Chitosan Particulate System As Vitamin C Carrier

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

Masters of Technology
in
Biotechnology

Submitted by

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in the dissertation entitled **"Chitosan Particulate System As Vitamin C Carrier"** in partial fulfillment of the requirement for the award of the degree of Masters of 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.

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CERTIFICATE

This is to certify that the dissertation entitled **"Chitosan Particulate System As Vitamin C Carrier"** submitted by Anushree in partial fulfillment of the requirement for the award of Degree of Masters of Technology in Biotechnology to Thapar university, Patiala, is a record of student's own work carried out by her 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 or Institute.

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ABSTRACT

Microencapsulation is a process of controlled drug delivery system that can improve the retention time of the nutrient in the food and allow controlled release at specific times, during food consumption or in the intestinal gut. It enables the controlled release of the ingredients by creating a barrier to avoid any chemical reaction. Microspheres are small sphere with uniform coating of polymer around it. They are very small in size but very effective in terms of number of dosage required. Microencapsulation technique offers potential advantages over conventional drug delivery systems and also, is established as an unique carrier systems for many pharmaceuticals as well as food ingredients.

The aim of the present work was the formulation and evaluation of L-Ascorbic acid encapsulated microspheres by ionotropic gelation method using chitosan as biopolymer and tripolyphosphate as cross linking agent. Chitosan (CTS) biopolymers have immense structural possibilities for chemical and mechanical modifications for being used as coating material in microspheres. They are biodegradable polymers which degrade in vivo either enzymatically or non-enzymatically to biocompatible and non-toxic byproducts. In addition to biodegradability they offer low production costs, biocompatibility, nontoxic nature and mucoadhesive property.

Vitamin C (L- Ascorbic acid), is an essential water soluble vitamin, has a variety of biological, pharmaceutical and dermatological functions. It acts as an effective antioxidant due to the presence of ene-diol moiety. It is very unstable to air, moisture, light, heat, and oxygen. Due to so many environmental, physical and chemical instability constrain it becomes challenging to develop such a drug delivery system for stabilized release of vitamin C.

Vitamin C encapsulated chitosan microspheres prepared was characterized for their percentage yield, morphology, particle size, swelling index, encapsulation efficiency and *in vitro* drug release rate. Release studies were done in buffer pH 1.2 and then subsequently in buffer pH 6.8. The release rate of drug was affected by the composition of cross linking agent, biopolymer composition and pH of cross linking agent solution. Microspheres of batch B15 showed high drug release profile, high encapsulation efficiency, high percentage swelling index by optimizing various parameters and also their morphology was good as compared to other batches.

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LIST OF ABBREVIATIONS

- TPP Tripolyphosphate
- STPP Sodium Tripolyphosphate
- AA Ascorbic acid
- SI Swelling index
- CTS Chitosan
- HCl Hydrochloric acid
- Conc. Concentration
- DW Distilled water
- NaOH Sodium hydroxide
- KCl Potassium Chloride
- KH_2PO_4 Potassium dihydrogen phosphate
- DD Degree of deacetylation
- PVA Poly vinyl alcohol
- DDS Drug delivery system
- CDDS Controlled drug delivery system
- OD Optical density

CHAPTER I

INTRODUCTION

INTRODUCTION

Vitamin C (ascorbic acid) is a water-soluble vitamin. It is essential for the manufacture of collagen protein, wound healing, healthy immune and nervous systems and as an antioxidant to help prevent diseases (Reavley, 1998). Vitamin C concentrations in the plasma and leukocytes rapidly decline during infections and stress. Supplementation of vitamin C was found to improve components of the human immune system such as antimicrobial and natural killer cell activities, lymphocyte proliferation, chemo taxis, and delayed type hypersensitivity (Wintergerst *et al.,* 2006).

It is a pharmaceutical bioactive compound which plays a major role in day to day life of human being. It is stable as a powder but this stability decreases when dissolved in water. Environmental factors such as temperature, pH, oxygen, metal ion, UV and x-ray affect its stability (Uddin *et al.*, 2001). Ascorbic acid is highly oxidative which creates problem in its bioavailability. Controlled drug delivery system may help to give highest retention of ascorbic acid in human body. Now a days functional food development is a keen research area for researchers as it helps in providing basic vitamins and nutrients required by human body to keep themselves away from various diseases. Ascorbic acid has wide application in food industry as well.

DRUG DELIVERY SYSTEM (DDS)

Drug delivery is the method or process of administering a pharmaceutical or bioactive compound to achieve a therapeutic effect in humans or animals. The design of effective drug delivery systems has recently become an integral part of the development of new pharmaceutical bioactive compounds. The goal is to provide a therapeutic quantity of compound to the proper site in the body in order to achieve the desired effect and maintain such effect for the entire period of treatment. Hence, research continuously keeps on searching for ways to deliver drugs over an extended period of time, with a well-controlled release profile. A new development namely, controlled drug release dosage forms, has evolved from the need for a prolonged drug effect, a better control of drug administration and the reduction of side-effects.

Controlled drug delivery system (CDDS)

Controlled drug delivery systems aim to improve the effectiveness of drug therapy (Langer *et al*., 1996). These systems modify several parameters of the drug: the release profile and capacity to cross biological carriers (depending on the size of the particle), biodistribution, clearance, and stability (metabolism), among others. In other words, the pharmacokinetics and the pharmacodynamics of the drug are modified by these formulations. Controlled release offers numerous advantages over conventional dosage forms. This approach increases therapeutic activity and decreases side effects, thus reducing the number of drug dosages required during treatment (Vilar *et al*.,2012). Controlled release drug delivery systems are being developed to address many of the difficulties associated with traditional methods of administration (Gaurav Tiwari *et al*., 2012) CDDS can be achieved by microencapsulation technique.

Microencapsulation is a technology that can improve the retention time of the drug in the body and allow controlled release at specific times. It is not a new technology. Microencapsulation technique has been utilised in the pharmaceutical industry for the past 30 years to offer controlled release of drugs to the body (Rosinski *et al.*, 2002). It is relatively new to the food industry and is finding use in maximising the retention of the bioactivity of the components during the processing and storage of the formulated product and delivering the desired bioactive components to the target site of the body (Korhonen, 2002).

More specifically, the microcapsule has the ability to preserve a substance in the finely divided state and to release it as occasion demands. These microcapsules may range from **submicrometer to several millimeters** in size and have a multitude of different shapes, depending on the materials and methods used to prepare them.

Various reasons for using microencapsulation technique for development of control drug delivery systems are :

(1) Encapsulation or entrapment can protect the core material from degradation by reducing its reactivity to its outside environment (e.g., heat, moisture, air, and light),

(2) Evaporation or transfer rate of the core material to the outside environment is decreased or retarded,

(3) The physical characteristics of the original material can be modified and made easier to handle,

(4) The product can be tailor to either release slowly over time or at a certain point (i.e., to control the release of the core material to achieve the property delay until the right stimulus),

(5) The flavor of the core material can be masked,

(6) The core material can be diluted when only very small amounts are required, yet still achieve a uniform dispersion in the host material, and

(7) It can be employed to separate components within a mixture that would otherwise react with one another. (Shahidi and Han, 1993).

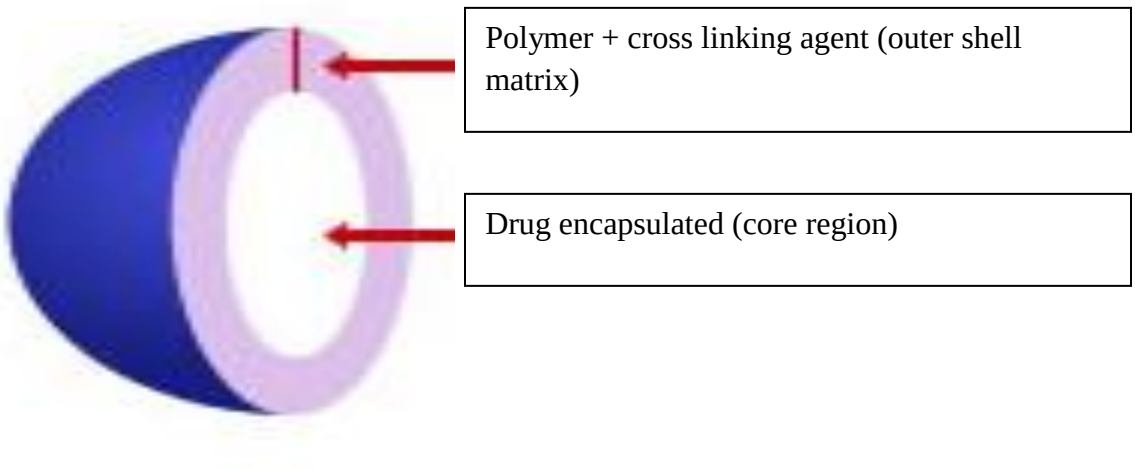


Figure 1: Diagram of representations of microcapsules in which continuous core surrounded by continuous shell.

The use of microencapsulated bioactive compound/drug allows them to be carefully tailored to the specific release site through the choice and microencapsulation variables, specifically, the method and drug-polymer ratio. The total amount of ingestion and the kinetics of release are variables that can be manipulated to achieve the desired result. Using innovative microencapsulation technologies, and varying the copolymer ratio, molecular weight of the polymer, etc., microcapsules can be developed.

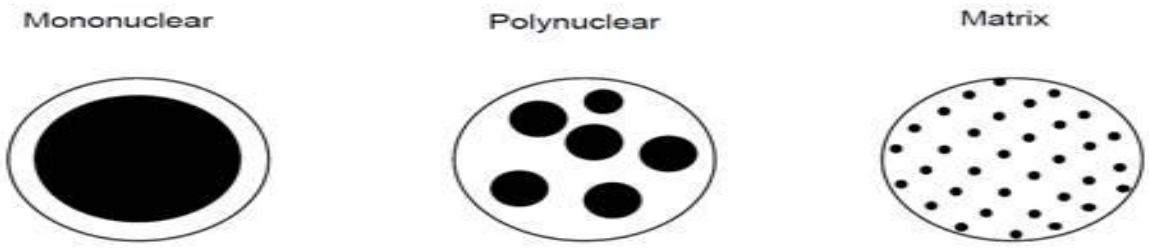


Figure 2: Different types of morphology of microsphere.

The various techniques used for the development of microspheres are ionic gelation method, spray drying, coacervation method, emulsification method etc.

IONIC GELATION TECHNIQUE

Ionic gelation is based on the ability of poly electrolytes to cross link in the presence of counter ions to form beads also called as microspheres. The microspheres are produced by dropping a drug-loaded polymeric solution into the aqueous solution of polyvalent cations. The cations diffuses into the drug-loaded polymeric drops, forming a three dimensional lattice of ionically cross linked moiety.

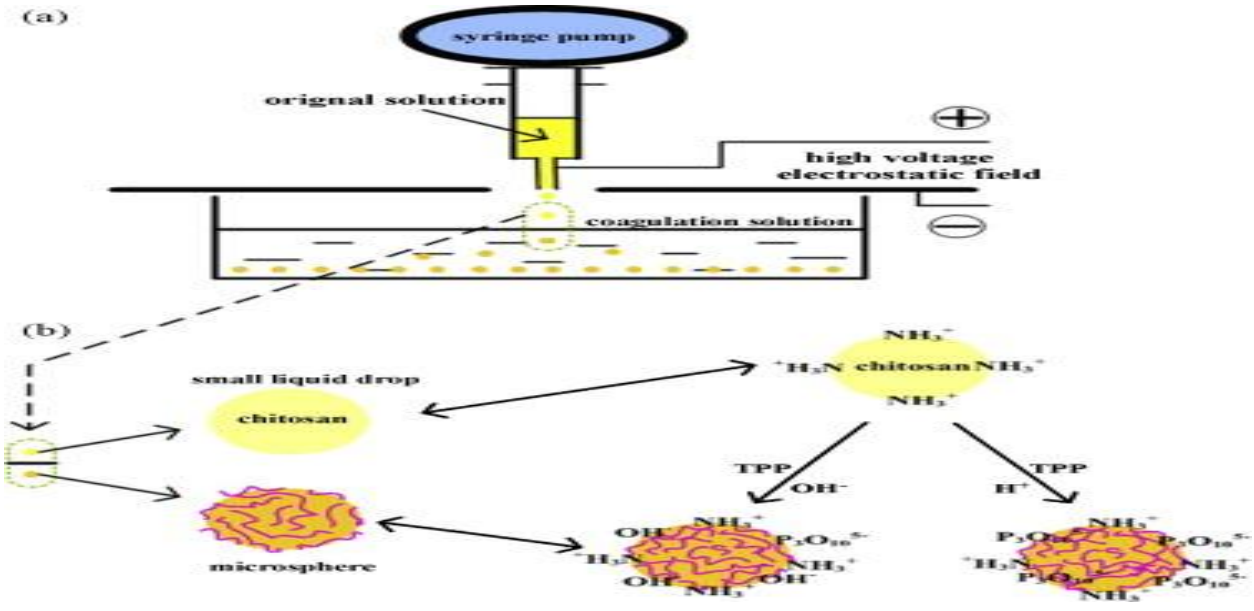


Figure 3 : Microspheres preparation. (a) Preparation process and (b) Microspheres formation (Ma L. *et al*, 2010)

BIODEGRADABLE SYSTEM: *The coating material*

Biopolymers are used as wall material for microencapsulating the food ingredient or drug because they are biodegradable, biocompatible and non-toxic. There are various types of biopolymers used for encapsulation. Biodegradable polymers, which undergo chain scission as part of their function and prior to removal from the body, play a more limited role than bio-stable polymers in medicines. Different types of polymers used are listed in table 1.

Table1: Different types of polymers used as carrier for microencapsulation.

A. SYNTHETIC POLYMERS	B. NATURAL POLYMERS
• **Nonbiodegradable** - acrolein ,epoxy polymer etc • **Biodegradable** polylactides and glycolides copolymer (PLGA) , polyanhydrides	• **Proteins** - albumin, gelatin etc. • **Carbohydrate** - chitosan , alginate etc. • **Chemically modified carbohydrate** - poly(acryl)dextran, poly(acryl)starch etc.

Although a number of synthetic biodegradable polymers have been developed for micro-encapsulation applications, the use of natural biodegradable polymers remains attractive because of their abundance in nature, good biocompatibility, and ability to be readily modified by the simple chemistry.

Natural polysaccharides have gained an important role for gel entrapment and micro-encapsulation, and for that different methods have been developed. The high degree of diversity in functional properties of these biopolymers has made natural polysaccharides indispensable partners for many applications. They can have an almost unlimited variety of chemical structures, with different sugar composition, anomeric configuration and position of linkages, repeating sequence, degree of polymerization, and charge density. Polysaccharides have attracted increasing interest as the natural biological and chemical carriers because they

are commercially available at low cost, are readily modified by simple chemical reactions for specific applications, and they exhibit a broad range of physicochemical properties. For example, chitosan, primarily composed of 2-amino-2-deoxy-β-D-glucopyranose (D-glucosamine), is obtained from chitin, the second most abundant natural polysaccharide. Chitosan and its derivatives have showed excellent biocompatibility, biodegradability, low immunogenicity, and biological activities The most widely used natural polysaccharides are obtained from higher plants and from seaweeds. In the former group, cellulose and starch are the most important together with pectins and gluco- and galactomannans, while in the latter, alginate, agar, chitosan and carrageenan are the best known bio polymers.

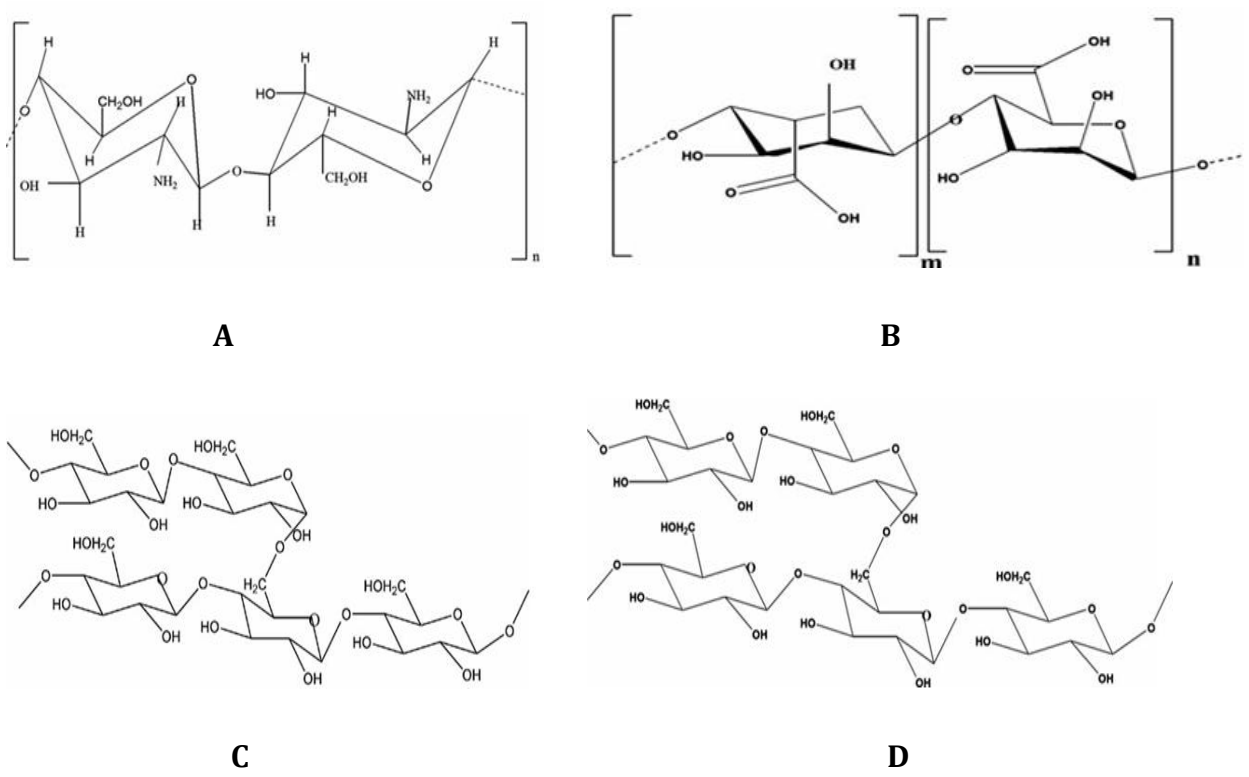


Figure 4: Natural polymers used for microencapsulation: (A) chitosan, (B) alginate, (C) starch and, (D) dextran.

Table 2 : Requirements for an ideal drug carrier.

Prerequisite for ideal drug carrier
1. **Increase of bioavailability**: The retention time of drug should be high for their greater therapeutic effect. 2. **Long release period**: The drugs are released over extended period and hence improve the patient's compliance and reduce the need for follow-up care. 3. **Constant drug plasma concentration**: The drug levels are maintained within a desired range and total dose can be reduced. 4. **Localized delivery of drug**: The product can be administrated directly at the site where drug action is needed and hence systemic exposure of the drug can be reduced.

CHAPTER II

OBJECTIVES OF STUDY

OBJECTIVES OF STUDY

1. To develop a drug delivery system for controlled release of vitamin C by varying the formulation variables and optimization of parameters.
2. To evaluate cross linked chitosan microspheres for various characteristics.
3. Analyzing the effect of pH on microencapsulation of vitamin C.
4. To compare encapsulation efficiencies and release profiles of vitamin C encapsulated microspheres.

CHAPTER III

REVIEW OF LITERATURE

REVIEW OF LITERATURE

- Bodmeier *et al*. (1989) described the production of chitosan beads by ionotropic gelation with TPP. They used chitosan with different viscosities dissolved in acetic acid, in which they dissolved/dispersed caffeine, salycilic acid, quinidine or sulfadiazine. They compared the drying process of the beads, and demonstrated that drug entrapment was higher when the beads were freeze dried instead of air dried.

- Y Nishioka et al (1990) produced glutaraldehyde cross linked chitosan microspheres that contained cisplatin. The drug loading efficiency increased markedly with an increasing chitosan and chitin content and the sustained release effect was enhanced with an increase in the chitosan content from 1% to 5% and chitin content from 0% to 1.5%.

- Kirby *et al*. (1991) studied the stabilization of ascorbic acid by microencapsulation in liposome. They concluded that encapsulation of vitamin C gives significant improvements to shelf life. This is true for both aqueous solutions and in the presence of common food components which would normally lead to its rapid degradation. They also concluded that ascorbic acid encapsulated in liposome has application in stabilizing vitamin C in aquaculture products and vitamin protection in infant food formulations.

- Davies *et al.*, (1991) studied that the pH value of the food affects the ascorbic acid as the oxidation rate of ascorbic acid is maximum at pH 5.0 and 11.5. UV and x-ray irradiation causes a free-radical photochemical oxidation under both aerobic and anaerobic conditions. Due to this oxidative effect that makes vitamin C such a good antioxidant.

- Aral et al. (1998) investigated a controlled release protein delivery system by using bovine serum albumin (BSA) as a model drug. Chitosan was reacted with sodium alginate in the presence of sodium tripolyphosphate for bead formation. Spherical beads were produced with diameter in the range 0.78-0.92mm and 13-90% encapsulation efficiency. Release properties of beads were affected by their BSA

content. It appeared that the encapsulation of BSA was affected by initial protein and sodium alginate concentrations and presence of pectin (1%) in the external phase.

- Trindade and Grosso (2000) studied the stability of ascorbic acid microencapsulated in granules of rice starch and in gum Arabic. They used spray drying as the microencapsulation technique and concluded that Ascorbic acid coated with starch containing 1% gelatin binding agent was not very stable at room temperature, higher temperature and high relative humidity. Ascorbic acid coated with gum Arabic remained relatively stable at room temperature and a change in temperature only reduced its stability slightly. Relative humidity differences did not alter the retention rate.

- Uddin *et al*. (2001) studied the effect of process variables on ascorbic acid characteristics. They chose four different encapsulation techniques – thermal phase separation, melt dispersion, solvent evaporation and spray drying. They concluded that microencapsulated product size decreased as the molecular weight increased. They also studied the release rate of ascorbic acid, which is defined as the ratio of ascorbic acid released to the solution to its initial encapsulated weight. They concluded that microencapsulated ascorbic acid could prevent the ascorbic acid color change by being highly stable, it could retard the core release rate and generally mask the acid taste. And Starch and β-cyclodextrin delayed the degradation of ascorbic acid when stored at 38^{o}C and relative humidity of 84%.

- K. A. Naidu (2003) studied the importance of ascorbic acid in day to day life. It is essential for collagen, carnitine and neurotransmitters biosynthesis. Most plants and animals synthesize ascorbic acid for their own requirement. However, apes and humans cannot synthesize ascorbic acid due to lack of an enzyme gulonolactone oxidase. Hence, ascorbic acid has to be supplemented mainly through fruits, vegetables and tablets. 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, immune-modulator and prevents cold etc.

- Padayatty *et al.* (2003) studied the antioxidant property of ascorbic acid. They reported that as an electron donor, vitamin C is a potent water-soluble antioxidant in humans. Antioxidant effects of vitamin C have been demonstrated in many experiments in vitro. Human diseases such as atherosclerosis and cancer might occur in part from oxidant damage to tissues. They showed that diets high in fruits and vegetables are associated with lower risk of cardiovascular disease, stroke and cancer, and with increased longevity. They studied the dose concentration of vitamin C in healthy people showed a sigmoidal relationship between oral dose and plasma and tissue vitamin C concentrations.

- V.R. Sinha *et al.* (2003) studied about the chitosan microsphere as a potential carrier for drugs in their review article. They concluded that reacting chitosan with controlled amounts of multivalent anion results in cross linking between chitosan molecules. The entrapment efficiency increases with increase in chitosan concentration. Release of drug from chitosan microsphere is dependent upon the molecular weight of chitosan, concentration of chitosan, drug content and density of cross linking. Various therapeutic agents such as anticancer, anti-diabetic, antibiotics, steroids, proteins etc. have been incorporated in chitosan microspheres to achieve controlled release.

- D.W. Lee *et al.* (2003) studied the preparation and release characteristics of polymer-coated (chitosan) and blended alginate microsphere. The plain alginate microsphere showed a fast release of drug as a result of the swelling and disintegration in simulated intestinal media. To prevent a rapid drug release, alginate microspheres were coated or blended with polymers. Chitosan coating and HPMC-blending provided an extended release of drug. Moreover, Chitosan-coated microspheres showed a smooth and round surface and HPMC blended microspheres exhibited a linear release profile. As the

amount of polymer in sodium alginate or coating solution increased, the drug release decreased.

- Rajesh Pandey and G. K. Khuller (2004) studied the chemotherapeutic potential of alginate-chitosan microsphere as drug carriers. This study described the formulation of alginate–chitosan microspheres for the controlled release of drug with the aim of reducing the dosing frequency as well as the dose.

- Rodgers (2004) studied the use of ready meals as carriers for nutraceuticals. They fortified cook-freeze mashed potato with encapsulated vitamin C. They looked at the effect of three process treatments: a control with no vitamin C addition, ordinary vitamin C addition (33 mg/100 g) and encapsulated vitamin C addition (50 mg/100 g). Results showed that ordinary vitamin C plus potato mash was reduced slightly in fresh and freeze processes and was greatly reduced to only 2-3 mg/100 g in chill and freeze chill processes. In contrast, the encapsulated vitamin C, which started off at higher concentrations, remained high in all processes. The greatest reduction was in chill and freeze chill processes, but not to the same degree as ordinary vitamin C plus potato mash and ~15 mg/100 g was still present. This shows that the microencapsulated vitamin C survived the process conditions better than ordinary vitamin C.

- Desai and Park (2005) did a study of encapsulating vitamin C in tripolyphosphate (TPP) cross-linked chitosan microspheres by spray drying. Its a relatively new process intended for oral delivery. Results showed a mean particle size of 6.1-9.0 µm which was influenced by the amount of cross-linking agent. Encapsulation efficiency was around 58% but decreased as the amount of tripolyphosphate solution increased.

- K.G.H Desai and H.J. Park (2005), studied the recent developments in microencapsulation of food ingredients. They discovered that microencapsulating food ingredients for controlled release application is a promising alternative to solve the

major challenges faced by the food industry. The main problem is the selection of appropriate method of encapsulation and the selection of encapsulating material.

- Wintergerst *et al*. (2006) studied the immune –enhancing role of vitamin C and zinc and effect on clinical conditions. They found that supplementation of vitamin C improves components of the human immune system such as antimicrobial and natural killer cell activities, lymphocyte proliferation, chemo-taxis, and delayed-type hypersensitivity. Likewise, zinc under nutrition or deficiency was shown to impair cellular mediators of innate immunity such as phagocytosis, natural killer cell activity, and the generation of oxidative burst. Therefore, both nutrients play important roles in immune function and the modulation of host resistance to infectious agents, reducing the risk, severity, and duration of infectious diseases.

- Devika Bhumkar and Varsha B. Pokharkar (2006) studied the effect of pH on cross-linking of chitosan with sodium tripolyphoshate (TPP). They concluded that the ionotropic gelation method for formation of cross linked chitosan particles can be easily modified from ionic cross-linking to deprotonation by adjusting the pH of TPP. Chitosan was ionically cross-linked with TPP at lower pH and by deprotonation mechanism at higher pH. The swelling of microsphere was also influenced by the pH of TPP.

- Hammad Umer *et al.* (2011) studied the complete process of microencapsulation and the various techniques used for the development of microspheres. They studied the potential advantages offered by microencapsulation than by the conventional drug delivery systems. They developed unique carrier system for the site-specific drug delivery system. They studied the various application of microencapsulation which can be attained in today's world.

- Vilar G. *et al.* (2012), studied the polymers and their uses in the drug delivery system. They found out that to achieve higher bioavailability of drug, polymers can be used as drug carriers. The polymeric systems are suitable for site-specific and time-controlled

delivery of drug. These types of pharmaceutical formulations are used to transport drugs that are rapidly degraded in the body fluids. The hydrophobic core of polymeric micelles facilitates the incorporation of hydrophobic drugs either through covalent or non-covalent bond formation.

CHAPTER IV

RESEARCH ENVISAGED

RESEARCH ENVISAGED

Microencapsulation is a process by which a core, i.e. bioactive or functional ingredient, is packaged within a secondary material to form a microcapsule. The secondary material, known as the encapsulant, matrix or shell, forms a protective coating or matrix around the core, isolating it from its surrounding environment until its release is triggered by changes in its environment. This avoids undesirable interactions of the bioactive with other food components or chemical reactions that can lead to degradation of the bioactive, with the possible undesirable consequences on taste and odor as well as negative health effects.

It is essential to design a microencapsulated ingredient with its end use in mind. This requires knowledge of (1) the core, (2) the encapsulant materials, (3) interactions between the core, matrix and the environment, (4) the stability of the microencapsulated ingredient in storage and when incorporated into the food matrix and (5) the mechanisms that control the release of the core.

4.1 Selection of core material: THERAPEUTIC AGENT

The core material is the material over which coating has to be applied to serve the specific purpose. Core material may be in form of solids or droplets of liquids and dispersions. The composition of core material can vary and thus furnish definite flexibility and allow effectual design and development of the desired microcapsule properties. A substance may be microencapsulated for a number of reasons. Examples may include protection of reactive material from their environment, safe and convenient handling of the materials which are otherwise toxic or noxious, taste masking, means for controlled or modified release properties means of handling liquids as solids, preparation of free flow powders and in modification of physical properties of the drug.

During the development of functional foods using microencapsulated food ingredients, the selection of ingredients and processes was traditionally based on empirical approaches. Ubbink and Kruger (2006) have suggested that an alternative concept is to use a retro-design approach that relies more on a fundamental understanding of the required performance of the ingredient

in a complex food environment. This approach encompasses an understanding of the effects of processing and the factors controlling the chemical and physical events that govern the stability and release properties of a microencapsulated product; however, the test of whether a microencapsulation system is suitably tailored for its end product application is its acceptance in the marketplace. The final product application must be the focus of the microencapsulated product development in order that the core is protected from various stresses during incorporation into the final product. It is important to ensure that when microencapsulation is used to deliver active ingredient, it provides a simple, efficient and cost-effective therapeutic effect.

4.2 ASCORBIC ACID: Vitamin C

Vitamin C, also known as ascorbic acid, a water-soluble nutrient found in some foods. In the body, it acts as an antioxidant, which helps in protecting the cells from the free radicals (Jacobs et al. 2001). Free radicals are those compounds which are formed when our bodies convert the food that we eat into energy. People are also exposed to free radicals in the environment from cigarette smoke, air pollution, and ultraviolet light from the sun.

The body also needs vitamin C to make collagen, a protein required for the healing of wounds. In addition, vitamin C improves the absorption of iron from plant-based foods and helps the immune system work properly to protect the body from various diseases. In fact, research indicates that vitamin C is the most commonly supplemented micronutrient in the United States, with typical dosages ranging from **60-1000 mg/day**. The popularity of vitamin C supplementation is likely a consequence of its purported health performance enhancing effects, coupled with its low toxicity even at very high dosages (2-4 grams/day). The lack of toxicity likely results from an increase in urinary excretion, coupled with a decrease in bioavailability for vitamin C, as dosing is increased.

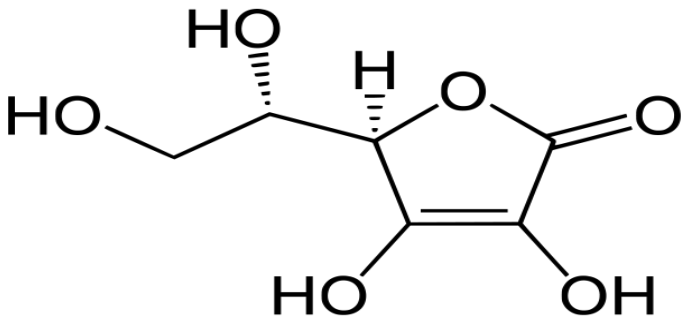


Figure 5 : Chemical structure of ascorbic acid.

4.2.1 PHYSICOCHEMICAL PROPERTIES OF ASCORBIC ACID

- Empirical formula: $C_6H_8O_6$
- Chemical name: 2-oxo-L-threo-hexono-1,4-lactone-2,3-enediol
- Molecular weight: 176.12 g/mol
- Crystalline form: Monoclinic
- Odor: odorless
- Melting point : 190-192^0 C
- Taste: Pleasant, sharp, acidic taste
- Exhibits maximal stability between pH 4 and 6
- Pharmacological action: as an antioxidant and as vitamin.

4.2.2 SOURCES OF ASCORBIC ACID

Ascorbic acid is widely distributed in fresh fruits and vegetables. It is present in fruits like orange, lemons, grapefruit, watermelon, papaya, strawberries, cantaloupe, mango, pineapple, raspberries and cherries. It is also found in green leafy vegetables, tomatoes, broccoli, green and red peppers, cauliflower and cabbage.

Most of the plants and animals 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, guinea pigs, fruit eating bats, apes and humans cannot synthesize ascorbic acid due to the absence of the **enzyme L-gulonolactone oxidase**. Hence, in humans ascorbic acid has to be supplemented through food and or as tablets/capsules etc.

4.2.3 BIONECESSITY OF VITAMIN C

Vitamin C, a representative water soluble vitamin, has a variety of biological, pharmaceutical and dermatological functions. Its various bio-necessities are as follows-

1. A condition due to a dietary deficiency of ascorbic acid (vitamin C), characterized by malaise, lethargy, and weakness. As the disease progresses, joints, muscles, and subcutaneous tissues may become the sites of hemorrhage. Ascorbic acid deficiency frequently develops into SCURVY in young children. It also commonly develops in chronic alcoholism.
2. Vitamin C plays important role in various other diseases like-

- **In cancer prevention and treatment:**

  The prevention and treatment of cancer considers different mechanisms of vitamin C activity, such as

(a) Enhancement of the immune system by increased lymphocyte production (Vohra and Khan 1990)

(b) Stimulation of collagen formation necessary for 'walling off' tumours (Shklar and Schwartz 1996)

(c) Inhibition of oncogenic micro-organisms (Zhang and Wakisaka 1997)

(d) Correction of an ascorbate deficiency, often seen in cancer patients (Dyke and Craven 1994)

(e) Enhancement of the effect of certain chemotherapy drugs (Kurbacher et al. 1996)

(f) Prevention of cellular free radical damage (Flagg et al. 1995), and

(g) Neutralization of carcinogenic substances (Block 1991).

- **Age related macular degeneration (AMD) and cataract**

  AMD and cataracts are two of the leading causes of vision loss in elder people. However research suggests that vitamin C combined with other nutrients might help keep early AMD from worsening into advanced AMD. Some studies shows that people who get more vitamin C from foods have lower risk of getting cataracts.

- **Common cold prevention**

  Pauling suggested that ingestion of 1–2 g of ascorbic acid effectively prevents/ ameliorate common cold. There has been a long-standing debate concerning the role of ascorbic acid in boosting immunity during cold infections. Ascorbic acid has been shown

to stimulate immune system by enhancing T-cell proliferation in response to infection (Campbell *et al.*, 1999).

3. Antioxidant property of vitamin C is able to reduce damage caused by oxidizing chemicals, such as free radicals. These oxidizing chemicals, sometimes called reactive oxygen species, or ROS, are the normal byproducts of the cellular reactions that take place inside the human body. Oxidizing agents are very unstable and damage cells by reacting with important molecules and changing how they function. Vitamin C reduces this damage by directly binding to oxidizing chemicals 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).
4. Vitamin C also plays a major role in regulating immune system of the body. Many types of immune cells are stimulated by vitamin C, including white blood cells. These white blood cells help body to fight infection by attacking and killing viruses and bacteria. Vitamin C also helps in increasing the level of antibodies in the body, which are another defense mechanism which are used by immune system to attack invading microbes.

4.2.4 Reason for microencapsulation of vitamin C

Vitamin C is very unstable to air, moisture, light, heat, and oxygen. It easily decomposes into biologically inactive compounds such as 2,3 -diketo-L-gulonic acid, oxalic acid, L-threonic acid, L-xylonic acid and L-lyxonic acid (Machlin 2001). Therefore, in order to overcome the associated drawbacks, microencapsulation of vitamin C was considered (Trindade and Grosso 2000; Uddin et al. 2001). The aim of this study is to encapsulate vitamin C in cross-linked chitosan microspheres for the delivery of vitamin C via an oral route. The rationality for microencapsulating vitamin C are following:

- Vitamin C encapsulation may reduce its sensitivity as it is highly sensitive to outside environment (e.g. heat, moisture, air and light).
- To increase retention time as vitamin C is excreted out from the body very easily
- To control the release of vitamin C as its solubility in water in very high but its absorption is very less.

- To modify the physical characteristics and make vitamin C handling easier.

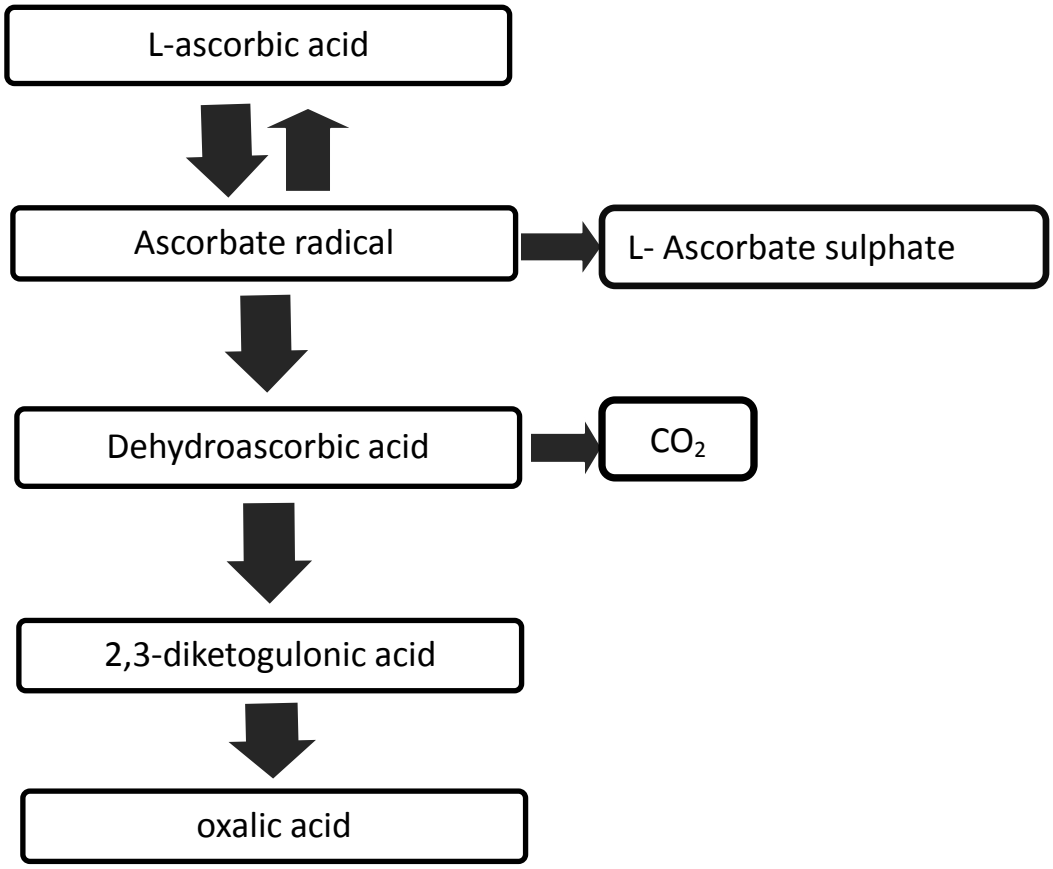


Figure 6: The flow diagram of the major metabolites of L-Ascorbic acid in humans (Machlin, 2001).

4.2.5 MECHANISM OF ACTION

In humans, an exogenous source of ascorbic acid is required for collagen formation and tissue repair by acting as a cofactor in the post translational formation of 4-hydroxyproline in -Xaa-Pro-Gly- sequences in collagens and other proteins. Ascorbic acid is reversibly oxidized to de hydroascorbic 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, conversion of folic acid to folinic acid, carbohydrate metabolism, synthesis of lipids and proteins, iron metabolism, resistance to infections, and cellular respiration.

4.2.6 PHARMACOKINETICS

The pharmacokinetic study on oral vitamin C conducted by Mark Levine and colleagues at NIH was initially 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. Their results in healthy humans found that vitamin C concentrations in plasma and cells were carefully, or tightly, controlled by multiple mechanisms acting together: bioavailability, or intestinal absorption; tissue accumulation; renal re absorption and excretion, and utilization rate as a function of homeostasis. Once oral intake of vitamin C exceeded 200 mg daily, the plasma concentration peak at 70~80 µM were obtained. Further increase in the dose did not provide obvious increase of concentration in plasma and in cells – the bioavailability drops, intracellular distribution saturated, and the renal excretion accelerated. However, when ascorbate was administered intravenously, tight control was bypassed, until renal excretion restored equilibrium – that could be hours depending on the dose (Levine *et al*, 1996).

Indeed, later researches found plasma ascorbate concentrations as high as 20-30mM were safely achieved with large dose of intravenous ascorbate (Chen *et al*, 2008). By contrast, oral intake did not provide plasma concentrations higher than 300 µM (Padayatty *et al*, 2004).

4.3 SELECTION OF POLYMERS

The development of polymeric controlled release system introduced a new concept in drug administration. These systems are less complicated and with high stability. Encapsulation in the polymer carrier eliminates the degradation of drugs; moreover, the release profile of the drugs can be controlled by properly choosing polymers. A polymer based on the C-C backbone is non-biodegradable. Biodegradable polymers commonly contain chemical linkages such as anhydride, ester, or amide bonds.

These polymers degrade in vivo either enzymatically or non-enzymatically to biocompatible and non-toxic byproducts. These can be further metabolized or excreted via normal physiological pathways. Biodegradable polymer not only have been extensively used in controlled delivery

systems, but also extended to medical devices (Leenslag *et al*., 1987), wound dressing (Hubbell, 1996), and for fabricating scaffolds in tissue engineering (Shi *et al*., 1996). In addition to biocompatibility, biodegradable polymers also offer other advantages including thermoplasticity, high mechanical strength, and controlled degradation rate.

4.4 CHITOSAN

Chitosan is a natural, cationic amino polysaccharide (pKa 6.5) copolymer of glucosamine and N acetyl glucosamine obtained by the alkaline, partial de-acetylation of chitin. It is the second most abundant natural polysaccharide and originates from shells of crustaceans. Chitosan is a biodegradable, biocompatible, positively charged nontoxic mucoadhesive biopolymer. Since chitosan contains primary amino groups in the main backbone that make the surfaces 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 (Artursson *et al*, 1994).

Chitosan has been reported to enhance drug permeation across the intestinal, nasal, and buccal mucosa (LueBen *et al*, 1997). 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. Chitosan is generally considered nontoxic and biodegradable, with an oral LD50 in mice of over 16 g/kg (Illum *et al*, 1998).

4.4.1 CHITOSAN SOURCES AND PRODUCTION

Chitin is found in the exoskeleton of some anthropods, 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 (Mw) and degree of deacetylation (DD).

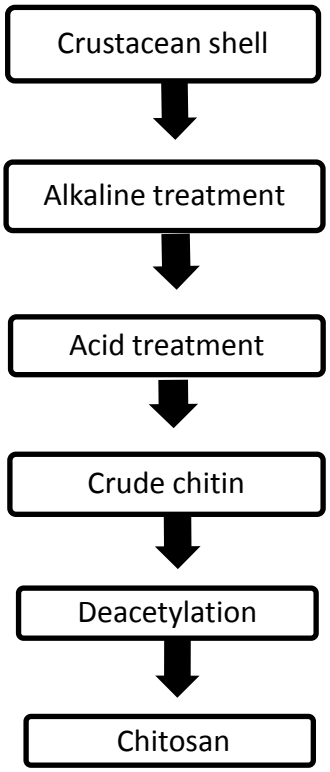


Figure 7: Flow diagram of chitosan production (Paul and Sharma, 2000).

4.4.2 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 positions of glucose rings. Chitosan is a 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.

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

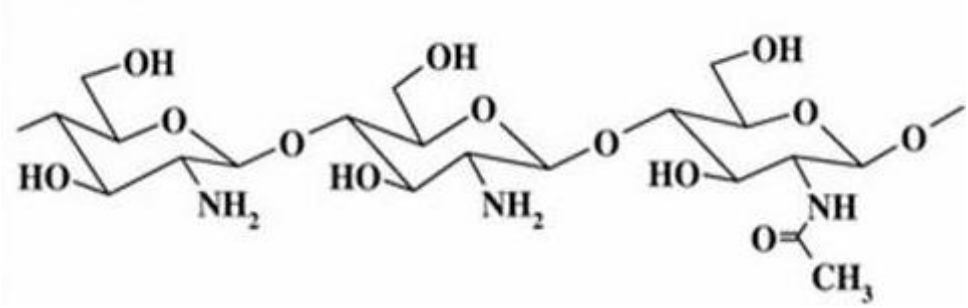


Figure 8 : Chemical structure of chitosan.

4.4.3 Physicochemical and Biological Properties of Chitosan

Chitosan is a semi crystalline polymer that exhibits polymorphism. Chitosan belongs to a series of polymers with different DD and Mw (Jayakumar *et al*, 2010), which are the two important physicochemical properties of chitosan. DD is defined as of the percentage of primary amino groups in the polymer backbone. The DD and Mw of chitosan can be altered by changing the reaction conditions during the manufacture of chitosan from chitin (typical commercial chitosan has a DD of 66–95%). 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 position of D-glucosamine residues, whereby polysaccharide is converted into polycation in acidic media.

Chitosan has a low solubility at physiological pH of 7.4 as it is a weak base (pKa 6.2–7). Adjusting solution pH to approximately 7.5 induces flocculation due to deprotonation and insolubility of the polymer (Jayakumar *et al*, 2009). Higher Mw chitosan of approximately 1,400 kDa demonstrates a stronger level of mucoadhesion than low Mw chitosan of 500–800 kDa, because the former has a higher level of viscosity. The viscosity of chitosan solution increases with an increase in chitosan concentration and DD but with a decrease in solution temperature and pH. It is known to possess a good complexing capacity. Chitosan can also complex with an oppositely charged polymer such as poly(acrylic acid), sodium salt of poly(acrylic acid), carboxymethyl cellulose, xanthan, carrageenan, alginate, pectin etc.

The biological properties of chitosan are given in table 3:

Table 3: Various biological properties of chitosan.

Biological properties of chitosan	
• Adsorption enhancer	• Antimicrobial activity
• Angiogenesis stimulation	• Analgesic action
• Biocompatible	• Antitumor activity
• Biodegradable	• Haemostatic
• Antioxidant	• Mucoadhesive
• Adsorption enhancer	• Macrophage activation

4.4.4 Drug Release and Release Kinetics

The release of drug from CTS based dosage form depends upon the morphology, size, density and extent of cross-linking of the particulate system, physicochemical properties of the drug as well as the polymer characteristics such as either it is hydrophilic or hydrophobic, gel formation ability, swelling capacity, mucoadhesive or bioadhesive properties and also on the presence of other excipient present in the dosage form. In vitro release of drug from the prepared dosage form in the dissolution media also depends upon volume of dissolution medium, pH and polarity, rate of stirring, temperature, sink condition and presence of enzyme. The release of drug from CTS particulate systems involves three different mechanisms: (a) erosion, (b) by diffusion and (c) release from the surface of particle. The release of drug mostly follows more than one type of mechanism. In case of release from the surface, adsorbed drug dissolves rapidly and it leads to burst effect when it comes in contact with the release medium (He *et al,*1998) observed that CTS based microspheres prepared by spray drying technique have shown burst release of cimetidine. The burst release of drug can be prevented by use of cross linking agents such as glutaraldehyde and formaldehyde or by washing microparticles with a proper solvent. Al-Helw *et al*., (1998) observed that a high release of the phenobarbitone in initial hours and drug release rate was dependent on the molecular weight of CTS and particle size of the microspheres. The microspheres prepared from high molecular weight CTS have shown slow release of drug as compared to those prepared from low molecular weight CTS.

This is due to the fact that high molecular weight CTS has lower solubility and formation of the high viscosity gel layer around the drug particles upon contact with the dissolution medium. Microspheres having the size range of 250-500 µm, the release of drug were 75-95% up to 3h but for particles having the size range of 500-1,000 µm, drug release was 56-90% in 5h. Kweon and Kang (1999) prepared the CTS-g-poly (vinyl alcohol) copolymer matrix to study the release pattern of prednisolone under various conditions. In this study drug release was controlled by the extent of PVA grafting, heat treatment or cross-link density. He observed that there was a linear relationship between the amount of drug release and square root of time indicating that release was based on diffusion mechanism.

4.5 CROSS LINKING AGENTS:

According to several studies, cross-linking the matrix using agents such as glutaraldehyde, NaOH and ethylene glycol diglycidyl ether could control the drug release from chitosan microspheres. However, these chemical cross-linking agents have the possibility of inducing undesirable effects. Chemically synthesised glutaraldehyde can cause irritation to mucosal membranes due to its toxicity. To overcome this disadvantage of chemical cross-linking, ionic cross-linking interaction are being applied. Ionic cross-linking with tripolyphosphate (TPP) produced chitosan beads, microspheres or nanospheres.

4.5.1 SODIUM TRIPOLYPHOSPHATE (STPP)

Sodium tripolyphosphate is an inorganic compound with formula $Na_5P_3O_{10}$. It is the sodium salt of the polyphosphate penta-anion, which is the conjugate base of triphosphoric acid. STPP is a preservative for seafood, meats, poultry, and animal feeds. In foods, STPP is used as an emulsifier and to retain moisture. Many governments regulate the quantities allowed in foods, as it can substantially increase the sale weight of seafood in particular. The United States Food and Drug Administration lists STPP as "generally recognized as safe."

STPP solution were used as crosslinking agent as it helps in making the membrane flexible and at the same time it can improve the chemical stabilities of chitosan membrane of the microspheres. Chitosan beads coss linked by a combination of tripolyphosphate and citrate ions

together, not only had a good shape, but also improved pH dependent drug release properties of chitosan microsphere (Shu and Zhu, 2002)

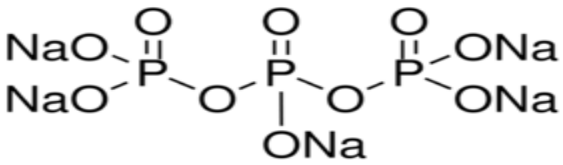


Figure 9: The chemical structure of sodium tripolyphosphate (STPP).

4.5.2 GLUTARALDEHYDE

Among the many available protein crosslinking agents, glutaraldehyde has undoubtedly found the widest application in various fields such as histochemistry, microscopy, cytochemistry, leather tanning industry, enzyme technology, chemical sterilization, and biomedical and pharmaceutical sciences. 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 in 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) and is more efficient than other aldehydes in generating thermally and chemically stable crosslinks (Nimni *et al*, 1987). In fact, studies of collagen crosslinking reactions with monoaldehyde (formaldehyde) and dialdehydes having chain lengths of two to six carbon atoms (glyoxal, malonaldehyde, succinaldehyde, glutaraldehyde, and adipaldehyde) demonstrated that the reactivity in this series is maximized at five carbons; thus glutaraldehyde is the most effective crosslinking agent (Bowes *et al*, 1968).

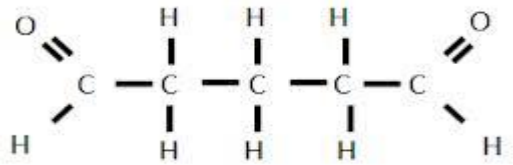


Figure 10: The chemical structure of glutaraldehyde.

CHAPTER V

EXPERIMENTAL WORK AND RESULTS

Experimental work

5.1 Experimental chemicals and equipments

Various chemical and equipments used for the preparation and evaluation of ionically cross linked chitosan microspheres are listed in table 4.

Table 4 : Experimental materials and equipments.

List of chemicals	List of equipments
• Chitosan (85% deacetylated, Sigma – Aldrich) • L- Ascorbic acid AR (LOBA Chemie ltd.) • Sodium Tripolyphosphate (Anhydrous) (STPP, LOBA Chemie ltd.) • Glacial acetic acid (1%) • Glutaraldehyde • Buffer solution at pH 1.2 and 6.8 • Conc. Hydrochloric acid (HCl) • Sodium hydroxide (NaOH) • Potassium dihydrogen phosphate • Potassium chloride (KCl) • Distilled water (DW)	• Analytical balance • pH meter • Magnetic stirrer • Nikon SMZ 800 microscope • UV-Visible spectrophotometer • Incubator • Shaking incubator • Disposable syringe • Glass beakers (50ml, 100ml, 150ml, 200ml, 250ml) • Measuring cylinder (100ml, 500ml) • Micropipette (T1000, T20)

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 at 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 at pH 6.8 (200ml)

50ml of 0.2M potassium dihydrogen phoshphate 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 upto 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 pH 1.2 and buffer of pH 6.8 respectively. From the above standard stock solution different ascorbic acid dilutions were prepared by using the formulae:

$$M1\ V1 = M2\ V2$$

The different concentrations which were prepared were 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml, 10 µg/ml by taking different aliquots from stock solution and making up the remaining volume to 10ml by adding distilled water, buffer of pH 1.2 and buffer of pH 6.8 respectively.

The absorbance of the different solution was measured at 244nm against distilled water or buffer at pH 1.2 and buffer at pH 6.8 respectively in triplicate respectively as blank in UV-Visible spectrophotometer. Standard curves were obtained by plotting the data.

Table 5: Standard curve data of L-Ascorbic acid in distilled water.

S. No.	Concentration (µg/ml)	Absorbance (A_{244})
1.	2	0.04
2.	4	0.096
3.	6	0.154
4.	8	0.201
5.	10	0.255

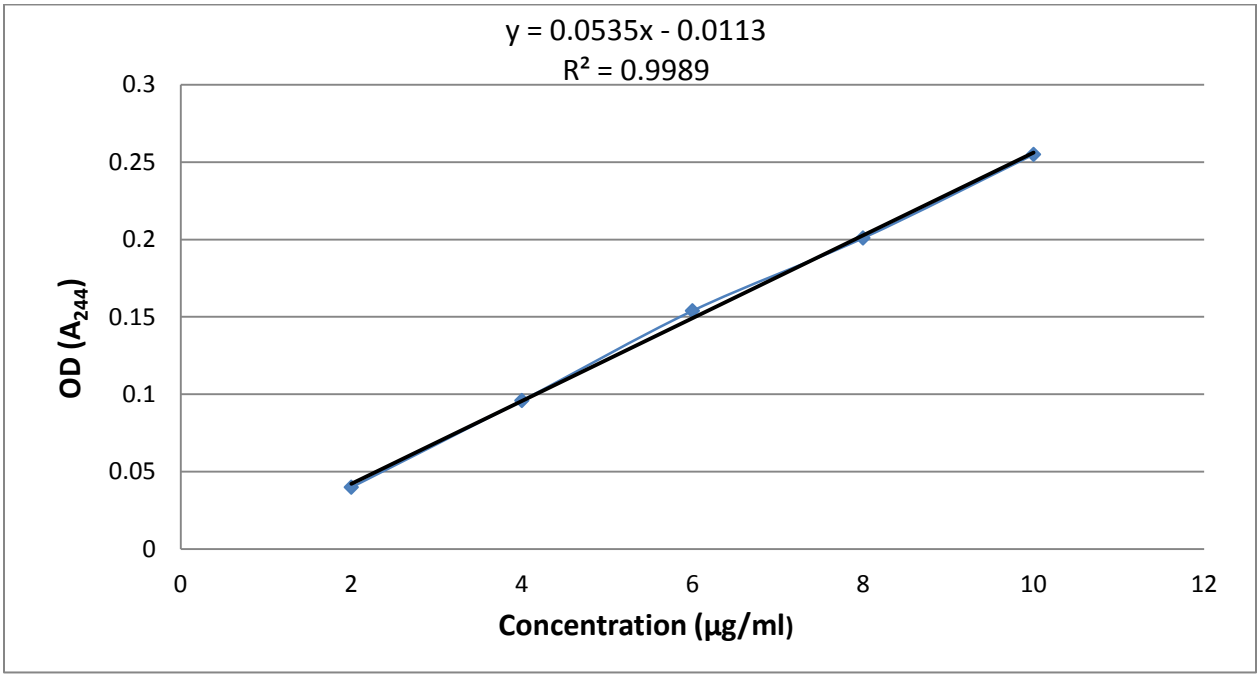


Figure 11: Standard curve of L-Ascorbic acid in distilled water.

Table 6 : Standard curve data of L-Ascorbic acid in buffer at pH 1.2.

S. No.	Concentration (µg/ml)	Absorbance (A_{244})
1.	2	0.121
2.	4	0.252
3.	6	0.374
4.	8	0.498
5.	10	0.618

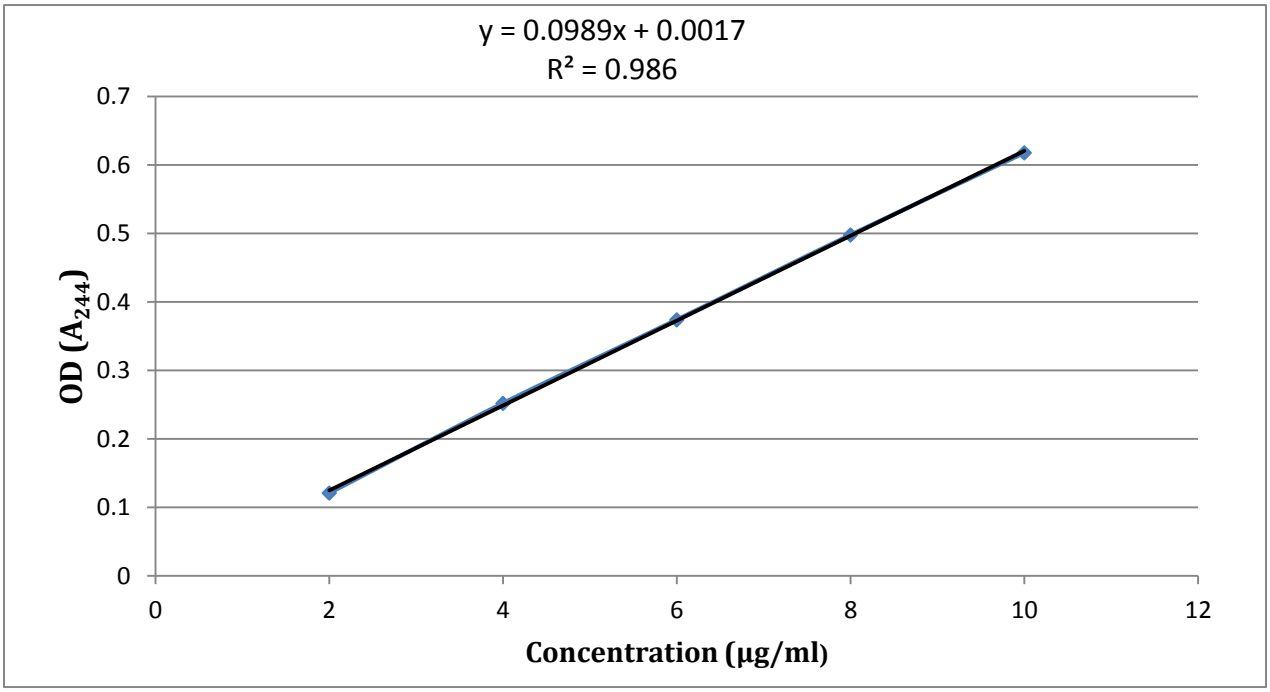


Figure 12: Standard curve of L-Ascorbic acid in buffer solution at pH 1.2.

Table 7 : Standard curve data of L-Ascorbic acid in buffer at pH 6.8

S. No.	Concentration (µg/ml)	Absorbance (A_{244})
1.	2	0.008
2.	4	0.019
3.	6	0.029
4.	8	0.048
5.	10	0.06

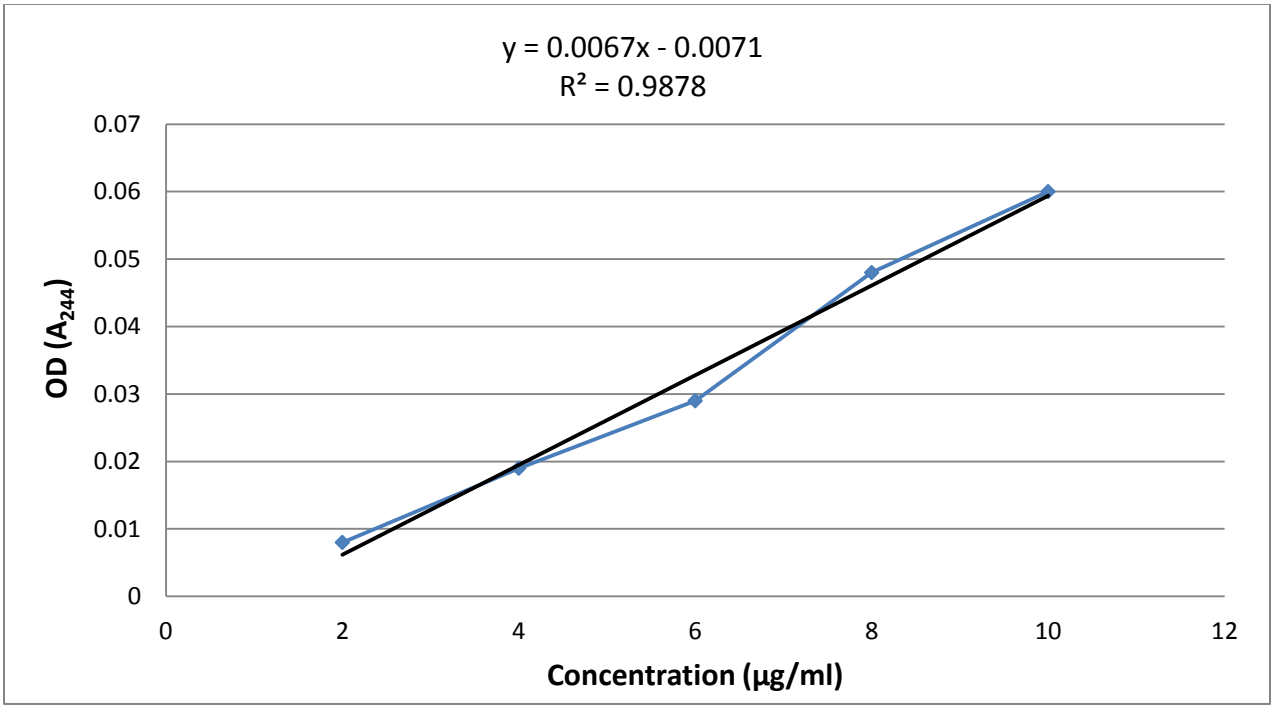


Figure 13: Standard curve of L-Ascorbic acid in buffer solution at pH 6.8.

5.3 Preparation of L-Ascorbic acid encapsulated chitosan microspheres

Biodegradable microspheres are widely used in drug delivery system. Biodegradable microspheres encapsulated L-Ascorbic acid was prepared by ionotropic gelation technique.

Ionotropic gelation technique is based on the ability of poly electrolytes to cross link in the presence of counter ions to form crosslinked microspheres. Microspheres are spherical crosslinked hydrophilic polymeric entity capable of extensive gelation and swelling in simulated biological fluids and the release of drug through it controlled by polymer relaxation. These crosslinked microspheres are produced by dropping a drug-loaded polymeric solution into the aqueous solution of polyvalent cations. The cations diffuse into the drug-loaded polymeric drops, forming a three dimensional lattice of ionically crosslinked moiety. Biomolecules can also be loaded into these microspheres under mild conditions to retain their three dimensional structure. In this technique, there has been a growing interest in the use of natural polymers as drug carriers due to their biocompatibility and biodegradability. The natural or semisynthetic polymers i.e. Alginates, Gellan gum, Chitosan, Pectin and Carboxymethyl cellulose are widely used for the encapsulation of drug by this technique (Mennini *et al*, 2008).

These natural polyelectrolytes contain certain anions/cations on their chemical structure, these anions/cations form meshwork structure by combining with the counter ions and induce gelation by cross linking. In spite of having a property of coating on the drug core these natural polymers also acts as release rate retardants.

In this technique Chitosan solutions were prepared at (1, 2 and 2.5% w/v) by dissolving chitosan in 10ml of 1% glacial acetic acid solution at room temperature. To the above prepared chitosan solution L-Ascorbic acid (1, 1.5 and 2% w/v) was added. The resultant mixed solution was dripped into the coagulation solution containing aqueous TPP solution by dripping it through a disposable syringe. The different solutions of TPP in water (1, 2, 2.5 % (w/v)) with different pH range 3, 4, 5, 6, 7, 8 and 9 were used for microsphere fabrication. The dropping rate and falling distance were kept constant. The solution was stirred for 15 minutes at 100 rpm followed by filteration and rinsing with deionized water. 1ml of 1% glutaraldehyde was added to stirring

solution. The microspheres obtained were air dried for twenty four hours followed by oven drying for six hours at 37^0 C.

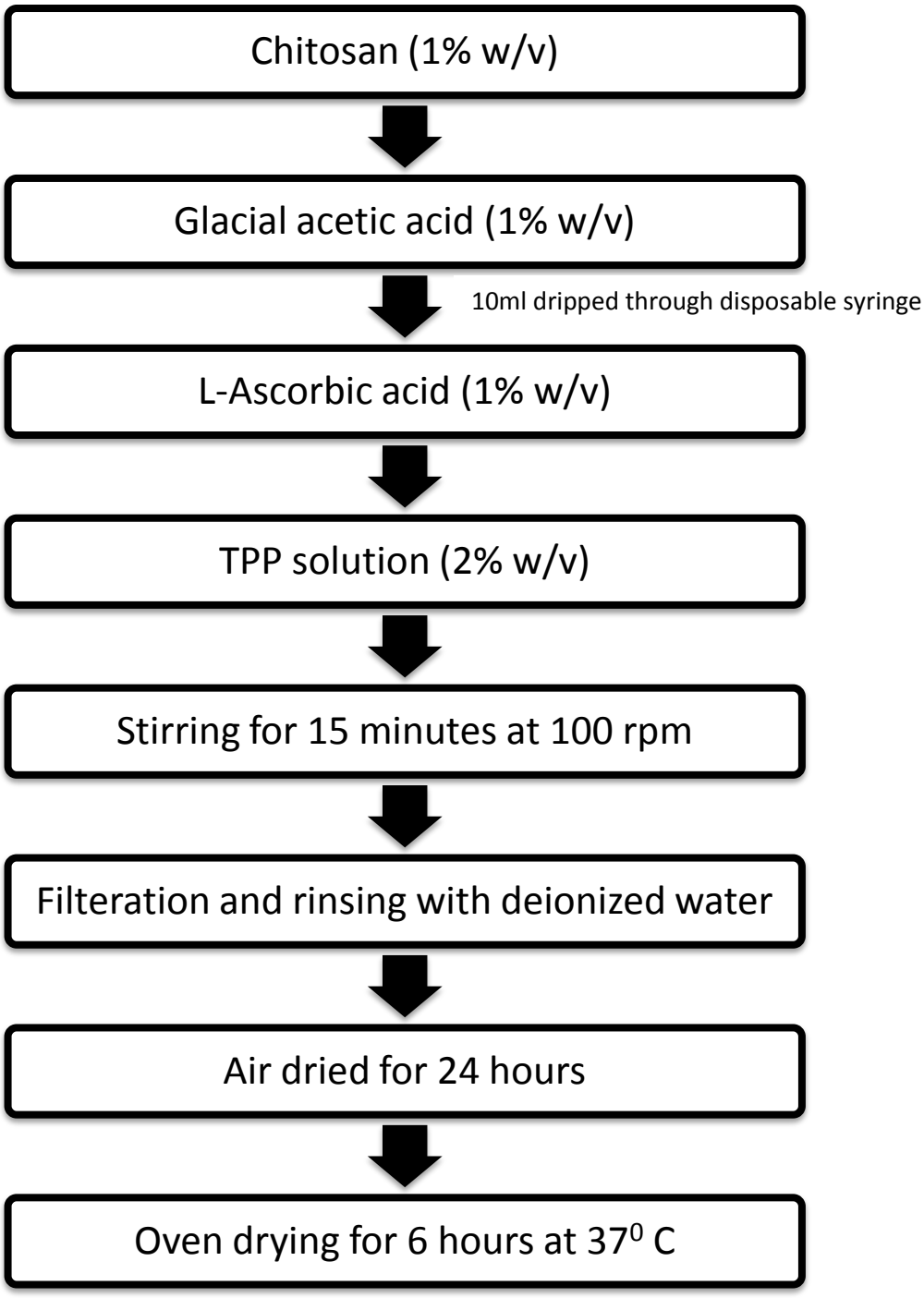


Figure 14: Flow diagram for ionotropic gelation technique (Poonam *et al*, 2012).

5.4 Formulation and process variables

Different batches of chitosan encapsulated L-Ascorbic acid was prepared by altering following variables:

- Volume of 1% glutaraldehyde solution (1-10ml)
- Concentration of polymer (1-2%)
- Concentration of vitamin C (1-2%)
- Concentration of cross linking agent (1-2%)
- pH of 2% TPP solution (3-9)

Table 8: Formulation of L-Ascorbic acid encapsulated chitosan microspheres by varying volume of 1% glutaraldehyde solution.

BATCH No.	CTS(%)	AA(%)	TPP(%)	GLUTARALDEHYDE (1%) VOLUME(ml)
B1	1	0	2	0
B2	1	0	2	1
B3	1	0	2	5
B4	1	0	2	10

Table 9: Formulation of L -Ascorbic acid encapsulated chitosan microspheres by varying chitosan concentration.

BATCH No.	CTS(%)	AA (%)	TPP(%)	GLUTARALDEHYDE (1%) VOLUME (ml)
B5	1	1	2	1
B6	1.5	1	2	1
B7	2	1	2	1

Table 10 : Formulation of L-Ascorbic acid encapsulated chitosan microspheres by varying TPP concentration.

BATCH No.	CTS(%)	AA(%)	TPP(%)	GLUTARALDEHYDE (1%) VOLUME (ml)
B8	1	1	1	1
B9	1	1	1.5	1
B10	1	1	2	1

Table 11 : Formulation of L-Ascorbic acid encapsulated chitosan microspheres by varying L-Ascorbic acid concentration.

BATCH No.	CTS(%)	AA(%)	TPP(%)	GLUTARALDEHYDE (1%) VOLUME (ml)
B11	1	1	2	1
B12	1	1.5	2	1
B13	1	2	2	1

Table 12 : Formulation of L-Ascorbic acid encapsulated chitosan microspheres by varying pH of 2% TPP solution.

BATCH No.	CTS(%)	AA(%)	TPP 2% pH	GLUTARALDEHYDE (1%) VOLUME (ml)
B14	1	1	3	1
B15	1	1	4	1
B16	1	1	5	1
B17	1	1	6	1
B18	1	1	7	1
B19	1	1	9	1

5.5 Evaluation parameters for chitosan encapsulated L-Ascorbic acid microspheres

All batches of L-ascorbic acid microspheres were evaluated for the following parameters.

1. Percentage yield
2. Equilibrium swelling studies
3. Average particle size
4. Encapsulation efficiency
5. *In vitro* release study

5.5.1 Percentage yield

Percentage yield is actual yield of microspheres formed from 10ml of drug – polymer matrix. The microspheres prepared were collected, dried and weighed. The percentage yield was calculated as:

$$\textbf{Percentage yield (\%)} = \frac{\textbf{weight of dried microspheres recovered}}{\textbf{weight of chitosan+ weight of TPP}} \textbf{X 100}$$

Table 13: Percentage yield of different batches of vitamin C encapsulated microspheres.

BATCH No.	Yield(mg)	Yield %
B5	430	20.48
B6	520	24.76
B7	580	27.62
B8	290	13.81
B9	170	8.09
B10	430	20.48
B11	430	20.47
B12	200	9.52

B13	160	7.62
B14	200	9.52
B15	240	11.43
B16	230	10.95
B17	200	9.52
B18	170	8.09
B19	430	20.48

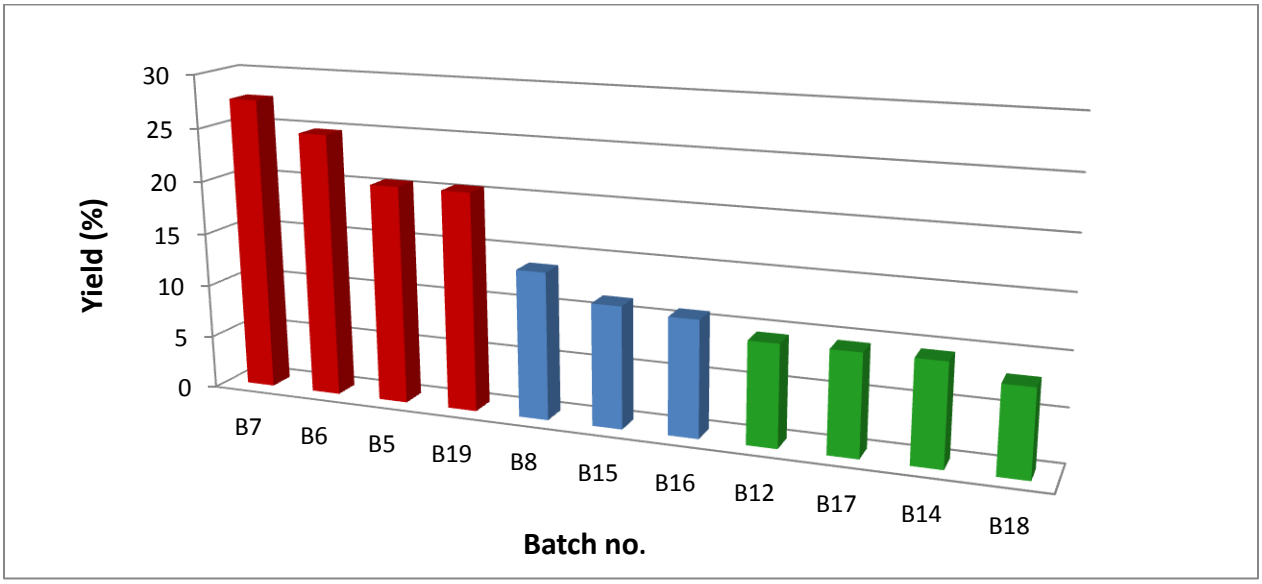


Figure15: Effect of various formulation parameters on percentage yield of vitamin C encapsulated chitosan microspheres.

5.5.2 Equilibrium swelling studies

The swelling ability of dried microspheres of chitosan microspheres was determined in distilled water, pH 1.2 buffer solutions and pH 6.8 buffer solutions. 20 mg of dried microspheres were immersed in 5ml of distilled water, pH 1.2 buffer and pH 6.8 buffer in different glass vials respectively. These immersed microspheres were kept at 37^0 C for 24 hours. Swollen microspheres were filtered, blotted and weighed immediately on an electronic balance. The percentage swelling index of microspheres at equilibrium was calculated by using the following formula:

$$\% \text{ Swelling Index} = \frac{w_f - w_i}{w_f} \text{ X } 100$$

Where, W_f = weight of swollen microspheres after 24 hrs.

W_i = initial weight of microspheres

Table 14: Percent swelling index of different batches of vitamin C encapsulated chitosan microspheres.

BATCH	SI(with DW) %	SI(with pH 1.2 buffer) %	SI(with pH 6.8 buffer) %
B1	47.43	76.7	40.2
B2	50.42	97.9	45.5
B3	47.30	84.3	43.1
B4	38.90	82.8	47.7
B5	35.56	96.8	46.9
B6	50.2	Completely dissolved	45.3
B7	45.20	Completely dissolved	50.2
B8	35.33	97.7	53.5
B9	45.62	96.2	37.6
B10	35.56	96.8	41
B11	35.56	96.8	36.4
B12	40.74	89.8	50.1

B13	35.48	86.3	30.6
B14	41.5	Completely dissolved	54.1
B15	49.6	95.4	49.9
B16	39.7	Completely dissolved	52.9
B17	39.3	96.4	46.9
B18	39.9	Completely dissolved	46.9
B19	35.56	96.8	45.3

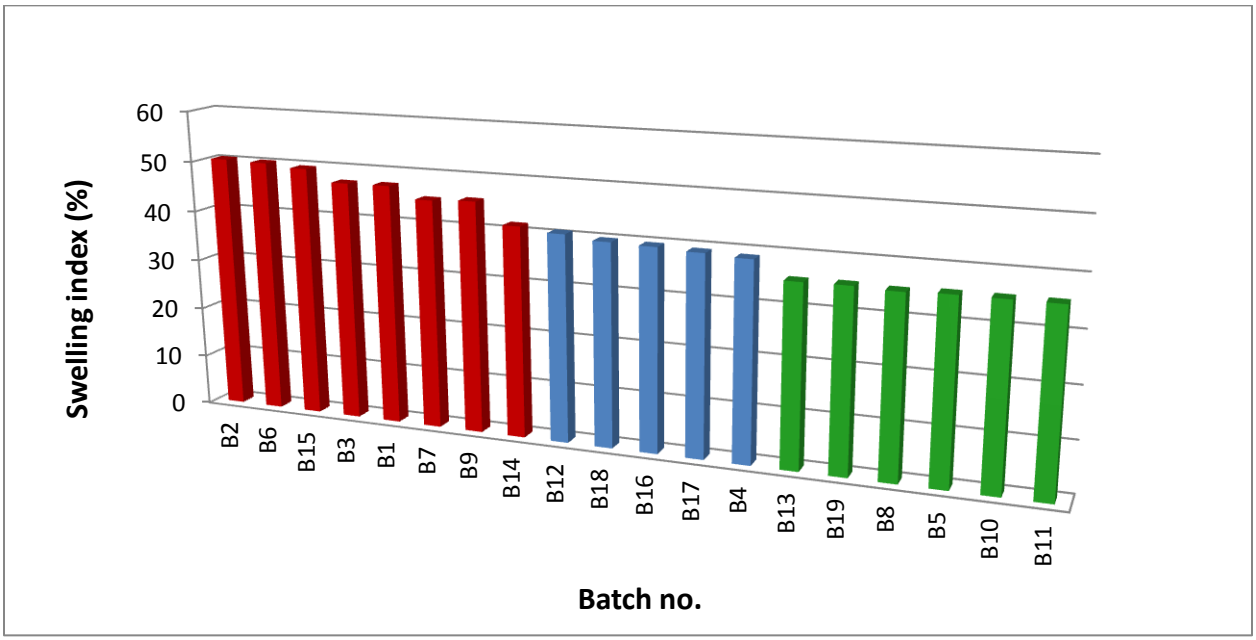


Figure 16: Effect of various parameters on percentage swelling index of vitamin C encapsulated chitosan microspheres in distilled water.

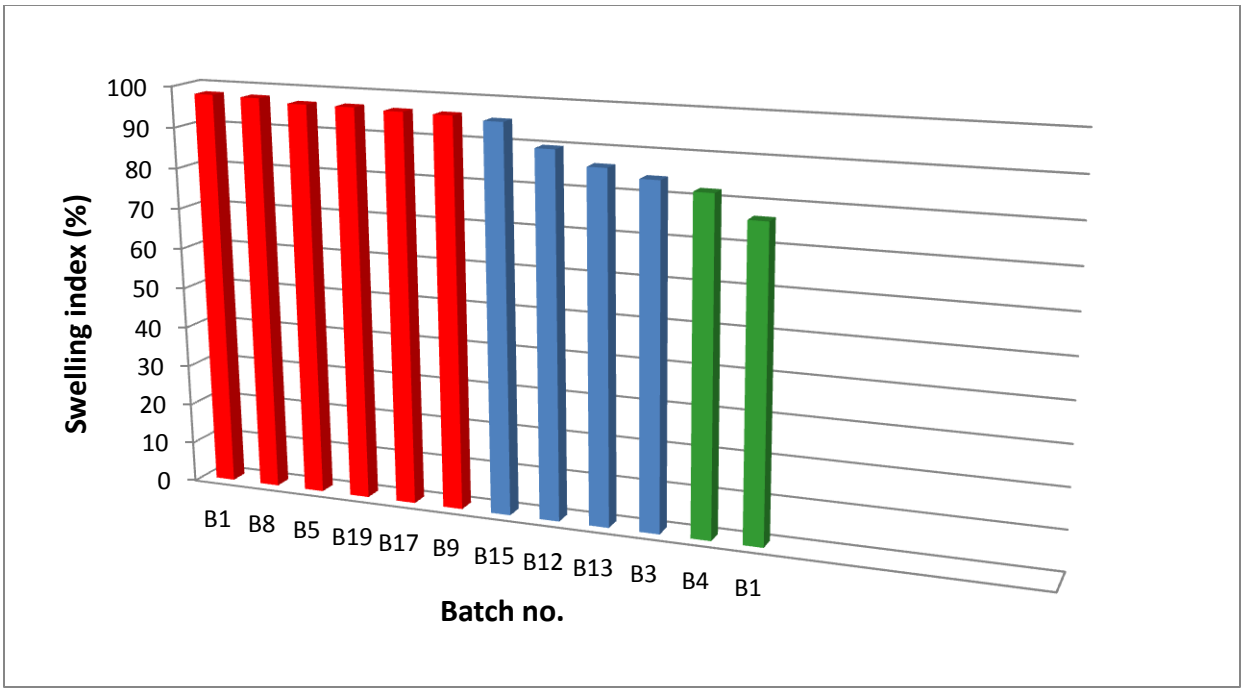


Figure 17: Effect of various formulation parameters on percentage swelling index of vitamin C encapsulated chitosan microspheres in pH1.2 buffer.

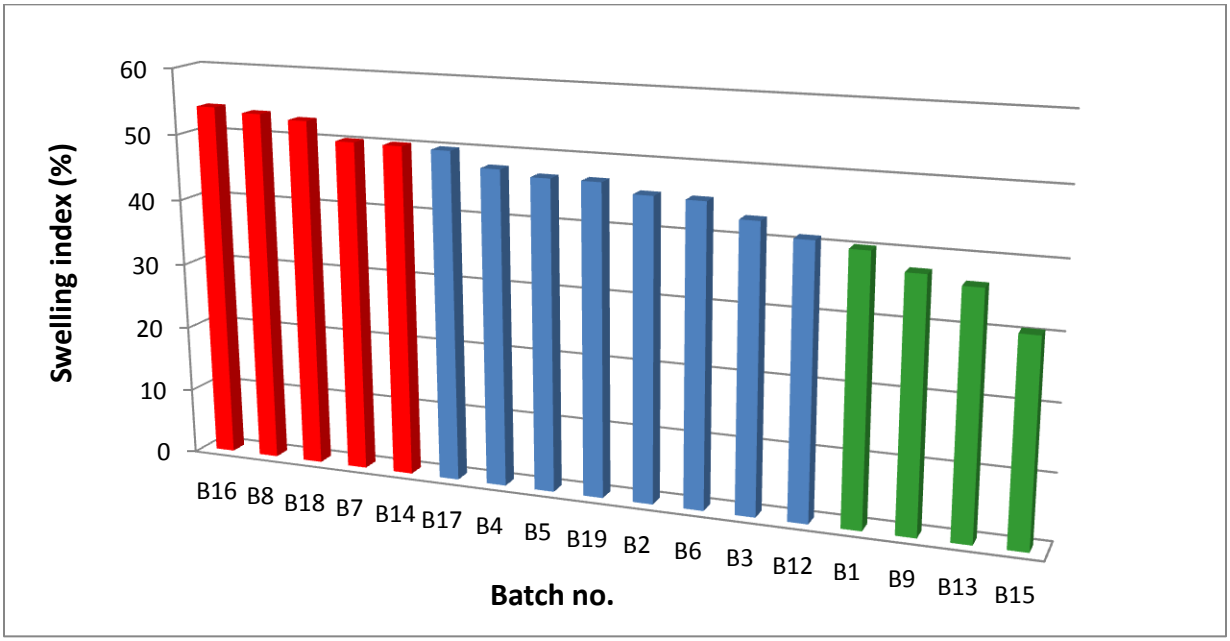
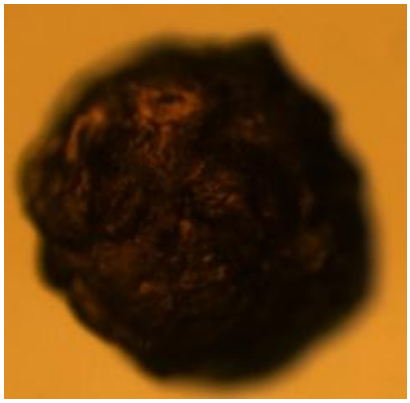


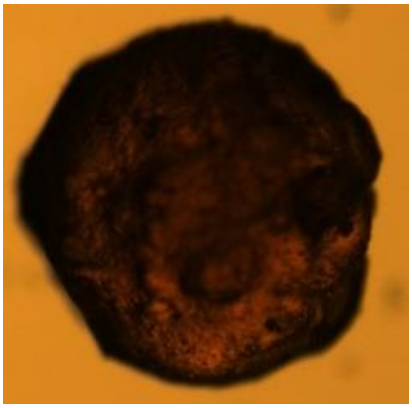
Figure 18: Effect of various formulation parameters on percentage swelling index of vitamin C encapsulated chitosan microspheres with pH 6.8 buffer.

5.5.3 Average particle size determination

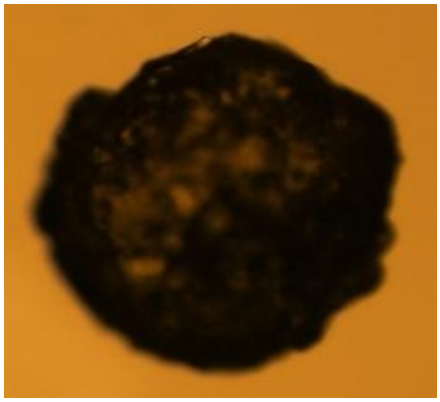
All the microspheres were evaluated with respect to their size using Nikon SMZ 800 optical microscope attached to computer for visual display of microspheres. The software which were used for acquiring live fast image of microspheres was NIS-element BR. The particle size of more than 50 microspheres was measured for each batch randomly by optical microscope. The average particle size of microspheres was determined by calculating the total size measured for microspheres which is divided by the number of microspheres i.e. 50. The morphological characteristics of vitamin C encapsulated chitosan microspheres were also observed using Nikon SMZ 800 optical microscope.



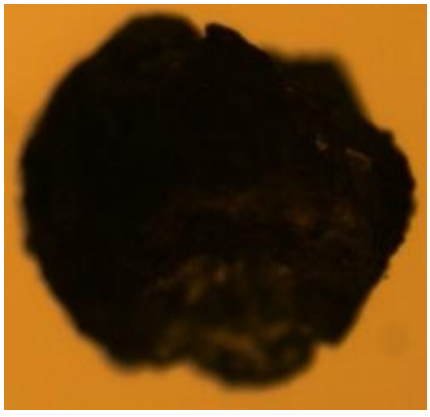
B14



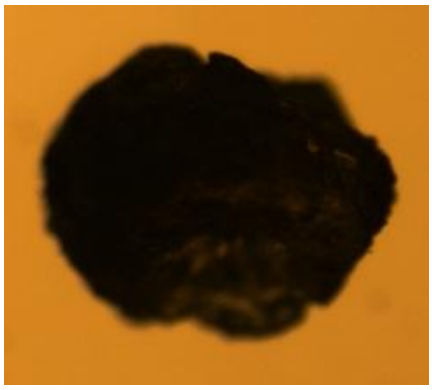
B15

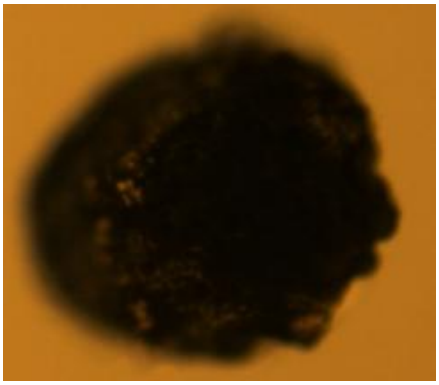


B16



B17





B18 **B19**

Figure 19: Morphology of vitamin C encapsulated chitosan microspheres at the different pH of 2% TPP cross linking agent (B14-B19).

Table 15: Average particle size of different batches of chitosan microspheres.

BATCH	Average particle size(µm)
B1	354.2
B2	401.1
B3	364.6
B4	323.7
B5	474.24
B6	444.08
B7	500.14
B8	362.53
B9	406.45
B10	474.24
B11	474.24
B12	406.24
B13	433.02
B14	445.34
B15	488.42
B16	361.48
B17	403.89
B18	336.03
B19	474.24

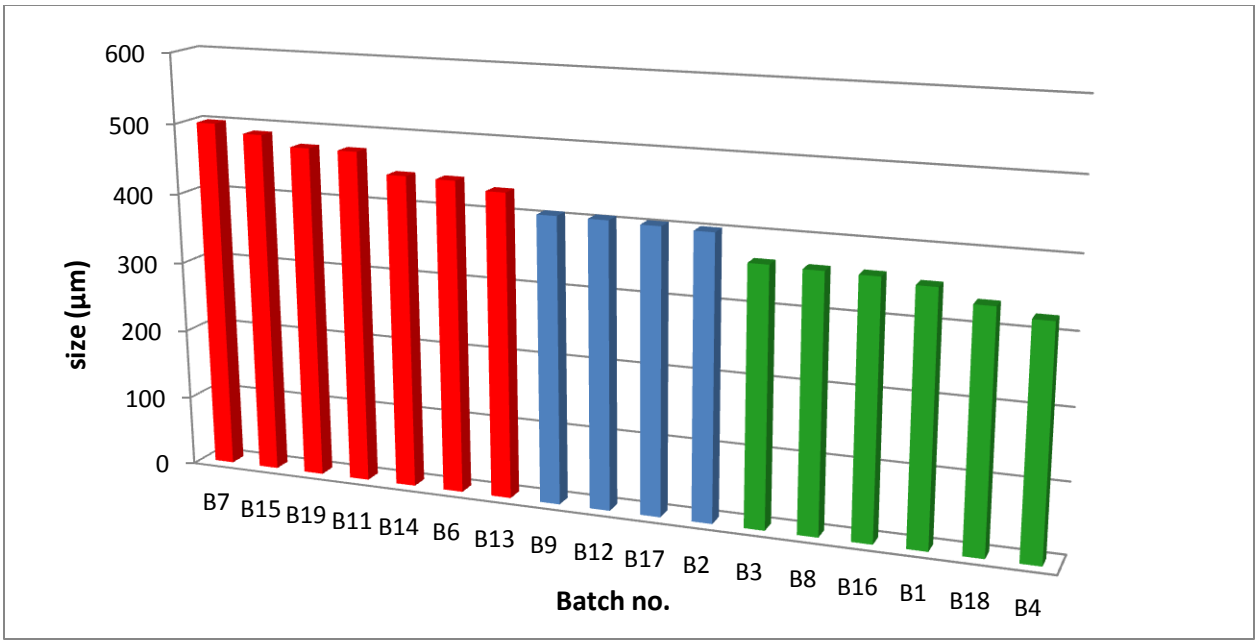


Figure 20: Effect of various formulation parameters on average size of chitosan microspheres.

5.5.4 Determination of encapsulation efficiency

Drug loading efficiency of vitamin C encapsulated chitosan microspheres was performed by accurately weighing 50mg of microspheres and then crushing them properly using pestle and mortar into fine powder. This powder was then suspended in 10 ml of pH1.2 buffer and its vitamin C absorbance was taken using UV spectrophotometer at 244nm. Then vitamin C concentration was calculated for each batch separately.

$$\textbf{Percentage encapsulation efficiency} = \frac{\textbf{Estimated drug content}}{\textbf{Theoretical drug content}} \textbf{ X 100}$$

Table 16 : Encapsulation efficiency of different batches of vitamin C encapsulated chitosan microspheres.

BATCH No.	ENCAPSULATION EFFICIENCY (%)
B5	76.25
B6	57.51
B7	67.4
B8	7.45

B9	29.31
B10	76.25
B11	76.25
B12	52.08
B13	59.49
B14	37.2
B15	53.34
B16	36.89
B17	37.88
B18	23.43
B19	76.25

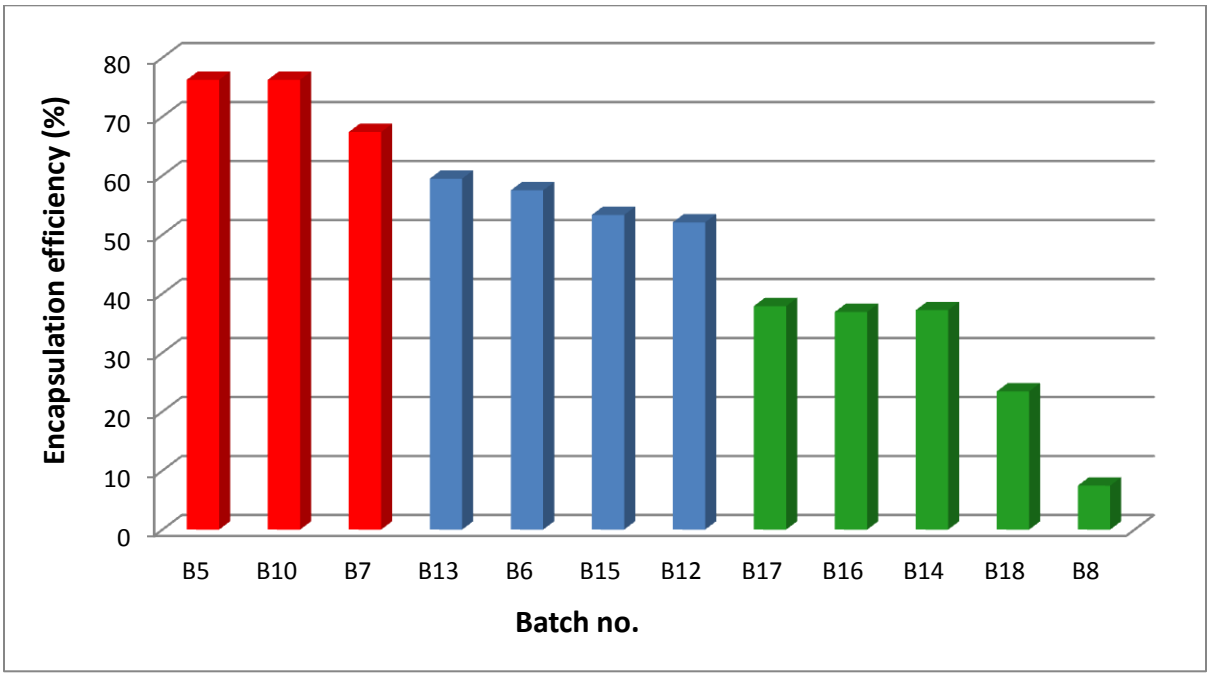


Figure 21: Effect of various formulation parameters on encapsulation efficiency of vitamin C encapsulated microspheres.

5.5.5 Evaluation of in vitro drug release study

50mg of vitamin C encapsulated chitosan microspheres were incubated in 100ml buffer of pH 1.2 in a 150 ml conical flask kept in a shaking incubator at 37^0 C and at 100 rpm. After 4 hours microspheres were filtered and transferred into 100ml buffer of pH 6.8 and incubated similarly at 37^0 C and at 100 rpm. Starting from 0 hour and at desired intervals of time 5ml sample was withdrawn and replaced with the same amount of fresh buffer respectively.

Drug released in the buffer medium at different time interval was assayed by measuring the absorbance at 244nm (λmax of vitamin C in 0.1N HCL) after suitable dilution using a UV spectrophotometer. Released concentration of vitamin C at different time interval was calculated and release profile of vitamin C encapsulated chitosan microspheres of various batches were studied.

Table 17: Drug release data of vitamin C encapsulated microspheres by varying chitosan composition (1-2 %).

	B5		B6		B7	
TIME (hours)	**Conc.(µg/ml)**	**Cumulative release (%)**	**Conc.(µg/ml)**	**Cumulative release (%)**	**Conc.(µg/ml)**	**Cumulative release (%)**
0	0.4	0.0	0.01	0.0001	0.1	0.0
0.5	5.2	0.0	0.8	0.008	1.6	0.02
1	23.2	0.02	3.6	0.05	6.3	0.1
1.5	39.6	0.07	6.4	0.11	10.7	0.2
2	55.1	0.1	9.2	0.21	14.8	0.4
3	83.5	0.3	14.5	0.48	22.6	0.9
4	111	0.5	19.8	0.87	29.8	1.5
5	507	1.5	86.0	2.64	132.6	4.6
6	537	2.5	89.1	4.47	136	7.7
7	560	3.6	91.1	6.36	136	10.9
8	570	4.2	92.5	8.28	139	12.5
24	583	5.4	93.0	9.25	140	15.9

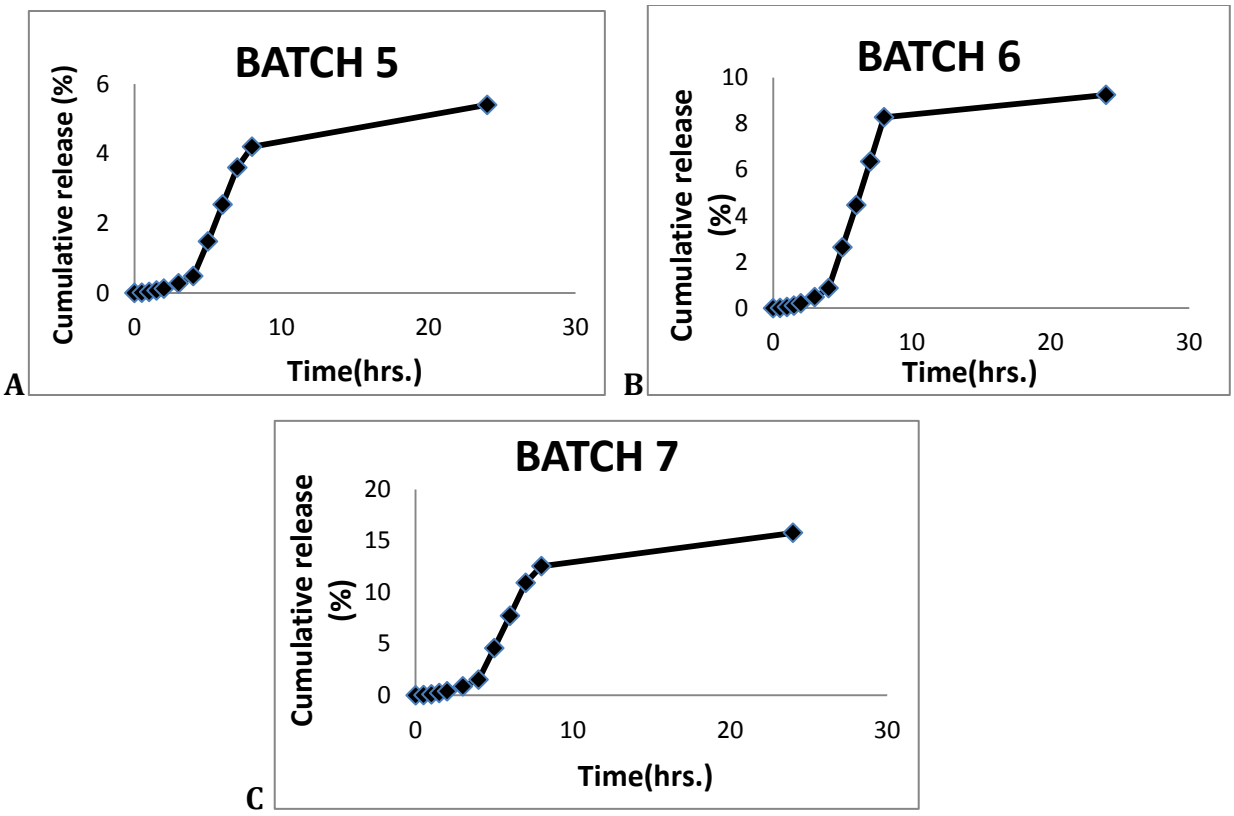


Figure 22(A-C) : Drug release curves of B5, B6 and B7 respectively.

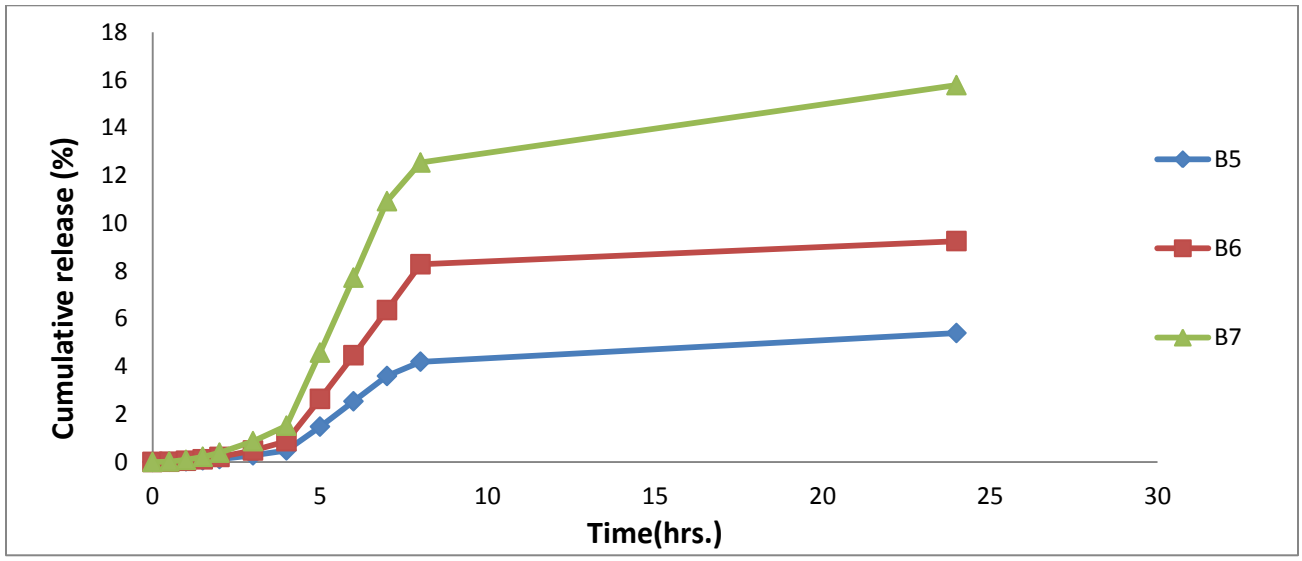
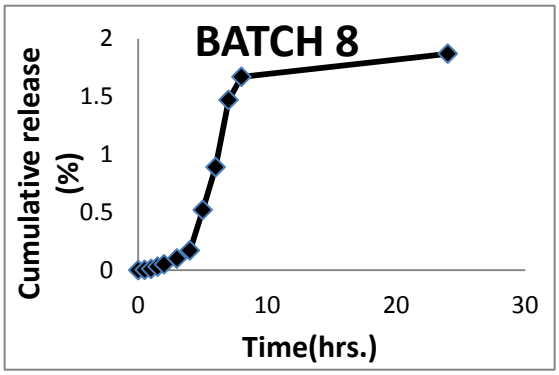


Figure 23: Vitamin C release profiles by varying chitosan concentration of batches (B5-B7).

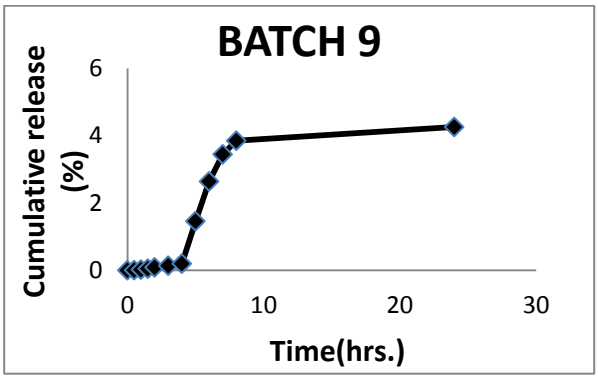
Table 18 : Drug release data of vitamin C encapsulated microspheres by varying TPP composition (1-2 %).

	Batch 8		Batch 9		Batch 10	
Time (hours)	Conc.(µg/ml)	Cumulative release (%)	Conc.(µg/ml)	Cumulative release (%)	Conc.(µg/ml)	Cumulative release (%)
0	0.1	0.0	0.2	0.0	0.4	0.0
0.5	0.8	0.0	1.1	0.00	5.2	0.0
1	3.1	0.0	4.6	0.02	23.2	0.03
1.5	5.3	0.0	8.1	0.05	39.6	0.07
2	6.9	0.1	11.4	0.1	55.1	0.1
3	10	0.1	14.7	0.1	83.5	0.3
4	13	0.2	17.8	0.2	111	0.5
5	61	0.5	112.9	1.5	507	1.5
6	65	0.9	117	2.6	537	2.5
7	67.9	1.5	118.6	3.5	560	3.6
8	68.8	1.7	119.3	3.9	570	4.2
24	200	1.9	119.9	4.3	583	5.4

A



B



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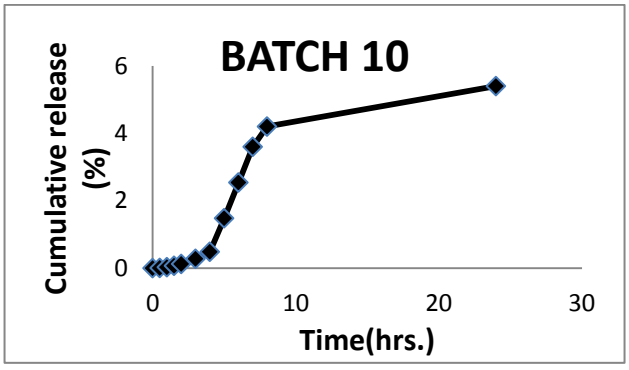


Figure 24(A-C) : Drug release curves of B8, B9 and B10 respectively.

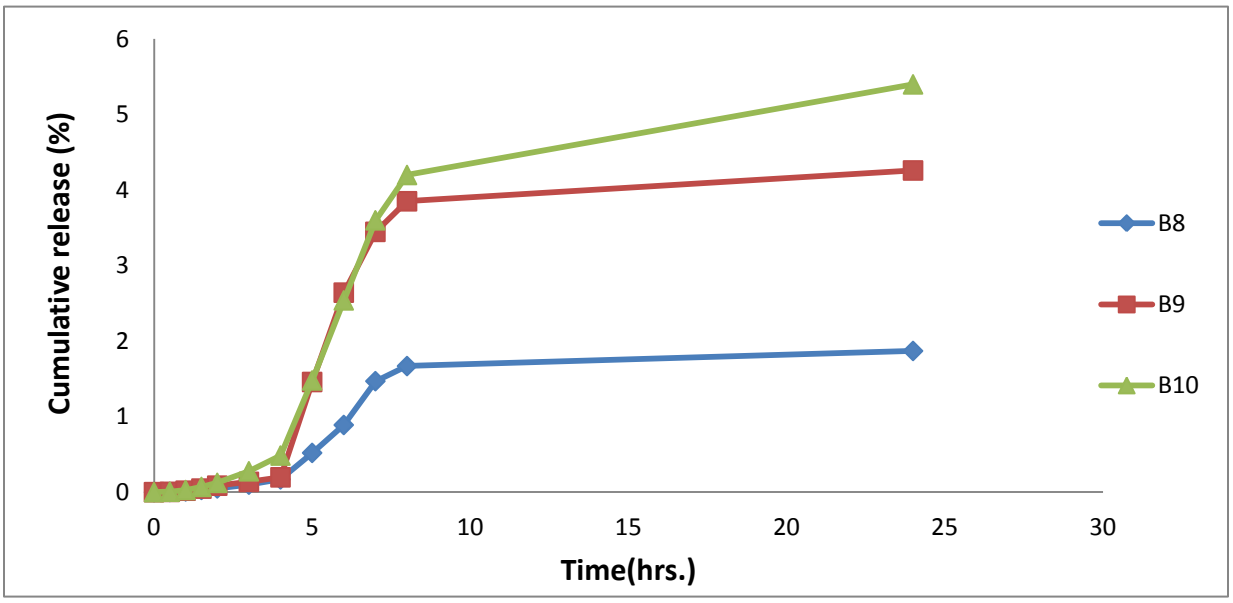


Figure 25: Vitamin C release profiles of chitosan microspheres by varying composition of TPP (1-2%) batches (B8-B10).

Table 19: Drug release data of vitamin C encapsulated microspheres by varying vitamin C composition (1-2 %).

	B11		B12		B13	
Time (hours)	Conc.(µg/ml)	Cumulative release (%)	Conc.(µg/ml)	Cumulative release (%)	Conc.(µg/ml)	Cumulative release (%)
0	0.4	0.0	0.2	0.0	0.1	0.0
0.5	5.2	0.0	1.2	0.0	1.7	0.0
1	23.2	0.0	4.8	0.0	6.8	0.0
1.5	39.6	0.0	8.4	0.1	11.8	0.1
2	55.1	0.1	11.8	0.1	16.5	0.2
3	83.5	0.3	18.4	0.2	25.4	0.3
4	111	0.5	24.5	0.4	33.6	0.5
5	507	1.5	111.7	1.3	157.7	1.5
6	537	2.5	116.4	2.2	160.2	1.9
7	560	3.6	120	3.7	162.8	3.0
8	570	4.2	122	4.6	164.5	4.6
24	583	5.4	122	5.1	165.3	5.6

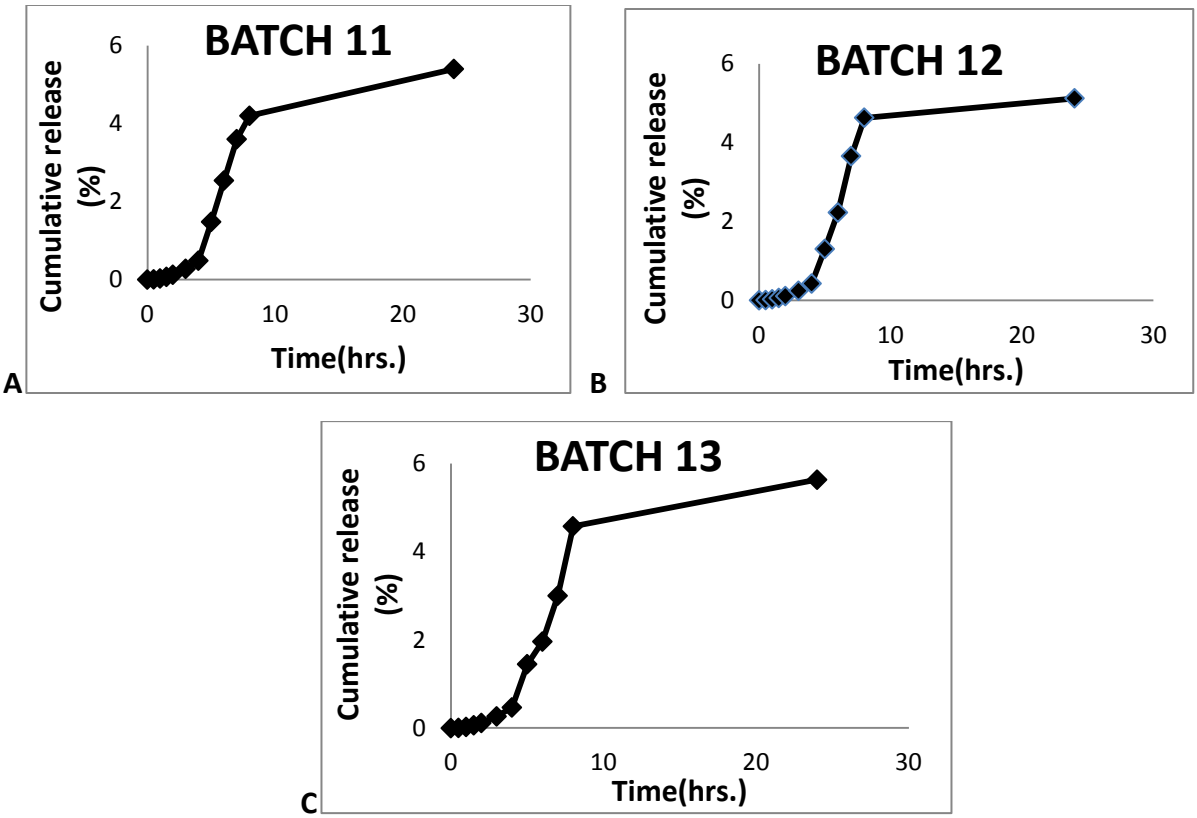


Figure 26(A-C) : Drug release curves of B11, B12 and B13 respectively.

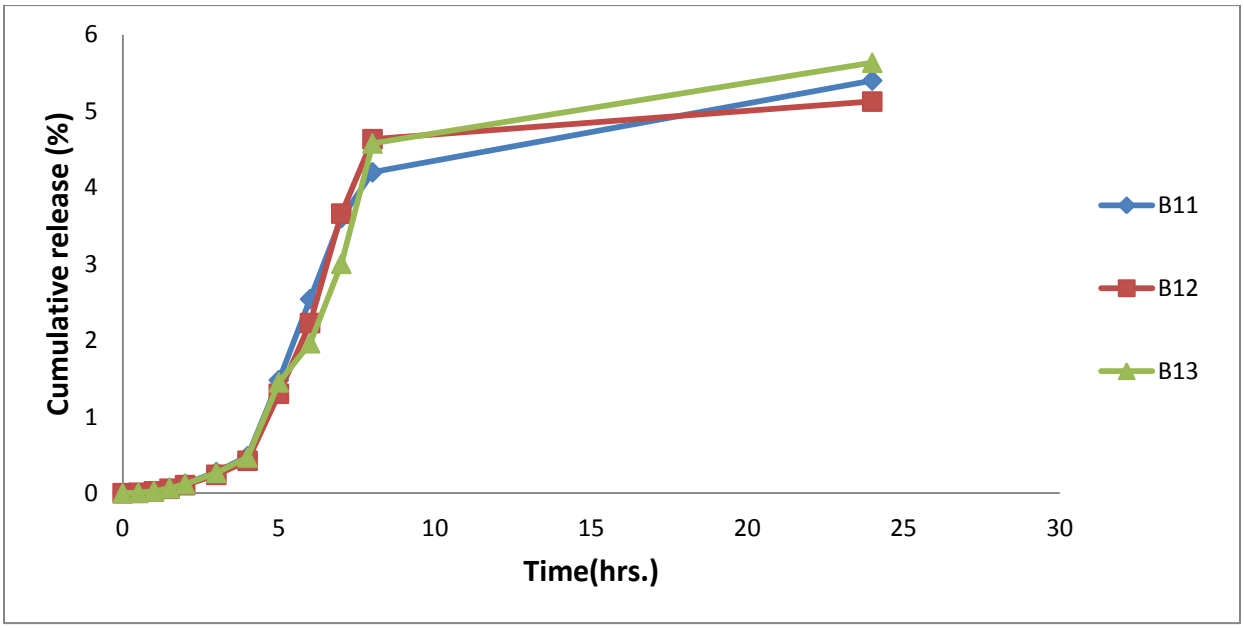
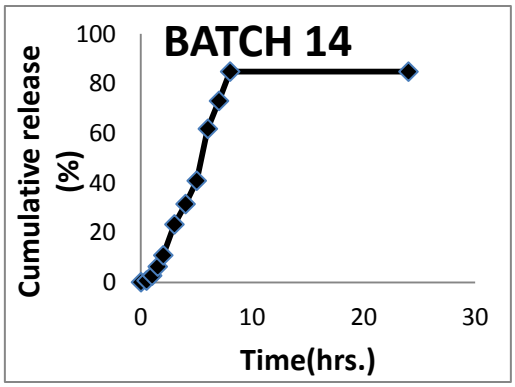


Figure 27 : Vitamin C release profiles of chitosan microspheres by varying composition of L-Ascorbic acid (1-2%) batches (B11-B13).

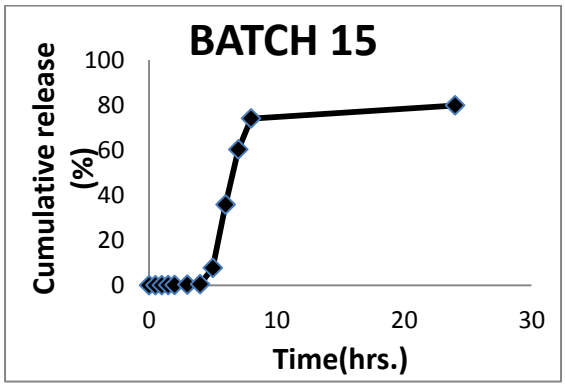
Table 20 : Drug release data of vitamin C encapsulated microspheres by varying pH of 2% TPP cross linking solution.

	B14		B15		B16	
Time (hours)	Conc.(µg/ml)	Cumulative release (%)	Conc.(µg/ml)	Cumulative release (%)	Conc.(µg/ml)	Cumulative release (%)
0	2	0.0	0.09	0.0	0.2	0.0
0.5	21	0.7	1.26	0.0	1.8	0.0
1	66	2.7	4.76	0.1	5.8	0.0
1.5	120	6.3	7.92	0.1	26	0.2
2	150	10.9	10.92	0.1	64	0.6
3	220	23.3	16.5	0.2	130	1.8
4	270	31.5	21.6	0.5	200	3.8
5	310	40.9	96	7.7	50.9	4.1
6	350	61.8	98	35.8	87.6	4.6
7	370	73.0	100	60.3	486	7.4
8	390	84.8	101	74.1	140	8.1
24	390	84.8	102	79.9	157	9.1

A



B



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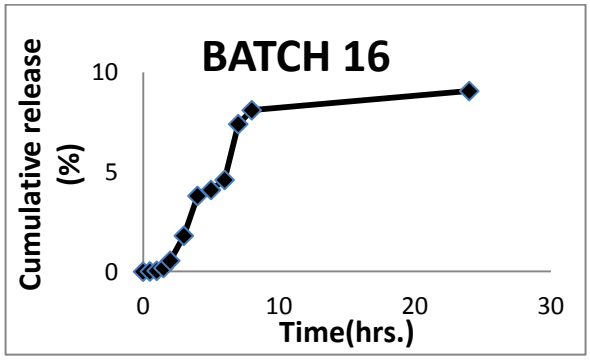

Figure 28(A-C): Drug release curves of batches B14, B15 and B16 respectively.

Table 21 : Drug release data of vitamin C encapsulated microspheres by varying pH of 2% TPP cross linking solution.

	B17		B18		B19	
Time (hours)	Conc.(µg/ml)	Cumulative release (%)	Conc.(µg/ml)	Cumulative release (%)	Conc.(µg/ml)	Cumulative release (%)
0	0.1	0.00	0	0	0.4	0.00
0.5	5.5	0.03	12	0.04	5.2	0.01
1	4.7	0.06	52	0.22	23.2	0.03
1.5	7	0.11	93	0.53	39.6	0.07
2	10	0.17	132	0.98	55.1	0.12
3	18	0.45	202	2.24	83.5	0.28
4	96	1.73	293	4.93	111	0.49
5	98.8	2.92	366	7.28	507	1.48
6	99.9	3.52	426	8.73	537	2.54
7	100	4.75	433	10.19	560	3.6
8	102	5.37	458	11.76	570	4.2
24	101	5.98	432	13.22	583	5.4

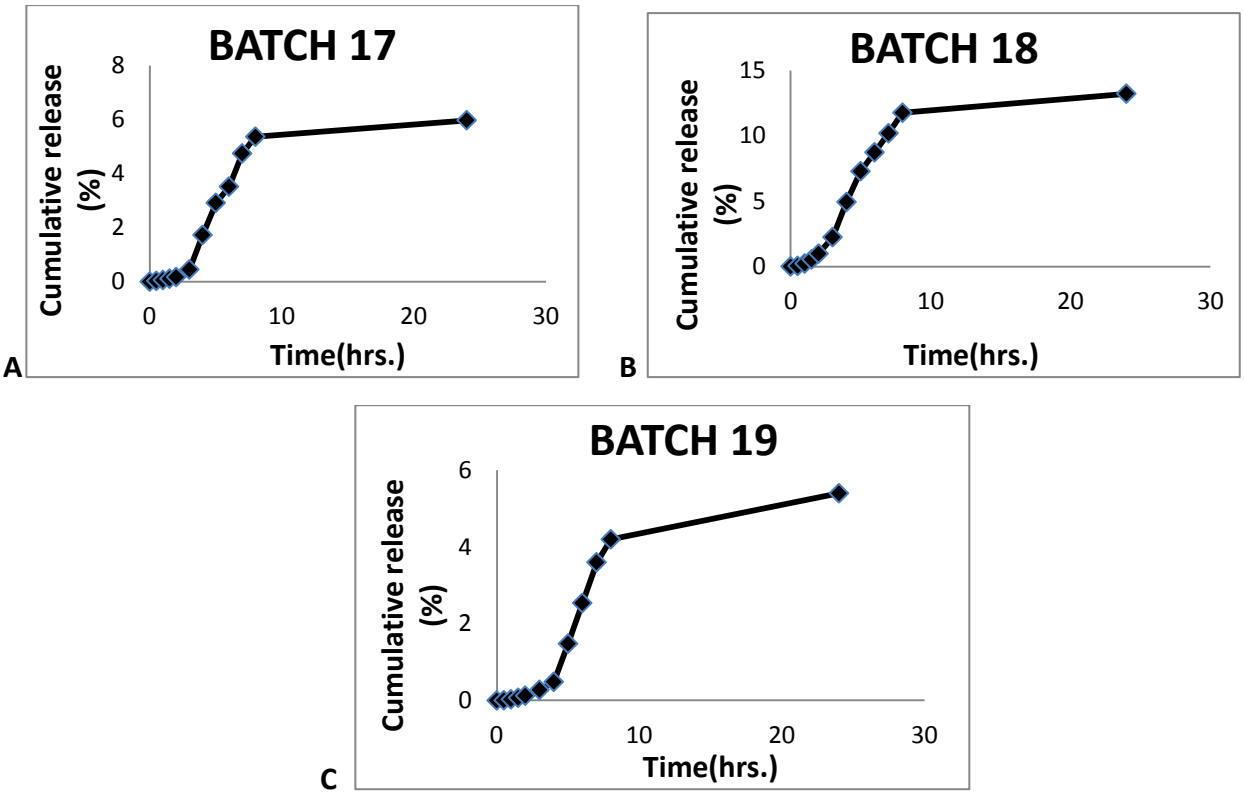

Figure 29(A-C) : Drug release curves for batches B17, B18 and B19 respectively.

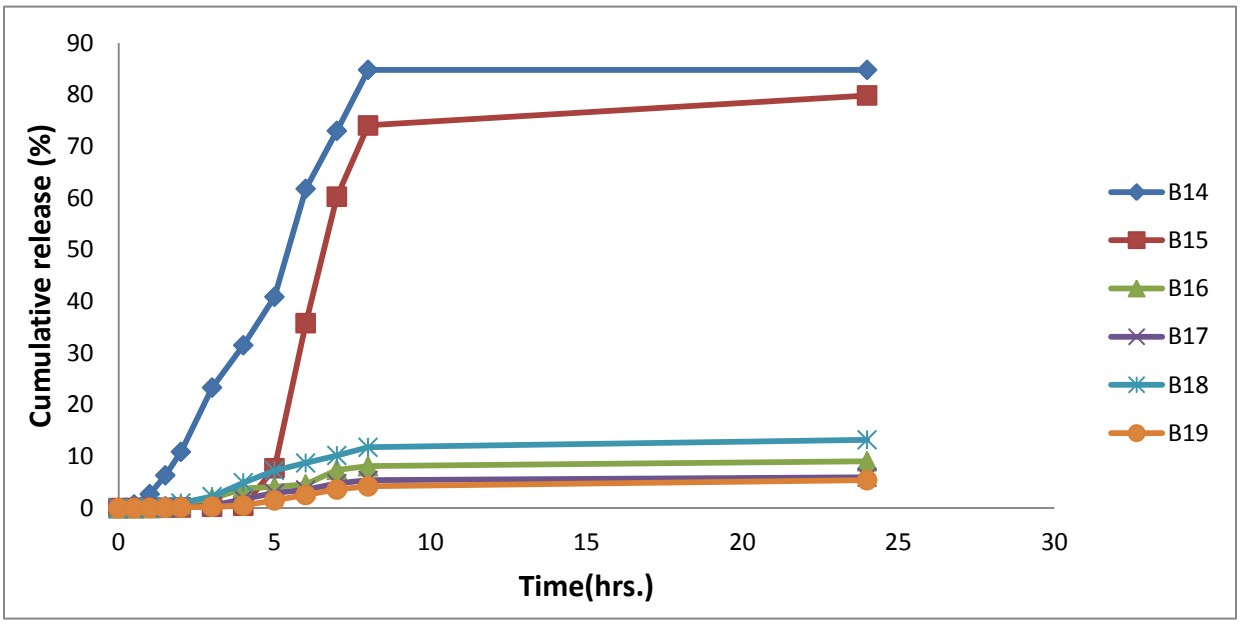

Figure 30 : Vitamin C release profiles of chitosan microspheres by varying pH of 2% TPP cross linking agent solution (4-9) batches (B14-B19).

CHAPTER VI

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Biodegradable microspheres are widely used for encapsulation of food ingredients as well as pharmaceutical compounds. These encapsulated microspheres provided a good barrier to highly reactive core material and also provide controlled release of core material. There are various techniques used for the preparation of these microspheres like spray drying, coacervation method which require sophisticated and costly instruments. But ionotropic gelation is found to be a simple method which uses simple instruments and core material could be easily entrapped in a completely aqueous environment under mild conditions. Hence ionotropic gelation method was selected for the preparation of vitamin C encapsulated chitosan microspheres. Chitosan with natural muco-adhesive properties allows designing of drug carrier systems that can bind to the intestinal mucosa, and thus improve the residence time and hence bioavailability. Hence chitosan being used as the coating material lived up to its properties.

The main aim of this study was to evaluate cross linked chitosan-L-Ascorbic acid microspheres for its controlled release properties and efficiency for vitamin C release and profoundly analyzing the effect of pH on microencapsulation of vitamin C. To achieve the same effect various parameters were studied viz. drug concentration, polymer concentration, cross linking agent concentration and pH, stirring speed and stirring time. The various batches prepared by varying the formulation parameters were then analyzed for their various characteristics like percentage yield, average particle size, swelling index, drug encapsulation efficiency, and *in vitro* drug release rates.

Table 8-12 indicates the batches of vitamin C encapsulated chitosan microspheres prepared by cross linking in sodium tripolyphosphate solution by varying various formulation parameters mentioned above.

Firstly the volume of 1% v/v glutaraldehyde was optimized so that the surface morphology of microsphere could be enhanced and also burst release of drug could be controlled for which B1- B4 of unloaded chitosan microspheres were prepared. Microspheres of batch B2 showed average size of 401.1µm which was highest of remaining batches. And also % swelling index of

batch B2 was 50.42, 97.9 and 45.5 in distilled water, buffer solution (pH1.2) and buffer solution (pH6.8) respectively. And hence the batch B2 prepared by using 1ml volume of glutaraldehyde (1% v/v) was considered to be the best batch compared to others.

The percentage yield of different batches of chitosan-ascorbic acid microspheres is tabulated in table 13. It varied from 7-27.62 %. The comparative study of percentage yield is shown by histogram in figure 16. Maximum percentage yield was obtained from B7 from 10ml of drug-polymer solution. B7 composition contained 2% chitosan, 1% ascorbic acid, 2% sodium tripolyphosphate and 1ml of 1% glutaraldehyde. Percentage yield decreased by decreasing the chitosan concentration, it increased by increasing TPP concentration and decreased by increasing ascorbic acid concentration. Yield % was obtained highest for the TPP solution at pH 9 i.e. B19 followed by B15 at pH 4.

The percentage equilibrium swelling index of different batches of microspheres is listed in Table 14. The swelling index in distilled water, buffer of pH 1.2 and buffer of pH 6.8 ranged between 35-50.4%, 76.7-97.7% and 30-54% respectively. The comparative study of swelling index in different medium are shown by histogram in figures 17, 18 and 19. Maximum swelling index was obtained from the batch B6 in distilled water having the composition of 1.5% chitosan, 1% ascorbic acid and 2% sodium tripolyphosphate solution. Batch B8 showed maximum swelling index in buffer of pH 1.2 having the composition 1% chitosan, 1% ascorbic acid and 1% sodium tripolyphosphate solution. Batch B14 showed maximum swelling index in buffer of pH 6.8 having the composition 1% chitosan, 1% ascorbic acid and 2% sodium tripolyphosphate solution at pH 3.

Swelling index decreased by increasing the 1% (v/v) glutaraldehyde volume. Swelling index obtained was highest for B6 containing 1.5% chitosan in distilled water but it completely got dissolved in buffer (pH1.2) and showed minimum swelling index in buffer (pH 6.8) in comparison to batches prepared by varying chitosan concentration. So batch B5 was considered best among batches produced by varying chitosan concentration.

Among the batches prepared by varying sodium tri-polyphosphate concentration, batch B10 was considered optimized containing 2% (w/v) sodium tripolyphosphate concentration. As it showed optimum percentage swelling index in distilled water, buffer (pH 1.2) as well as in

buffer (pH 6.8). Similarly batches prepared by varying ascorbic acid concentration, batch B11 containing 1% ascorbic acid was considered best in comparison to batches B12 and B13 due to their optimized in distilled water and in both buffers. Swelling index was higher for batch B15 in comparison to batches produced by varying pH of 2% sodium tripolyphosphate solution as it showed maximum swelling index in distilled water as well as in buffer (pH 6.8). Batch B15 was considered to have maximum swelling index in comparison to all the batches as it showed very good swelling index in all three medium respectively.

The average particle size of chitosan microspheres is listed in Table 15. It varied from 323-500 µm. The comparative study of average particle size is shown by histogram in figure 21. Minimum size of microspheres was obtained from B4 of unloaded chitosan microspheres and batch B18 of vitamin C encapsulated chitosan microspheres. B18 had the composition of 1% chitosan, 1% ascorbic acid and 2% sodium tripolyphosphate solution of pH 7. While maximum average particle size was seen in batch B7 having composition 2% chitosan, 1% ascorbic acid and 2% sodium tripolyphosphate. Average particle increased by increasing the polymer concentration and cross linking agent concentration.

Average particle size of batch B15 was 488.42 µm which was highest in comparison to batches prepared by varying the pH of 2% TPP cross linking agent solution.

The morphology of vitamin C encapsulated chitosan microspheres was determined under NIKON SMZ 800 light microscope. The photos obtained from it are shown in figure 20. The batches obtained by varying the pH after optimizing the concentration of chitosan, ascorbic acid and sodium tripolyphosphate are shown in photos. These microspheres are spherical in shape having an outer darker zone of polymer encapsulating the core material i.e. vitamin C. Batch B15 showed the even surface morphology of microspheres having a spherical shape in comparison to other batches.

Encapsulation efficiency of various vitamin C encapsulated chitosan microspheres is listed in Table 16. It ranged between 7.45 - 76.25 %. The comparative study of encapsulation efficiency is shown by histogram in Figure 22. Maximum encapsulation efficiency was observed in batches B5 and B19 having the compostion 1% CTS, 1%AA, 2% STPP and 1% CTS, 1% AA, 2% STPP

solution at pH 9 respectively. Drug encapsulation efficiency increased by increasing the concentration of sodium tripolyphosphate from 1-2%. This can be attributed to the surface irregularities of the chitosan microspheres observed with a lower concentration of cross linking agent. Damaged microspheres with surface irregularities, fragmentation or holes are likely to cause the loss of a substantial amount of vitamin C during the ionotropic gelation microencapsulation process.

There was no significant result obtained by either increasing or decreasing the concentration of chitosan as well as ascorbic acid. It showed maximum value for the batch B19 followed by batch B15 in comparison to batches prepared by varying the pH of sodium tripolyphosphate solution. The reason for low drug encapsulation may be due to the high instability of drug. The instability of drug was tried to overcome by making it stable by varying the pH of sodium tripolyphosphate solution. It showed that Vitamin C was maximum stable at either pH 9 or pH 4. As reported by NCBI Ascorbic acid showed maximum stability at pH between 4 and 6. Hence batch B15 microspheres tend to show the best drug encapsulation efficiency in comparison to other batches.

The cumulative release rate study of different batches of vitamin C encapsulated microspheres was performed in buffer at pH 1.2 and then subsequently with buffer at pH 6.8. Both of these buffers were chosen for studying the release rate of vitamin C as they simulated close to the physiologically gastrointestinal conditions of the human body. Microencapsulation provides the key functionality for controlled release of food ingredients at the right place and the right time. A timely and targeted release improves the effectiveness of food additives, broadens the application range of food ingredients and ensures optimal dosage; thereby improving the cost effectiveness for the food manufacturer (Augustin *et al*., 2001).The dissolution behavior of chitosan microspheres was dependent on pH of the medium.

The in vitro drug release rates of different batches of vitamin C encapsulated chitosan microspheres are listed in Table 17 – 21. The comparative study of drug release curves is shown in figures 24, 26, 28 and 31.

It was observed that there was a delay in release of drug in buffer at pH 1.2 in all the batches. It can be attributed that the leaching of drug from the microspheres was delayed due to the outer

layer of chitosan which degraded very slowly at this pH and hence subsequent release rate of drug. The dissolution of vitamin C encapsulated chitosan microspheres in phosphate buffer pH 6.8 was rapid. This is obvious because of greater solubility of vitamin C in the aqueous medium.

It was observed that the release rate of vitamin C from tripolyphosphate-chitosan microspheres increased with the increasing concentration of cross-linking agent solution. The increased release rate of vitamin C from tripolyphosphate chitosan microspheres may be due to decreased sphericity of microspheres. As the concentration of sodium tripolyphosphate increased, it provided good surface morphology to the microspheres. There was increase in release rate of vitamin from chitosan microspheres batches prepared by increasing the concentration on chitosan. But there was no significant change in the release rate of vitamin C from chitosan microspheres batches prepared by varying the concentration of ascorbic acid.

On varying the pH of tripolyphosphate solution, maximum release rate was observed as compared to all the other batches. Batch B4 release rate was 84.8% followed by batch B15 batch which showed release rate of 79.86 %. It can be concluded that drug release rate is dependent on the percentage swelling index and drug encapsulation efficiency; if the percentage swelling index and encapsulation efficiency of drug is high then drug release rate will be high.

CHAPTER VII

CONCLUSION

CONCLUSION

L-Ascorbic acid encapsulated chitosan microspheres cross linked with TPP were prepared by ionotropic gelation method. Chitosan showed promising role in controlled drug delivery system from being a natural biodegradable polymer to providing good barrier to the core material from the surrounding environment. This helped in retaining the Vitamin C for longer duration within the human body.

Stable vitamin C encapsulated chitosan microspheres were prepared by using suitable pH and composition of TPP solution. Microspheres of different size, loading efficiency, percentage swelling index, and surface morphology could be obtained by varying TPP composition, AA composition, CTS composition and also by varying pH of TPP solution.

The particle size and encapsulation efficiency ranged between 323-500 µm and 24-76 % respectively. The yield of different batches ranged between 7-27.62 %. The percentage swelling index in distilled water, buffer of pH 1.2 and buffer of pH 6.8 ranged between 35-50.4 %, 76-97% and 30-54% respectively. The cumulative drug release was highest for batch 15 and its morphology was also good as compared to other batches. Batch 15 microspheres compositions was CTS(1%), AA(1%) and 1ml gltaraldehyde which were prepared at pH4 of STPP(2%) solution.

From the above studies based on varying the formulation variables, it may be concluded that ionotropic gelation method using natural biopolymers can be used to encapsulate broad range of food ingredients and pharmaceutical compounds for their controlled release/ delivery. This technique has numerous advantages as it is mild, effective method where harsh and harmful chemicals are not used and hence safer for human consumption.

Microspheres with high encapsulation efficiency, good morphology, high swelling index and high drug release rate can be prepared by optimizing the various parameters which are used for preparing these microspheres like TPP concentration, drug concentration, chitosan concentration and pH of TPP solution. All these parameters with their best value was observed in the B15 vitamin C encapsulated chitosan microspheres having the composition 1% CTS, 1% AA and 2% TPP solution at pH 4.

CHAPTER VIII

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REFERENCES

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