Evaluation and Characterization of L-ascorbic acid encapsulated chitosan microspheres.

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

Masters of Technology
In
Biotechnology

Submitted by:

Darshleen Kaur Madan
Reg. no. 601304005

Under the supervision of

Mrs. M. Vasundhara
Assistant Professor, DBT

Department of Biotechnology

Thapar University

Patiala 147004

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Certificate

This is to certify that the dissertation entitled **"Evaluation and characterization of L-ascorbic acid encapsulated chitosan-microspheres."**, submitted by Darshleen Kaur Madan in partial fulfillment of the requirement for the award of the Degree of Masters in Technology in Biotechnology to Thapar University, Patiala, is a record of student's own work carried under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University.

Vasundhara

Mrs. M. Vasundhara

Supervisor
Department of Biotechnology
Thapar University
Patiala

Dr. Dinesh Goyal

Head of Department
Department of Biotechnology
Thapar University
Patiala

Dr. S. S. Bhatia

Dean
(Academic Affairs)
Thapar University
Patiala

ii

Candidate's Declaration

I hereby declare that the work presented in the dissertation entitled **"Evaluation and characterization of L-ascorbic acid encapsulated chitosan-microspheres."** submitted in partial fulfillment of the requirement for the award of the degree of Master in Technology in Biotechnology, is an authentic record of my own work during the period of one year, 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.

Darshleen Kaur Madan

Place: Thapar University, Patiala

Date: 15-Jul-2015

Darshleen Kaur Madan

601304005

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

Vasundhara

Mrs. M. Vasundhara

Supervisor
Department of Biotechnology
Thapar University
Patiala

Dr. Dinesh Goyal

Head of Department
Department of Biotechnology
Thapar University
Patiala

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Abstract

Microencapsulation technique employed to design controlled drug release enhances the safety, efficacy and reliability of the drug therapy. The objective is to regulate drug release rate in order to provide relief to patients from following a dosage regime. These control release methods help in enhancing the stability, bioavailability, and half-life of drug. The controlled and localized release reduces the risks of manifestation of any side-effects. There are many methods for preparation of microspheres with wide range of size depending on the application requirement.

The aim of the present study has been evaluation and characterization of microspheres by water-in-oil emulsification method using chitosan as polymer and glutaraldehyde as cross-linking agent. Chitosan is chosen as the polymer for this study. It is a natural, non-toxic, biocompatible and biodegradable polymer with a great potential for pharmaceutical and biomedical applications. Glutaraldehyde is used as cross-linking agent due to its commercial availability and low cost. L-ascorbic acid (vitamin C) is selected for this study as it a very important vitamin with many emerging applications, especially in cancer treatment.

L-ascorbic acid encapsulated chitosan microspheres were prepared by dropping the chitosan-drug solution in the light liquid paraffin and span-80 emulsion, followed by periodical addition of glutaraldehyde. The system was subjected to constant stirring at high rpm. The effect of varying polymer-drug ratio on the preparation and properties of microspheres were studied. Microspheres were characterized by their percentage yield, particle size and morphology, percentage swelling index, drug encapsulation efficiency, and *in vitro* release.

Blank chitosan microspheres were also prepared by the same method, minus the drug. The effect of varying polymer concentration was studied. These microspheres were also characterized by their percentage yield, particle size and morphology. Blank microspheres were loaded with L-ascorbic acid on the basis of their percent swelling index under different pH conditions. Their drug encapsulation efficiency and *in vitro* release were studied.

The drug loading by incubation method was less than the loading in cross-linking method. The polymer to drug ratio affects the drug yield, particle size and morphology, encapsulation efficiency and drug release profile of microspheres. After studying various parameters it was examined that B6 was the best batch having polymer to drug ratio 2.5:1 i.e. 2.5% (w/v) chitosan and 1% (w/v) L-ascorbic acid.

List of Tables

List of Figures

Contents

Chapter 1: Introduction

The objective of research and development throughout the technology sphere is to ease human life. In pharmaceutical technology one aspect can be achieved by drug delivery systems that can precisely control the release rates or target drugs to a specific body site. Hence intend to improve efficacy and safety of the product, to match with patient's convenience and compliance. This type of formulations is commercially economic.

Drugs are rarely administered as pure chemical substances and almost always given in formulated drug delivery systems. These can vary from relatively simple to complex drug delivery systems through the use of various excipients in the formulations. The efficacy of drug can be significantly affected by the method of drug delivery.

The main purpose of drug delivery system is not only to deliver a biologically active compound in a controlled manner but also to maintain drug level in the body within therapeutic window (Coelho et al., 2010).

Conventional drug administration methods, including pills, tablets, capsules, injections, ointments, creams etc., do not usually provide controlled release or target specificity, which is neither economic nor safe. In many cases, conventional drug delivery provides abrupt increase of drug concentration in the body, following a relatively short period at the therapeutic level, drug concentration eventually drops off until re-administration.

Efficiency of many drugs is often limited by their potential to reach the site of therapeutic action. In conventional dosage forms only a small amount of drug administered reaches to the target site, while the majority of the drug is distributed throughout the body in accordance with its biochemical and physicochemical properties. Hence, development of a drug delivery system that optimizes the therapeutic action of the drug while reducing its toxic effects *in vivo* is a challenging task (Tiyaboonchai, 2013).

Novel drug delivery systems are rational drug delivery systems which improve drug potency with greater safety, controls drug release to provide sustained therapeutic effect (sustained release or controlled release dosage) and is potential to target a desired site (targeted/smart drug delivery). With advances in biotechnology, genomics, and combinatorial chemistry, a wide variety of new, more potent and specific therapeutics are being created. Because of common problems such as low solubility, high potency, and/or poor stability of many of the

new drugs, the means of drug delivery can impact efficacy and potential for commercialization as much as the nature of the drug itself. Thus, there is a corresponding need for safer and more effective methods and devices for drug delivery.

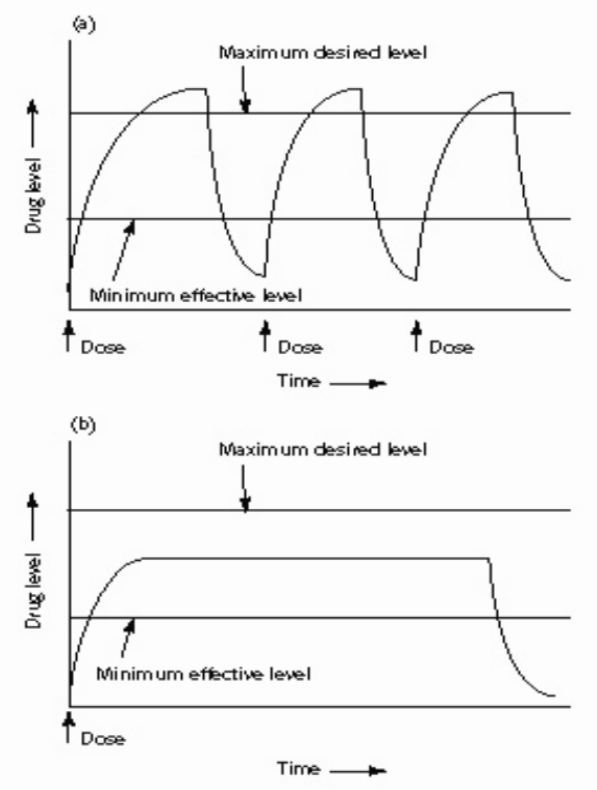

Figure 1: Drug level in the blood with (a) conventional drug dosing and (b) controlled-delivery dosing.

With conventional methods, the drug level in blood follows the profile shown in figure 1(a), in which repeated administration is required to keep the effect of drug sustained. The key point of drug administration methods is that the blood level of the drug should remain in the therapeutic window. In controlled release drug delivery system designed for long term administration to attain a constant release profile as shown in figure 1(b), in the therapeutic window, for an extended period of time (Shaik et al., 2012).

Advantages of controlled release drug delivery system:

1. Therapeutic advantage: maintenance of steady plasma level of the drug over a prolonged time period, hence increasing the bioavailability.
2. Safety: reduction in adverse side effects as controls pharmacokinetics to remain within therapeutic window.

3. Patient comfort and compliance: reduction in dosing frequency.
4. Reduction in healthcare cost: the total cost of controlled release product could be comparable with the conventional release product (Ummadi et al., 2013).

With the intersection between study of polymers and material science, carrier technology offers an intelligent approach of drug delivery by coupling the drug to a suitable carrier particle. Current study areas in this field include drug loaded liposomes, biodegradable microspheres, nanoparticles, micelles and drug polymer conjugates.

Microspheres constitute an important part of this sort of drug delivery system, because of their small size and efficient carrier characteristics they provide high bioavailability. Presently there is a wide range of biocompatible polymers for production of such microspheres. The ideal size ranges between 1 to 200 μm and their spherical morphology may differ with the material and method of preparation. Administration of drugs via such systems is advantageous as microspheres can be ingested, injected or inhaled (Sakagami and Byron, 2005). They can be tailored for desired release profiles and with few modifications can provide site-targeted release.

Rationale for microspheres as controlled drug delivery system:

1. They facilitate accurate delivery of small quantities of potent drug and reduced concentration of drug at site other than the target organ or tissue and hence reduce chances of toxicity.
2. They provide protection for unstable drug before and after administration, prior to their availability at the site of action. Figure 2 shows the entrapment in microsphere and encapsulation in microsphere.

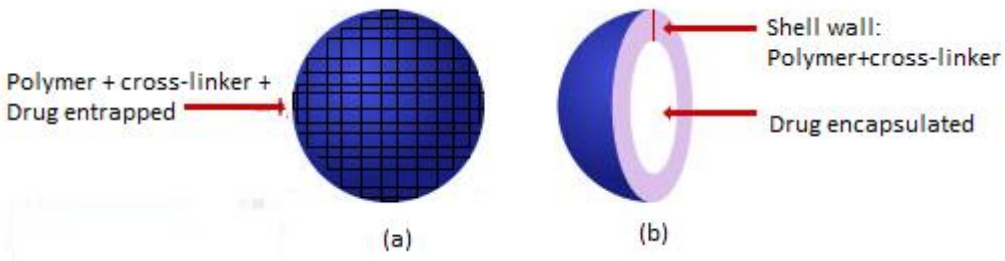

Figure 2: Entrapment in a microsphere (a), Encapsulation in a microsphere (b).

3. They provide the ability to manipulate the *in vivo* action of the drug, pharmacokinetic profile, tissue distribution and cellular interaction of the drug.
4. They enable controlled release of drug and hence no peaks and troughs in concentration of drug (such as Narcotic, Antagonist, Steroid hormones).
5. Aid in dispersion of water insoluble substance in aqueous media.

In spite of all these significant advantages, there are certain challenges that need to be contemplated on:

1. Dose dumping: rapid release of potent drugs with narrow therapeutic index may cause adverse effects.
2. Poor *In vitro In vivo* correlation: despite excellent *in vitro* release characteristics it may give rise to unsatisfactory drug absorption *in vivo*.
3. Patient variation: absorption of drug released from the dosage form may vary among individuals.
4. Economic factors: the cost of such preparations may be high from those of standard formulations
5. Drug selection: the drug to be encapsulated need to confirm a certain rationale.
6. Process conditions: change in temperature, pH, solvent addition, agitation and evaporation may influence the stability of core particles.
   (Satish et al., 2013)

Pharmaceutical and biomedical applications of microspheres

1. Microencapsulated products currently in market: aspirin, vitamins, metformin, potassium chloride, progesterone, and contraceptive hormone combinations.
2. Microspheres have also found potential applications as injection, or inhalation products (Gaspar et al., 2013).
3. Vaccine delivery such as hepatitis, influenza, diphtheria.
4. Oral delivery of easily degraded drugs: gene therapy with DNA plasmids; delivery of insulin (Patel et al., 2011).
5. Paramedical uses of microcapsules include bandages with microencapsulated anti-infective substances.
6. Taste and odor masking.

Microsphere based drug delivery systems allows drug release tailored to a specific treatment site with the combination of various drug-polymer formulations. Using innovative microencapsulation technologies, and by varying various factors such as copolymer ratio, molecular weight of polymer etc., microspheres can be developed into an optimal drug delivery system with desired release profile of the drug encapsulated. As microspheres are small in size their surface to volume ratio is large and is advantageous for controlled release of insoluble drugs (Sinha et al., 2004).

The ideal drug delivery system should be biocompatible, mechanically strong, inert, comfortable and safe for the patient, with high drug loading efficiency, and easy to fabricate and sterilize (Seenivasan, 2012).

It has been observed that microspheres are better choice of drug delivery system than many other types of drug delivery systems as it has the advantage of target specificity and better patient compliance. Its applications are enormous as they are not only used for delivering drugs but also for imaging tumors, detecting biomolecular interactions etc. In future microspheres will have an important role to play in advancement of medical field (Hire and Derle, 2014).

There are a lot of applications of microspheres not only in pharmaceuticals as drug delivery system but also in food industry; it can contain flavor, vitamins, fragrances and cooling agents; in agriculture for microbial control and fertilizing soil. With the sophisticated study of polymers, drugs and other components, along with the emerging technologies this has a great potential to be explored.

Chapter 2: Objectives of study

- To prepare L-ascorbic acid encapsulated chitosan-microspheres by emulsification method.
- To perform optimization of microspheres by varying formulation parameters.
- To study the characteristics and drug release profiles of the prepared microspheres.

Chapter 3: Review of Literature

Huguet and Dellacherie, (1996) encapsulated BSA (MW:67,000), human haemoglobin (MW: 64,500) and dextran in calcium alginate beads coated with chitosan. The effect of the structure of encapsulated materials on their release was studied.

Genta et al., (1997) prepared acyclovir-loaded chitosan microsphere using emulsification technique which were characterized for their morphology and physicochemical characteristics by *in vitro* dissolution tests and *in vivo* ocular administration in rabbits. Results showed that diameter of 90% of the particles is $\leq$ 25 μm and the drug was homogeneously dispersed inside the microspheres. The *in vitro* dissolution profiles slower than that of the raw drug. *In vivo* results showed prolonged high concentrations of acyclovir and increased area under the curve.

Jameela et al., (1998) prepared smooth, highly spherical, cross-linked progesterone-loaded chitosan microspheres in the size range of 45-300 μm with glutaraldehyde as cross-linking agent. An aqueous dispersion medium consisting of liquid paraffin and petroleum ether was prepared and stabilized using sorbitan sesquioleate. *In vitro* release studies indicated a prolonged release over 40 days. *In vivo* studies by administering intramuscular injections in rabbit showed that a plasma concentration of 1 to 2 ng/ml was maintained up to 5 months without a high 'burst effect'.

Genta et al., (1998) studied the influence of glutaraldehyde on drug release and mucoadhesive properties of chitosan microspheres. The aim of this study was to develop a new simple *in vitro* technique based on electron microscopy in order to study the effect of polymer cross-linking density on mucoadhesive properties of the chitosan microspheres. With the results from SEM and TEM it was confirmed that high affinity for mucin of uncross-linked chitosan microspheres and thus their bioadhesive properties. Further observation was made that the bioadhesive characteristic was low for glutaraldehyde cross-linked chitosan microspheres.

Riberio et al., (1999) studied microencapsulation of lipophilic drugs in chitosan-coated alginate microspheres. Chitosan was applied to increase the mechanical strength and stabilize the microspheres in simulated intestinal media.

Monteiro and Airoldi, (1999) studied the interaction of chitosan-glutaraldehyde in a homogeneous system. The modified polymer was characterized by means of carbon, hydrogen and nitrogen elemental analysis, scanning electron microscopy, X-ray diffractometry, nuclear magnetic resonance, infrared and Raman spectroscopy. It was observed that degree of crystallinity and particle size decreased as the amount of glutaraldehyde was increased in the polymer. The conclusion was that the physical and chemical properties are not just affected by the dissolution of the natural chitosan.

Ravi Kumar, (2000) reported that microparticles have potential in the application of administrating therapeutic molecules. He discussed novel properties that have been developed to increase efficiency of drug delivery, improve release profiles and drug targeting.

S. Puttipipatkhachorn et al., (2001) studied drug physical state and drug-polymer interaction on drug release from chitosan matrix films. Four different grade of chitosan (molecular weight and degree of deaceylation) were used to cast chitosan film with salicylic acid as model acid and theophylline as basic drug. Crystalline characteristics, thermal behavior, drug-polymer interaction and drug release behavior of films were analyzed. It was suggested that swelling property, dissolution characteristics of polymer films, pKa of drugs and drug-polymer interactions were important factors governing drug release patterns from chitosan films.

Ko J. A. et al., (2002) fabricated chitosan microparicles with tripolyphosphate by ionic cross-linking. The particle size was found out to be in the range from500 to 710μm with encapsulation efficiency more than 90%. With decrease in pH of tripolyphosphate and increase in molecular weight of chitosan, the microspheres had more spherical and smooth morphology. With decrease in molecular weight and concentration of chitosan, release behavior was increased.

Naidu, (2003) studied importance of ascorbic acid. It was discussed that how it is essential for collagen, carnitine and neurotransmitters biosynthesis. Many health benefits were also listed. US routine drug allowance b/w 100-120 mg/day.

Dhawan and Singla, (2003) devised nifedipine-chitosan microspheres using emulsification phase-separation method. It was found to have higher encapsulation efficiency and excellent swelling properties. These microspheres exhibited faster release at low loading compared to high loadings. It was further deduced that with increase in polymer content the value of

diffusion exponent characteristic of release mechanism(n) approached one, which is indicative of zero order.

Hejazi and Amiji, (2003) summarized the recent applications of chitosan in oral and/or buccal delivery, stomach-specific drug delivery, intestinal delivery, and colon-specific drug delivery. They inferred that chitosan appears to be a promising material for GI drug and gene delivery applications as many derivatives and formulations are being examined.

Dubey and Parikh, (2004) studied the effects of process parameters such as stirring speed, concentration of chitosan and concentration of drug on preparation of microspheres using chemical denaturation method. The morphology, particle size, encapsulation efficiency and *in vitro* release studies were analyzed. It was concluded that formation of chitosan microspheres is helped by the use of differential stirring. While an increase in the concentration of water-soluble drug may help to increase encapsulation efficiency and drug load over a large concentration range, the effect is limited in case of water insoluble drugs.

Sinha et al., (2004) reviewed chitosan microspheres as a potential carrier for drugs., studied biodegradable properties, methods of preparation and applications, encapsulation of drugs, factors affecting encapsulation efficiency and release kinetics.

Desai and Park, (2005) encapsulated vitamin C in TPP cross-linked chitosan microspheres by spray drying method. The mean particle size ranged from 6.1 – 9 μm. The influence of the concentration of cross-linking agent was observed on morphology, encapsulation efficiency, zeta potential and release rate.

Desai et al., (2005) studied the characteristics of vitamin C encapsulated TPP-chitosan microspheres affected by molecular weight of chitosan. It was observed that with increase in molecular weight of chitosan the release rate decreased.

Varshosaz et al., (2006) prepared chitosan microspheres for insulin nasal delivery by emulsification cross-linking method. The effect of chitosan quality, cross-linker process and amount were studied on the morphology, particle size, loading efficiency, flow and release. The *in vivo* studies were conducted for the same in diabetic rats. The result showed that chitosan microspheres of insulin are absorbable from nasal route.

Yeom et al., (2006) investigated the effects of mega-dosage of vitamin C on cancer patients' health related quality of life. The study was conducted on 39 terminal cancer patients by administering each with a 10 g dose twice a day. There was an improve in score that measured the quality of life. However, its anti-cancer effects are still controversial.

Wang et al., (2006) prepared uniform-sized chitosan microspheres containing insulin by membrane emulsification and a two-step solidification process using TPP. The solidification conditions of the two step process were optimized by investigating its effects on morphology of microspheres, encapsulation efficiency (EE), drug activity and release profile *in vitro*. The determined optimized conditions include pH value of aqueous phase and TPP solution was 3.5-4.0, the molar ratio of amino group of chitosan to aldehyde was 1:1 and cross-linking time of glutaraldehyde was 60 min.

Kulkarni et al., (2007) formulated ibuprofen encapsulated chitosan microspheres with glutaraldehyde as cross-linker via different methods and varying the process conditions: concentration of cross-linking agent, polymer to drug ratio, stirring speed in order to optimize the microsphere size, degree of swelling, drug entrapment efficiency, and release rates. FTIR confirmed absence of any chemical interactions. The optical microscopy revealed the size range of 30-200 μm and SEM studies revealed a smooth surface and spherical shape of microspheres. It was also concluded that with decrease in concentration of cross-linker there was an increase in the swelling ratio whereas extended cross-linking lowered entrapment efficiency. The *in vitro* drug release was controlled and extended up to 10 hrs.

Beppu et al., (2007) studied the effect of glutaraldehyde cross-linking of chitosan membrane on ion permeability and water absorption. Diffusion experiments confirmed some subtle changes in permeability of ions. The results indicated that chemical modification with glutaraldehyde turns chitosan more hydrophobic.

Hickey et al., (2008) studied the pharmacokinetics of oral vitamin C. The purpose was to test whether plasma vitamin C levels, following oral doses in supplemented volunteers, are tightly controlled and subject to a maximum in the region of 220 μM/l, as suggested by previous researchers for depleted subjects. It was concluded that repeated doses could sustain levels well above the formerly assumed maximum.

Cantoni et al., (2010) made an effort to formulate and simultaneously evaluate sustained release tablets containing antioxidants such as vitamin A, vitamin C, vitamin E, zinc and selenium for treatment of AIDS.

Jyothi et al., (2010) reviewed microencapsulation techniques, factors influencing encapsulation, various process conditions, different manufacturing parameters and techniques were discussed.

Singh A., (2011) reported that biopolymers are choice of research in pharmaceuticals as excipients in formulation of drug delivery system because of its low toxicity, biodegradability, stability and renewable nature.

Mikirova et al., (2012) researched on the effect of high-dose intravenous vitamin C on inflammation in cancer patients. The study was conducted on 45 cancer patients. The results supported the hypothesis that high dose intravenous ascorbate treatments may reduce inflammation in cancer patients.

Vilar et al., (2012) studied polymers and there uses in drug delivery system, contemplated on studies to attain high bio-availability, site specific and time controlled drug delivery system. It was concluded that biopolymers are the trusted source for development of controlled drug delivery system.

Comunian et al., (2013) fabricated solid lipid microcapsules containing ascorbic acid using microfluidic technique. The morphology was analyzed by SEM. Encapsulation efficiency, particle size and stability of ascorbic acid was evaluated. The result indicated the enormous potential of the designed vehicle to prevent its degradation in a food product.

X. L. Tian et al., (2013) researched on synthesis and characterization of chitosan-vitamin C complex with the help of fourier transformed infrared spectroscopy (FTIR), differential scanning calorimeter and H-NMR. Results showed that the scavenging activity on O_2 by chitosan-vitamin C was stronger than that by chitosan and at low concentrations (<0.5 m/ml) it was stronger for the complex than that of vitamin C but with concentrations >0.1 mg/ml, its scavenging activity was lower than that of vitamin C.

Chapter 4: Research Envisaged

Microencapsulation is a process by which an active pharmaceutical ingredient i.e. a drug is packed within a secondary material to form microspheres. This secondary material is termed as the matrix or shell that protects the drug from surrounding environment until its release is triggered by alteration in its environment. This obviates undesirable interactions of the drug with other bioactive components present in the body. In order to tailor microspheres with desired attributes the knowledge of certain things is crucial, including (a) the drug to be encapsulated, (b) the encapsulating material, (c) interactions between the drug, matrix and the environment, (d) the stability of the preparation, (e) the mechanism that control the release of the core.

4.1 Selection of drug

The selection of a drug for designing a controlled release product is quite challenging as the efficiency of many drugs in often limited due to the many factors, such as, the patient's diseased state, physiochemical and biological properties of drug, pharmacokinetics and pharmacodynamics etc. Hence selection of drug requires consideration of number properties.

4.1.1 Physicochemical properties of drug

1. Dose size: therapeutic dose size of the drug should be low.
2. Partition Coefficient: extremes in partition coefficient of a drug are undesirable.
3. Aqueous solubility: extremes in aqueous solubility of a drug are undesirable.
4. Drug stability: drug used for sustained drug delivery should be stable over the entire length of the gastro-intestinal tract.
5. Molecular size: large molecules will show small diffusion coefficients and may be unsuitable for a sustained release system.

4.1.2 Biological properties of drug

1. Biological half-life: a drug with a short half life requires frequent dosing and this makes it a desirable candidate for sustained release formulations.
2. Absorption: drugs that are slowly absorbed with variable absorption rate are poor candidates.
3. Distribution: drugs with high apparent volumes of distribution are poor candidates.
4. Therapeutic index: drugs with narrow therapeutic index require precise control over the blood levels of drug placing a constraint on controlled release dosage forms.

4.2 L-Ascorbic acid

L-Ascorbic acid, commonly known as vitamin C, is a water-soluble organic compound related to glucose. It is essential for collagen, carnitine and neurotransmitters biosynthesis. The current US recommended daily allowance (RDA) for ascorbic acid ranges between 100–120 mg/per day for adults. Many health benefits have been attributed to ascorbic acid such as antioxidant, anti-atherogenic, anti-carcinogenic, immunomodulator and prevents cold etc. (Naidu, 2003).

Inside body, Vitamin C acts as an antioxidant, which helps in protecting cells from free radicals. Its biologically active form, Vitamin C, functions as a reducing agent and coenzyme in several metabolic pathways. It is essential in synthesis of collagen, carnitine, and neurotransmitters and also in the synthesis and catabolism of tyrosine. It finds applications in preventing lung cancer, in preventing or slowing the process of cataract, in treating some chronic diseases such as rheumatoid arthritis and also a common remedy for common cold. It has beneficial effects on skin cells, and some studies have shown that vitamin C may help prevent and treat ultraviolet (UV)-induced photo-damage.

A severe deficiency (lack) of vitamin C in the diet causes scurvy, a disease with symptoms of extreme weakness, lethargy, easy bruising, and bleeding. The lack of vitamin C in patients with scurvy makes collagen thinner in texture; when vitamin C is given, collagen becomes thicker again.

Most of the plants and animals can synthesize ascorbic acid from D-glucose or D-galactose. A majority of animals produce relatively high levels of ascorbic acid from glucose in liver. However, bats, guinea pigs, apes and humans cannot synthesize ascorbic acid due to the mutation in L-gulonolactone oxidase gene. Hence, in humans, it is essential to supplement ascorbic acid through food or as a drug. There is a wide range of fruits and vegetables rich in Vitamin C. It is present in fruits like orange, lemons, grapefruit, papaya, mango, strawberries, cherries, raspberries, watermelon and pineapple. It is also found in green leafy vegetables, broccoli, tomatoes, green and red pepper, cauliflower and cabbage.

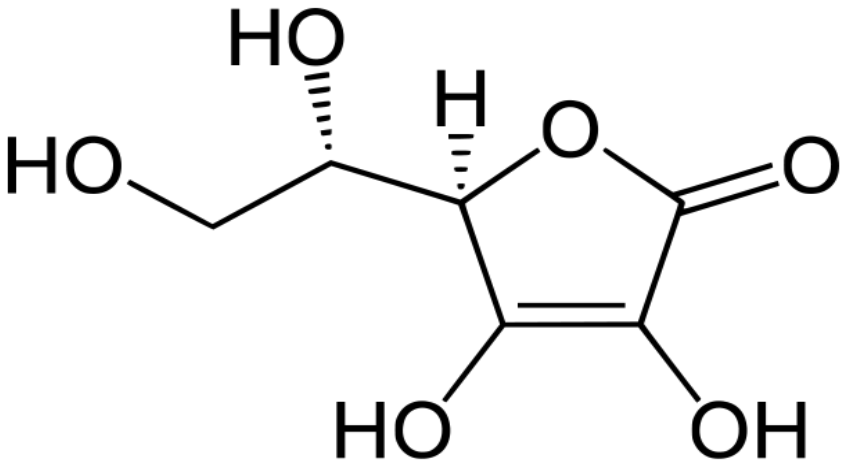

Figure 3: Chemical structure of ascorbic acid.

4.2.1 Physicochemical properties of L-ascorbic acid

1. Empirical formula: $C_6H_8O_6$
2. IUPAC name: 5-(1,2-dihydroxyethyl)-3,4-dihydroxy-5-furan-2-one
3. Molecular weight: 176.12 g/mol
4. Crystalline form: Monoclinic
5. Odor: Odorless
6. Melting point: 190-192°C
7. Taste: Pleasant, sharp, acidic taste
8. Exhibits maximal stability between pH 4 and 6
9. Pharmacological action: as an antioxidant and as vitamin.

4.2.2 Applications of ascorbic acid

L-Ascorbic acid has many essential roles in various biochemical reactions and potential to cure many other diseases and conditions, some of which are discussed as following:

A. Bio-necessity of L-Ascorbic acid

1. Prevents scurvy:

   A condition due to dietary deficiency of ascorbic acid is characterized by lethargy, malaise and weakness. As the disease progresses, joints, muscles, and subcutaneous tissues may become the sites of hemorrhage. Its deficiency frequently develops scurvy in children.

2. Reduce oxidative damage:

   Antioxidant property of L-ascorbic acid reduces the damage caused by oxidizing chemicals, such as free radicals. These oxidizing chemicals are the common byproducts of the cellular reactions that take place inside human body. These entities are very unstable and cause damage to the cells by reacting with the important molecules and interfering with their functionality. L-Ascorbic acid reduces this damage by directly binding to oxidizing chemical and converting them to less harmful molecules. Reducing oxidative damage can have many benefits for body, including reducing cancer and heart disease (Padayatty et al., 2003).

3. Regulates immune system:

   L-Ascorbic acid plays a major role in regulating immune system of the body. Many types of immune cells are stimulated by L-ascorbic acid including white blood cells. It also helps in increasing the level of antibodies in the body. Vitamin C has an antibacterial and anti-viral effect due to interaction with metal transition ions creating a selective pro-oxidant environment that kills or inactivates pathogens through the production of peroxide, without causing significant toxicity to healthy cells. (Melissa Ge et al., 2008)

B. Ascorbic acid as modern drug

L-ascorbic acid now a days is synonymous with the term ‘modern drug’ as it has a variety of biological, pharmaceutical and dermatological functions. It is a commonly known fact that its deficiency causes scurvy.

1. Vitamin C and Cancer:

The treatment of cancer considers different mechanisms of L-ascorbic acid, such as neutralization of carcinogenic substances (Block 1991), correction of ascorbate deficiency, prevention of cellular free radical damage (Flagg et al.,1995), enhancement of effect of certain chemotherapy drugs (Kurbacher et al., 1996), inhibition of gastric carcinoma promoting micro-organisms (Zhang et al., 1997).

In some studies, conducted on humans, it has been indicated that in terminal cancer patients treated with high-dose vitamin C indicated subjective improvement in quality of life (Yeom et al., 2007), as well as improvements in physical, mental, and emotional functions,

symptoms of fatigue, insomnia, nausea and vomiting, pain and appetite loss (Takahashi et al., 2012).

2. Remedy to common cold:

   In 1970s Nobel laureate Linus Pauling concluded from a placebo-controlled trials that Vitamin C has potential to prevent and alleviate common cold. Over a two dozen new trials were undertaken thereafter. It has been a subject of controversy ever since. In a recent study by Harri Hemila and others, it was concluded that with a regular dosage of 0.2 g per day, Vitamin C had no effect on common cold incidence in the ordinary population. However, it had a modest but consistent effect in reducing the duration and severity of common cold symptoms and also with participants exposed to short periods of extreme physical stress vitamin C halved the common cold risk. (Hemila et al., 2008)

3. Photoprotection:

   L-Ascorbic acid limits the damage induced by ultraviolet (UV) light exposure. It is not a "sunscreen", its antioxidant activity protects against UV-induced damage caused by free radicals (Darr D et al., 1992) UV light decreases vitamin content of skin depending on the intensity and duration of UV exposure. In cultured keratinocytes the addition of vitamin C reduces UV-related DNA damage and lipid peroxidation, limits the release of pro-inflammatory cytokines, and protects against apoptosis (Tebbe B et al., 1997). In addition to its antioxidant functions, vitamin C regulates the synthesis of the structural protein collagen. The role of vitamin C in the hydroxylation of collagen molecules is well characterized. Hydroxylation of collagen is necessary for its extracellular stability and support of the epidermis (Peterkofsky, 1991)

4. Age-related macular degeneration (AMD) and cataract:

   The prevalence and effects of age-related macular degeneration and cataract are increasing dramatically in senile population. A synergistic effect of vitamin C with other nutrients, such as vitamin E, is recommended for AMD but not cataract (Seddon, 2007). However, some studies show that people who get more vitamin C from food have lower risk of getting cataract.

4.2.3 Rationale for microencapsulation of L-ascorbic acid.

It is very unstable to air, moisture, heat, light, and oxygen. It gets oxidized into biologically inactive compounds such as oxalic acid, 2,3-diketo-L-gulonic acid, oxalic acid, L-threonic acid, L-lyxonic acid and L-xylonic acid (Desai and Park, 2005). Therefore, in order to overcome the associated drawbacks, microencapsulation was considered (Trindade and Grosso 2000; (Uddin and Hawlader,, 2001). The rationality for microencapsulation of L-ascorbic acid are explained as following:

a. To reduce the sensitivity of L-ascorbic acid against the environmental factors, such as, heat, moister, light and air.
b. To increase the retention time of L-ascorbic acid as its half-life is low. (30mins)
c. To control the release of L-ascorbic acid as its solubility in water is very high but its absorption is very low.

4.2.4 Mechanism of action

In humans, an exogenous source of L-ascorbic acid is required for collagen formation and tissue repair by acting as cofactor in the post translational formation of 4-hydroxyproline in –Xaa-Pro-Gly- sequences in collagens and other proteins. L-Ascorbic acid is reversibly oxidized to dehydroascorbic acid in the body. These two forms of the vitamin are believed to be important in oxidation-reduction reactions. The vitamin is involved in tyrosine metabolism, carbohydrate metabolism, iron metabolism, conversion of folic acid, synthesis of lipids and proteins, resistance to infections, and cellular respiration.

4.2.5 Pharmacokinetics

The pharmacokinetic study on oral administration of vitamin C conducted by Mark Levine and colleagues at NIH was purposed to identify a dose-plasma concentration relationship for oral vitamin C ingestion, as a pre-requisite to study the dose-function relationship and to determine the optimal intake of this vitamin. The results in healthy volunteers indicated that the drug concentration in plasma and cells were carefully controlled by multiple mechanisms acting together: bioavailability, tissue accumulation, renal re-absorption and excretion, and utilization rates as a function of homeostasis. When the oral intake of vitamin C was made to exceeded 200mg/day, the plasma concentration peaked at 70-80μM. Further increase in dose did not provide obvious increase of concentration in plasma and in cells: the bioavailability

drops, intracellular distribution saturated, and renal excretion accelerated. However, when ascorbate was administered intravenously, tight control was bypassed, until renal excretion restored equilibrium which can tale hours depending on the dose (Levine et al., 1996).

In later studies it was found that plasma ascorbate concentrations as high as 20-30 mM were safely achieved with large dose of intravenous ascorbate (Chen et al., 2008) and for oral intake, plasma ascorbate concentration did not reach higher that 300 μM (Padayatty et al., 2004).

4.3 Selection of polymers

A polymer is a large molecule composed of smaller units called monomers that are bonded together. For long term drug delivery applications polymers with biodegradable characteristics have been proven to be useful. Biodegradable polymers are highly desirable in such applications as they degrade in the body to biologically inert and compatible molecules, which are further metabolized or excreted via normal physiological pathways. Incorporating drugs in such polymers, dosage forms that release the drug over a prolong time period can be prepared. Hence, these biodegradable polymers offer a novel approach for developing sustained release drug delivery system.

The five key advantages that polymeric products can offer are: sustained drug delivery of drug, localized delivery of drug, stabilization of the drug, release rate which is less dependent of drug properties and steadier release rate with time (Jones David, 2007).

4.4 Chitosan

Chitosan is a natural, cationic amino polysaccharide copolymer of glucosamine and N-acetylglucosamine obtained by alkaline, partial deacetylation of chitin. It is the second most abundant natural polysaccharide and originates from shell of crustaceans such as crabs, shrimps and lobsters. It is a biodegradable, biocompatible, positively charged nontoxic mucoadhesive biopolymer. As chitosan contains primary amino groups in the main backbone that make the surface positively charged in biological fluids, biodegradable nano/microparticles can be readily prepared by treating chitosan with a variety of biocompatible polyanionic substances such as sulfate, citrate, and tripolyphosphate (Pillai et al. 2009). These unique features of chitosan have stimulated development of delivery systems for a wide range of biological agents.

It has been reported to enhance drug permeation across the intestinal, nasal, and buccal mucosa. Chitosan microspheres have arisen as a promising candidate in oral or other mucosal administration for improving the transport of bio macromolecules such as peptides, proteins, oligonucleotides, and plasmids across biological surfaces. This is because chitosan microspheres can improve the drug adsorption via the paracellular route. It is generally considered nontoxic and biodegradable.

4.4.1 Chitosan structure

The structure of chitosan is very similar to that of cellulose [made up of β(1-4)-linked D-glucose units], in which there are hydroxyl groups at C-2 position of glucose rings. Chitosan is a linear copolymer polysaccharide consisting of β(1-4)-linked 2-amino-2-deoxy-D-glusose (D-glucosamine) and 2-acetamide-2-deoxy-D-glucose(N-acetyl-D-glucosamine) units.

The biodegradability and biological role of chitosan is frequently dependent on the proportions of N-acetyl-D-glucosamine and D-glucosamine residues. In figure 4 deacetylation of chitin to chitosan is shown. The term chitosan is used to describe a series of polymers of different molecular weight and deacetylation, defined in terms of the percentage of primary amino groups in the polymer backbone (Rinaudo, 2006) the deacetylation of typical commercial chitosan usually ranges between 70 to 95 % , and the molecular weight between 10 and 1,000 kDa.

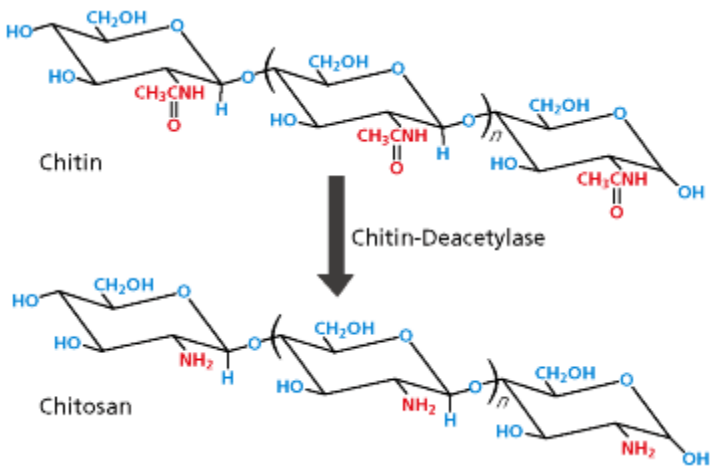

Figure 4: Deacetylation of chitin to chitosan.

4.4.2 Chitosan sources and production

Chitin is found in the exoskeleton of some arthropods, insects, and some fungi. Commercial sources of chitin are the shell wastes of crab, shrimp, lobster etc. Chitosan is usually prepared by the deacetylation of chitin. The conditions used for deacetylation will determine the average molecular weight and degree of deacetylation. Figure 5 shows a flow diagram for chitosan production.

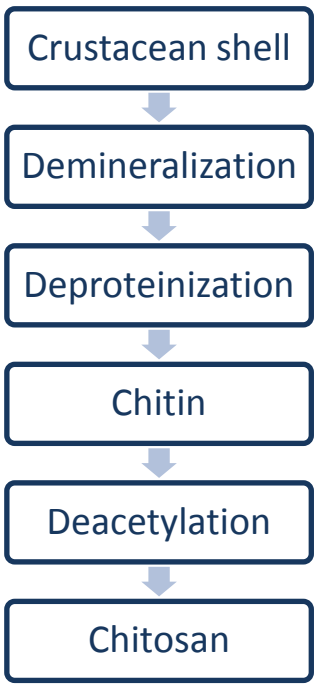

Figure 5: Flow diagram of chitosan production.

4.4.3 Physicochemical and biological properties of chitosan

Chitosan is semi-crystalline polymer that exhibits polymorphism. It belongs to a series of polymers with different degree of deacetylation and molecular weight, which are two important properties of chitosan. Degree of deacetylation is defined as the percentage of primary amino groups in the polymer backbone. The degree of deacetylation and molecular weight of chitosan can be altered by changing the reaction conditions during the manufacturing of chitosan from chitin. Chitosan appears as off-white, odorless flakes.

Chitosan has low solubility at physiological pH of 7.4 as it is a weak base (pKa 6.2-7). It induces flocculation due to deprotonation and insolubility of polymer (Jayakumar et al., 2010). It is readily soluble in dilute acids such as acetic acid, formic acid etc. (Ravi Kumar, 2000). The solubilization occurs through protonation of amino groups on the C-2 position of D-glucosamine residues, whereby polysaccharide is converted into polycation in acidic media.

The viscosity of chitosan solution increases with the increase in chitosan concentration and degree of deacetylation. It also increases with decrease in solution temperature and pH. Hence, higher molecular weight chitosan of approximately 1,400 kDa demonstrates a stronger mucoadhesive than low molecular weight chitosan of 500-800 kDa as the former has higher level of viscosity.

Chitosan is an highly basic polysaccharide with unique property of polyoxysalt formation, ability to form films, chelate metal ions and optical structural characteristics. (Ravi Kumar, 2000)

4.4.4 Applications of chitosan

The following tables summarize various chitosan applications. Table 1 summarizes the potential biomedical applications along with the chitosan characteristic attribute contributing to the respective application. Table 2 and Table 3 discuss the Principle applications and the specific characteristic for applications of chitosan in hair care respectively (Rinaudo, 2006).

Table 1: Principle properties of chitosan in relation to its use in biomedical applications.

S. no.	**Potential biomedical applications**	**Principle characteristics**
1.	Surgical sutures	Biocompatible
2.	Dental implants	Biodegradable
3.	Artificial skin	Renewable
4.	Rebuilding of bone	Film forming
5.	Corneal contact lenses	Hydrating agent

6.	Time release drugs for animals and humans	Nontoxic, biological tolerance
7.	Encapsulation material	Hydrolyzed by lyzosyme Wound healing properties

Table 2: Principle applications of chitosan

S.No.	**Industry**	**Applications**
1.	Agriculture	Defense mechanism in plants, stimulation of plan growth, seed coating, frost protection, time release of fertilizers and nutrients into the soil.
2.	Water & waste treatment	Flocculent to clarify water (drinking water, pools), removal of metal ions, ecological polymer (eliminate synthetic polymers), reduce odors.
3.	Food and beverages	Not digestible by human (dietary fiber), bind lipids (reduce cholesterol), preservative, thickener and stabilizer for sauces, protective, fungi-static, antibacterial, coating for fruit.
4.	Cosmetics	Maintain skin moisture, treat acne, improve suppleness of hair, reduce static electricity in hair, tone skin, oral care (toothpaste, gum).
5.	Biopharmaceutics	Immunologic, antitumoral, hemostatic and anticoagulant, healing, bacteriostatic.

Table 3: Specific characteristics for applications of chitosan in hair care.

Properties	**Uses**
Aqueous solution interacting with negatively charged hair (electrostatic interaction) Antistatic effect (due to hydrophobic character), maintains moisture in low humidity and hair style in high humidity. Removing sebum and oils from hair (hydrophobic) Antibacterial and antifungal activity Thickening polymer Role in surfactant stability; stabilize emulsion Make hair softener, increase mechanical strength Protect elastic film on hair, increasing their softness	Shampoos Hair tonics Rinses Permanent wave agents Hair colorants Lacquers Hair sprays Time release delivery (chitosan beads and gels)

4.5 Cross-linking agents

Cross-linking agents induce the formation of chemical links between molecular chains to form a three-dimensional network of polymers and also improves cohesion, adhesion and thermal stability. According to several studies, cross-linking the matrix using agents such as glutaraldehyde, NaOH, ethylene glycol diglycidyl ether etc. could control the drug release from chitosan microspheres.

4.5.1 Glutaraldehyde

Glutaraldehyde is a linear 5-carbon dialdehyde. It is a clear, pale straw, pungent oily liquid that is soluble in water, alcohol as well as organic solvents. It is mainly available in concentration range of 2% to 25% (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). As the cross-linking is mainly influenced by the size and type of the cross-linking agent and fuctional group of the polymer, it provides with higher encapsulation. Chemical structure of glutaraldehyde is shown in figure 6.

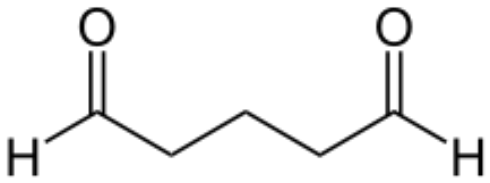

Figure 6: Chemical structure of Glutaraldehyde.

Glutaraldehyde is commercially produced by the oxidation of cyclipentene and by Diels-Alder reaction of acrolein and methyl vinyl ether followed by hydrolysis. It exists in the form of hydrates, which have several structures (Eral et al. 1974), shown in figure 7.

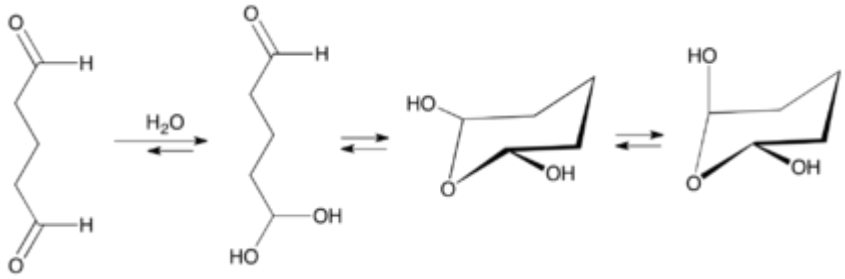

Figure 7: Structures of glutaraldehyde.

4.6 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 in oil type emulsion). The molecular formula of span 80 is $sC_{24}H_{44}O_6$ and molecular weight is 428.6 g/mol. The chemical structure of span 80 is shown in figure 8.

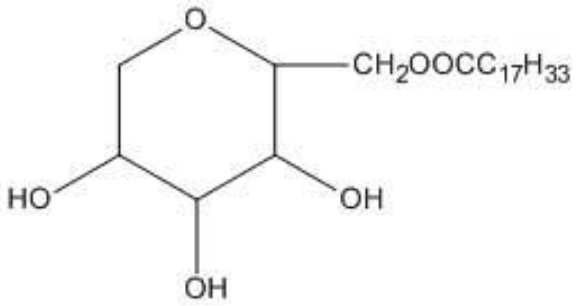

Figure 8: Chemical structure of span 80.

4.6.1 Applications

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

4.7 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.

4.7.1 Applications

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

Chapter 5: Experimental Work

5.1 Experimental chemicals and equipments

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

Table 4: Experimental Materials

Experimental chemicals	
▪ Chitosan (> 75% deacetylated, Sigma-Aldrich) ▪ L-Ascorbic acid ▪ Glutaraldehyde (25%) ▪ Light liquid paraffin oil ▪ Sorbitan monooleate (Span 80)	▪ Acetic acid ▪ Buffer (pH 1.2) ▪ Buffer (pH 6.8) ▪ Hexane ▪ Distilled Water

Table 5: Experimental equipments

Experimental equipments	
▪ Mechanical stirrer ▪ Magnetic stirrer ▪ Magnetic bead ▪ Analytical Balance ▪ Optical microscope ▪ UV-Visible Spectrophotometer ▪ Incubator(s) ▪ Shaking Incubator ▪ Nikon SMZ 800 microscope	▪ pH meter ▪ Disposable syringe ▪ Glassware ▪ Measuring cylinder (25ml, 100ml, 500ml, 1000ml) ▪ Micropipette (200l, 1ml) ▪ Funnel ▪ Filter paper ▪ Petriplates

5.2 Preparation of standard curves of L-Ascorbic acid (Vitamin C)

Concentration of L-Ascorbic acid in the respective solution was estimated by measuring their absorbance at 244nm with the help of UV-Visible spectrophotometer. The solution was prepared in distilled water, pH 1.2 buffer solution and pH 6.8 buffer solution. The absorbance was taken in triplicates and with the help of Microsoft Office Excel 2007 standard graphs were plotted. The R-squared value and equation were generated.

5.2.1 Preparation of buffer pH 1.2 (200ml)

50ml of 0.2M potassium chloride (KCl) solution was prepared. 85ml of 0.2M hydrochloric acid (HCl) solution was prepared. Both the solutions were mixed together. And the final volume was made up to 200ml by adding remaining volume of distilled water.

5.2.2 Preparation of buffer pH 6.8 (200ml)

50ml of 0.2 M potassium dihydrogen phosphate solution was prepared. 22.4ml of 0.2M sodium hydroxide (NaOH) solution was prepared. Both the solutions were mixed together. And the final volume was made up to 200ml by adding remaining volume of distilled water.

5.2.3 Preparations of standard curves

Standard solution was prepared by dissolving 100mg of L-ascorbic acid in distilled water, buffer of pH 1.2 and buffer of pH 6.8 each. From the above standard stock solution different drug dilutions were prepared by using the formulae:

$$M1V1=M2V2$$

The different concentrations which were prepared were 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml, and 10 µg/ml by taking different aliquots from stock solution and making up the remaining volume to 10ml with respective buffer solution. The absorbance of the different solution was measured in triplicate at 244nm against the respective buffer solution as blank in UV-Visible spectrometer. Standard curves for water, buffer of pH 1.2 and buffer of 6.8 were obtained by plotting the respective data.

Table 6: Standard curve data of L-Ascorbic acid in distilled water.

S. No.	Concentration (µg/ml)	Absorbance (A_{244})
1.	2	0.038
2.	4	0.094
3.	6	0.148
4.	8	0.198
5.	10	0.243

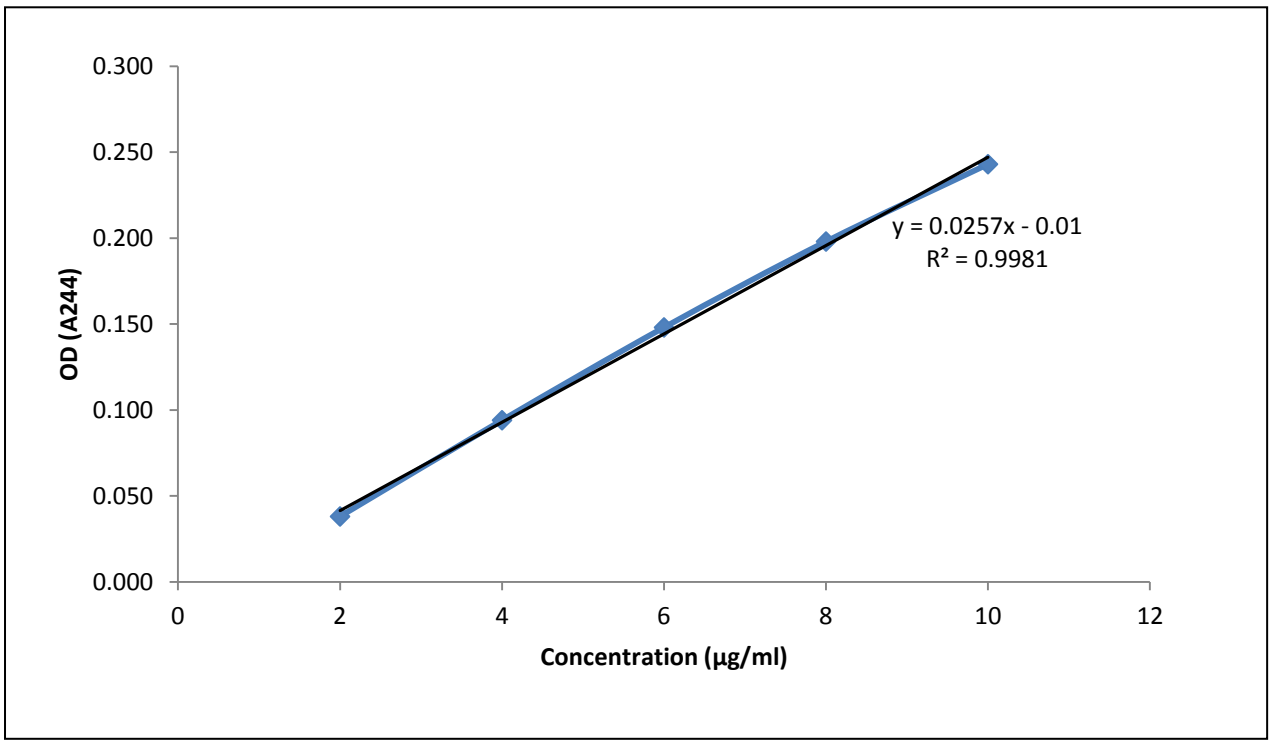

Figure 9: Standard curve of L-ascorbic acid in distilled water.

Table 7: Standard curve data of L-Ascorbic acid in buffer pH 1.2.

S. No.	Concentration (µg/ml)	Absorbance (A_{244})
1.	2	0.076
2.	4	0.172
3.	6	0.257
4.	8	0.334
5.	10	0.409

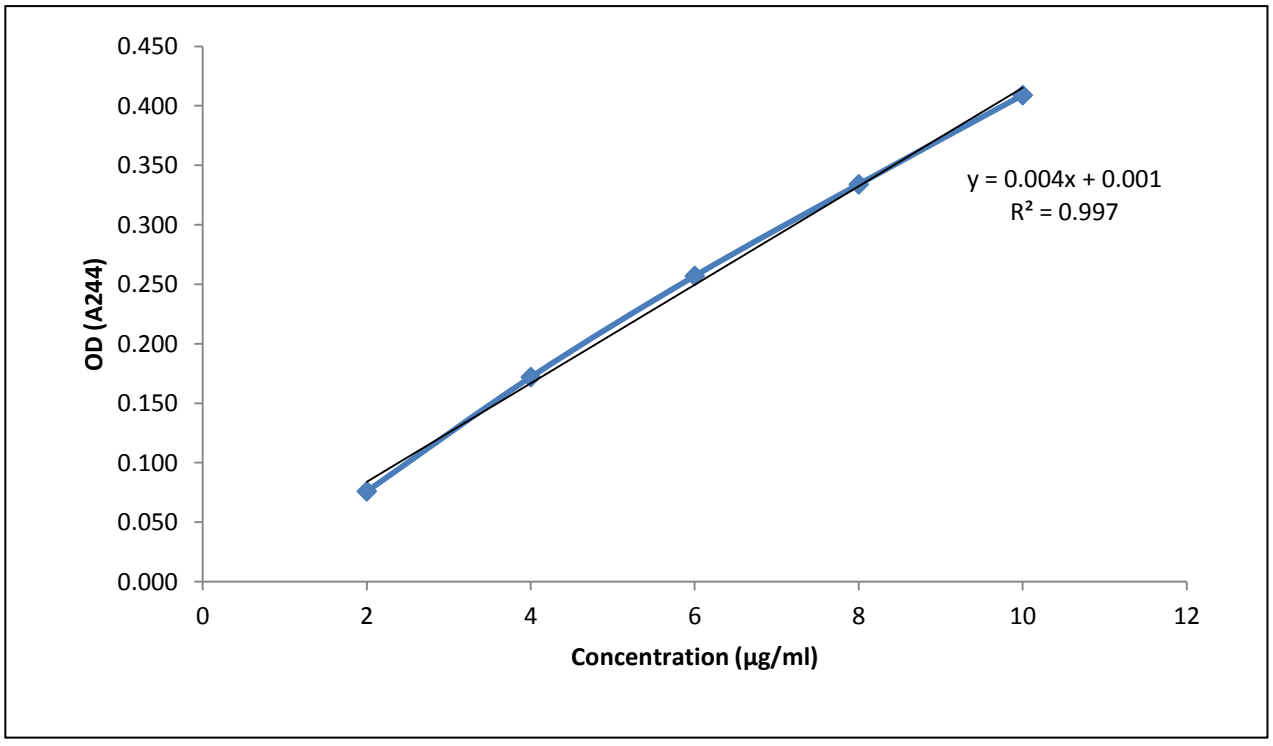

Figure 10: Standard curve of L-ascorbic acid in buffer pH 1.2.

Table 8: Standard curve data of L-Ascorbic acid in buffer pH 6.8.

S. No.	Concentration (µg/ml)	Absorbance (A_{244})
1.	2	0.073
2.	4	0.122
3.	6	0.179
4.	8	0.237
5.	10	0.308

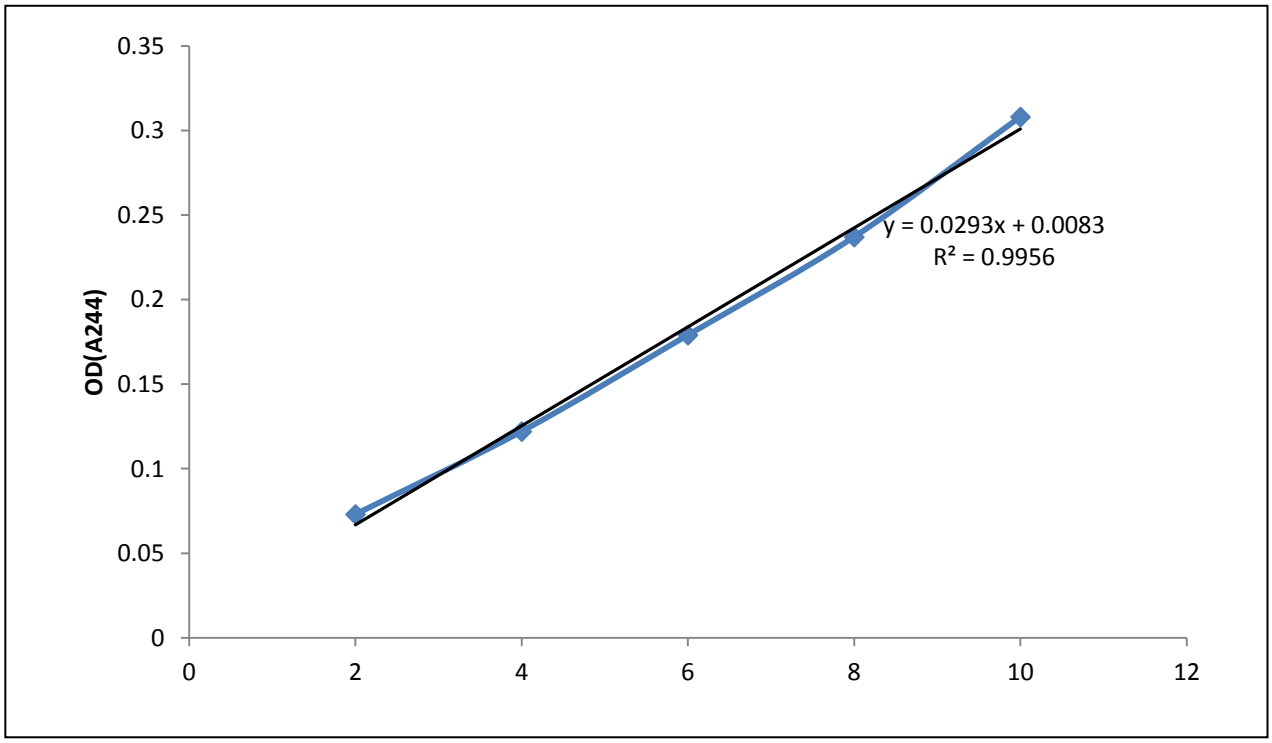

Figure 11: Standard curve of L-ascorbic acid in buffer pH 6.8.

5.3 Preparation of chitosan microspheres

Chitosan microspheres were prepared by emulsification cross-linking method (Akbuka et al. 1994). This method utilizes the reactive functional amine group of chitosan to cross-link with the aldehyde groups of the cross-linking agent. Two sets of chitosan microspheres were prepared: (a) L-ascorbic acid encapsulated microspheres and (b) Blank microspheres.

a. L-ascorbic acid encapsulated microspheres

In this method, chitosan solution (1%, 1.5%, 2%, 2.5% w/v) was prepared by dissolving chitosan in 2% v/v of acetic acid at room temperature. The drug, L-ascorbic acid (0%, 0.5%, 1%, 1.5%) was dissolved directly into the above prepared chitosan solution. 20ml solution was dropped from a constant through a disposable syringe needle into gently agitating 125ml light liquid paraffin containing 0.5% span 80 as emulsifier. The dropping rate and falling distance were kept constant (in case of blank no drug was added). The above water-in-oil emulsion was stirred for 15 minutes. Then glutaraldehyde (25% v/v) was added drop wise at an interval of 15 minutes, for an hour. The total volume of glutaraldehyde added was 2.5ml. The solution was stirred for two and a half hours, followed by filtration and rinsing with hexane. Microspheres obtained were air dried overnight for 24 hours followed by oven drying at 28°C.

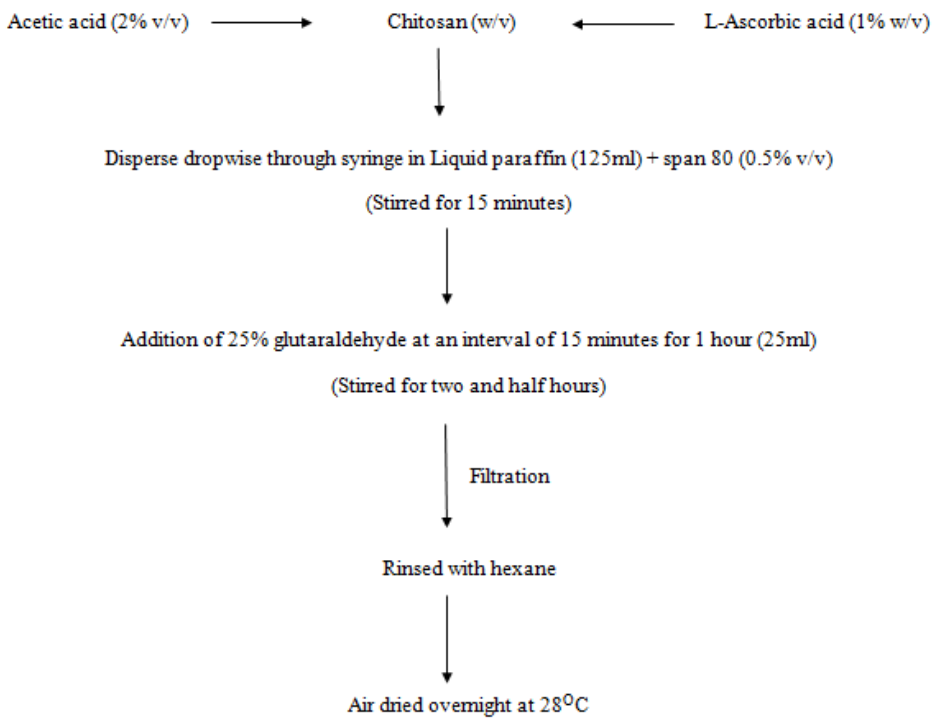

Figure 12: Process flowchart for the preparation of chitosan microspheres.

Different batches of cross-linked chitosan microspheres were prepared by altering the following formulation variables:

Table 9: Formulation and process parameters for preparation of different batches of microspheres.

Concentration of chitosan	(1%, 1.5%, 2%, 2.5% w/v)
Drug concentration	(0%, 0.5%, 1%, 1.5%,w/v)
Volume of 25% v/v Glutaraldehyde	2.5ml
Volume of chitosan-drug solution	20ml
Total volume of solution	147.5ml
Stirring time	2.5 hours
Temperature	28-30°C

Table 10: Formulation of L-ascorbic acid encapsulated chitosan microspheres.

Batch No.	**Conc. of Chitosan (%w/v)**	**Polymer to drug ratio**	**Conc. of drug (% w/v)**	**Volume of glutaraldehyde (25% v/v) (ml)**	**Stirring time (hours)**	**Volume of chitosan-drug solution (ml)**
B1	1.5	3:1	0.5	2.5	2.5	20
B2	2	4:1	0.5	2.5	2.5	20
B3	2.5	5:1	0.5	2.5	2.5	20
B4	1.5	1.5:1	1	2.5	2.5	20
B5	2	2:1	1	2.5	2.5	20
B6	2.5	2.5:1	1	2.5	2.5	20
B7	1.5	1:1	1.5	2.5	2.5	20
B8	2	1.3:1	1.5	2.5	2.5	20
B9	2.5	1.7:1	1.5	2.5	2.5	20

b Blank microspheres

Encapsulation of L-ascorbic acid in blank microspheres by varying chitosan concentration (P1: 1.5%, P2: 2%, P3: 2.5% w/v) was performed under different pH conditions. The blank microspheres were suspended in ascorbic acid solution of different pH (3,5,7,9) for 4 hours. These microspheres were then filtered, followed by hexane wash and were dried at 28°C.

Table 11: Formulation of blank cross-linked chitosan microspheres.

Batch No.	Conc. of Chitosan (%w/v)	pH	Volume of glutaraldehyde (25% v/v) (ml)	Stirring time (hours)
P1a	1.5	3	2.5	2.5
P1b	1.5	5	2.5	2.5
P1c	1.5	7	2.5	2.5
P1d	1.5	9	2.5	2.5
P2a	2	3	2.5	2.5
P2b	2	5	2.5	2.5
P2c	2	7	2.5	2.5
P2d	2	9	2.5	2.5
P3a	2.5	3	2.5	2.5
P3b	2.5	5	2.5	2.5
P3c	2.5	7	2.5	2.5
P3d	2.5	9	2.5	2.5

Chapter 6: Results and discussion

Microspheres were prepared by emulsification cross-linking method. In emulsification cross-linking method, water-in-oil emulsion was prepared by emulsifying the chitosan aqueous solution in oil phase. The method is sophisticated, require accurate handling and measurements. The equipments used must be properly calibrated and the solutions prepared must be homogenous in nature.

The aim of this investigation was to study the influence of varying drug concentration, polymer concentrations, stirring rate, pH and other related parameters on cross-linked chitosan microspheres encapsulated with L-ascorbic acid.

Different batches of microspheres were prepared by altering the formulation variables and process conditions. All the batches of chitosan based microspheres were evaluated for the following properties:

1. Percentage yield
2. Average particle size and morphology (SEM)
3. Equilibrium swelling studies
4. Encapsulation efficiency
5. *In vitro* release studies

6.1 Evaluation of L-ascorbic acid encapsulated chitosan microspheres.

6.1.1 Percentage yield

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

$$\% \text{ Yield} = \frac{\text{Wt of dried microspheres}}{(\text{wt of polymer} + \text{drug} + \text{cross-linker})} \times 100$$

The percentage yield data for the batches obtained is discussed in table 12 and the histogram comparison is shown in figure 13.

Table 12: Percentage yield of different batches of chitosan based microspheres.

Batch No.	Yield (g)	Percentage yield (%)
B1	0.90	29.37
B2	1.11	35.31
B3	1.51	46.34
B4	0.91	28.99
B5	1.11	34.14
B6	1.39	41.51
B7	0.82	25.34
B8	1.11	33.23
B9	1.45	41.91

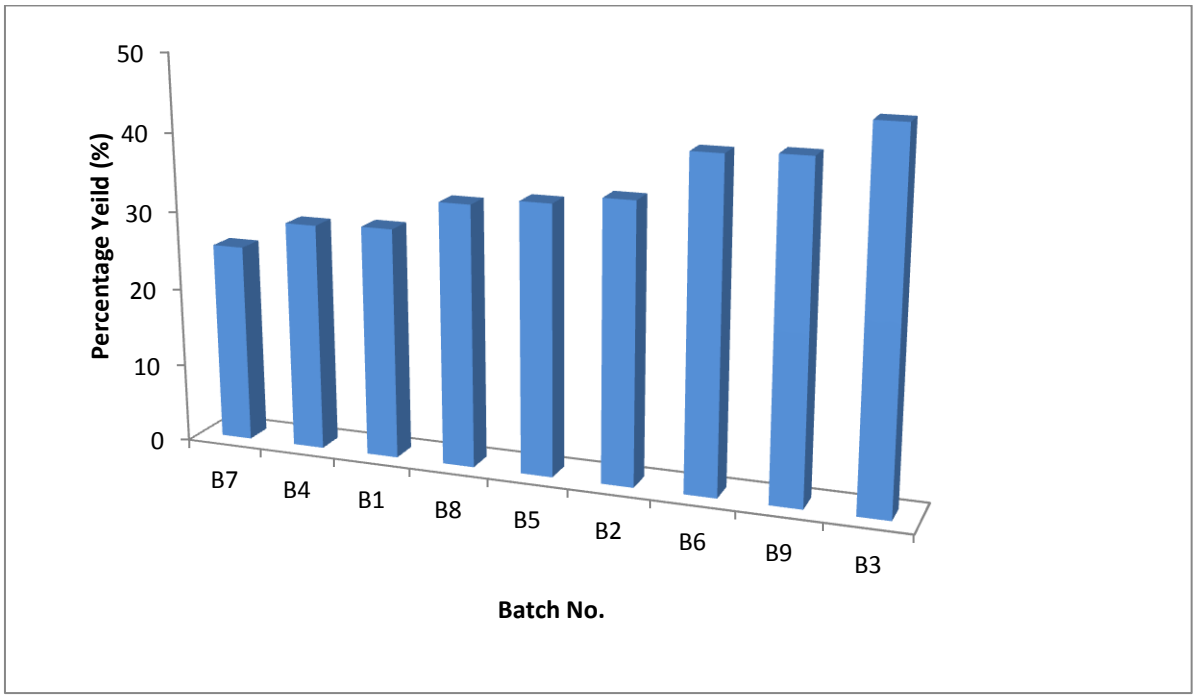

Figure 13: Effect of various formulation parameters on percentage yield of L-ascorbic acid encapsulated chitosan microspheres.

The percentage yield ranged from 25.34% to 41.91%. It was observed that there was increase in percentage yield with increase in chitosan concentration. This difference in percentage yield due to change in concentration of chitosan was also observed in blank batches; P1=31.25%, P2=34.25; P3= 37.23%. Increase in drug concentration also had some significant effect. The percentage yield was decreasing with increase in drug concentration. However the effect of change in drug concentration was less as compared to the effect caused by chitosan. As the polymer to drug ratio increased, the percentage yield also increased, with maximum yield obtained for batch B3 with 2.5% chitosan and 0.5 % drug.

6.1.2 Average particle size

The particle size of the microspheres was measured directly by "Nikon SMZ 800 light microscope". The chitosan microspheres were mounted on the stage of microscope and diameter of microspheres was measured with the help of ocular scale. The data for average particle size is reported in table13 and comparative histogram is shown in figure 14.

Table 13: Average size of different batches of chitosan microspheres.

Batch No.	Average size (µm)
B1	48
B2	90
B3	94
B4	59
B5	67
B6	73
B7	134
B8	118
B9	115

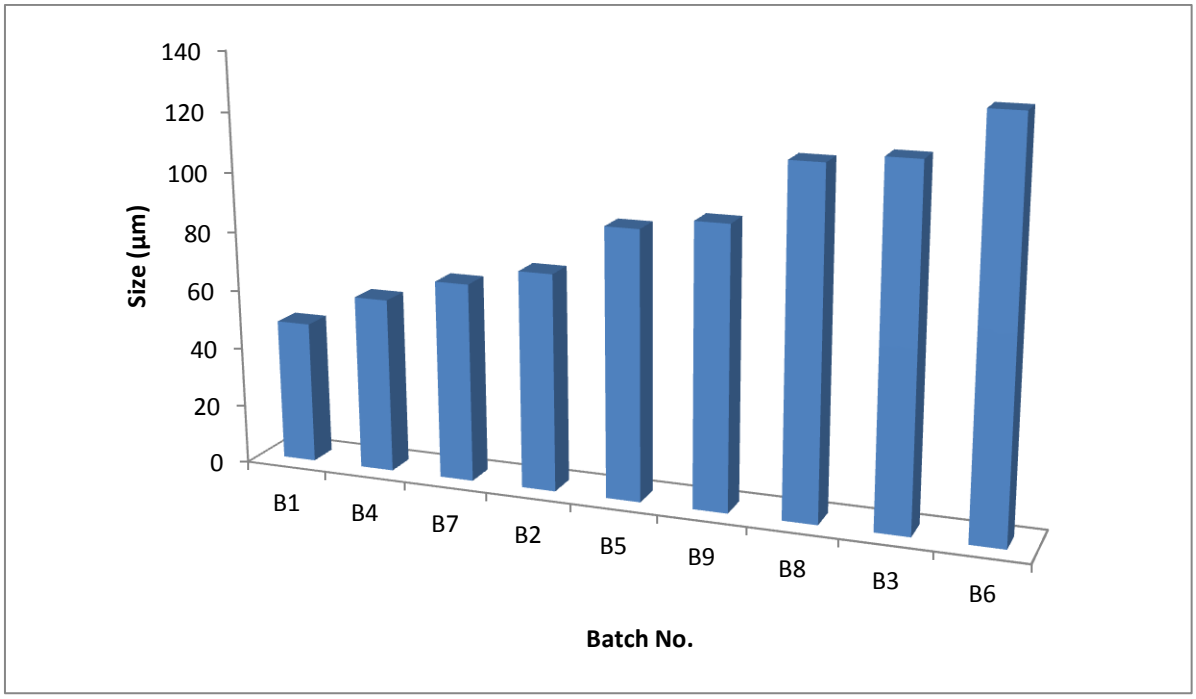

Figure 14: Effect of formulation on the size of microspheres.

6.1.2.1 Morphology

The size of microspheres was confirmed with the scanning electron microscopy and their morphology was recorded. Some of the observations are shown in figure 15. The surface of the microspheres encapsulated with L-ascorbic acid was observed to be rough as compared to the blank microspheres. The surface also gets smoother with the difference in concentration of drug and concentration of polymer. That is with higher polymer to drug ratio the surface gets smoother.

The effect of stirring was also recorded on the size of microspheres. A batch was prepared by varying the stirring rate from 500 rpm to 1000 rpm (100rpm every 30 minutes) during their preparation period. A wide range of sizes was obtained from this arrangement, shown in figure 16.

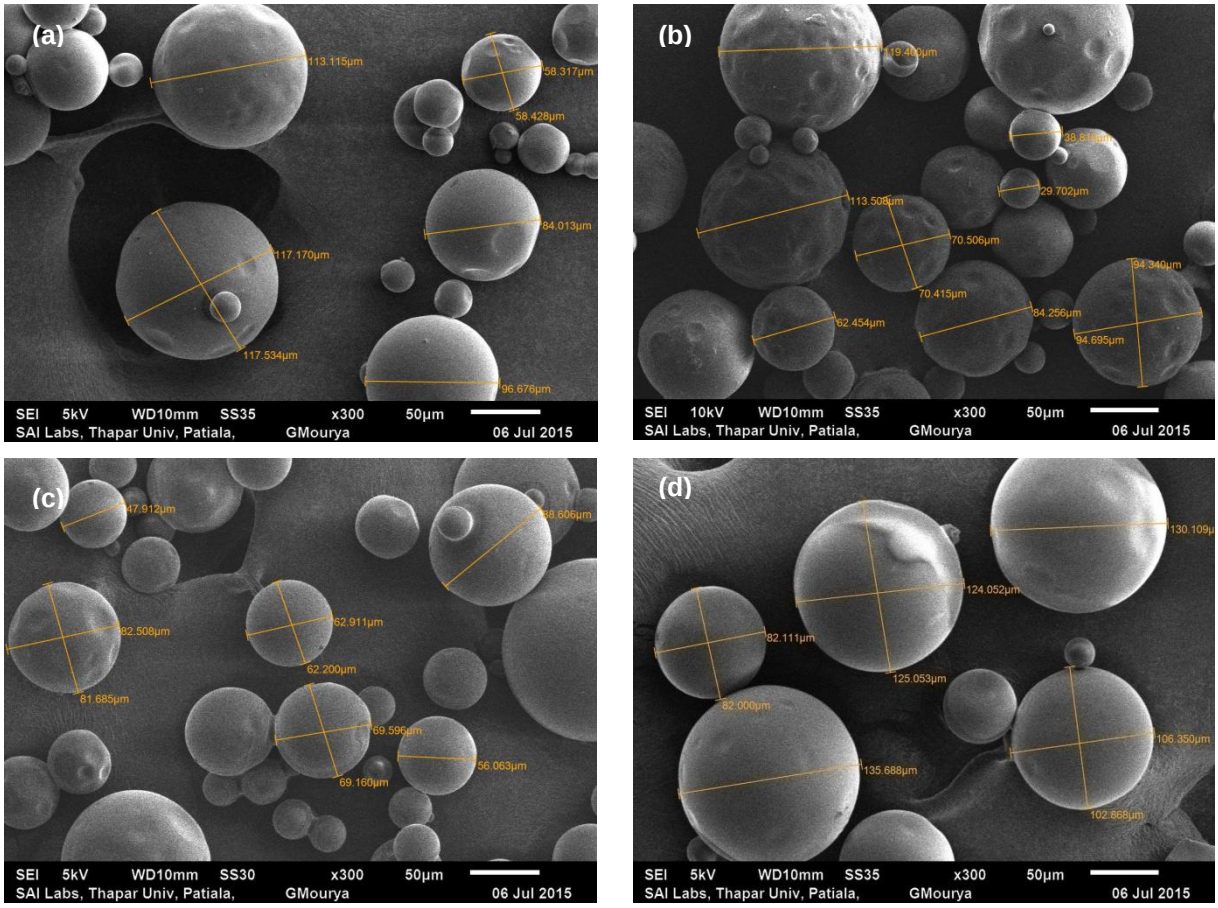

Figure 15: Size and morphology of L-ascorbic acid encapsulated chitosan microspheres: (a)B2, (b)B3, (c)B5, (d)B9.

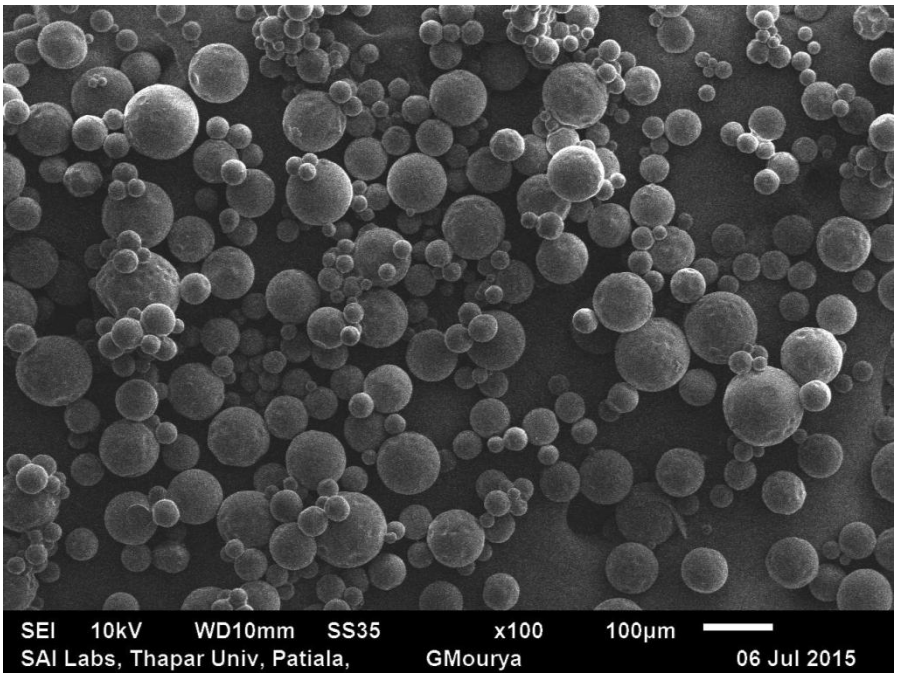

Figure 16: Effect of stirring rate on the size of microspheres.

6.1.3 Equilibrium swelling studies

The swelling ability of dried microspheres of chitosan microspheres was determined in distilled water (DW), buffer pH 1.2 and buffer pH 6.8 in different glass vials respectively. These immersed microspheres were kept at 37°C for 24 hours. Swollen microspheres were filtered, blotted and weighed immediately on an analytical balance. The percentage swelling index of microspheres at equilibrium was calculated by using the following formula:

$$\%Esw = \frac{Wo\text{-}We}{We} \times 100$$

Where,
Wo= Initial weight of microspheres.
We= Weight of microspheres at equilibrium.

The data for swelling index in water, buffer pH 1.2, and buffer pH 6.8 is reported in table 14 and their respective comparative histogram shown in figures 17, 18 and 19.

Table 14: Percentage swelling index of different batches of L-ascorbic acid encapsulated chitosan microspheres.

Batch No.	%Esw (with DW)	%Esw (buffer pH 1.2)	%Esw (buffer pH 6.8)
B1	37.5	65.2	33.1
B2	32.2	67.8	43.5
B3	24.2	43.4	21.9
B4	40.7	48.8	35.9
B5	38.8	71.1	46.5
B6	22.2	40.8	30.8
B7	36.1	62.1	32.4
B8	30.6	66.9	40.3
B9	15.6	35.5	22.5

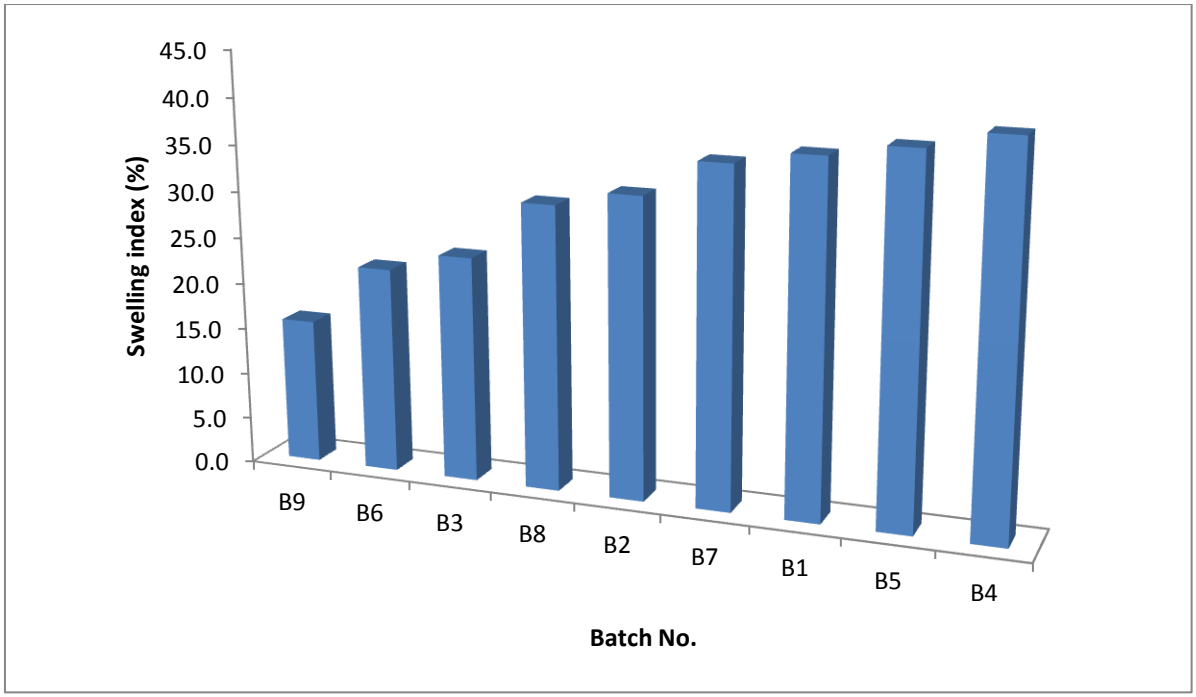

Figure 17: Effect of various parameters on percentage swelling index of L-ascorbic acid encapsulated chitosan microspheres in distilled water.

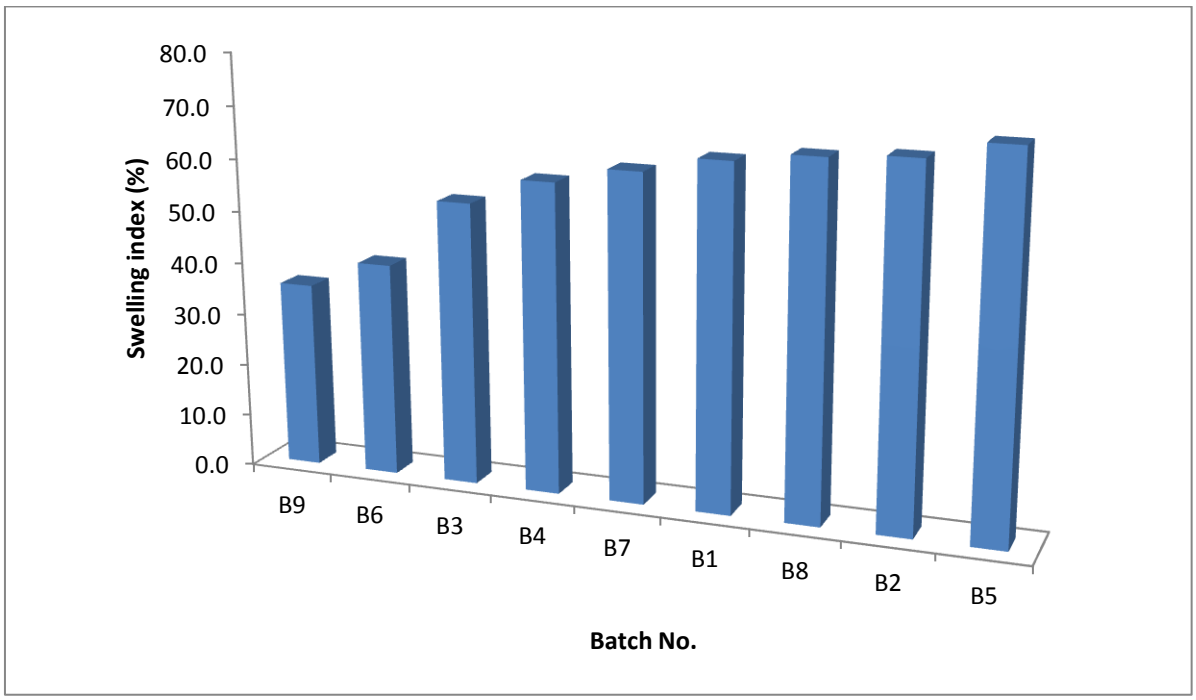

Figure 18: Effect of various parameters on percentage swelling index of L-ascorbic acid encapsulated chitosan microspheres in buffer pH 1.2.

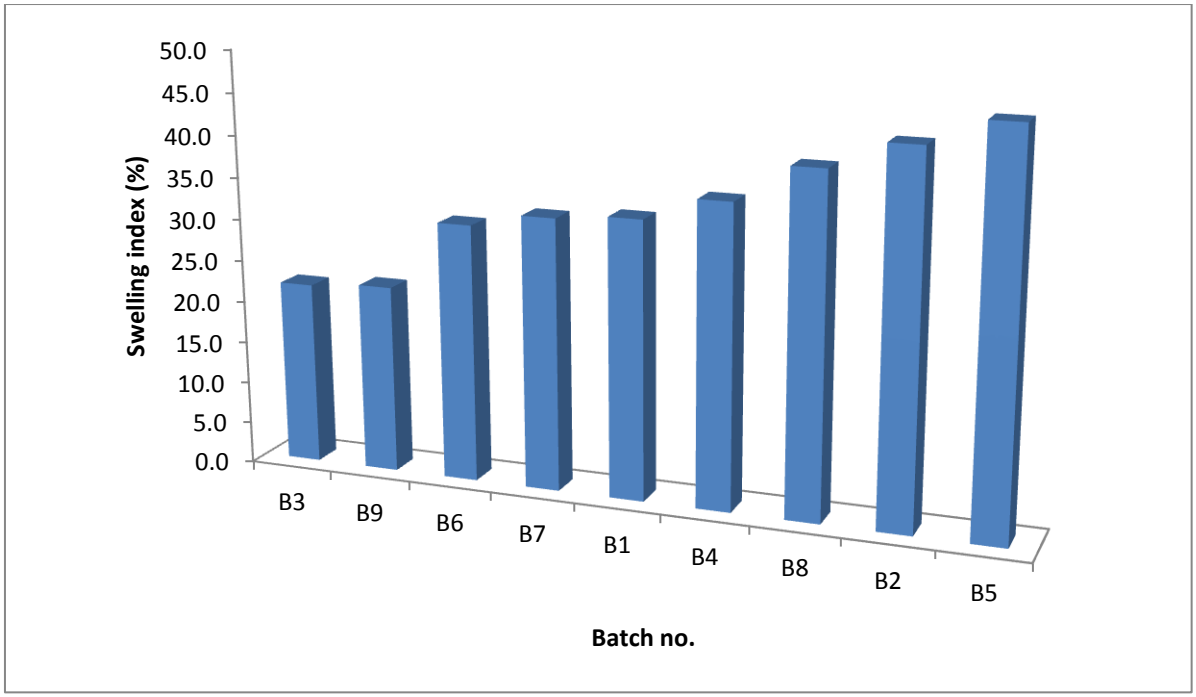

Figure 19: Effect of various parameters on percentage swelling index of L-ascorbic acid encapsulated chitosan microspheres in buffer pH 6.8.

At low pH (pH 1.2), the swelling index is recorded to be more as compared to the swelling index in distilled water and pH 6.8. This is because at low pH the protonation of amino groups present in chitosan take place. This leads to repulsion in the chains of the polymer resulting into dissociation of secondary interactions such as intramolecular hydrogen bonding and hence allowing more water into the microspheres.

6.1.4 Determination of encapsulation efficiency

20 mg of L-ascorbic acid encapsulated chitosan microspheres were crushed properly into fine powder using pestle and mortar. This powder was then suspended in 10ml of buffer pH 1.2 and its L-ascorbic acid absorbance was taken using UV spectrophotometer at 244nm. Then drug concentration was calculated for each batch separately.

$$\% \text{ Encapsulation efficiency} = \frac{\% \text{ Drug loading}}{(\% \text{ Theoretical loading})} \times 100$$

The encapsulation efficiencies obtained for the respective batches is reported in table 15 and the comparative histogram in figure 20.

Table 15: Encapsulation efficiency for different batches of L-ascorbic acid encapsulated chitosan microspheres.

Batch No.	Encapsulation efficiency (%)
B1	46.58
B2	83.42
B3	88.81
B4	30.40
B5	58.25
B6	82.40
B7	30.48
B8	45.83
B9	79.77

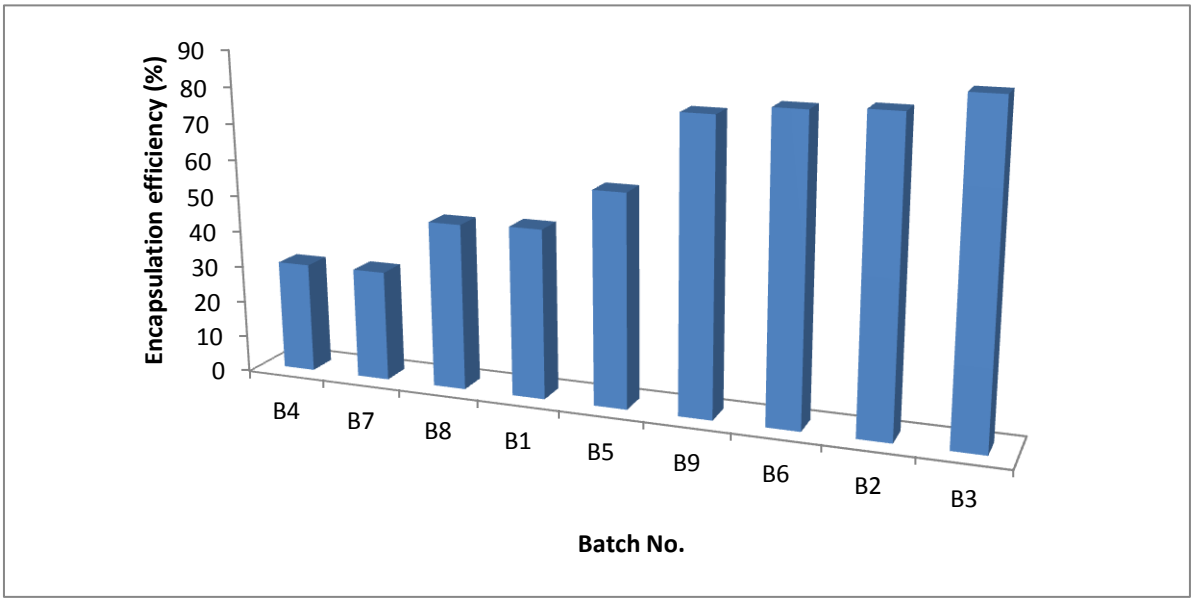

Figure 20**:** Effect of various formulation parameters on encapsulation efficiency of L-ascorbic acid encapsulated microspheres.

The trend observed in the encapsulation of drug in the microsphere is that the encapsulation efficiency increases with increase in chitosan concentration. With increase in chitosan concentration the cross-linking increases resulting in higher entrapment of drug molecules in the surface gel matrix as well as in the core of microspheres.

However the polymer to drug ratio also interferes in drug encapsulation. It was observed that higher the polymer to drug ratio more will be the encapsulation efficiency. Higher polymer concentration as compared to drug concentration allows greater entrapment as well as encapsulation efficiency (B2), vice versa is also true i.e. if the concentration of both, the polymer and the drug are comparable then it lowers the encapsulation efficiency (B4, B7).

6.1.5 *In vitro* release studies

In accordance with the physiological gastrointestinal conditions, the *in vitro* release study was performed in buffer of pH 1.2 followed by in buffer of pH 6.8. The dissolution behavior of cross-linked chitosan microspheres was dependent on pH. They swell in buffer of pH 1.2 but didn't show much change in buffer of pH 6.8. An initial burst 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 can be attributed to the diffusion of the drug entrapped in core of microspheres. It was also observed that with change in polymer to drug ratio the release profile varied. In buffer of pH 1.2, protonation of amino groups breaks the hydrogen bonds, leading to weaker electrostatic interactions and hence causes swelling of microspheres and higher release of drug. However in buffer of pH 6.8, there is strong attractive force between phosphate and chitosan, which causes slower release of drug.

50mg of drug loaded chitosan microspheres were incubated in 100ml buffer of pH 1.2 in a 150 ml conical flask in a shaking incubator at 37°C at 100 rpm. After four hours microspheres were filtered and transferred into 100 ml buffer of pH 6.8 and again incubated in similar conditions as before. Starting from 0 hour and at desired intervals of time 3ml sample was withdrawn and replaced with the same amount of fresh buffer respectively. Released concentration of L-ascorbic acid at different time interval was calculated and release profile of the batches was studied.

Table 16: Percentage cumulative release studies of the batches B1 to B9.

S.no.	Time (hrs)	% cumulative release								
		B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**
1	0	0	0	0	0	0	0	0	0	0
2	0.5	45.33	50.67	42.94	60.00	40.00	46.84	62.61	41.30	67.07
3	1	42.69	55.52	49.88	70.97	44.53	56.68	72.15	59.13	75.43
4	2	51.91	63.62	56.26	77.91	49.87	63.26	77.82	64.79	79.24
5	3	58.19	71.19	58.14	88.11	56.70	77.57	81.10	61.41	84.50
6	4	61.70	73.41	59.32	90.91	62.61	86.85	87.05	81.54	93.10
7	4.5	72.85	76.26	61.89	91.02	63.21	88.17	88.91	82.99	93.60
8	5	75.94	75.71	61.89	91.14	64.38	88.27	88.66	82.54	93.82
9	6	75.58	75.87	61.89	90.80	64.04	90.26	89.52	82.52	93.37
10	7	75.11	75.42	62.12	90.56	63.77	87.74	88.12	81.35	93.02
11	8	75.55	76.05	61.82	90.55	64.15	88.08	87.82	81.31	92.93
12	24	78.69	76.05	62.28	92.16	66.66	88.56	88.87	82.54	93.97

The data of percent cumulative release rate is presented in table 16. The release profiles recorded for each batch is depicted in graphical form in figure 21. The comparison of the release profiles with change in polymer concentration and keeping the drug concentration constant is illustrated in figure 22. The comparison with respect to change in concentration of drug and constant concentration of polymer are depicted in figure 23.

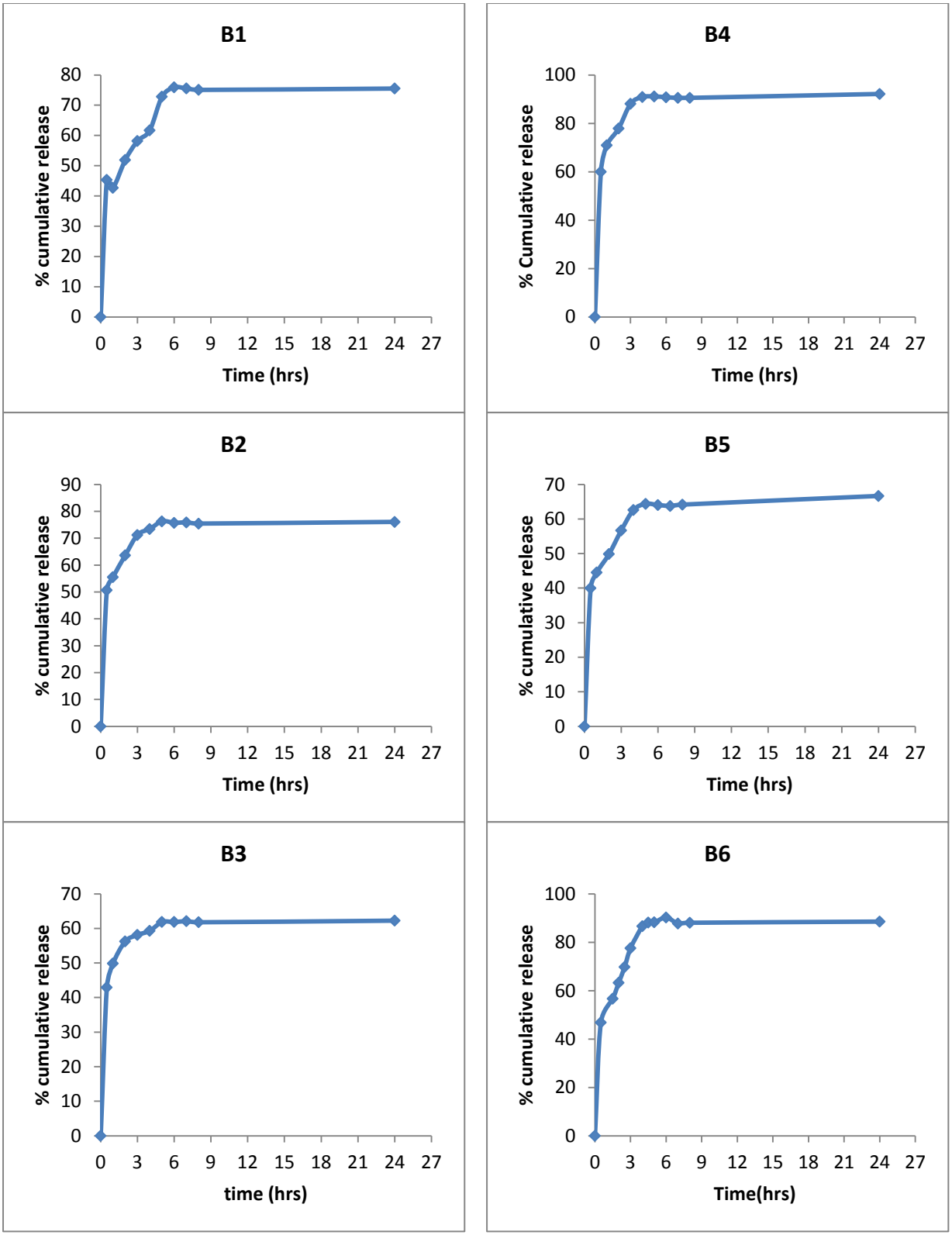

Figure 21(a): Drug release profiles of L-ascorbic acid encapsulated chitosan microspheres (B1 to B6).

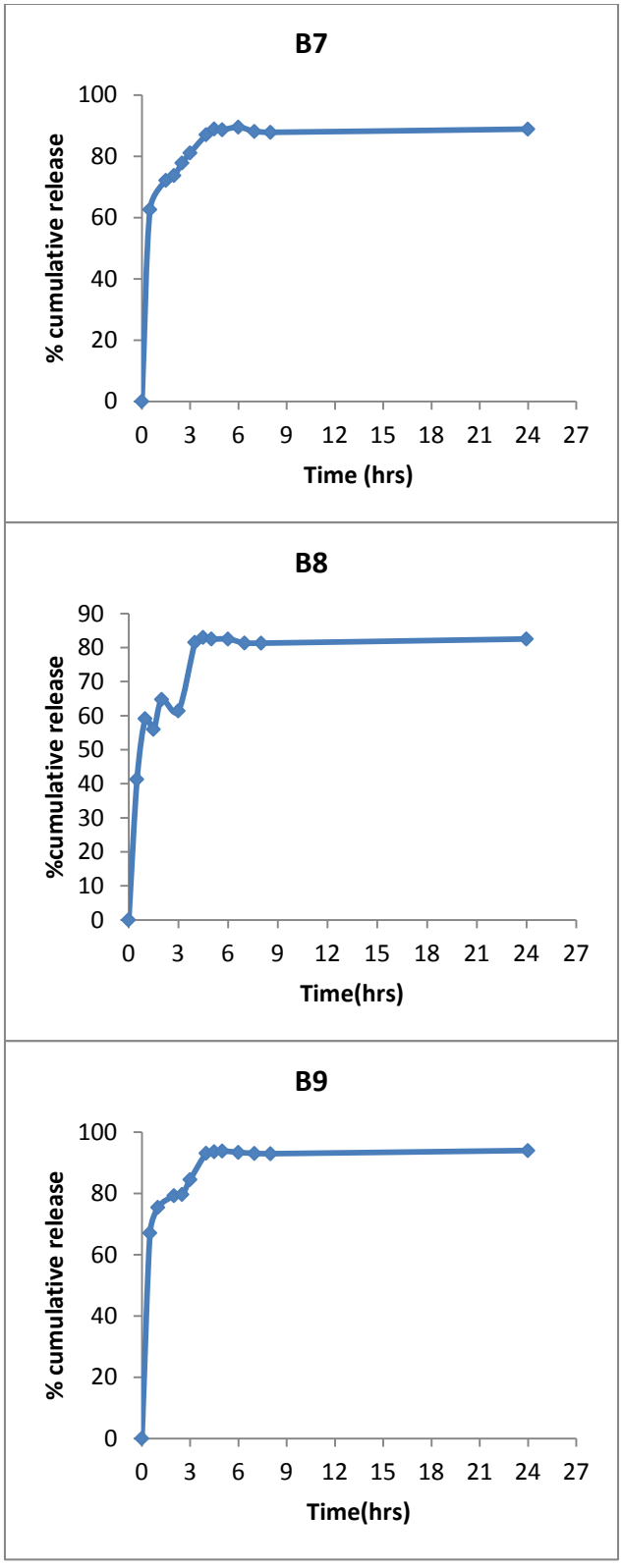

Figure 21(b): Drug release profiles of L-ascorbic acid encapsulated chitosan microspheres (B7 to B9).

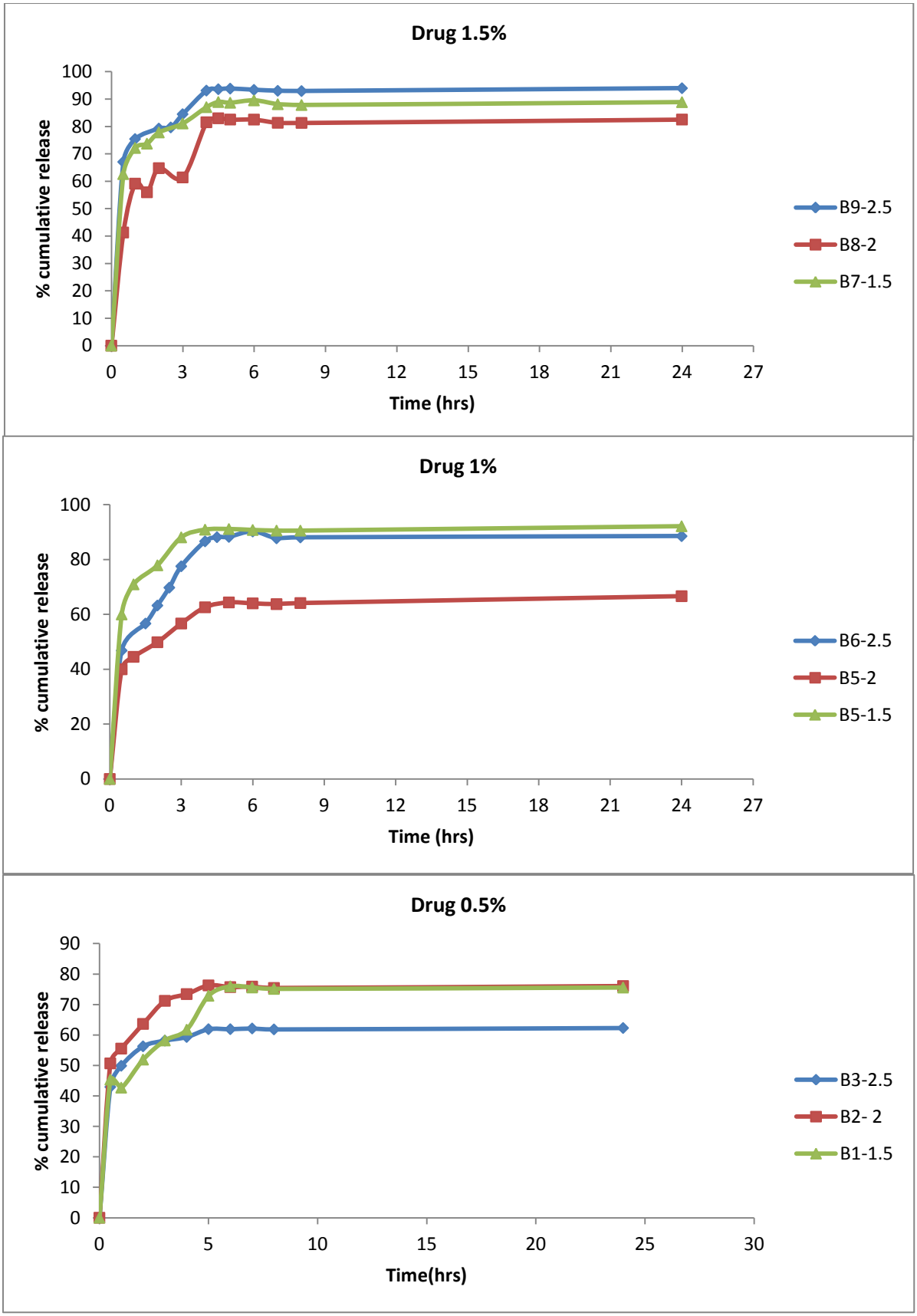

Figure 22: Drug release profile comparison of different polymer concentration keeping drug concentration constant.

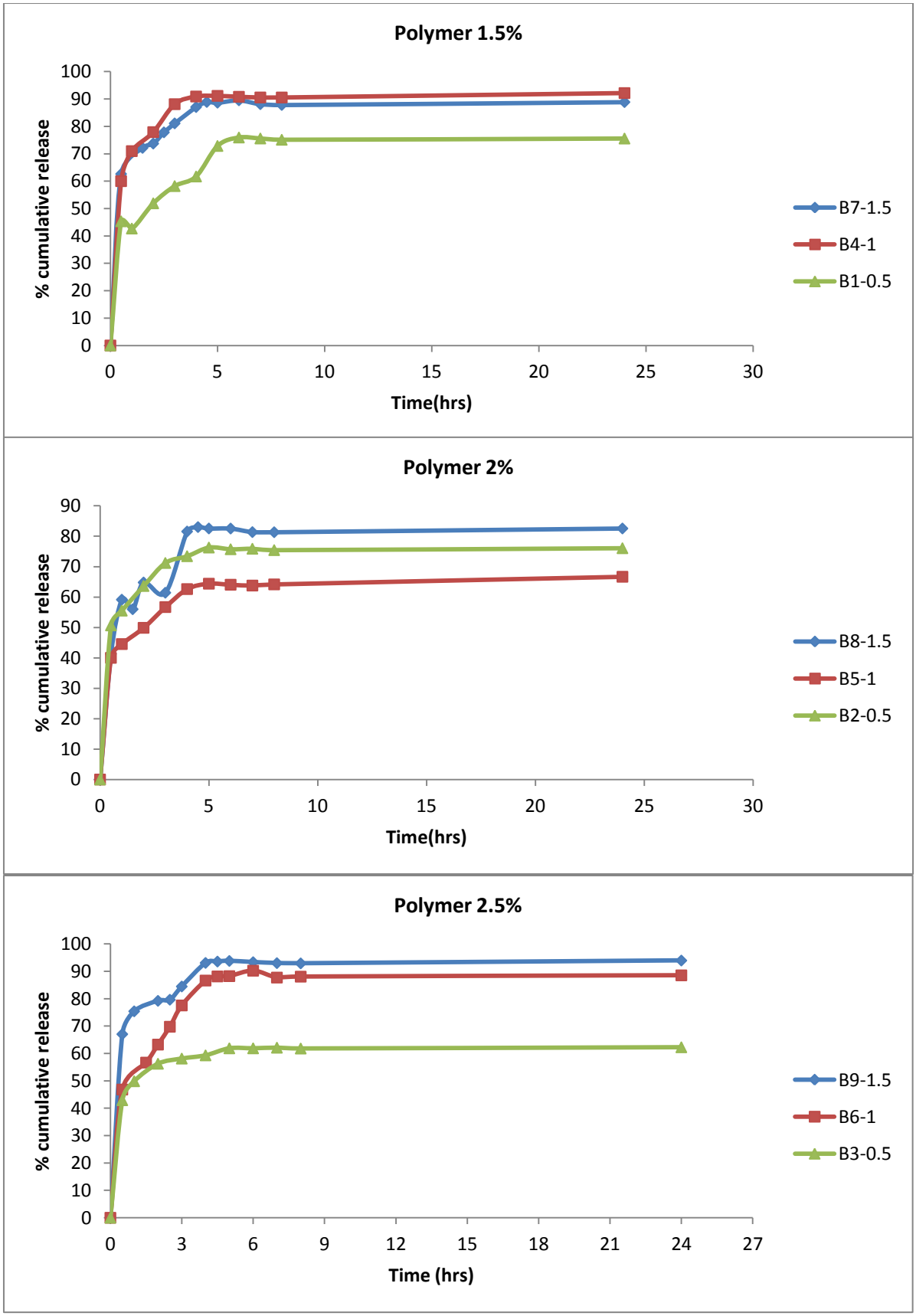

Figure 23: Drug release profile comparison of different drug concentration keeping polymer concentration constant.

The drug release profile depends on various factors such as concentration of polymer, drug encapsulation, environmental conditions etc. Initially burst effect was recorded for all the batches as seen in figure 21. This is due to the release of drug entrapped in surface matrix of microspheres and also attribute to high encapsulation efficiency. This burst effect was recorded maximum for B9 i.e. 67.07% followed by B7 (62.61%) and B4 (60.00%). In B7 and B4 the polymer to drug ratio is 1:1 and 1.5:1 respectively, which contributes to the high burst effect.

It can be inferred from the release profiles that with decrease in concentration of drug the release profile for respective polymer concentration changes. For polymer concentration 2.5% (w/v) the values of release kinetics increase with increase in encapsulated drug efficiency. Similar trend was observed for polymer concentration 1.5% (w/v). However for polymer concentration 2% (w/v) this trend was not observed. For B5 and B6 with polymer to drug ratio 2:1 and 2.5:1 respectively, the release rate is low which is caused by low diffusion of drug molecules from highly cross-linked microspheres.

6.2 Evaluation of blank chitosan microspheres.

6.2.1 Percentage yield

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

$$\% \text{ Yield} = \frac{\text{Wt of dried microspheres}}{(\text{wt of polymer} + \text{drug} + \text{cross-linker})} \times 100$$

Table 17: Percentage yield of different batches of blank chitosan microspheres.

Batch No.	Yield (g)	Percentage yield (%)
P1	0.8983	31.25
P2	0.9762	34.25
P3	1.0984	37.23

The range of yield varied from 31.25 to 37.23. It was observed that the yield of blank microspheres increased with increase in polymer concentration. More the polymer concentration more will be the cross-linking induced by glutaraldehyde resulting in higher yield of microsphere formation.

Various attempts of preparation of microspheres with chitosan concentration 1% (w/v) were also made. The end product was aggregated flakes with no defined shape. This can be due to low level of cross-linking within the matrix.

6.2.2 Average particle size

The particle size of the microspheres was measured directly by "Nikon SMZ 800 light microscope". The chitosan microspheres were mounted on the stage of microscope and diameter of microspheres was measured with the help of ocular scale. The size of the blank chitosan microsphere ranged from 124 μm to 165 μm. The size increased with increase in chitosan concentration.

Table 18: Average sizes of different batches of blank chitosan microspheres.

Batch No.	Average size (µm)
P1	124
P2	144
P3	165

6.2.2.1 Morphology

The size of microspheres was confirmed with the scanning electron microscopy and their morphology was recorded. Some of the observations are shown in figure 24. The surface of the blank chitosan microspheres was smooth as compared to the surface of chitosan microspheres encapsulated with L-ascorbic acid, which was rather rough.

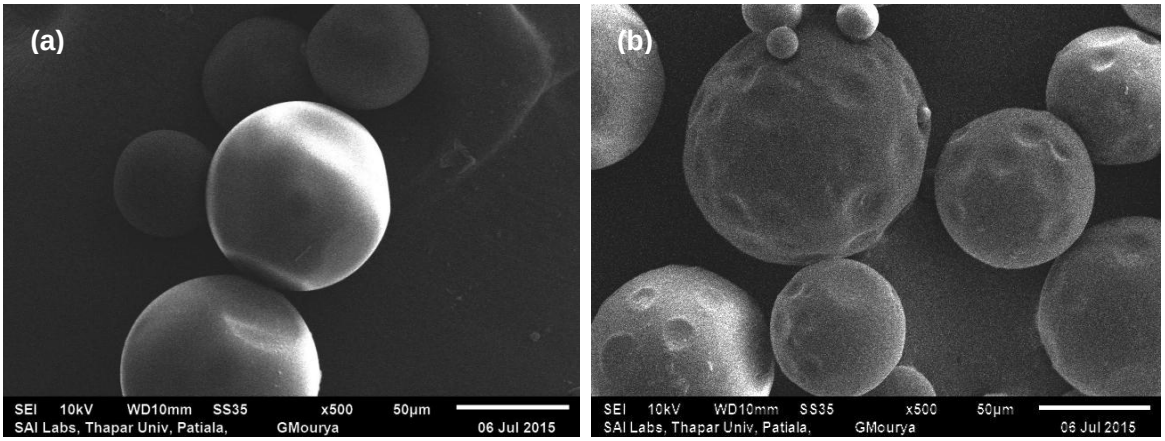

Figure 24: Comparison of the morphology of (a) blank chitosan microspheres(P2) and (b) L-ascorbic acid encapsulated chitosan microspheres (B2).

6.2.3 Equilibrium swelling studies

The swelling studies of blank microspheres were conducted in different pH (1.2, 3, 5, 6.8, 7, 9) conditions in different glass vials respectively. These immersed microspheres were kept at 37°C for 24 hours. Swollen microspheres were filtered, blotted and weighed immediately on an analytical balance. The percentage swelling index of microspheres at equilibrium was calculated by using the following formula:

$$\%Esw = \frac{We\text{-}Wo}{We} \times 100$$

Where,
Wo = Initial weight of microspheres
We = Weight of microspheres at equilibrium

Table 19: Percentage swelling index of different batches of blank chitosan microspheres.

S. no.	Conc. of Chitosan (%w/v)	pH	% Esw
1	**1.5**	9	39.76
2		7	41.35
3		6.8	35.06
4		5	52.94
5		3	62.12
6		1.2	65.16
7	**2**	9	23.66
8		7	33.99
9		6.8	45.50
10		5	62.34
11		3	68.05
12		1.2	70.06
13	**2.5**	9	12.28
14		7	18.37
15		6.8	27.54
16		5	48.45
17		3	50.25
18		1.2	51.92

At low pH i.e. at pH 1.2, the swelling index is recorded to be more as compared to the swelling index in distilled water and pH 6.8. This is because at low pH the protonation of amino groups present in chitosan take place. This leads to repulsion in the chains of the polymer resulting into dissociation of secondary interactions such as intramolecular hydrogen bonding and hence allowing more water into the microspheres. With increase in pH the amino group remain deprotonated, repulsion in the polymer chains diminish allowing shrinking.

6.2.4 Determination of encapsulation efficiency

Depending on the swelling studies, L-ascorbic acid solution was made for different pH (3,5,7,9). 150mg of microspheres of each concentration were immersed in the drug solution of different pH each for 5 hours. These microspheres were then dried and evaluated for various factors.

20 mg of L-ascorbic acid encapsulated chitosan microspheres were crushed properly into fine powder using pestle and mortar. This powder was then suspended in 10ml of pH 1.2 buffer and its L-ascorbic acid absorbance was taken using UV spectrophotometer at 244nm. Then drug concentration was calculated for each batch separately.

$$\% \text{ Encapsulation efficiency} = \frac{\% \text{ Drug loading}}{(\% \text{ theoretical loading})} \times 100$$

The data recorded for drug encapsulation efficiencies at different pH is reported in table 20. The comparative histogram for these is illustrated in figure 25.

Table 20: Encapsulation efficiency of L-ascorbic acid loaded chitosan microspheres.

Batch No.	Encapsulation efficiency (%)
P1a	21.60
P1b	12.00
P1c	9.80
P1d	4.10
P2a	20.60
P2b	13.10
P2c	10.80
P2d	4.40
P3a	11.70
P3b	10.40
P3c	6.20
P3d	3.70

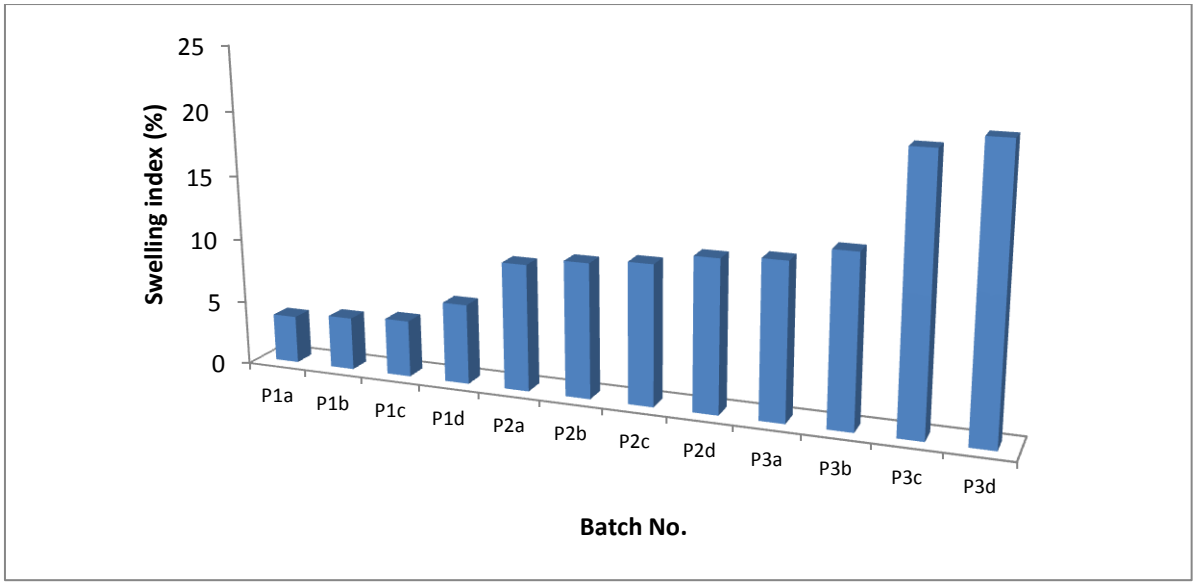

Figure 25: Effect of various parameters on encapsulation efficiency of L-ascorbic acid in blank microspheres.

The encapsulation efficiency was recorded to decrease with increase chitosan concentration. This is because with increase in polymer concentration the cross-linking gets dense and so inhibits the diffusion of drug molecule into the microsphere.

The encapsulation efficiency was higher at the lower pH, which can be attributed from swelling index. Higher the swelling index is more the drug entered into the core of blank microspheres.

6.2.5 *In vitro* release studies

In accordance with the physiological gastrointestinal conditions, the *in vitro* release study was performed in buffer pH 1.2 followed by in buffer pH 6.8. The dissolution behavior of cross-linked chitosan microspheres was dependent on pH. The swell in buffer pH 1.2 but didn't show much change in buffer pH 6.8. An initial burst 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 can be attributed to the diffusion of the drug entrapped in core of microspheres. It was also observed that with change in polymer to drug ratio the release profile varied. In buffer pH 1.2, protonation of phosphate ions breaks the hydrogen bonds, leading to weaker electrostatic interactions and hence causes swelling of microspheres and higher release of drug. However in buffer pH 6.8, there is strong attractive force between phosphate and chitosan, which causes slower release of drug.

50mg of drug loaded chitosan microspheres were incubated in 100ml buffer pH 1.2 in a 150 ml conical flask in a shaking incubator at 37°C at 100 rpm. After four hours microspheres were filtered and transferred into 100 ml buffer pH 6.8 and again incubated in similar conditions as before. Starting from 0 hour and at desired intervals of time 5ml sample was withdrawn and replaced with the same amount of fresh buffer respectively. Released concentration of L-ascorbic acid at different time interval was calculated and release profile of the batches was studied. The data thus obtained is presented in table 21, 22 and 23. The release profiles are shown in figure 26, 28 and 30. The comparative release profiles are depicted in figure 27, 29 and 31.

Table 21: Percentage cumulative release studies of the batches P1a to P1d.

S.no.	**Time (hrs)**	**% Cumulative release**			
		P1a	**P1b**	**P1c**	**P1d**
1	0	0	0	0	0
2	0.5	19.91	8.33	3.06	14.63
3	1	21.89	19.57	18.67	19.95
4	2	24.25	27.24	26.06	49.66
5	3	31.88	43.30	31.38	57.56
6	4	34.73	57.94	47.86	69.98
7	4.5	34.85	58.29	47.86	69.98
8	5	35.05	58.53	49.12	70.31
9	6	34.93	58.99	49.72	70.32
10	7	34.99	59.70	50.87	70.99
11	8	34.86	60.87	52.87	71.02
12	24	35.18	66.42	57.29	73.71

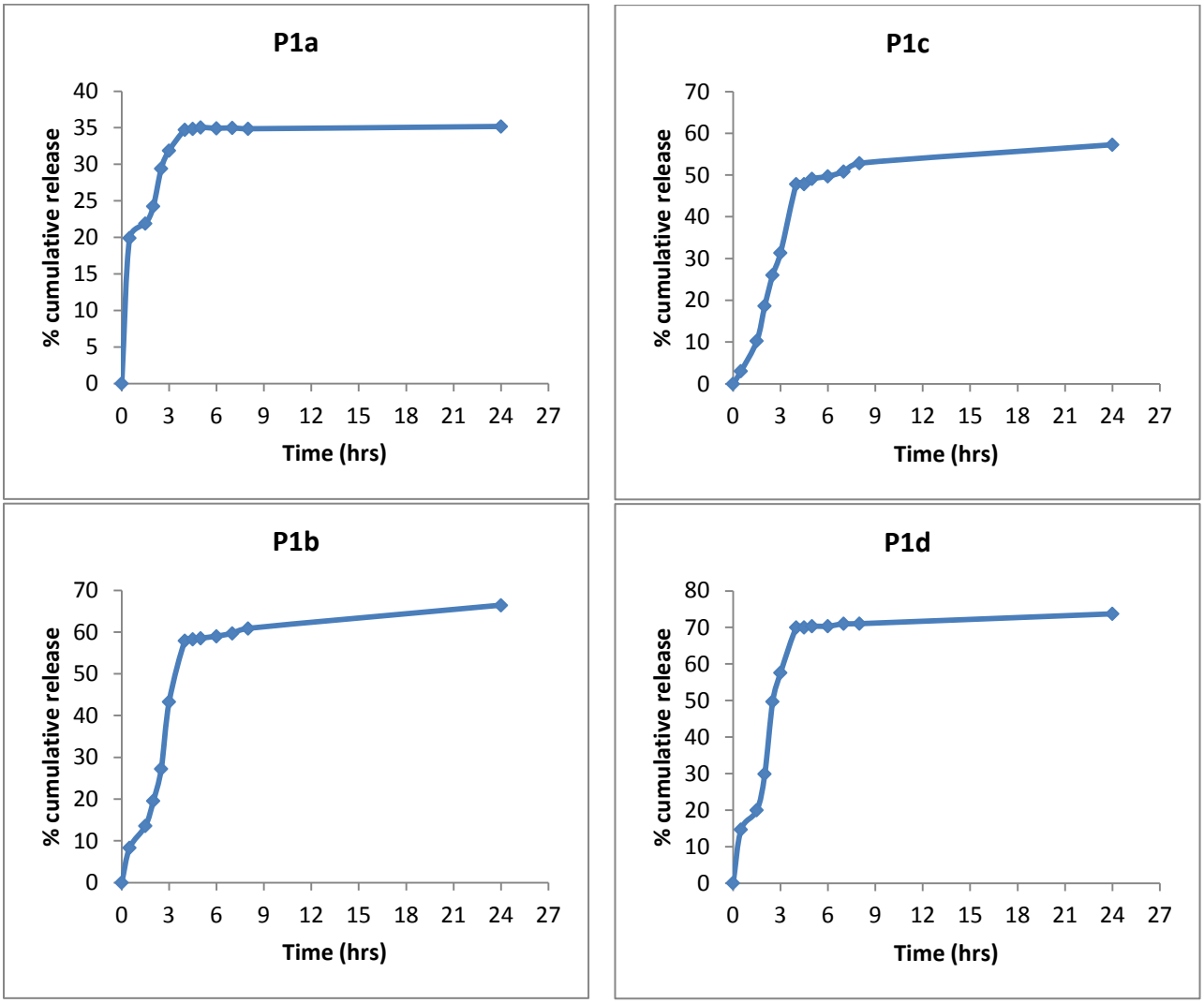

Figure 26: The drug release profiles of L-ascorbic acid loaded chitosan microspheres from P1a to P1d.

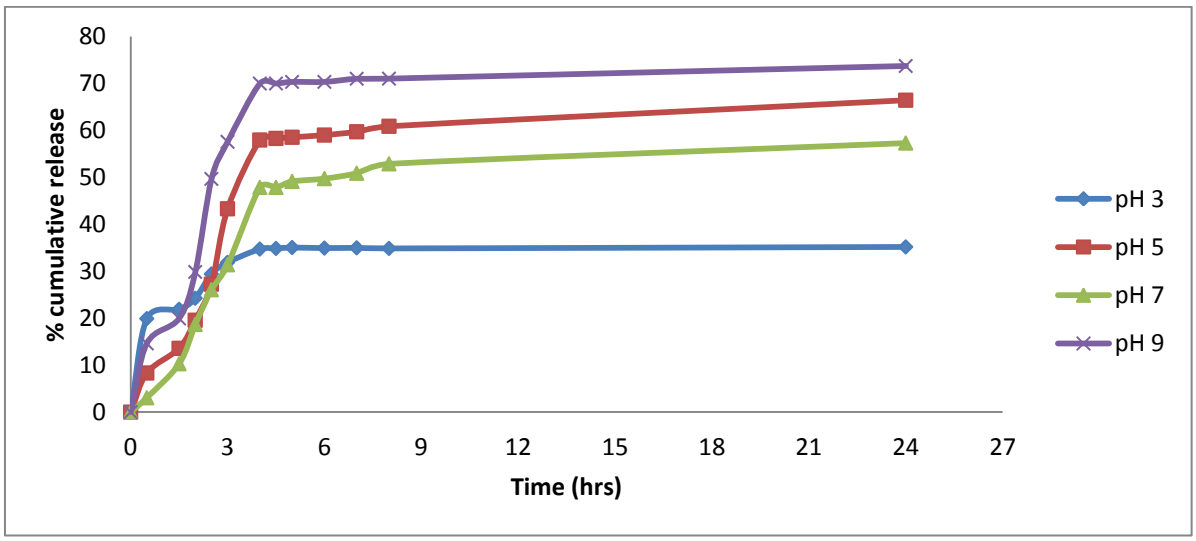

Figure 27: Effect of pH on drug release profile of L-ascorbic acid loaded chitosan microspheres with constant polymer concentration for batches P1a to P1d.

Table 22: Percentage cumulative release studies of the batches P2a to P2d.

S.no.	Time (hrs)	% Cumulative release			
		P2a	P2b	P2c	P2d
1	0	0	0	0	0
2	0.5	24.27	27.48	36.54	11.36
3	1	33.25	44.34	43.40	20.80
4	2	37.38	51.69	46.46	36.98
5	3	51.19	68.08	65.02	53.50
6	4	63.15	79.07	71.13	67.48
7	4.5	63.08	78.78	71.13	68.10
8	5	63.48	79.19	71.27	68.12
9	6	64.30	79.52	71.40	69.38
10	7	63.32	80.58	71.54	69.73
11	8	64.46	80.72	71.81	70.05
12	24	66.47	83.14	75.27	76.96

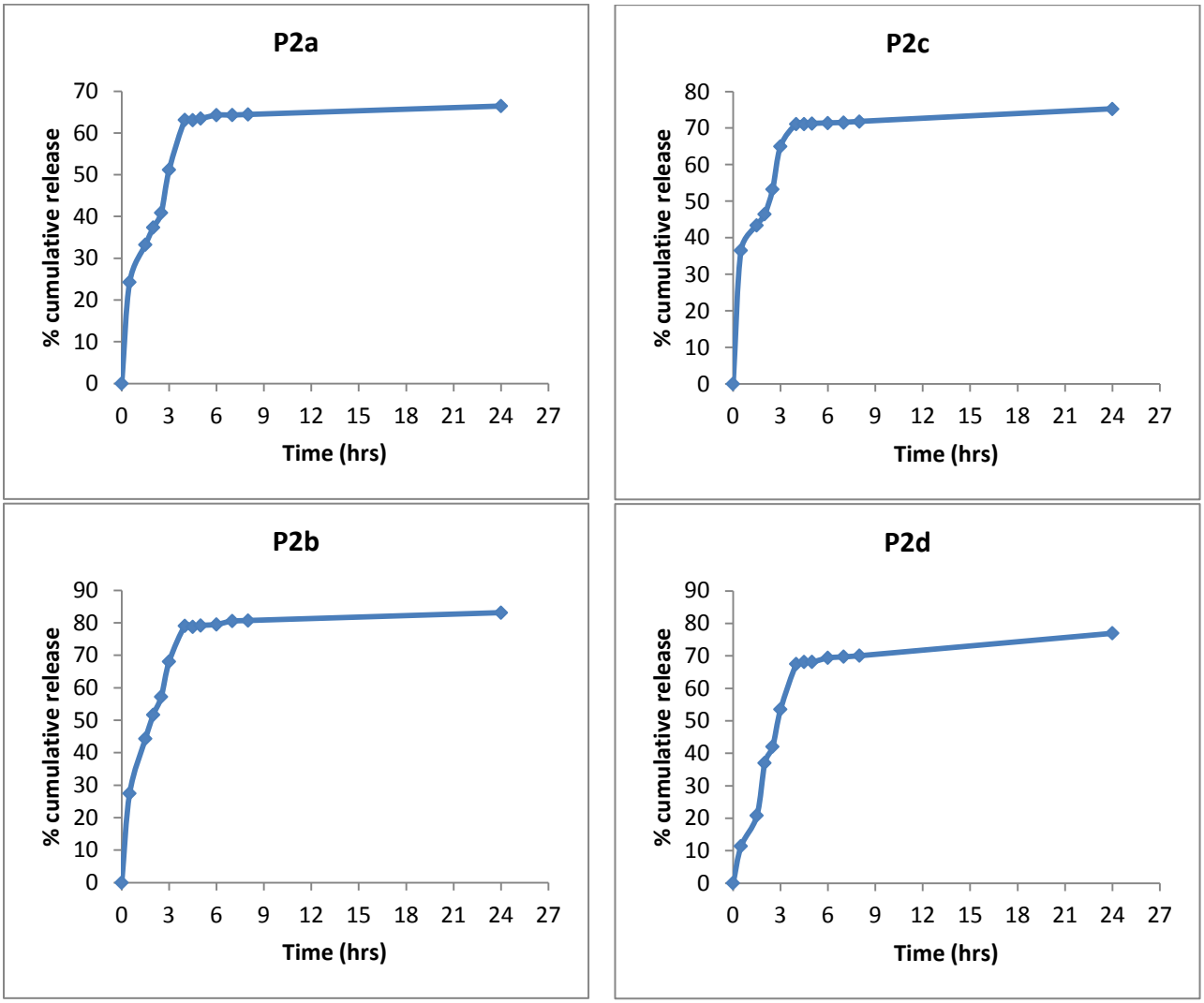

Figure 28: The drug release profiles of L-ascorbic acid loaded chitosan microspheres from P2a to P2d.

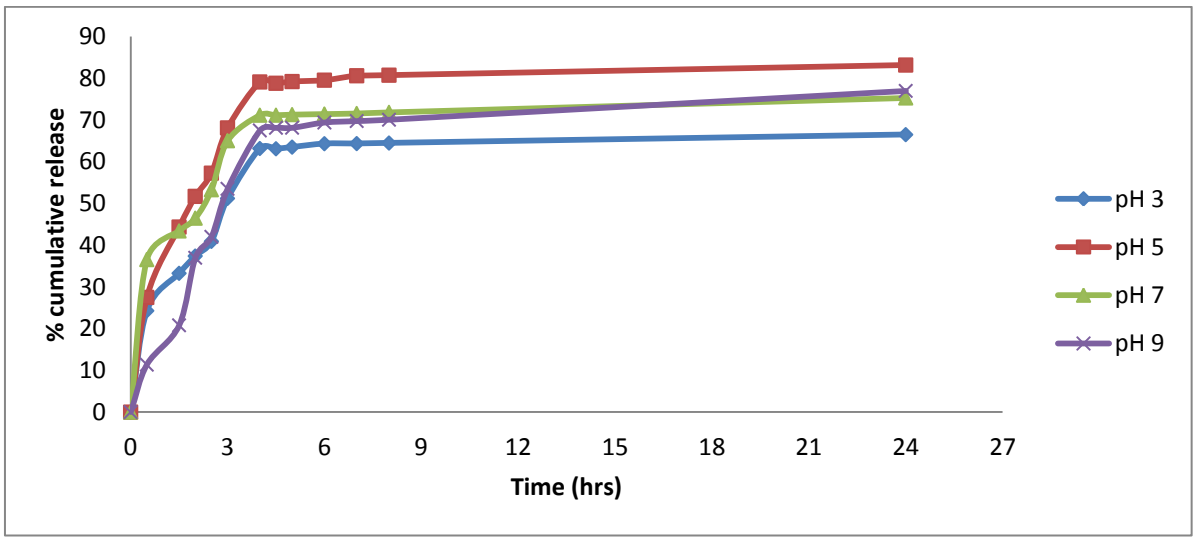

Figure 29: Effect of pH on drug release profile of L-ascorbic acid loaded chitosan microspheres with constant polymer concentration for batches P2a to P2d.

Table 23: Percentage cumulative release studies of the batches P3a to P3d.

S.no.	Time (hrs)	% Cumulative release			
		P3a	P3b	P3c	P3d
1	0	0	0	0	0
2	0.5	15.36	17.31	24.19	31.91
3	1	24.25	24.56	29.76	37.13
4	2	33.91	39.18	27.97	45.89
5	3	41.08	47.31	49.60	50.28
6	4	54.10	59.22	70.81	50.40
7	4.5	53.63	58.56	69.92	50.70
8	5	54.21	59.20	70.78	51.00
9	6	54.58	59.35	70.81	51.01
10	7	56.12	59.36	71.01	51.60
11	8	56.40	59.62	71.48	51.91
12	24	57.82	62.02	73.27	54.85

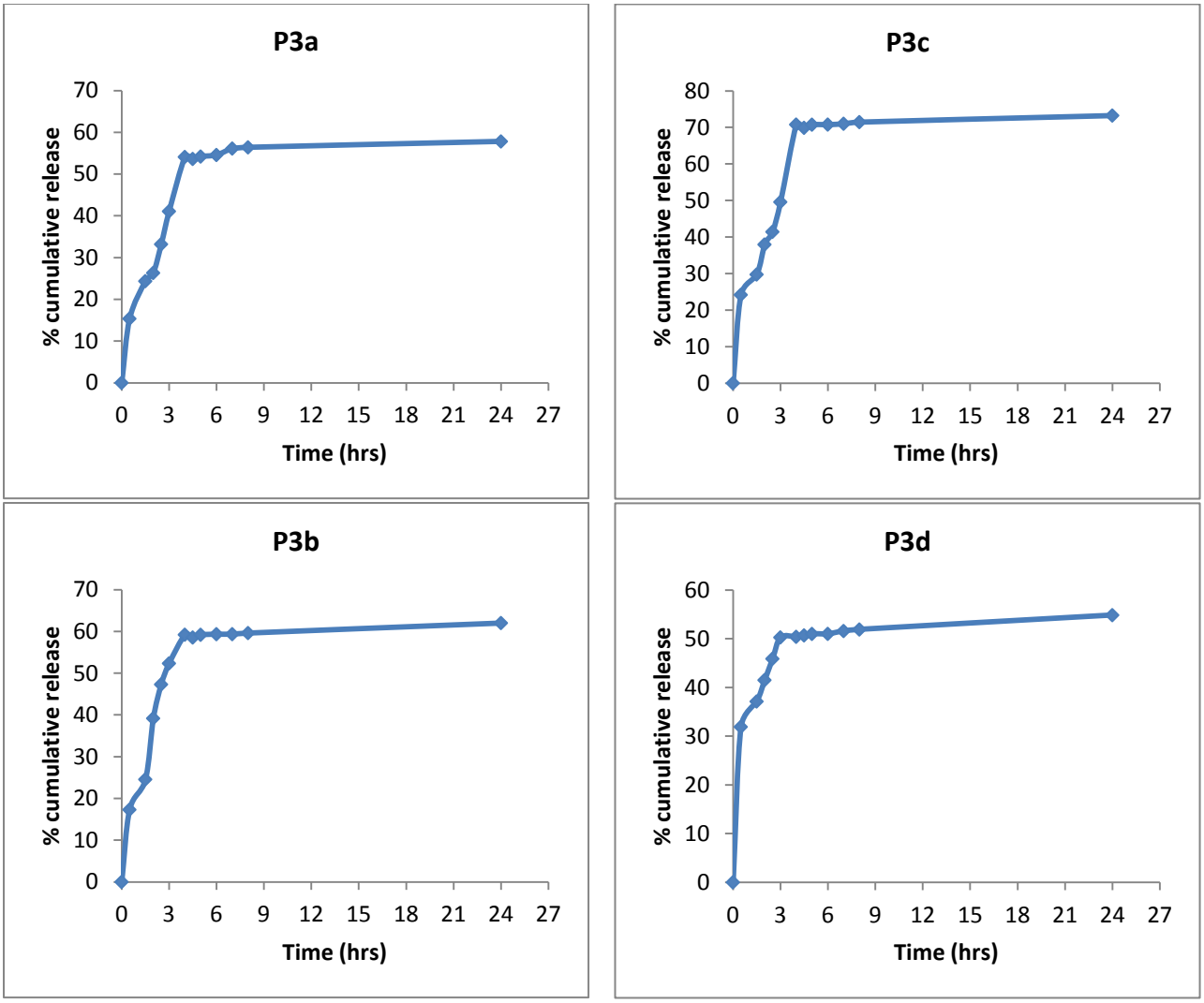

Figure 30: The drug release profiles of L-ascorbic acid loaded chitosan microspheres from P3a to P3d.

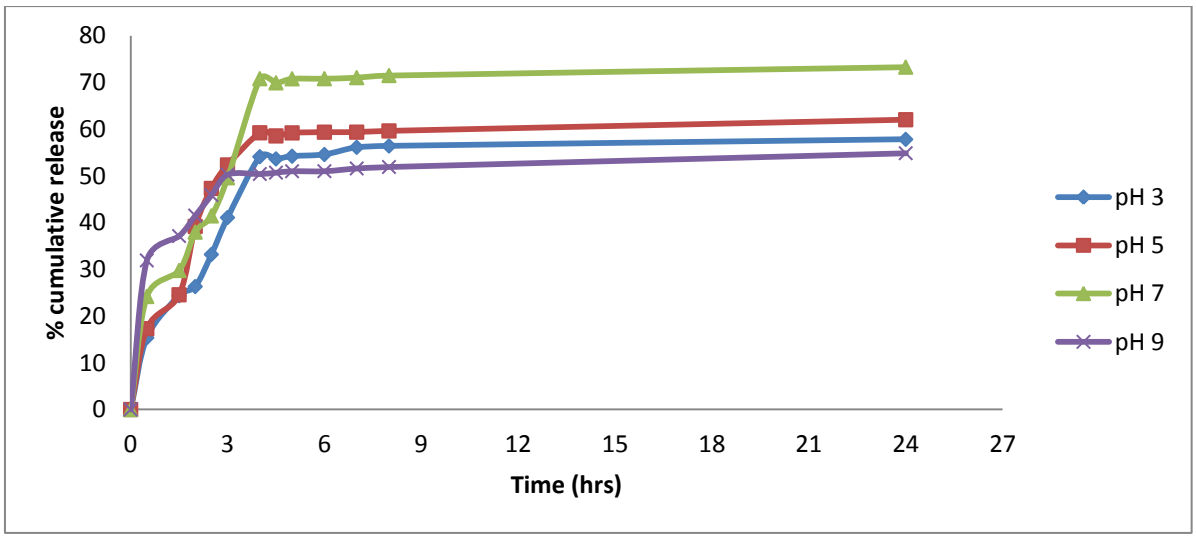

Figure 31: Effect of pH on drug release profile of L-ascorbic acid loaded chitosan microspheres with constant polymer concentration for batches P3a to P3d.

The drug release profiles for these microspheres vary in accordance with the drug loading efficiency and level of cross-linking. The burst effect was observed may be because some of the drug molecules adhered to the surface of microsphere during drug loading.

The release profile was sustained and steady for the microspheres which were loaded with L-ascorbic acid with pH 3 solution conditions. For batches prepared at pH 7 and pH 9, the percent cumulative release curve has higher values as compared to the batches prepared at pH3 and pH5. However there is deviation from this trend which is due to various drug loading efficiency or difference in cross-linking level.

The encapsulation efficiency of the blank microspheres is less than the encapsulation efficiency of the L-ascorbic acid encapsulated microspheres. This is because in first set the drug was added in polymer solution and the drug molecules entrapped while cross-linking of matrix during stirring period. But in the blank microspheres cross-linking had already taken place and the only loading possible was depending on the swelling index at different pH.

The preparation of chitosan microspheres with the polymer concentration of $\leq$ 1% lead to formation of aggregated flakes with no definite shape. Polymer concentration > 2.5% led to a highly viscous solution even in 2% acetic acid, which was difficult to dispersed dropwise out of a syringe. With increase in concentration of the drug, L-ascorbic acid, the formation of microspheres was obstructed. An increase in concentration of L-ascorbic acid the pH of solution varies significantly resulting in lowering the viscosity of chitosan solution which effects the cross-linking of matrix during stirring period.

Chapter 7: Conclusion

L-ascorbic acid encapsulated chitosan microspheres with glutaraldehyde as cross-linking agent were prepared by water-in-oil emulsification method in order to design a controlled release drug delivery system to achieve release of the drug at the desired rate over extended period of time to maintain therapeutic level in blood. Chitosan has proved to be a promising option of polymer for preparation of such drug delivery system as it is biocompatible and biodegradable, it protects the core material from environmental conditions and hence is not only suitable for formulation of drug formulations but is also favorable for various biomedical applications.

Various formulations were applied for designing these microspheres. Microspheres with different morphology, size, encapsulation efficiency, percentage swelling index and stability were obtained by varying the polymer-drug ratio, stirring rate, and drug loading conditions.

The particle size and encapsulation efficiency ranged between 48-165 μm and <10-88 % respectively. The percentage yield ranged between 25.34-46.33 %. the percentage swelling index in distilled water, buffer of pH 1.2 and buffer of pH 6.8 ranged between15.6-38.8 %, 35.3-67.8 %and 22.5-46.5 % respectively. The cumulative drug release was best observed in B5, B6 and B8. B5 was formulated with 2% (w/v) of chitosan and 1% (w/v) of L- ascorbic acid; B6 was formulated with 2.5% (w/v) of chitosan and 1% (w/v) of L- ascorbic acid and B8 was formulated with 2% (w/v) of chitosan and 1.5% (w/v) of L- ascorbic acid.

From the studies conducted on varying formulation and process parameters, it may be concluded that water-in-oil emulsification method using natural biopolymer can be used to encapsulate drugs like L-ascorbic acid which are not very stable or have a short half-life. Besides this technique has numerous applications in pharmaceutical, cosmetics, paper and food industries.

The microspheres were evaluated for different parameters and best batch was B6 having polymer to drug ratio 2.5:1 i.e. 2.5% (w/v) chitosan and 1% (w/v) L-ascorbic acid. Its percent yield was 41.51%, average particle size was 73 μm and drug encapsulation efficiency was 82.40%. B6's drug release rate profile was steady and consistent.

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Chapter 8: References

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List of Publications:

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