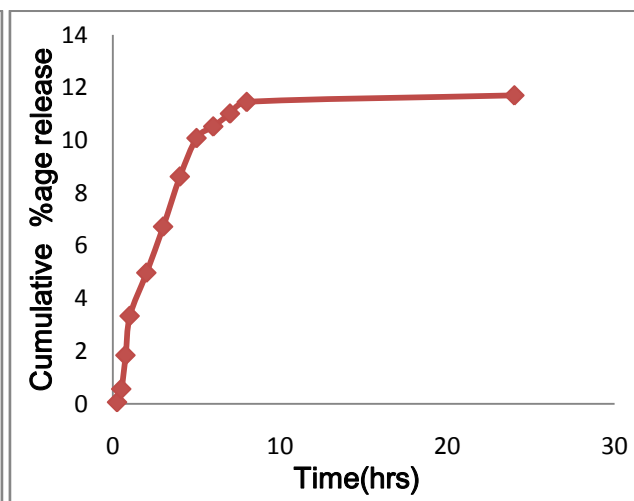
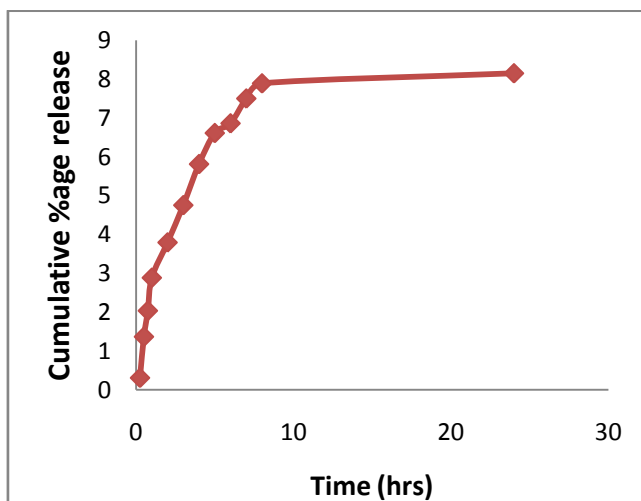


Fig. 24(i): Drug release curve of B9

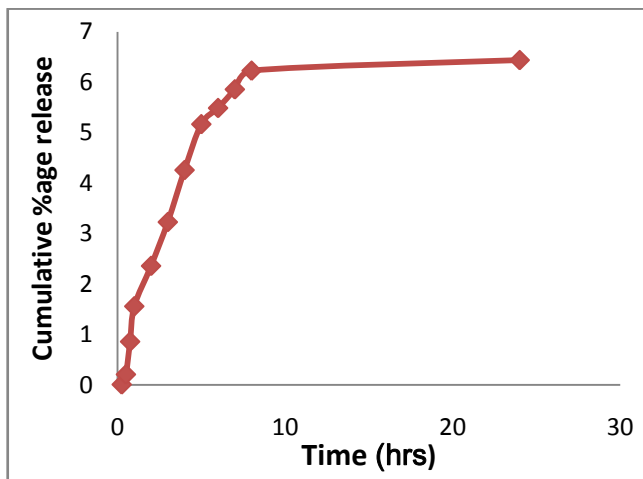


Fig. 24(j): Drug release curve of B10

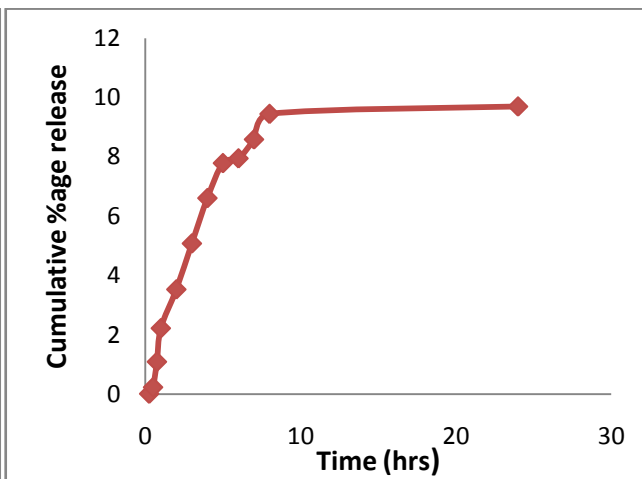


Fig. 24(k): Drug release curve of B11

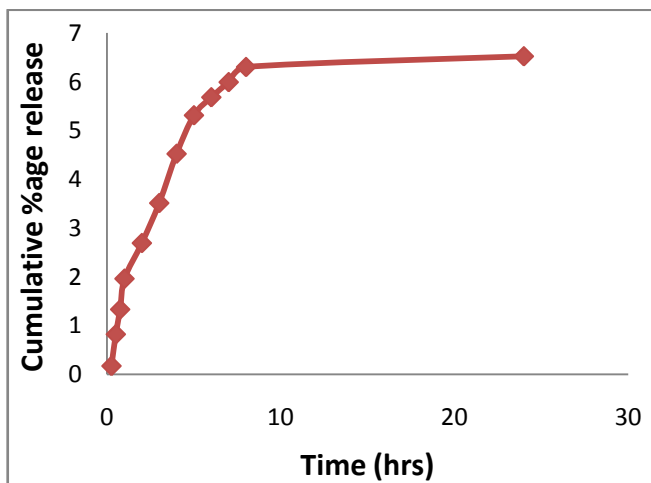


Fig. 24(l): Drug release curve of B12

Fig. 24(m): Drug release curve of B13

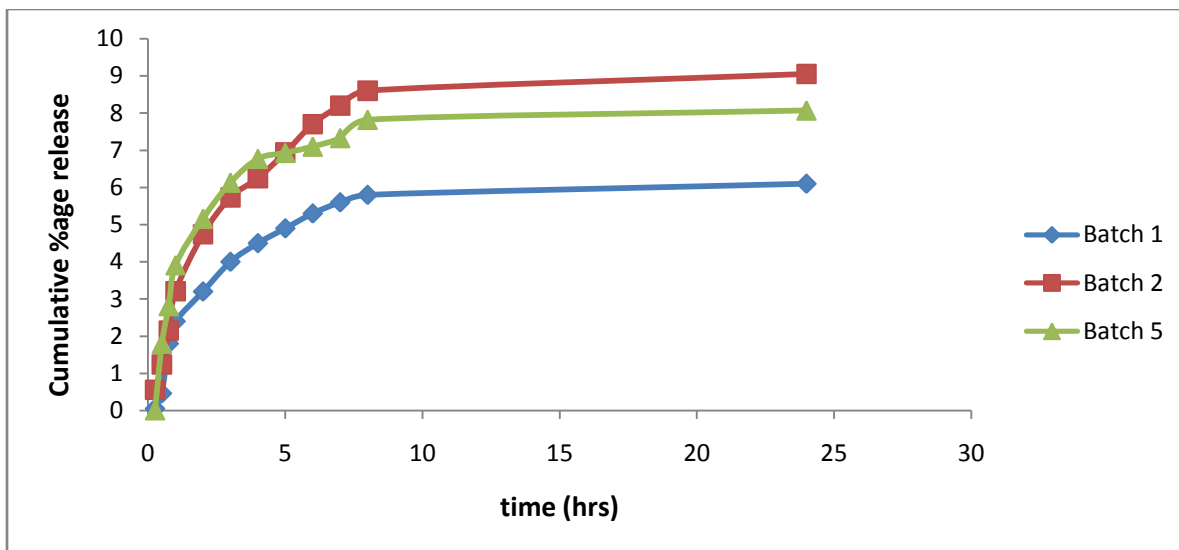


Figure 25: The drug release profiles of chitosan hydrogels with 25% glutaraldehyde conc.

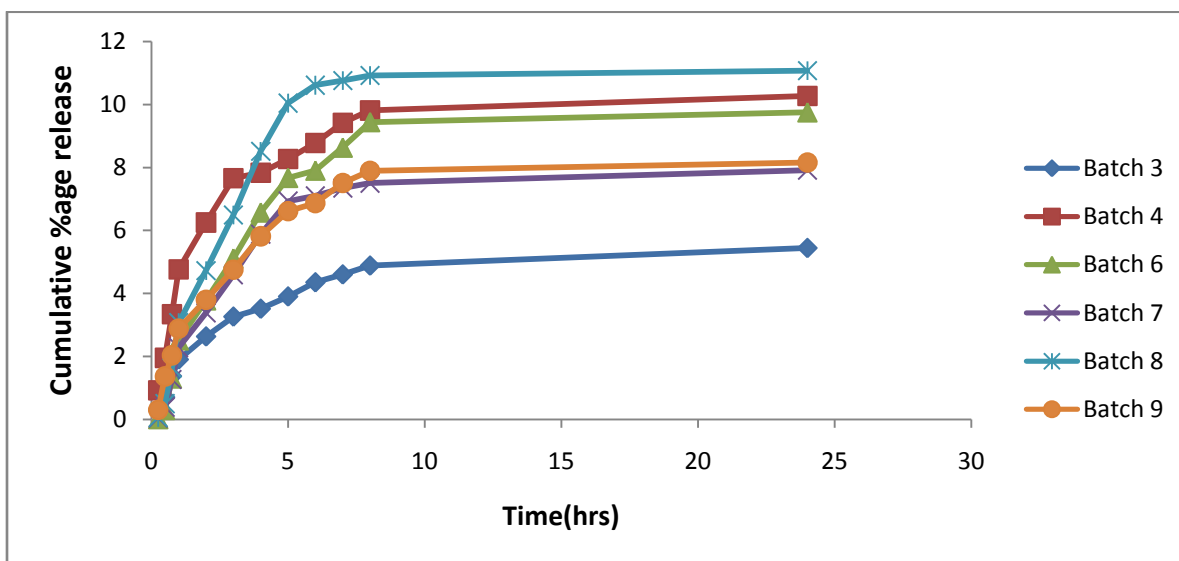


Figure 26: The drug release profiles of chitosan hydrogels with 50% glutaraldehyde conc.

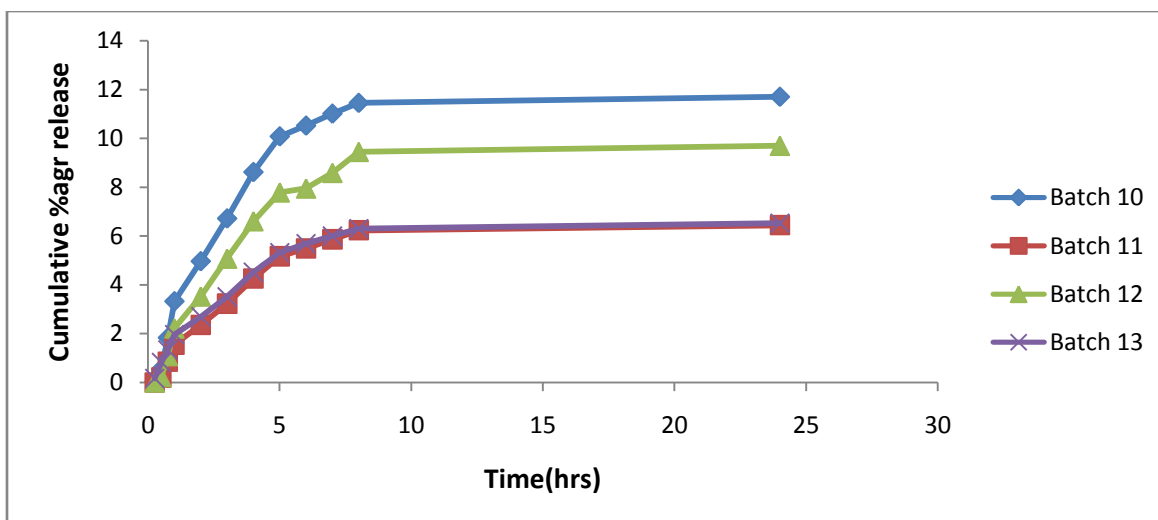


Figure 27: The drug release profiles of chitosan hydrogels with 40% and 60% glutaraldehyde conc.

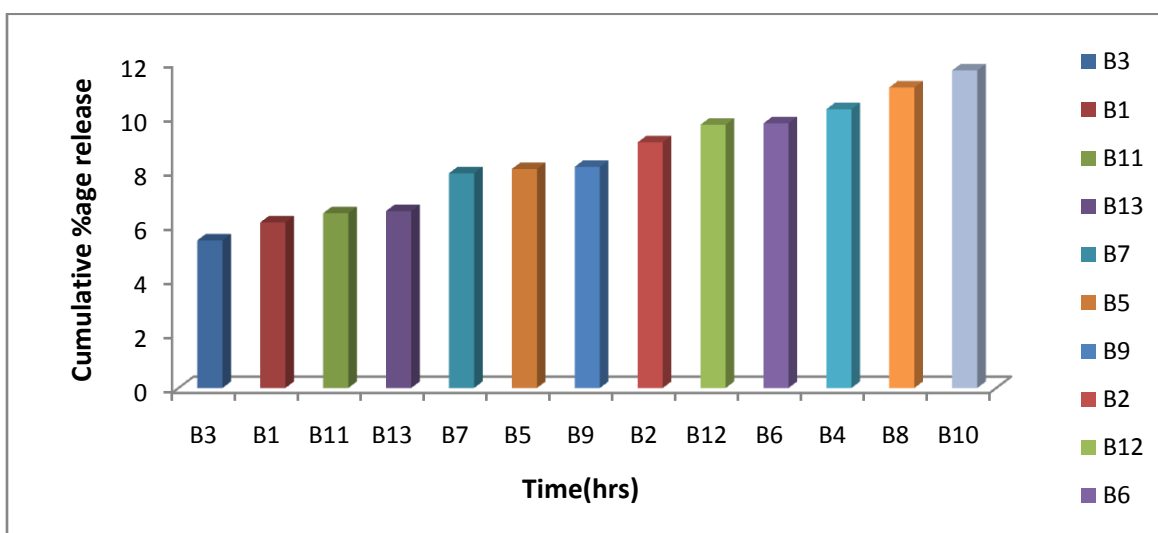


Figure 28: Cumulative %age release of drug from hydrogels after 24 hours

The cumulative percentage of drug release varied from 5.44% to 11.7%. This variation was observed due to the varying glutaraldehyde concentration. Maximum cumulative percentage release was observed in batch 10, having 40% (1ml) glutaraldehyde concentration. If the volume of glutaraldehyde increases then there is decrease in the drug release, as it promotes the formation of large number of inner cavities in the hydrogels, which does not allow the drug to diffuse in or out from the hydrogels.

If the volume of 40% glutaraldehyde increased to 2 ml then the cumulative percentage drug release decreased to 6.44%. Similar decrease of cumulative percentage is observed in the batch

having 60% glutaraldehyde concentration. If the volume increases from 1ml to 2 ml then the percentage drug release also decreased from 9.7 to 6.52%. In the batches, where 50% glutaraldehyde concentration was used, the cumulative percentage drug release also depends upon the volume of the glutaraldehyde solution. Increasing the volume of glutaraldehyde solution from 0.2 ml to 2 ml, the drug release also decreases from 11.07 to 7.91. But when the concentration of glutaraldehyde was 25%, cumulative percentage release increased with increase in volume.

Chitosan hydrogels with 0.5% w/v chitosan concentration were also prepared but did not form a gel and remained in liquid form.

C

HAPTER 6

CONCLUSION

CONCLUSION

The controlled release drug delivery system aims to release the drug at the desired rate over extended period of time to maintain therapeutic level in blood. Thus for controlled release delivery, carriers such as hydrogels and microspheres have been prepared using chitosan as a polymer because it offers many advantages. First, chitosan is considered as a safe material as it is a natural polymer possessing biocompatible and biodegradable properties. Secondly, it is a water soluble polymer which has an ideal property as drug delivery carrier; therefore, mild and simple preparation methods can be applied. Thus, chitosan based carriers are suitable for a broad category of drugs including macromolecules and labile drugs.

The present study has demonstrated the possibility of efficiently encapsulating ampicillin into crosslinked chitosan carriers of controlled size by using emulsion cross-linking method for microspheres and chemical crosslinking methods for hydrogels. After studying various parameters of microspheres, it was examined that highest encapsulation efficiency and highest percentage release was achieved in the batch 1 having chitosan conc. 1%(w/v), drug concentration 1%(w/v), glutaraldehyde conc. 25% v/v, 20 ml volume of chitosan solution. Good morphology and stability was observed. In hydrogels, it was examined that highest encapsulation efficiency and highest percentage release was achieved in batch 10, having glutaraldehyde concentration 40% v/v, chitosan concentration 1% w/v, drug concentration 1% w/v and 20 ml chitosan solution.

The optimization of various parameters in the preparation of both hydrogels and microspheres, such as chitosan concentration, stirring time, glutaraldehyde concentration and volume of drug chitosan concentration plays a very important role in obtaining microspheres/hydrogels of high yield, high encapsulation efficiency and high drug release rate. Though glutaraldehyde is a toxic cross-linking agent but its effect can be reduced by successive washing with distilled water and by adding glutaraldehyde in minimum concentration.

Microspheres showed better results as compared to hydrogels, as they were having uniform and spherical shape, high equilibrium swelling, high encapsulation efficiency and high

cumulative release of drug. Constant, steady, and reproducible release profiles were obtained with microspheres as compared to hydrogels. Thus, they can be used as prospective drug release carriers.

Future opportunities

- Microspheres and hydrogels provide massive advantages regarding drug targeting, delivery with additional potential to combine diagnosis and therapy.
- Can be employed in Anti-tumor therapy, gene therapy, AIDS therapy, radiotherapy.
- Can be used as carriers for delivery of vaccines and as vesicles to pass blood brain barrier.

C **HAPTER 7**

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REFERENCES

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