CHITOSAN MICROSPHERES AND FILMS USED IN CONTROLLED RELEASE

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ABSTRACT

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Thalassemia, a genetic blood disorder, occurs as a result of deformations of hemoglobin structures. Patients with thalassemia develop iron overload from chronic blood transfusions and require regular iron chelation to prevent potentially fatal iron-related complications. Deferiprone is a commercially available drug used as iron chelator for the treatment of thalassemia but the very long-term effectiveness is not clearly known yet. Therefore, some studies were carried out to find effective alternative drugs or delivery methods for treatment of thalassemia. Controlled delivery, which offers safer, more convenient, and more effective means of

administering actives, seems promising with this respect. Chitosan, a natural biopolymer produced from deacetylation of chitin, has a variety of promising pharmaceutical uses and is presently considered as a novel carrier material in drug delivery systems.

In this study, chitosan microspheres having different degree of deacetylation (DDA) and containing Deferiprone were prepared by oil/water emulsion method and by crosslinking with gluteraldehyde. Particle size, SEM, and in vitro drug release analysis were performed. The average sizes of the prepared microspheres increased with increasing degree of deacetylation of chitosan and with decreasing crosslinking degree. In vitro drug release studies showed that, the release rate of Deferiprone increased as the crosslinking degree increased, contrary to the expectations. This is explained by the crystalline structure of lightly crosslinked chitosan which have ordered and dense structure causing slower release rate for Deferiprone compare to highly crosslinked structures.

In the second stage of the study, chitosan films hardened with gluteraldehyde were prepared by film casting method. IR, DSC and mechanical analysis were performed. For the films with various crosslinking degrees, it was found that ultimate tensile strength (UTS) values differed from 50.6 MPa to 102.7 MPa, mean elastic modulus values differed from 3328.7 MPa to 3790.1 MPa and strain at break (SAB) values differed from 2.1% to 4.3%.

Keywords: Thalassemia, Deferiprone, chitosan, microsphere, film, controlled release

ÖZ

KONTROLLÜ SALIMDA KULLANILAN KİTOSAN MİKROKÜRELER VE FİLMLER

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Talasemi, hemoglobin yapılarında oluşan bozukluklar sonucunda ortaya çıkan, genetik bir kan hastalığıdır. Talasemi hastalarında kronik kan naklinden dolayı demir birikmesi meydana gelmekte ve olası, ölümcül, demirle ilişkili komplikasyonları önlemek için düzenli olarak alınan demir tutuculara ihtiyaç duyulmaktadır. Deferipron, talasemi hastalığının tedavisinde demir tutucu olarak kullanılan, ticari olarak piyasada bulunan bir ilaçtır, ancak, uzun süreli etkisi henüz net olarak bilinmemektedir. Bu sebeple, daha etkili alternatif ilaçlar ya da salım yöntemlerinin bulunabilmesi için bir takım çalışmalar sürmektedir. Bu açıdan, ilaçların uygulanmasında, daha güvenilir, daha elverişli ve daha etkili bir yöntem

olan kontrollü salım, ümit vericidir. Kitinin deasetilasyonuyla elde edilen, doğal bir biyopolimer olan kitosan, gelecek vaat eden çok çeşitli farmasotik kullanıma sahiptir ve günümüzde ilaç taşıyıcı sistemler için yeni taşıyıcı materyal olarak kabul edilmektedir.

Bu çalışmada, farklı deasetilasyon derecesine sahip Deferipron yüklü kitosan mikroküreler, gluteraldehit ile çapraz bağlananarak yağ/su emülsiyon metoduyla hazırlanmıştır. Partikül boyutu, SEM ve in vitro ilaç salım analizleri gerçekleştirilmiştir. Hazırlanan mikrokürelerin ortalama boyutları, kullanılan kitosanın deasetilasyon derecesinin artışıyla ve mikrokürelerdeki çapraz bağlanma derecesindeki azalma ile artış göstermiştir. In vitro ilaç salım çalışmalarında, beklenilenin tersine, çapraz bağlanma derecesi arttıkça, Deferipron salım hızının arttığı görülmüştür. Bu durum, yüksek çapraz bağlanma derecesine sahip kitosanla kıyaslandığında, Deferipron'un salım hızının yavaşlamasına neden olan düzenli ve sık yapıdaki az çapraz bağlanmış kitosanın kristal yapısıyla açıklanabilir.

Çalışmanın ikinci aşamasında, gluteraldehitle sağlamlaştırılmış filmler, döküm metoduyla hazırlanmıştır. Filmlerin IR, DSC ve mekanik analizleri gerçekleştirilmiştir. Farklı çapraz bağlanma derecelerine sahip filmler için, gerilme direnci değerleri 50.6 MPa ile 102.7 MPa arasında, elastik modulus değerleri 3328.7 MPa ile 3790.1 MPa arasında, maksimum uzama oranları ise 2.1% ile 4.3% arasında değişmiştir.

Anahtar kelimeler: Talasemi, Deferipron, kitosan, mikroküre, film, kontrollü salım

TO MY DEAR FAMILY

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INTRODUCTION

1.1 Thalassemia

Thalassemia is the name of a group of genetic blood disorders which is also called as Cooley's Anemia or Mediterranean Anemia. Thalassemia occurs as a result of deformations of hemoglobin structures. Hemoglobin is the oxygen-carrying component of the red blood cells. It consists of two different proteins, which are an alpha and a beta proteins. If the body doesn't produce enough of either of these two proteins, the red blood cells do not form properly and cannot carry sufficient oxygen to the cells. The result is anemia that begins in early childhood and lasts throughout life.

Thalassemia is not a single disorder but a group of hemoglobin related disorders that affect the human body in similar ways. Therefore, it is important to understand the differences between the various types of thalassemia which are explained in the following section [1].

1.1.1 Types of Thalassemia

1.1.1.1 Alpha Thalassemia

People whose hemoglobin does not produce enough alpha protein have alpha thalassemia. It is commonly found in Africa, the Middle East, India, Southeast Asia, southern China, and occasionally the Mediterranean region. There are four types of alpha thalassemia that range from mild to severe in their effect on the body.

***Silent Carrier State*:** This condition generally causes no health problems because the lack of alpha protein is so small that the hemoglobin functions normally. It is called "silent carrier" because of difficulty of its detection. Silent carrier state is "diagnosed" by deduction when an apparently normal individual has a child with hemoglobin H disease or alpha thalassemia trait.

Hemoglobin Constant Spring: This is an unusual form of Silent Carrier state that is caused by a mutation of the alpha globin. It is called Constant Spring after the region of Jamaica in which it was discovered. As in silent carrier state, an individual with this condition usually experiences no related health problems.

***Alpha Thalassemia Trait* or *Mild Alpha Thalassemia*:** In this condition, the lack of alpha protein is somewhat greater. Patients with this condition have smaller red blood cells and a mild anemia, although many patients do not experience symptoms.

However, mild thalassemia is often mistaken for iron deficiency anemia and iron supplements that have no effects on the anemia are prescribed.

Hemoglobin H Disease: In this condition, the lack of alpha protein is great enough to cause severe anemia and serious health problems such as an enlarged spleen, bone deformities and fatigue. It is named for the abnormal hemoglobin H (created by the remaining beta globin) that destroys red blood cells.

***Hemoglobin H-Constant Spring*:** This condition is more severe than hemoglobin H disease. Individuals with this condition tend to have a more severe anemia and suffer more frequently from enlargement of the spleen and viral infections.

Homozygous Constant Spring: This condition is a variation of hemoglobin H-Constant Spring that occurs when two Constant Spring carriers pass their genes on to their child (as opposed to hemoglobin H Constant Spring, in which one parent is a Constant Spring Carrier and the other a carrier of alpha thalassemia trait). This condition is generally less severe than hemoglobin H Constant Spring and more similar to hemoglobin H disease.

***Hydrops Fetalis* or *Alpha Thalassemia Major*:** In this condition, there are no alpha genes in the individual's DNA, which causes the gamma globins produced by the fetus to form an abnormal hemoglobin called hemoglobin barts. Most individuals with this condition die before or shortly after birth. In some extremely rare cases where the condition is discovered before birth, in utero blood transfusions have

allowed the birth of children with hydrops fetalis who then require lifelong blood transfusions and medical care [2].

1.1.1.2 Beta Thalassemia

People whose hemoglobin does not produce enough beta protein have beta thalassemia. It is found in people of Mediterranean descent, such as Italians and Greeks, and is also found in the Arabian Peninsula, Iran, Africa, Southeast Asia and southern China [1].

There are three types of beta thalassemia that also range from mild to severe in their effect on the body.

***Thalassemia Minor* or *Thalassemia Trait*:** In this condition, the lack of beta protein is not great enough to cause problems in the normal functioning of the hemoglobin. A person with this condition simply carries the genetic trait for thalassemia and will usually experience no health problems other than a possible mild anemia. As in mild alpha thalassemia, the small red blood cells of the person with beta thalassemia minor are often mistaken as a sign of iron-deficiency anemia and incorrectly iron supplements are prescribed.

***Thalassemia Intermedia*:** In this condition the lack of beta protein in the hemoglobin is great enough to cause a moderately severe anemia and significant health problems, including bone deformities and enlargement of the spleen. However, there is a wide range in the clinical severity of this condition, and the borderline between

thalassemia intermedia and the most severe form, thalassemia major, can be confusing. The deciding factor seems to be the amount of blood transfusions required by the patient. Generally, patients with thalassemia intermedia need blood transfusions to improve their quality of life, but not in order to survive.

***Thalassemia Major* or *Cooley's Anemia*:** This is the most severe form of beta thalassemia in which the complete lack of beta protein in the hemoglobin causes a life-threatening anemia that requires regular blood transfusions and extensive ongoing medical care. These extensive, lifelong blood transfusions lead to iron-overload which must be treated with chelation therapy to prevent early death from organ failure.

1.1.1.3 Other Forms of Thalassemia

In addition to the alpha and beta thalassemias, there are other related disorders that occur when the gene for alpha or beta thalassemia combines with an abnormal or mutant gene.

***E Beta Thalassemia*:** Hemoglobin E is one of the most common abnormal hemoglobins. It is usually found in people of Southeast Asian ancestry, such as Cambodians, Vietnamese and Thai. When combined with beta thalassemia, hemoglobin E produces E beta thalassemia, a moderately severe anemia which is similar in symptoms to beta thalassemia intermedia.

***Sickle Beta Thalassemia*:** This condition is caused by a combination of beta thalassemia and hemoglobin S, the abnormal hemoglobin found in people with sickle cell disease. It is commonly found in people of Mediterranean ancestry, such as Italians, Greeks and Turks. The condition varies according to the amount of normal beta globin produced by the beta gene. When no beta globin is produced by the beta gene, the condition is almost identical with sickle cell disease. The more beta globin produced by the beta gene, the less severe the condition [2].

1.1.2 Treatment of Thalassemia

1.1.2.1 Blood Transfusions and Chelation Therapy

The most common treatment for all major forms of thalassemia is red blood cell transfusions. These transfusions are necessary to provide the patient with a temporary supply of healthy red blood cells with normal hemoglobin capable of carrying the oxygen that the patient's body needs. While thalassemia patients were given infrequent transfusions in the past, clinical research led to a more frequent program of regular blood cell transfusions that has greatly improved the patients' quality of life. Today, most patients with a major form of thalassemia receive red blood cell transfusions every two to three weeks, amounting to as much as 52 pints of blood a year [1,2]. The blood transfusions creates iron overloading and toxicity on the patients. Actually iron is essential for life: all living cells, whether prokaryotic or eukaryotic, need a supply of iron for reduction of oxygen (respiration), reduction of carbon dioxide (photosynthesis), reduction of dinitrogen or other fundamental

biological processes. But excessive amounts of iron may become very toxic to the human body and, eventually, is fatal for vital cell structures.

Iron overload may be defined as an excess in total body iron stores. The normal iron concentration in the human body ranges between 40 and 50 mg / kg of body weight. Most of this iron is present in hemoglobin and in myoglobin: all the rest is stored as ferritin or as its less accessible form, hemosiderin. Only a few hundred milligrams of iron are stored in enzymes such as cytochrome c oxidase which, however, are essential to human life. Humans have very limited capacity for excretion of excess iron: in particular they lack any effective means to protect cells and tissues against iron overload. As a consequence, any increase in iron intake may cause in a short time an increase in body iron stores [3]. Because there is no natural way for the body to eliminate iron, the iron in the transfused blood cells builds up in a condition known as "iron overload" and becomes toxic to tissues and organs, particularly the liver and heart. Iron overload typically results in the patient's early death from organ failure [2].

Iron chelators are used in medicine to protect patients from the consequences of iron overload and iron toxicity in organs and tissues. The ideal chelator for treating iron overload in humans should act as a selective depletor of iron, should be efficiently absorbed by the gastrointestinal tract, could not cross the blood–brain and placental barriers and should lack or have a low toxicity [3]. Desferal and Deferiprone are the commercially available drugs used as iron chelators for the treatment of thalassemia.

1.1.2.1.1 Desferal

Desferal, which is also called as Desferrioxamine, N-[5-[3-[(5-Aminopentyl) hydroxycarbamoyl] propionamido] pentyl] -3- [[5-(N hydroxyacetamido) pentyl] carbamoyl] propionohydroxamic acid monomethanesufonate, is one effective drug used in treatment of thalassemia (Figure 1.1). For removal of excess iron, patients may undergo the difficult and painful infusion of Desferal [3]. A needle is attached to a small battery-operated infusion pump and worn under the skin of the stomach or legs five to seven times a week for up to twelve hours. Desferal binds iron by chelation. Chelated iron is later eliminated, reducing the amount of stored iron.

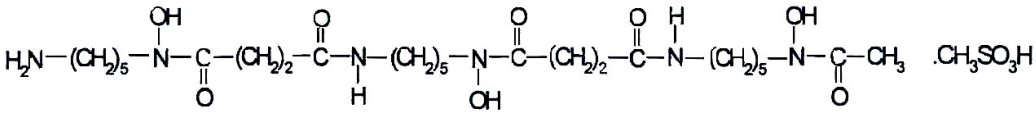


Figure 1.1 Structure of Desferal

Compliance with Desferal is vital to the thalassemia patient's long term survival. However, many patients find the treatment so difficult that they do not keep up with it or abandon treatment altogether. Lack of compliance with chelation therapy leads to accelerated health problems and early death. To combat the compliance problem, researchers are at work on less stressful new chelators that can improve patient compliance [2,4].

1.1.2.1.2 Deferiprone

Deferiprone, 1,2-dimethyl-3-hydroxypyridin-4-one [4], is a member of the family of hydroxypyridinones, of molecular weight 39 Da (Figure 1.2). By virtue of its low molecular weight, deferiprone possesses high absorption efficiency and it is efficiently absorbed from the human intestinal tract.

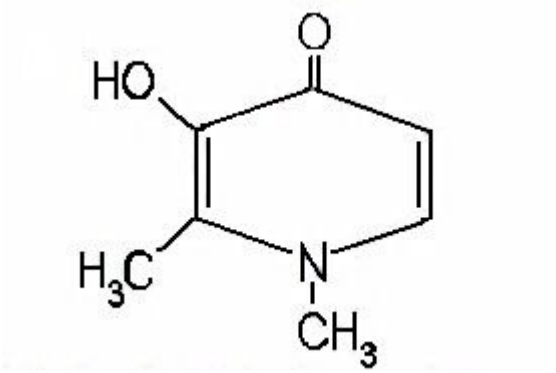


Figure 1.2 Structure of Deferiprone

Deferiprone was originally synthesized by Robert Hider and his colleagues at Essex University, patented in 1982 as an alternative to Desferal in the treatment of iron overload [5] and the early biological assessments were performed at University College Hospital in London. Deferiprone, in common with all hydroxypyridinones, forms 5-membered chelate rings in which iron is bound by two oxygen atoms.

Deferiprone is metabolized in the liver, where it undergoes rapid conversion to nonchelating metabolites. The major metabolite of Deferiprone in man is the glucuronide which, as a result of conjugation of the 3-hydroxyl function, is unable to bind iron. The majority of Deferiprone–iron complex is excreted in the urine (70%), while little iron is excreted in the feces.

It is estimated that more than 6000 patients in 40 countries have received deferiprone since the initiation of clinical trials. There are groups of patients in India, Switzerland and Cyprus who have been taking deferiprone daily since 1989. India was the first country where Deferiprone has been registered in 1994 and now there are more patients on Deferiprone than Desferal. Iron chelation therapy with deferiprone, in a large series of subjects affected by thalassemia intermedia, showed a reduction of liver iron stores and a normalization of serum ferritin levels. This observation was followed by the report of a significant decline in liver iron concentration in patients affected by thalassemia major, during a 5-year treatment with Deferiprone. The ability of Deferiprone to remove excess iron not only from liver cells but also from heart cells has been recently demonstrated in vitro. This observation was followed by evidence of reduction in cardiac iron stores, evaluated by magnetic resonance imaging, in patients with β-thalassemia major during long-term treatment with Deferiprone [3].

Since the administration of Desferal creates discomfort on many patients and since the very long-term effectiveness of Deferiprone is not clearly known yet, some studies were carried out to find effective alternative drugs or delivery methods for the treatment of thalassemia [4,6]. Use of natural and biocompatible polymers which are capable of adsorbing excess iron, are the candidates for an effective chelator [7,8], or production of a long term controlled release system for Desferal or Deferiprone can be effective alternative approaches.

1.2 Controlled Release Systems

Controlled release may be defined as a technique or method in which active chemicals are made available to a specified target at a rate and duration designed to accomplish an intended effect.

1.2.1 Drug Delivery

Conventionally, active agents are most often administered to a system by nonspecific, periodic application. For example, in medical treatment, drugs are introduced at intervals by ingestion of pills or liquids or by injection. The drugs then circulate throughout much of the body, and the concentration of the active agent rises to high levels, system-wide, at least initially. Both by injection and orally, the initially high concentrations may be toxic and cause side effects both to the target organ and neighboring structures. As time passes, the concentration diminishes, owing to natural metabolic processes, and a second dose must be administered to prevent the concentration from dropping below the minimum effective level (Figure 1.3). This situation is very inconvenient and difficult to monitor, and careful calculations of the amount of residual active agent must be made to avoid overdosing. The close attention required, together with the fact that large amounts of the drug are lost in the vicinity of the target organ, make this type of delivery inefficient and costly. In addition, side effects owing to drug misdirected to nontarget tissues are also possible.

In Figure 1.4, drug levels in blood are shown versus time when the drug is applied by various ways. The ideal controlled release rate is a constant concentration, that is effective but not toxic and, maintained for the desired time. Advantages of these systems for therapeutic agents are; reproducible and achieves prolonged constant delivery rate, convenience of less frequent administrations, and reduced side effects because the dose does not exceed the toxic level [9].

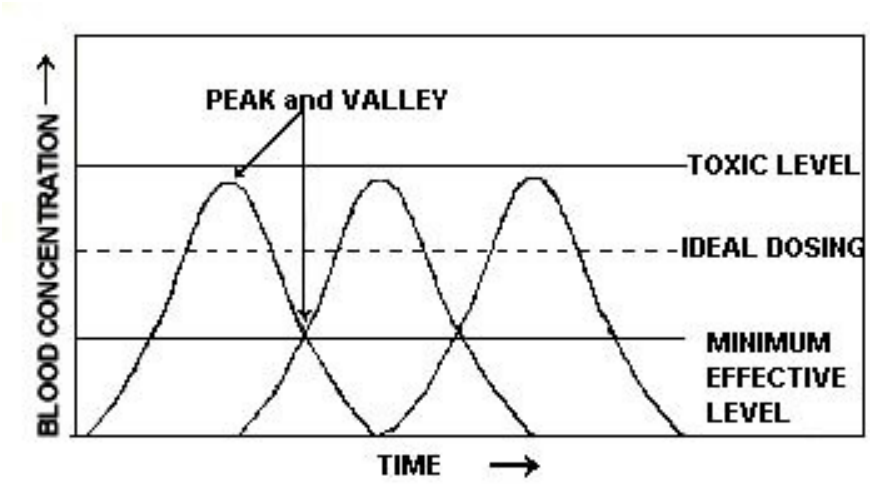


Figure 1.3 Hypothetical blood level pattern from a conventional multiple dosing schedule, and the idealized pattern from a controlled release system.

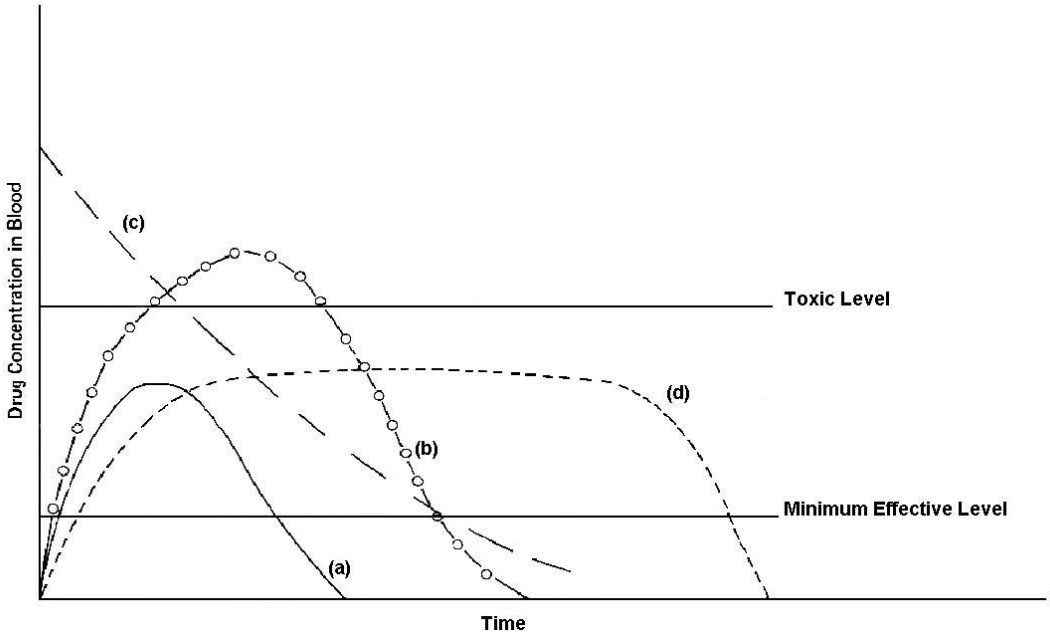


Figure 1.4 Typical drug level versus time profile. (a) Standard oral dose. (b) Oral overdose. (c) i.v. injection. (d) Controlled release ideal dose.

The large and growing variety of pharmaceuticals on the market and in development require versatile delivery systems that can adapt to the needs of particular applications, especially the capacity to generate the required delivery rates and, perhaps, variation of delivery rate over time. For example, many therapeutics require a constant release rate for varying durations from several weeks [10-12]. Such zero-order release is a long-sought goal of controlled-release drug delivery, but has been difficult to achieve for many pharmaceuticals. In contrast, variable drug release rates can be beneficial for many important indications. Intermittent high doses of antibiotics may alleviate evolution of resistance in bacteria, and discontinuous administration of vaccines often enhances the immune response.

Microparticle drug delivery systems may provide the needed versatility. Drug release rates can be controlled through the choice of polymer chemistry [13] (e.g. polymer composition, co-monomer ratios, molecular weight, etc.) or variation of the microparticle formulation parameters, and thus the physical characteristics of the resulting particles [14,15].

Controlled drug delivery is useful for the drugs which have short half-life, high systemic toxicity, and high cost.

Potential advantages of controlled release systems include;

(i) localized delivery of the drug to a particular body compartment, thereby lowering the systemic drug level;

(ii) preservations of medications that are rapidly destroyed by the body (this is particularly important for biologically sensitive molecules such as proteins);

(iii) reduced need for follow-up care;

(iv) increased comfort; and

(v) improved compliance [16].

1.2.2 Controlled Release Mechanisms in Drug Delivery Systems

An ideal controlled release mechanism for a device is the one which exhibits a zero order drug release, i.e. the release of drug is independent of time. However as the drug levels inside the device deplete, the rate of release also goes down. Thus most

drug delivery devices often show two phases of drug release: An initial phase (which may or may not be linear) and a second phase which relates to the rapid depletion of the drug from the device. A well designed drug delivery device would show a zero order release in its initial phase.

Depending on the mode of delivery (transdermal, pulmonary or polymeric), the mechanisms involved in the controlled release of the drug from the device may vary. For example, transdermal delivery involves diffusion of the drug through the skin. This diffusion can be enhanced using external driving forces such as electrical gradients or ultrasonic techniques. Polymeric implants involve either one or a combination of the following three mechanisms:

(i) Diffusion

(ii) Bioerosion

(iii) Osmosis

Out of these three mechanisms, diffusion of the drug through the polymer matrix almost always occurs to a certain extent. For devices employing diffusion and/or osmosis, parameters such as size of the drug molecules, porosity of the polymer matrix, degree of crosslinking and swelling characteristics of the polymer play an important role during the design of the drug delivery device [17-19]. On the other hand bioeroding systems include polymers which have active ingredients attached to them via labile bonds in which case the reactivity of the linkage becomes important, or the surface eroding polymers wherein hydrophobicity of the polymer as well as

the lability of the interchain linkages govern the controlled release characteristics [20].

1.3 Polymers for Drug Delivery

Various synthetic as well as natural polymers have been examined in drug delivery applications. If the polymer matrix does not degrade inside the body, then it has to be surgically removed after it is depleted of the drug. Hence to avoid the costs as well as risks associated with multiple surgeries, the polymer used as depot should be biologically degradable. Thus for a polymer used as a drug delivery matrix, it has to satisfy the following criteria:

(i) It has to be biocompatible and degradable (i.e. it should degrade in vivo to smaller fragments which can then be excreted from the body).

(ii) The degradation products should be nontoxic and should not create an inflammatory response.

(iii) Degradation should occur within a reasonable period of time as required by the application.

1.3.1 Biologically Degradable Polymers

Biologically degradable polymers can be loosely defined as that class of polymers which degrade to smaller fragments due to chemicals present inside the body. Thus two types of degradable polymers can be included under this definition,

biodegradable polymers and bioabsorbable polymers. Biodegradation in the strictest terms is defined as enzymatically promoted degradation. Hence biodegradable polymers are the ones which degrade to smaller fragments by enzymes present in the body. Bioabsorbable polymers on the other hand are those which degrade in the presence of other chemicals in the body.

1.4 Chitin and Chitosan

Chitin is 2-acetamido-2-deoxy-β-D-glucose and was described for the first time by Braconnot (1811) who was professor of Natural History, director of the Botanical Garden and member of the Academy of Sciences of Nancy, France. In the pursuit of his researches on mushrooms, he treated *Agaricus volvaceus* and other mushrooms with diluted warm alkali and isolated chitin, possibly slightly contaminated with proteins. He stated that "fungine seems to contain more nitrogen than wood" and concluded that it is "a quite distinct substance among those identified in plants".

Odier (1823) in an article on insects wrote: "It is most remarkable to find out in the insect structure the same substance that forms the structure of the plants". He called this substance chitin from the Greek term (tunic, envelope); even though he failed to detect nitrogen in chitin, he established for the first time a relationship between the insect cuticle and plant tissue [21].

In 1894, Hoppe-Seyler fused potassium hydroxide at 180ºC with chitin and obtained a product with diminished acetyl content, that he called chitosan [22]. Chitosan was

also studied by Araki [23], and von Furth and Russo concluded that three out of four acetyl groups can be removed from chitin [24]. Rigby in his patent on chitosan preparation presented a treatment of chitin with 40% aqueous solution of sodium hydroxide, for 4 hr at 110ºC [25,26].

Most of the research work carried out on chitin concerns the amino group, which is of course the most important function of the macromolecule. In chitin the amino group is acetylated, thus chitin is an amide of acetic acid; in chitosan the amino group is free and therefore chitosan is a primary amine. However, chitin can not be sharply distinguished from chitosan, because fully acetylated and fully deacetylated chitins do not normally occur in nature and are difficult to prepare.

The term chitin refers to a polymer of N-acetylglucosamine where a minority of the acetyl groups has been lost, while the term chitosan refers to a deacetylation product obtained from chitin, where most of the acetyl groups have been removed. Experimentally, chitosan can be distinguished from chitin because of its solubility in dilute acids. The amino groups of chitin and chitosan are exceptionally stable in 50% sodium hydroxide, even at 160ºC, at which most amines liberate ammonia or yield degradation products [27].

Ideally, the monomer of chitin is 2-acetamido-2-deoxy-β-D-glucose, while the monomer of chitosan is 2-amino-2-deoxy- β-D-glucose; for practical purposes, however, the extent of deacetylation in both the natural and the modified polymers has to be measured. The official name of chitin (a fully acetylated product)is: (1→4)-

2-acetamido-2-deoxy-β-D-glucan and the official name of chitosan (a completely deacetylated product) is: (1→4)-2-amino-2-deoxy-β-D-glucan (Figure 1.5).

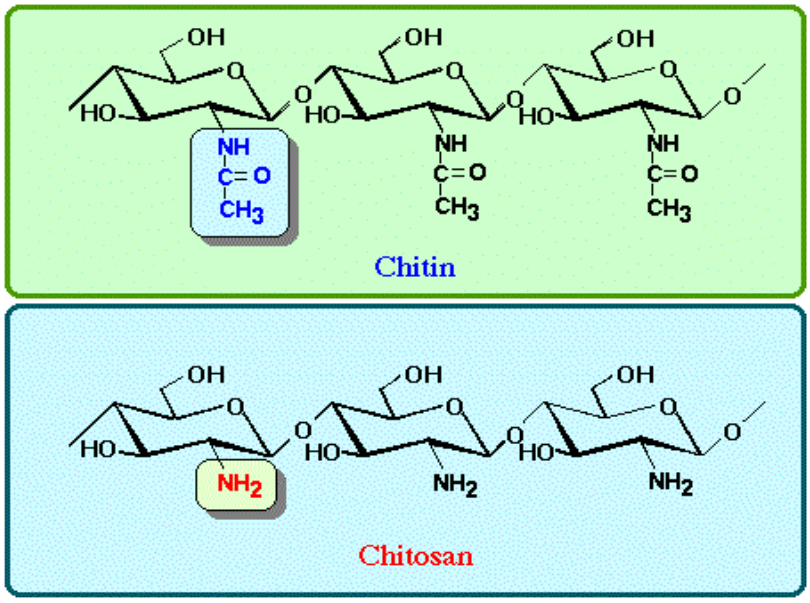


Figure 1.5 Chemical structure of Chitin and Chitosan

1.4.1 Preparation of Chitin and Chitosan

Harsh chemical treatments are usually required to remove calcium carbonate and protein from raw chitinous material. The raw material most abundantly available includes crab shells, shrimp shells, and prawn wastes. The regions of the world where crustacea are abundant are especially the United States of America, India, Thailand, Malaysia, Philippines, South Africa and Mexico. Chitin can also be obtained from sources other than marine wastes; large quantities of fungi currently grown in fermentation systems producing organic acids, antibiotics and enzymes, constitute a potential source of chitin.

Several procedures have been developed through the years; they are at the basis of the chemical processes for industrial production of chitin and chitosan [28] (Figure1.6).

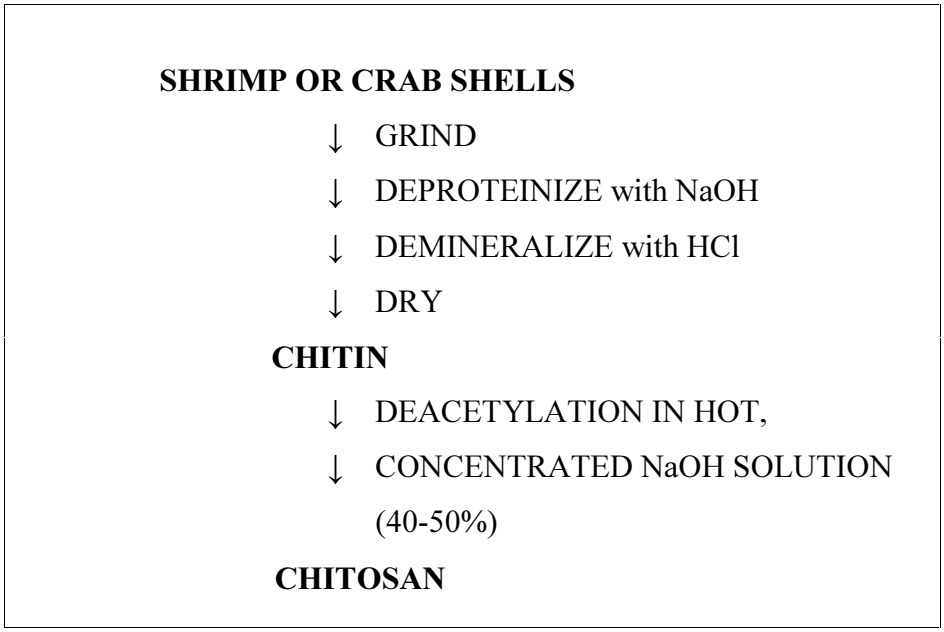


Figure 1.6 Preparation of Chitin and Chitosan

1.4.2 Important Characteristics of Chitosan

1.4.2.1 Cationic Properties

Many of chitosan's uses depend upon its cationic nature as given in Table 1.1. Chitosan is a linear polyelectrolyte at acidic pH's. It has a high charge density, one charge per glucosamine unit. Since many materials carry negative charges (e.g. proteins, anionic polysaccharides, nucleic acids, etc.), the positive charge of chitosan interacts strongly with negative surfaces to give an electric neutrality. Chitosan is an excellent flocculent due to its vast number of $-NH_3^+$ groups that can interact with

negatively charged colloids. Chitosan adheres readily to neutral polymers such as hair and skin, which are composed of negatively charged mucopolysaccharides and proteins. Chitosan forms complexes with many metal ions. Thus, it is useful in chelating iron, copper and magnesium and can also be used to remove toxic heavy metal ions such as silver, cadmium, mercury, lead, nickel and chromium [7,8,29].

Table 1.1 Cationic Properties of Chitosan

- Linear Polyelectrolyte
- High charge density
- Excellent flocculent
- Adheres to negatively charged surfaces
- Substantive to hair, skin
- Chelates metal ions

- Iron (Fe), Copper (Cu)
  - Toxic metals (Cd, Hg, Pb, Cr, Ni)
  - Radionucleides (Pu, U)

1.4.2.2 Biological Properties

Many of chitosan's biomedical applications rely on its non-toxic and biodegradable properties (Table 1.2). Chitosan has been shown to facilitate wound healing [30-33], reduce serum cholesterol levels and stimulate the immune system [34]. Chitosan, when coated on seeds, results in increased crop yields, apparently due to chitosan inducing a protective response by the germinating plant.

Table 1.2 Biological Properties of Chitosan

● Biocompatible - non-toxic - biodegradable - natural polymer	● Bioactive - wound healing accelerator - reduce blood cholesterol levels - immune system stimulant

1.4.2.3 Chemical Properties

Chitosan, being a high molecular weight polymer, is a linear polyamine whose amino groups are readily available for chemical reactions and salt formation with acids (Table 1.3). Since chitosan can be viewed as a cellulose derivative, the primary (C-6) and secondary (C-3) hydroxyl groups can be used to make derivatives. The scientific literature contains many interesting chemical derivatives of chitosan with commercial potential [35,36].

Table 1.3 Chemical Properties of Chitosan

- Linear polyamine (poly D-glucosamine)
- Reactive amino groups
- Reactive hydroxyl groups (C3-OH, C6-OH)

1.4.2.4 Solution Properties

Chitosan has rather specific solution properties. First, when in the free amine form, chitosan is not soluble in water at neutral pH's. At acidic pH's, the free amino groups ($-NH_2$) become protonated to form cationic amine groups ($-NH_3^+$). Table 1.4 lists some key solution behavior of the two amine forms of chitosan [37].

Table 1.4 Solution Properties of Chitosan

Free Amine ($-NH_2$)	Cationic Amine ($-NH_3^+$)
- Soluble in acidic solutions	- Soluble at pH's<6.5
- Insoluble at pH's>6.5	- Forms viscous solutions
- Insoluble in H_2SO_4	- Solutions shear thinning
- Limited solubility in H_3PO_4	- Forms gels with polyanions
- Insoluble in most organic solvents	- Will remain soluble in some alcohol-water mixtures

1.4.3 Commercial Products of Chitosan

The type of chitosan used commercially is determined by its physical form (flake, powder, solutions), its purity (ultrapure, standard, industrial) and its molecular form (high to low viscosity, percent deacetylation (>75%), free amine or acid salt), and ultimately the cost (Table 1.5).

Table 1.5 Commercial Products of Chitosan

Unique Characteristics	Application Area
PROTASAN™ - Ultrapure quality - Powder - Water soluble - Chitosan-acid salt	- In vivo biomedical uses - Any application requiring very high purity
SEA CURE-PLUS™ - High quality - Powder - Water soluble - Chitosan-acid salt	- Pharmaceutical/medical - Cosmetics/personal care
SEA CURE™ - High quality - Flake/powder - Free amine form - Low, medium, high viscosity grades	- Enzyme/cell immobilization - Pharmaceutical/medical - Membranes - Cosmetics/personal care - Food related
PRO FLOC™ - Flake/powder - Free amine form - Self-dissolving blends available - Low, medium, high viscosity grades	- Agriculture uses - Industrial uses - Metal recovery - Waste treatment - Detoxification

1.4.4 Application Areas of Chitosan

1.4.4.1 Clarification and Purification of Water and Beverages

Since chitosan is a natural polymer, it is preferred over synthetic polymer flocculants which may contain hazardous monomers. In conjunction with the Division of Animal Feed, Department of Health and Human Service, and the U.S. Food and Drug Administration (FDA); the Association of Animal Feed Control Officials, Inc., (AAFCO) has issued the following description of chitosan as a flocculent for recovering proteinaceous material: " Chitosan is a cationic carbohydrate polymer intended for use as a precipitation agent of proteinaceous material for food processing plants. It is chemically derived by deacetylation of the naturally occuring chitin in crab and shrimp shells. Proteinaceous material coagulated with chitosan must have safety and efficacy data approved before it can be registered or offered for sale" [38]. According to the U.S. Environmental Protection Agency, chitosan is acceptable for potable water applications when used within the stated rates and under the restrictions listed [Protan Laboratories, Inc., Chitin and Chitosan-General Properties and Applications, PLI-002, Redmond, WA,1987] [38]:

1. A bed of flaked chitosan may also be used as filter medium in potable water treatment.
2. Whereas deacetylated chitin is newly proposed as a potable water treatment chemical, and whereas such new chemicals do not fulfill the requirement of "virtual identity", chitosan is acceptable for use only on an experimental or pilot basis, under conditions which are carefully controlled by the appropriate state.

1.4.4.2 Chelation

Since chitosan is an excellent chelator of many harmful metals (e.g. copper, nickel, chromium, cadmium, manganese, cobalt, lead, mercury, zinc, uranium and silver), a sharp increase in interest in using chitosan to detoxify hazardous wastes has developed [21]. Chitosan's ability to chelate iron, as well as its flocculating activity, makes it a very useful pool and spa clarifier.

1.4.4.3 Biotechnology

Chitosan has been used to immobilize cells containing useful enzymes and to immobilize enzymes directly [39]. Cells and enzymes can merely be entrapped or adsorbed onto ionotropic chitosan gels formed by mixing chitosan solutions with solutions of anionic polymers (e.g. polyphosphate, alginates, pectin, carrageenan). Alternatively, chitosan beads can be crosslinked to render them insoluble before attaching the enzymes. By attaching protein antigens or antibodies to chitosan, affinity type columns can be fabricated [38,40]. Biotechnological applications of chitosan are given in Table 1.6.

Table 1.6 Biotechnological Applications of Chitosan

● Immobilization of Enzymes and Cells
- entrapment and absorption
- crosslinked with dialdehydes (e.g. gluteraldehyde)
- charge complexes with anionic polymers
● Chitosan Gels
- stable to phosphate buffers, Na^+ and K^+ ions
- easily prepared at room temperature
- non-toxic
- high cell loads / activities
● Purify / Recover Biologicals

1.4.4.4 Pharmaceutical

There are several reasons why chitosan is being actively pursued by pharmaceutical companies (Table 1.7) [41,42]. First, it has many useful as well as biological characteristics that allow it to be used in wound healing (Table 1.8) [30-33]. In drug delivery, it is chitosan's biocompatibility and its ability to be totally absorbed in vivo (e.g. lysozyme) that allow its use in bioerodible and orthopedic devices [43].

Table 1.7 Pharmaceutical Applications of Chitosan

- Wound Healing
- Drug delivery
- Bioengineering material
  - orthopedic
  - contact lens
- Cholesterol reducing agent
  - investigative new drug (IND) application
  - human feeding trails

Since chitosan has been shown to be effective in lowering serum cholesterol when added to the diet, human feeding trials are scheduled requiring the filing of an investigative New Drug (IND) with FDA [44].

Table 1.8 Chitosan Applications: Wound Healing

Key Properties	Key Functions
- Biocompatible - Accelerate wound healing - Absorb liquids	- Reduce fibroplasia (scar tissue) - Hemostatic - Form protective film/coating
Variety of Useful Forms	Potential Uses
- Powder - Paste/salve - Solution/gel - Film - Fiber - Spray	- Bandages - Sutures - Synthetic skin - Eye bandage

In addition, chitosan has a variety of promising pharmaceutical uses and is presently considered as a novel carrier material in drug delivery systems, as indicated by the large number of studies published over the last few years.

As a pharmaceutical excipient, chitosan has been added for sustained release and to improve the dissolution of poorly soluble drugs. Films of crosslinked chitosan glutamate were studied for their potential use in the coating of pellets and tablets in the development of sustained-delivery systems [45]. Chitosan was also utilized in a spray-drying process to produce controlled drug release particles [46]. The in vitro studies showed that an important cosideration is the optimum degree of deacetylation of chitosan as it affected the release characteristics from these matrices. Chitosan in a capsule form has been studied for colonic delivery [47]. Muranishi's group coated chitosan capsules to deliver 5-aminosalicylic acid (5-ASA) to the colonic region using a rat model [47]. The results suggested that colon-specific drug delivery could be achieved through the use of the chitosan capsules.

Molecules such as bovine serum albumin, diphtheria toxoid (DT) and bisphosphonates have been successfully incorporated into chitosan microspheres. Jameela *et al.* [48] showed that, in preliminary immunogenicity studies in rats using DT-loaded chitosan microspheres, the antibody titres were relatively constant over a five-month period. Chitosan microspheres that contained bisphosphonates were implanted in the tibialis muscle of rats to treat several pathological conditions associated with bone destruction. The controlled release of antiresorption and

anticalcification agents from the coated microspheres effectively inhibited bioprosthetic tissue calcification in the rat subdermal model.

The poor bioavailability of topically applied ophthalmic drugs implies a necessity for frequent instillation to achieve therapeutic effect. This inconvenience could be overcome by a prolonged release of the drug in the corneal area. Use of chitosan-based colloidal suspensions in *in vivo* studies showed a significant increase in ocular drug bioavailability [49].

The development of new carrier systems for gene delivery represents an enabling technology for treating many genetic disorders. Promising results were reported in the formation of complexes between chitosan and DNA [50]. Also there is a growing interest in the development of nasal delivery systems for many drugs, including peptides and proteins. Several studies have reported the use of chitosan as a safe nasal-delivery system for proteins [51].

Modified chitosans were reported to display a growth-inhibitory effect on tumor cells. This property was employed by Ouchi *et al*. [52] by conjugating chitosan or chitosamino-oligosaccharide (COS) to 5-fluorouracil (5FU) in order to provide a macromolecular system with strong antitumor activities and reduced side effects.

1.5 Aim of the Study

A very active drug and a very effective delivery method are important points of thalassemia treatment. Today the commonly used and the most effective drugs are Desferal and Deferiprone. Use of natural and biocompatible polymers which are capable of adsorbing excess iron, are the candidates for an effective chelator [7,8]. On the other hand, production of controlled release systems for the drugs Desferal or Deferiprone are other effective alternative approaches.

The aim of the study is to prepare Deferiprone carrying controlled release systems for the treatment of thalassemia. For this purpose chitosan was chosen as the support material since it has unique polymeric cationic and biocompatible character and easily gel and film forming properties.

Deferiprone carrying systems in the form of microspheres with different crosslink densities were prepared and release kinetics were examined. Chitosan films with different crosslinking degrees were also prepared in order to search mechanical properties of the prepared films. It is known that biodegradability together with biocompatibility and suitable mechanical properties is found only in a small group of materials and chitosan is among these materials. Therefore, mechanical properties (modulus of elasticity, tensile strength, and percent strain at break) were examined for a possible design of a controlled release system.

CHAPTER II

EXPERIMENTAL

2.1 MATERIALS

Materials used in this study and their manufacturers are listed in Table 2.1

Table 2.1 Materials and manufacturers

Materials	**Manufacturers**
Chitin	Sigma, USA
Chitosan (DDA=85%)	Sigma, USA
Chitosan (DDA=75%)	Prepared
Acetic Acid (99-100%)	Atabay Kimya, Turkey
Glutaraldehyde (50%)	BDH, UK
Deferiprone	Aldrich, Germany
Tween 80	Acros Organics
Methanol	Merck, Germany
Diethyl ether	Fluka, Switzerland
Ethanol	J.T.Baker
Acetone	Merck, Germany
Sodium hydroxide	Merck, Germany
Hydrochloric Acid	Merck, Germany
Hydrobromic Acid (48%)	Fischer, USA
Sodium phosphate	Fischer, USA
Di-sodium hydrogen phosphate-2-hydrate	Riedel
Mangan dioxide	Aldrich, Germany
Dialysing tube	Sigma, USA
Corn oil	Ülker, Turkey
Millipore	Millex, France

0.01M Phosphate buffer solution with pH 7.4 was prepared by dissolving 533.87 mg sodium phosphate and 1091.37 mg disodium hydrogen phosphate-2-hydrate in 1L distilled water.

2.2 METHODS

2.2.1 Purification of Chitin

1 M NaOH and 1 M HCl solutions were prepared for the purification of chitin. 10 gram chitin that may contain foreign matter was refluxed for 5 hours in 100 mL, 1 M NaOH at 80°C. The precipitated chitin was separated, washed with distilled water and digested in 100 mL, 1 M HCl for 15 hours at room temperature. The acid and alkali treatments were repeated 4 times. And then chitin is decolorized by refluxing with acetone at 55°C for 9 hours [53].

2.2.2 Preparation of Chitosan from Purified Chitin

In order to prepare chitosan, purified chitin was refluxed in 500 mL, 47% NaOH at 110°C for varying time periods to obtain chitosan with different degrees of deacetylation. The obtained chitosan was washed with distilled water many times till neutralization (controlled by litmus paper) and then dried in vacuum desicator at 50°C [54].

2.2.3 Determination of Degree of Deacetylation

500 mg of prepared chitosan was taken and dissolved in 100 mL 0.5 M HBr solution prepared from 9 M HBr by dilution. Then 50 mL 9 M HBr was added on the solution to form precipitate. It was centrifuged for 30 minutes and precipitated chitosan salt was separated. The salt was washed 3 times with methanol until neutralization. Then it was washed with diethylether several times and then dried. The dried chitosan was again washed 3 times with methanol (about 15 mL). Then 100 mL diethylether was added and filtered. During filtration methanol and diethylether treatments were repeated 2 times to remove adsorbed water. The obtained chitosan salt was dried in vacuum desiccator and in order to obtain degree of deacetylation (DDA), 100 mg of the dried chitosan salt was dissolved in 50 mL of distilled water. 2 drops of phenol phtalein was added as indicator and the solution was titrated with 0.1 M NaOH [55]. This procedure was repeated 3 times and degree of deacetylation values were calculated from the given equations as an average value;

$$M_{NaOH} \times V_{NaOH} = m^{1}_{chitin} / MW_{\ chitin\ unit}$$

$$m^{1}_{chitin} / m_{chitosan} \times 100 = DDA\ \%$$

where M_{NaOH} is the molarity of NaOH used, V_{NaOH} is the volume of NaOH needed for titration, $MW_{\ chitin\ unit}$ is the formula weight of one unit in chitin molecule (203.13 g/mole), m^{1}_{chitin} is the calculated weight of chitin and $m_{chitosan}$ is the weight of

chitosan (in g =100 mg) used in the preparation of the solution that was titrated and DDA is the degree of deacetylation value of chitosan.

2.2.4 Preparation of Microspheres

Two different chitosans, one with 75% degree of deacetylation, and the other with 85% degree of deacetylation, were used for microsphere preparations. Chitosan solutions (3%, w/v) were prepared by dissolving chitosan in 5% (v/v) acetic acid. 10 mL chitosan solution was dispersed in 50 mL corn oil, with 0.5 mL Tween 80, to form water-in-oil emulsions. Solution was stirred at 1000 rpm for 30 minutes and 2 mL gluteraldehyde was added at 15 minutes intervals twice by stirring at room temperature. The reaction was carried out for 5 hours with stirring. Then the microspheres were filtered off, washed several times with acetone and then with diethylether and dried at 50˚C for 12 hours.

2.2.4.1 Preparation of Drug-loaded Microspheres

In order to prepare Deferiprone loaded microspheres, 10 mg Deferiprone was dissolved in 1 mL distilled water and added into the chitosan solution at the beginning of the microsphere preparation process. Then the same procedures were applied as described previously. Various types of microspheres prepared in this study are given in Table 2.2.

Table 2.2 Prepared Chitosan Microspheres

Name of the Chitosan Microsphere	Degree of Deacetylation of Chitosan (%)	Concentration of Chitosan (%w/v)	Concentration of Gluteraldehyde (%v/v), 2 mL	Amount of Deferiprone (mg)
CM75/10	75	3	10	0
CM85/10	85	3	10	0
CMD75/10	75	3	10	10
CMD75/1	75	3	1	10
CMD75/0.01	75	3	0.01	10
CMD85/10	85	3	10	10
CMD85/1	85	3	1	10
CMD85/0.1	85	3	0.1	10
CMD85/0.01	85	3	0.01	10

2.2.5 Characterization of Microspheres

2.2.5.1 Morphological Analysis

The morphology of microspheres was examined by a scanning electron microscope (SEM, Jeol Model 6400). For this purpose, the samples were coated with gold under vacuum and their scanning electron micrographs were obtained.

2.2.5.2 Particle Size Analysis

Particle size analysis were performed on samples of microspheres suspended in acetone using Malvern Mastersizer S Version 2.15 equipment. The average size and size distribution curves of microspheres were obtained.

2.2.6 Preparation of Chitosan Films

Chitosan solutions were prepared by dissolving 100 mg chitosan (DDA=85%) in 10 mL of 5% aqueous acetic acid solution at ambient temperature with stirring. Solutions (10 mL) were put into plastic petri dishes (diameter = 9 cm) and films were obtained after evaporation of water at room temperature. The thickness of the films were about 0.10 mm. Chitosan films were crosslinked with different concentrations of gluteraldehyde to obtain films of various degrees of crosslinking (Table 2.3). The concentrations of gluteraldehyde solutions were; 1%, 0.1%, 0.01% (v/v). 1 mL of each solution was added to 10 mL 1% (w/v) chitosan solution and stirred for 30 minutes prior to putting the solutions into molds.

Table 2.3 Prepared chitosan films

Name of the Chitosan Film	Degree of Deacetylation of Chitosan (%)	Concentration of Chitosan (%w/v)	Concentration of Gluteraldehyde (%v/v), 1 mL
CF	85	1	0
CF/0.01	85	1	0.01
CF/0.1	85	1	0.1
CF/1	85	1	1

2.2.7 IR Analysis

To observe the structural changes of chitin and chitosan with various degree of deacetylation, and to elucidate the crosslinkage of chitosan films, IR analysis were performed by using Perkin Elmer 1600 Series FTIR.

2.2.8 Differential Scanning Calorimetry (DSC) Analysis

DSC thermograms of chitosans with different degrees of deacetylation and chitosan films were obtained by using DuPond 2000 Differential Scanning Calorimeter. Samples were heated at a scanning rate of 10˚C/min by using dry nitrogen flow. Heating curves were obtained to determine the thermal behaviours.

2.2.9 In-vitro Release Studies

In-vitro Deferiprone release profiles from microspheres were obtained by using dialysis method. For this purpose 30 mg microspheres, loaded with deferiprone, were placed into a dialysis tube (molecular weight cut off 12000 D), then suspended in 10 mL phosphate buffer solution (PBS) with pH=7.4. Then they were stayed in shaking water bath at 37°C. At certain time intervals dissolution medium was withdrawn and immediately replaced with equal volumes of fresh PBS. The removed solution was stirred at 25°C for 3 hours with 1 mg MnO_2, in order to obtain Deferiprone-MnO_2 complex for making Deferiprone visible at UV- spectrophotometry. Unreacted MnO_2 was removed by centrifugation. The solution was filtered using 0.45 μm millipore filters and the obtained clear solutions were analyzed spectrophotometrically at λ=278 nm in order to determine the amount of released Deferiprone. Calibration curve for Deferiprone is given in Appendix A.

2.2.10 Mechanical Tests

Tensile tests were carried out for the prepared chitosan films crosslinked with different amount of gluteraldehyde (GA) and were cut as rectangular strips. The gage length was 30±2 mm and width was 10 mm for each sample. The thickness of each specimen was measured at two ends and at the middle by a micrometer. The average of these three values was used in calculations. At least 10 experiments were carried out for each type of films and average values were calculated.

LLOYD LRX 5K (LLOYD Instrument, ENGLAND), equipped with a 100 N load cell, was used for mechanical testing experiments. The mechanical test machine was under the control of a computer running program WindapR. During measurement, the film was pulled by top clamp at a rate of 3 mm/min. The tensile load applied on the specimen was continuously recorded by the computer. The tensile strength for each specimen was obtained from the equation:

$$\rho = F/A$$

Where ρ is the tensile strength, F is the maximum load applied just before rupture and A is the initial area of the specimen. The load deformation curve was converted to stress-strain curve, where stress is the load per unit area (F/A as pascal) and strain is deformation per unit length ($\Delta l/l_0$, where l_0 is the initial length and Δl is the change in the length). Slope of the straight line exist in elastic region of the stress-strain curve is accepted as the elastic modulus of the specimen.

Statistical Analysis

Statistical analysis were assessed using one way ANOVA. When ANOVA produced significant differences, the group or groups that caused the difference was/were determined by using Student's t-test.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Deacetylation of Chitin

Deacetylation of chitin takes place preferentially in the amorphous region of chitin then proceeds from the edge to the inside of the crystalline region [56]. Reaction scheme for deacetylation is given in Figure 3.1.

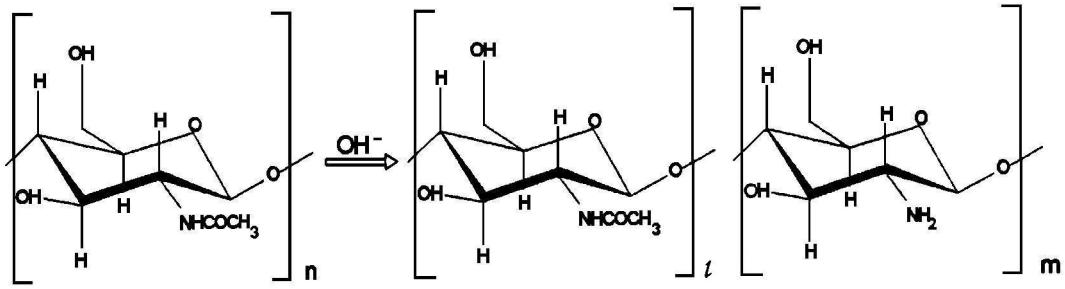


Figure 3.1 Deacetylation reaction

In this study purified chitin was deacetylated by refluxing with concentrated NaOH for 88 h and converted to chitosan which has 75% degree of deacetylation (DDA). On the other hand, chitosan obtained from Sigma (DDA=85%) was further

deacetylated and for the resultant chitosan DDA was measured as 90%. These results are tabulated in Table 3.1.

Table 3.1 Deacetylation Degrees of Chitosan Samples

Origin	Reaction time with NaOH	Reaction temperature (°C)	Degree of Deacetylation (%DDA)
Purified Chitin	88 hours	110	**75**
Pure Chitosan	-	-	**85**
Pure Chitosan	20 hours	110	**90**

3.2 Chitosan Microspheres

3.2.1 Effect of Crosslinker on Size and Shape of the Microspheres

Chitosan microspheres were prepared by using gluteraldehyde (GA) as crosslinker. Aldehydes can react with the amino groups of proteins even in the presence of water at body temperature, forming a Schiff base. GA has two reactive aldehyde groups in one molecule and hence has been used as a crosslinker of collagen, other proteins, and biological soft tissues [57]. Crosslinking reaction between chitosan and GA is shown in Figure 3.2.

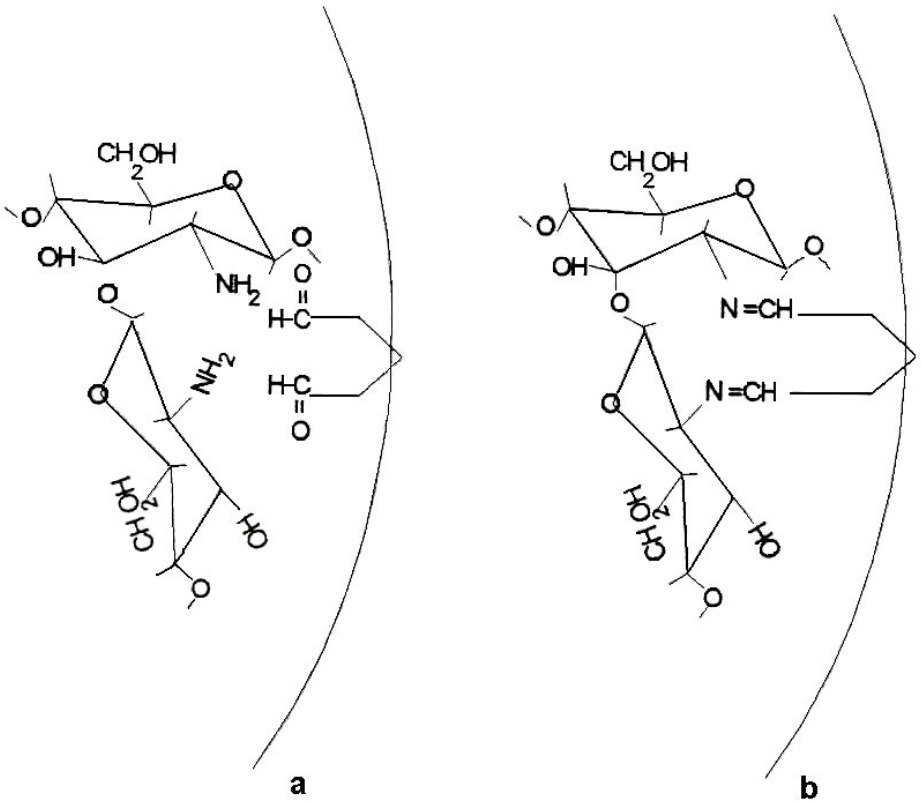
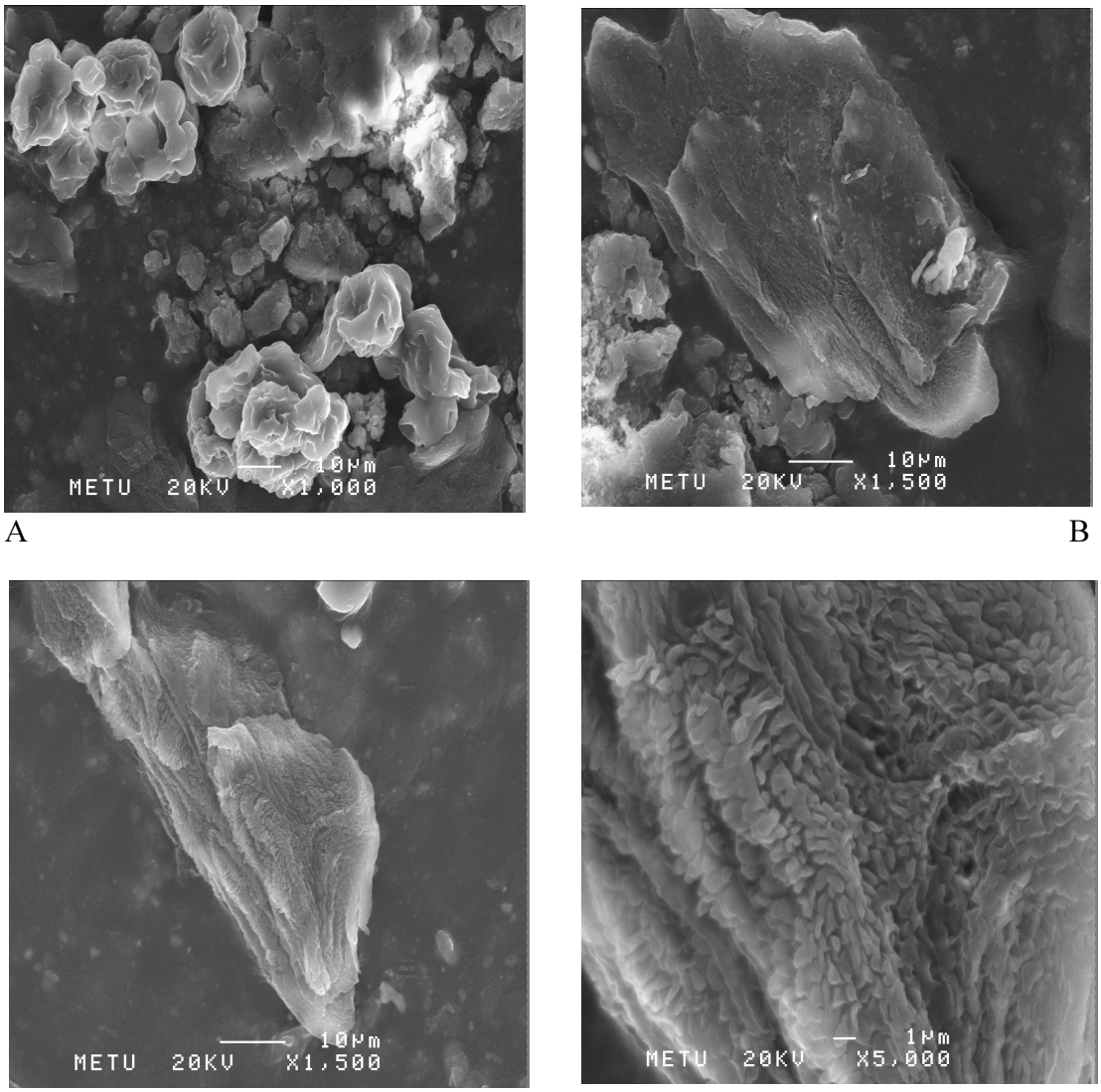


Figure 3.2 Crosslinking mechanism of the microspheres with gluteraldehyde

a) Gluteraldehyde and chitosan before crosslinking reaction

b) Covalent bond formation between chitosan and gluteraldehyde

In order to obtain various crosslinking degrees, GA solutions with different concentrations were used in the preparation of microspheres. As the GA concentration was increased, color changed from pale yellow to brownish due to a sequence of interchain crosslinking formation and the intensity of the color increased as the gelation proceeded. On the other hand, as the concentration of the crosslinker was increased, bond formation between the microspheres and aggregation was observed. SEM micrographs show the differences in the structures of the microspheres prepared by using chitosan with different DDA values, and various GA concentrations (Figures 3.3 to 3.9).



A B

C D

Figure 3.3 Chitosan microspheres (DDA=75%) prepared with 0.01% GA

For the chitosan microspheres prepared with chitosan having 75% DDA, it can be seen from Figure 3.3A that when the concentration of the crosslinker (GA) was 0.01%, the obtained microspheres were not homogeneous and there were particulate crystalline structures (Figures 3.3B and 3.3C). The close up of the crystalline part is shown in Figure 3.3D. SEM micrographs show that this very low concentration of GA is not enough to overcome intermolecular attractions between chitosan molecules and crystalline particles still exist in the structure. But when the GA

concentration was increased to 1%, the obtained microspheres were spherical in shape demonstrating rough and porous surfaces as seen in Figure 3.4.

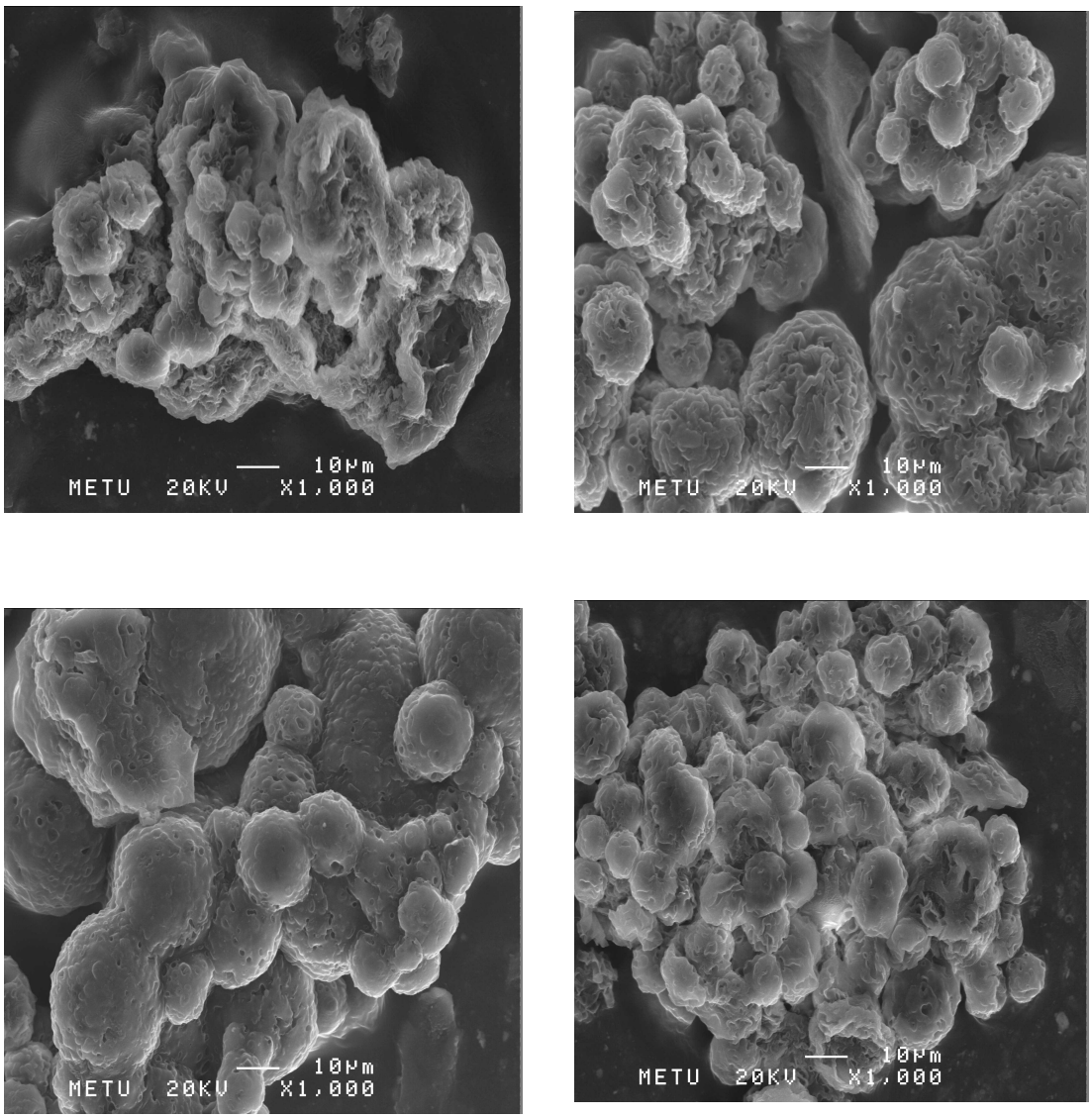


Figure 3.4 Chitosan microspheres (DDA=75%) prepared with 1% GA

Further increase in the concentration of GA up to 10%, led the formation of proper microspheres as shown in Figure 3.5.

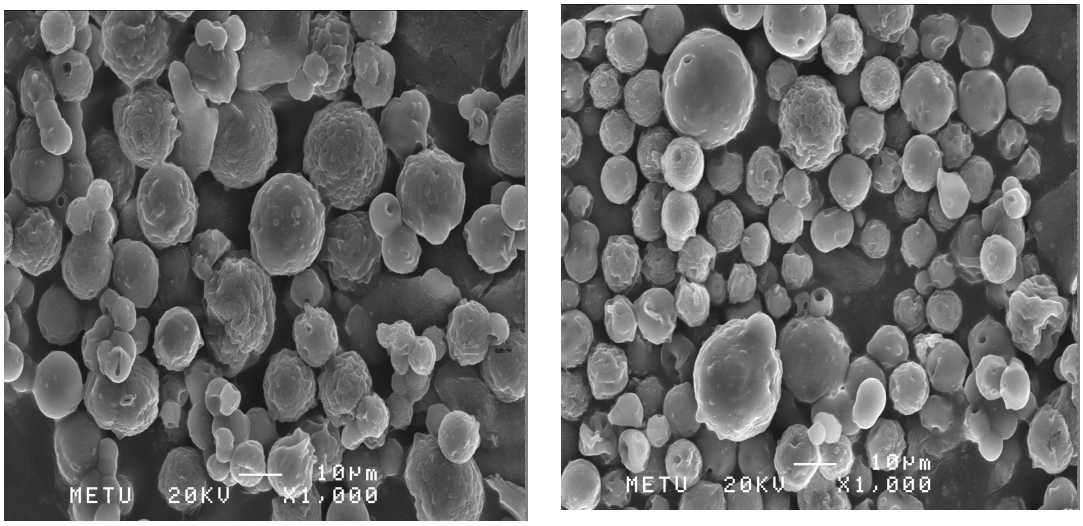


Figure 3.5 Chitosan microspheres (DDA=75%) prepared with 10% GA

Microspheres prepared with chitosan having 85% DDA were more proper in spherical shape. Figure 3.6 shows these microspheres prepared with 0.01% GA. When the concentration of GA was increased to 0.1%, aggregation occurred (Figure 3.7). This is the result of surface crosslinking taking place between deacetylated groups of chitosan molecules. Crosslinking and macromolecules between the microspheres can be clearly observed in Figure 3.7.

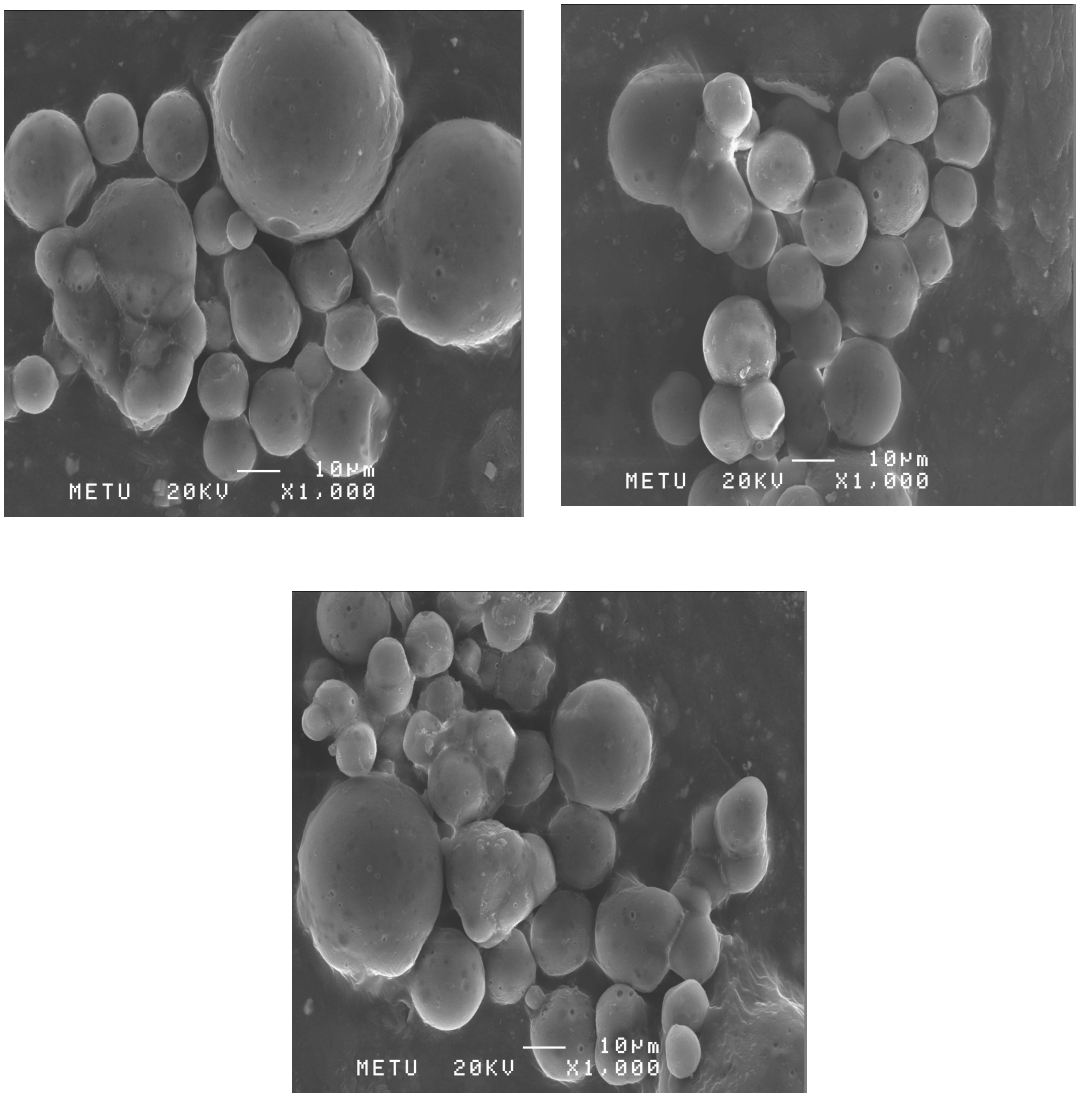


Figure 3.6 Chitosan microspheres (DDA=85%) prepared with 0.01% GA

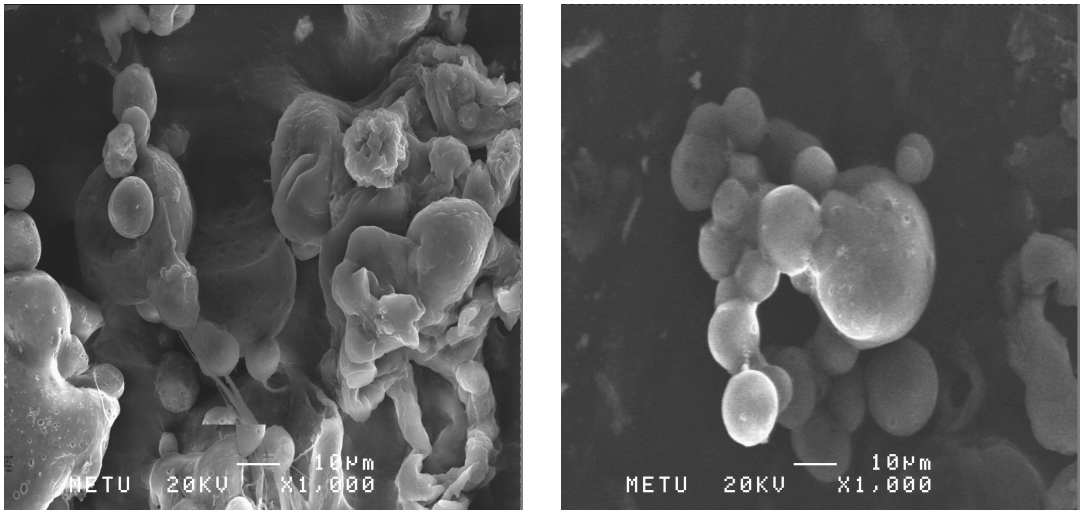


Figure 3.7 Chitosan microspheres (DDA=85%) prepared with 0.1% GA

When GA concentration was increased to 1% and 10%, proper spherical microspheres formed but the sizes of the microspheres were not homogeneous (Figures 3.8 and 3.9). The microspheres prepared with 1% or 10% GA, adherence of the smaller microspheres onto the bigger ones was observed. The microspheres with bigger size might be formed by the encapsulation of aggregated small microspheres.

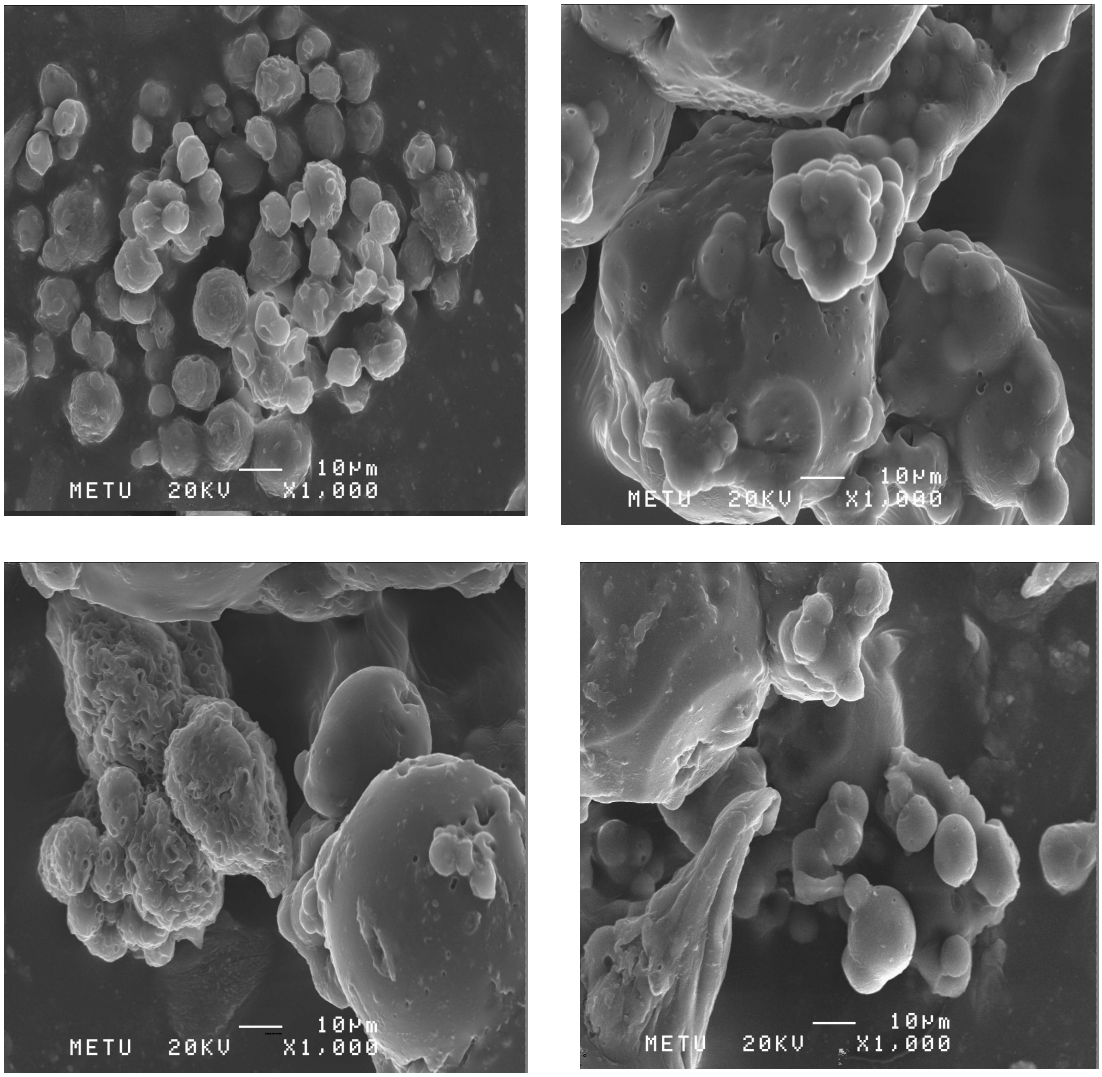


Figure 3.8 Chitosan microspheres (DDA=85%) prepared with 1% GA

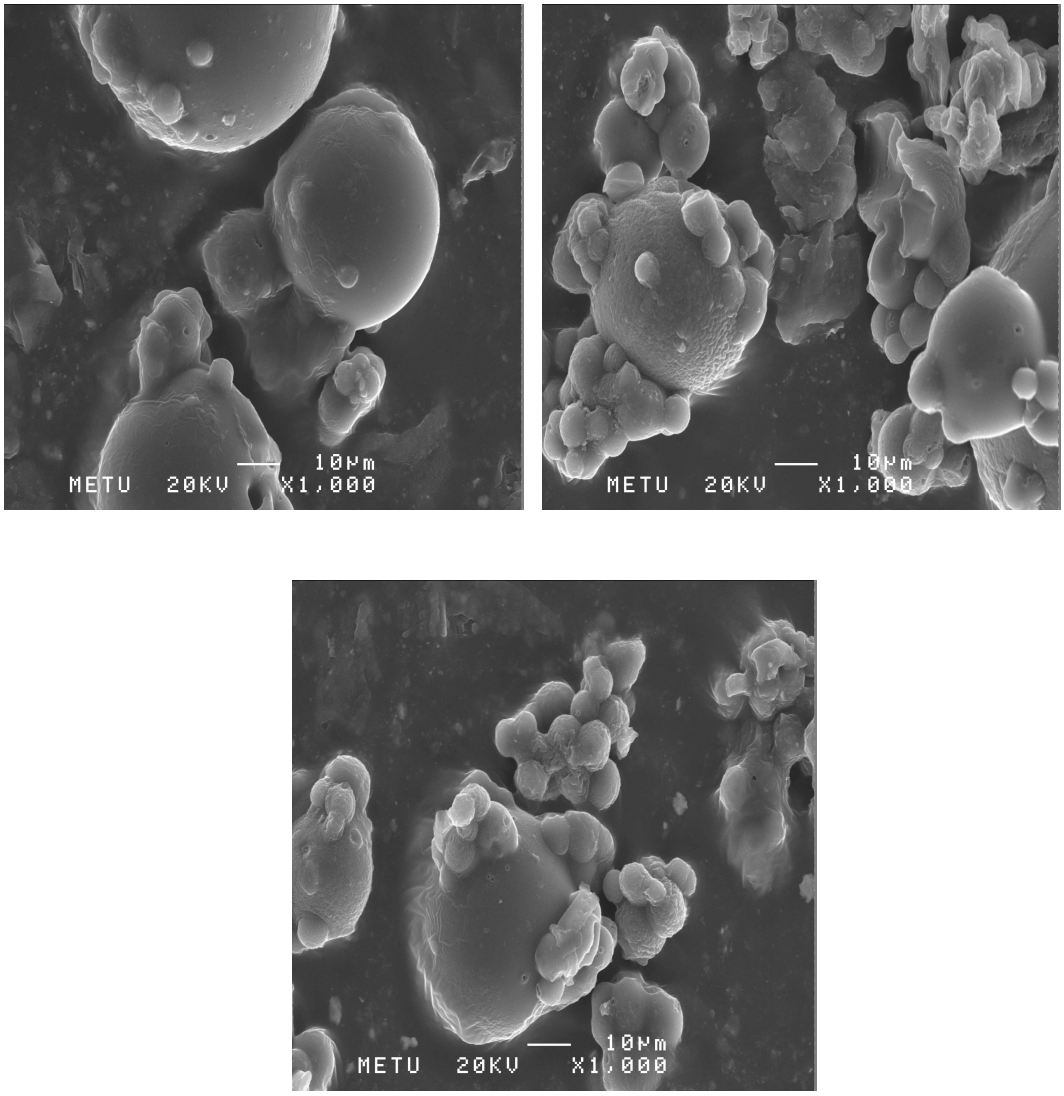


Figure 3.9 Chitosan microspheres (DDA=85%) prepared with 10% GA

SEM micrographs demonstrate that, there are some certain optimum values of DDA value of chitosan and concentration of GA in the preparation of proper and homogeneous microspheres.

3.2.2 Particle Size Analysis of Microspheres

Particle size distribution curves of the microspheres are given in Appendix B. Average sizes and the differences in the sizes of microspheres can be seen from Table 3.2 and Figure 3.10, respectively.

Table 3.2 Sizes of different chitosan microspheres

Type of microspheres	Average size (μm)
85/10	48.53
85/1	82.16
85/0.1	98.76
85/0.01	104.57
75/10	30.75
75/1	66.09
75/0.01	83.57

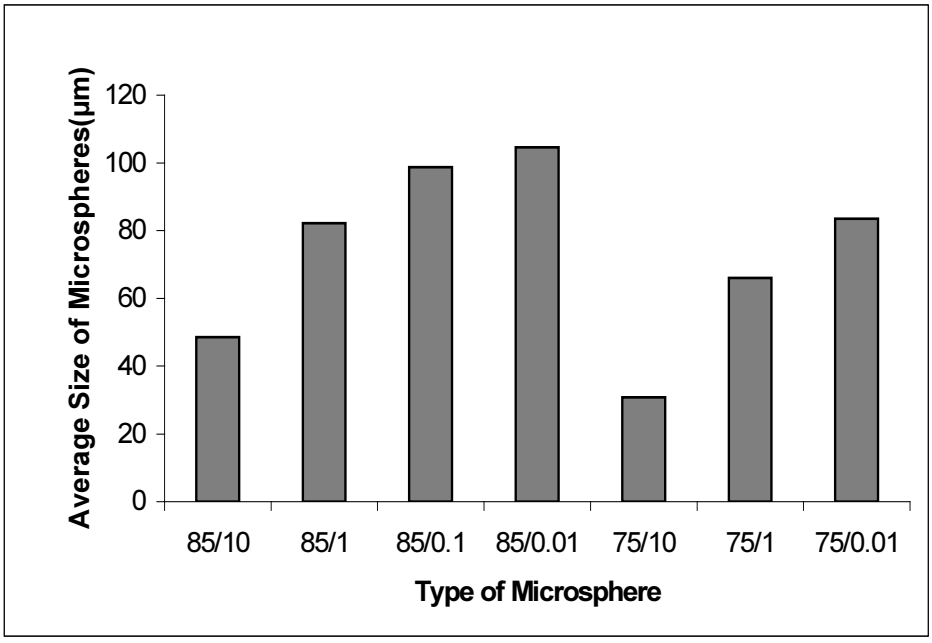


Figure 3.10 Size distribution of microspheres

Preparation parameters of the microspheres (stirring rate, crosslinking degree, degree of deacetylation of the chitosan, etc.) affect the particle size of the microspheres. Previously it was shown that as the stirring rate increases, average size of the microspheres decreases [8]. In this study, a constant stirring rate of 1000 rpm was applied.

Particle size analysis of microspheres showed that crosslinking degree affects the size of the microspheres. Varying sizes of microspheres according to the concentration of crosslinker are shown in Figure 3.11. Increase in the concentration of crosslinker caused a decrease in the average mean diameters of the microspheres from 104.57μm to 48.53μm for microspheres with 85% DDA and 83.57μm to 30.75μm for microspheres with 75% DDA.

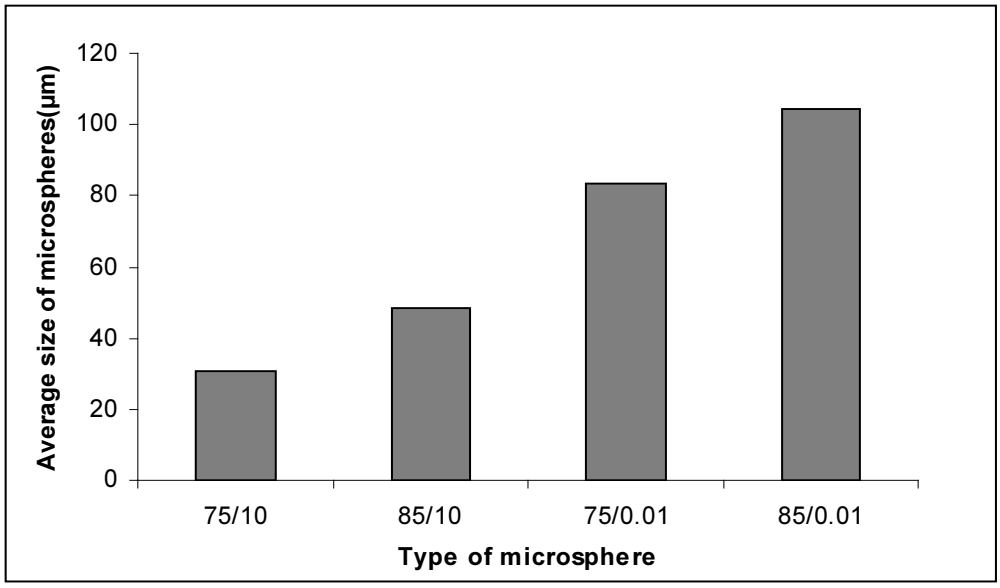


Figure 3.11 Effect of crosslinker concentration on the size of the microspheres

Degree of deacetylation is another factor affecting the sizes. It was observed that as DDA increases, viscosity of the chitosan solution increases, causing formation of bigger microspheres.

As seen from the SEM micrographs (Figures 3.3 to 3.9) generally aggregation occurred and the sizes were not homogeneous. The sizes given up to now were the volume mean diameter of the microspheres. The percentage sizes of the microspheres are given in Table 3.3.

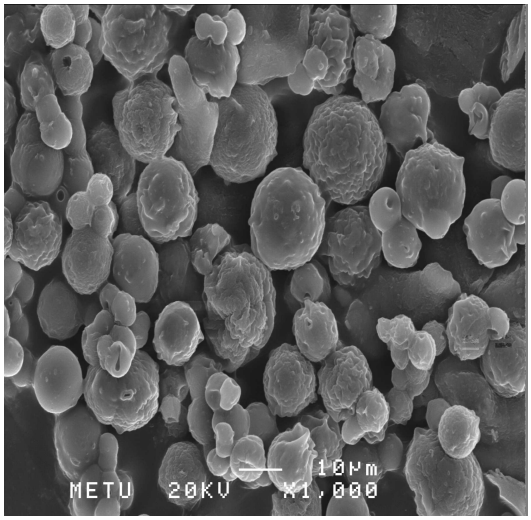
Table 3.3 Particle size analysis results

Type of microspheres	D (v,0.1) (μm)	D (v,0.5) (μm)	D (v,0.9) (μm)	VMD (μm)	SMD (μm)
85/10	25.67	46.50	74.81	48.53	26.86
85/1	33.17	72.00	132.28	82.16	38.35
85/0.1	43.54	72.67	121.20	98.76	39.39
85/0.01	33.09	75.55	216.16	104.57	38.88
75/10	16.47	28.96	47.94	30.75	26.00
75/1	26.07	57.09	118.58	66.09	36.15
75/0.01	28.27	76.59	150.63	83.57	32.61

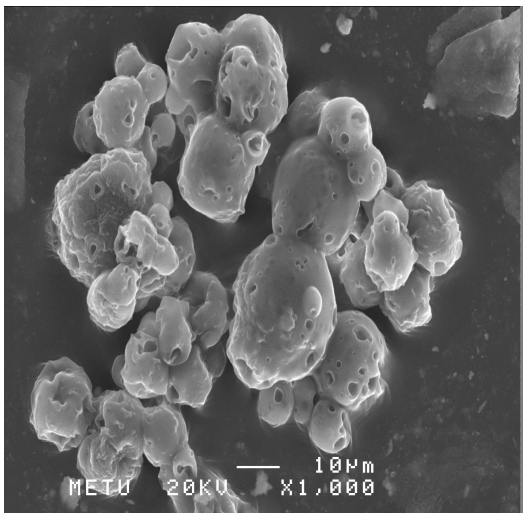
D (v,0.1) is the size of particle for which 10% of the sample is below this size.
D (v,0.5) is the size of particle at which 50% of the sample is smaller and 50% is larger than this size. This value is also known as the mass median diameter (MMD).
D (v,0.9) gives a size of particle which 90% of the sample is below this size.
D (4,3) is the volume mean diameter (VMD).
SMD is the surface mean diameter [D(v,3.2)] also known as the Sauter mean.

3.2.3 Drug Loading to Microspheres

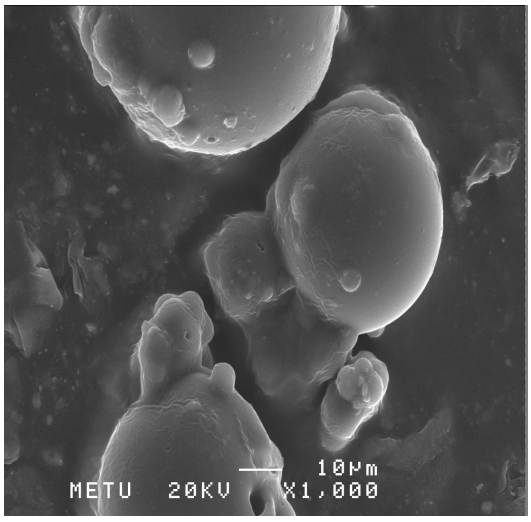
Deferiprone was loaded to microspheres during the preparation process. SEM micrographs demonstrated that, the sizes and the shapes of the microspheres were not affected by loading Deferiprone (Fig. 3.12).



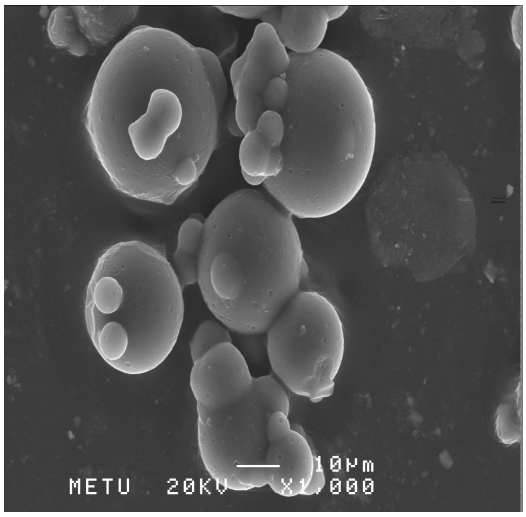
A



B



C



D

Figure 3.12 Chitosan microspheres (A) CM75/10 (B) CMD75/10 (C) CM85/10 (D) CMD85/10

3.2.4 In Vitro Release Studies

UV spectrum of Deferiprone showed maximum absorption at 278 nm. This wavelength was used for the determination of Deferiprone in the preparation of calibration curve and in the detection of amount of Deferiprone released from chitosan microspheres. Release behaviour of Deferiprone from chitosan microspheres prepared with chitosan having 75% DDA and 85% DDA are given in Figure 3.13 and 3.14, respectively.

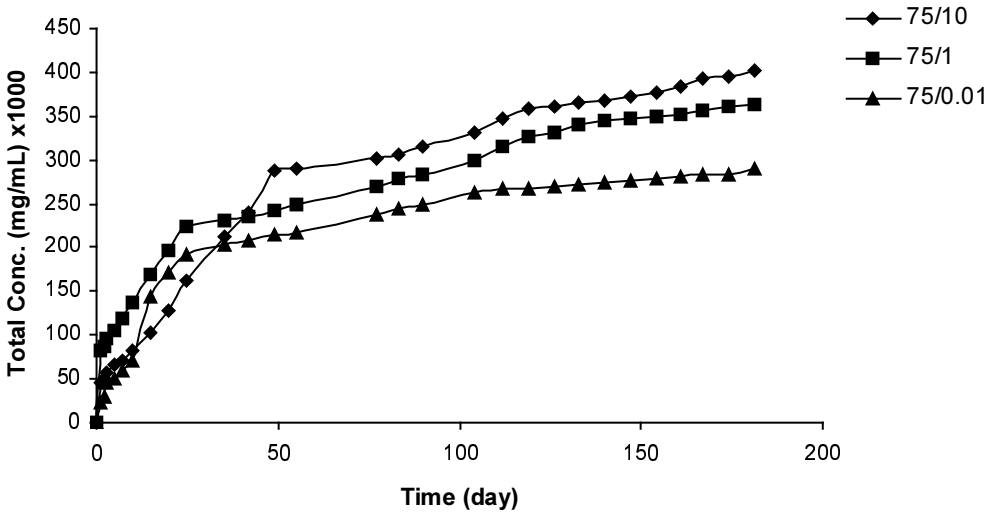


Figure 3.13 Release of Deferiprone from chitosan (DDA=75%) microspheres

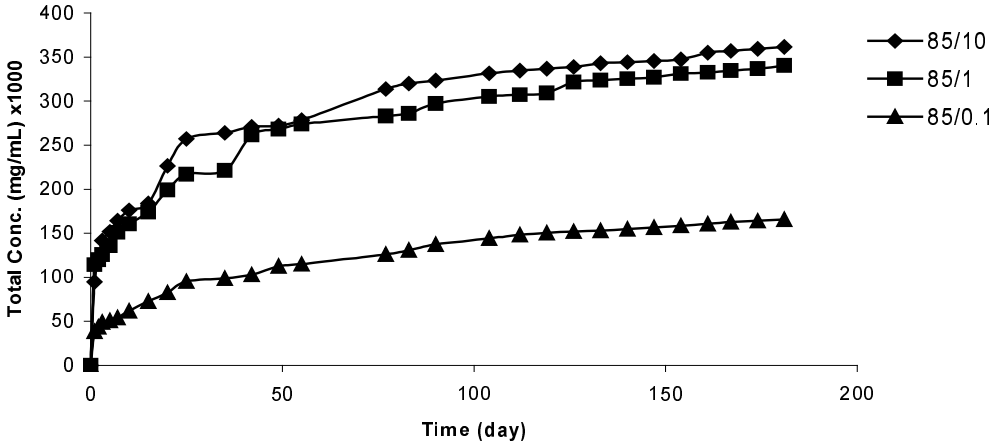


Figure 3.14 Release of Deferiprone from chitosan (DDA=85%) microspheres

It was observed that, as the crosslinking degree increased, the release rate of the drug also increased contrary to expectations. This unexpected result can be explained by the crystalline structure of chitosan [58,59]. When high amount of crosslinker was added into the reaction medium, formation of crosslinks between chitosan molecules adversely affects the proper crystal orientation in the structure. The disorientation between the chitosan chains forms larger pores in the structure of microspheres, which leads higher release rates from the highly crosslinked microspheres. The initial fast release can be attributed to the release of drug that was adsorbed on the surface of particles.

It can be seen from Table 3.4 that release trend of Deferiprone also slightly depends on the degree of deacetylation of chitosan. As the DDA of chitosan increased, the release became slower. This might be due to loose gel structure of chitosan with low degree of deacetylation. For CMD75/10, 40.1% of loaded Deferiprone was released, while for CMD85/10, it was decreased to 36.2%. The released amount of

Deferiprone was 36.3% for CMD75/1, 29.1% for CMD75/0.01, 34.0% for CMD85/1 and 16.6% for CMD85/0.1.

Table 3.4 Released amount of Deferiprone from different type of microspheres

Type of MS	Released Deferiprone (%)
75/10	40.1
85/10	36.2
75/1	36.3
85/1	34.0
75/0.01	29.1
85/0.1	16.6

Another explanation for slower release from chitosan microspheres with high DDA, is the difference in the sizes of microspheres. Microspheres with low DDA were smaller in size compared to the microspheres with high DDA due to viscosity differences of the solutions. For these samples with 75% DDA, area to volume ratio is higher than that of samples with 85% DDA, and this lead to faster release.

The amount of Deferiprone that was entrapped, the maximum amount that was released from microspheres and the calculated encapsulation efficiencies are given in Table 3.5. Taking the total released amount of Deferiprone as 100% for all microspheres, percent release of Deferiprone is given in Figure 3.15. Encapsulation efficiencies were 36.2%, 34.0% and 16.6% for CMD85/10, CMD85/1 and CMD85/0.1, respectively. And for the microspheres prepared with chitosan having 75%DDA, Deferiprone encapsulation efficiencies were found as 40.1% for CMD75/10, 36.3% for CMD75/1 and 29.1% for CMD75/0.01.

Table 3.5 Amount of Deferiprone entrapped in different types of microspheres

Type of MS	Theoretical amount of Deferiprone (mg)	Total entrapped Deferiprone (mg)	Encapsulation efficiency (%)
CMD85/10	1	0.362	36.2
CMD85/1	1	0.340	34.0
CMD85/0.1	1	0.166	16.6
CMD75/10	1	0.401	40.1
CMD75/1	1	0.363	36.3
CMD75/0.01	1	0.291	29.1

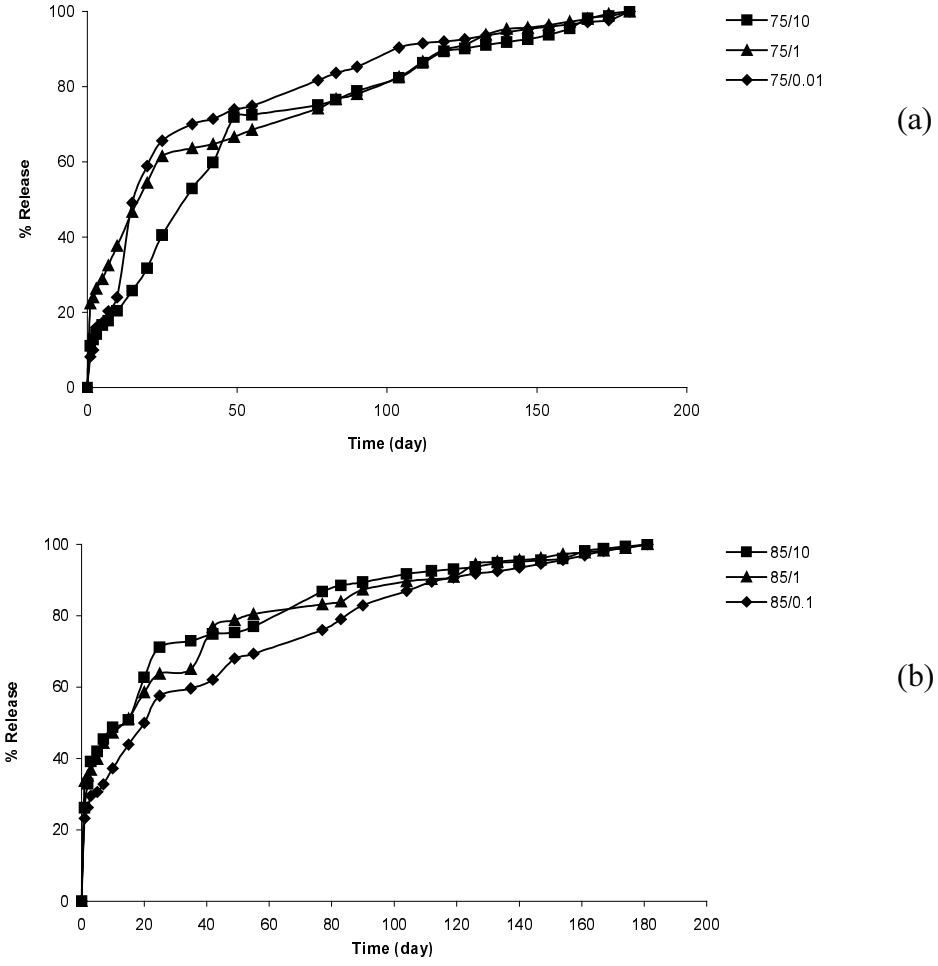


Figure 3.15 Percent Deferiprone release from chitosan microspheres, with (a) DDA=75% and (b) DDA=85% , by taking total released amount of Deferiprone as 100%

Encapsulation efficiencies found to be low because of the high solubility of the drug in aqueous medium.

3.3 Chitosan Films

Prepared chitosan films were all homogeneous and transparent. The thicknesses of the dry films were about 15μm.

3.3.1 Infrared Analysis

Infrared spectra of chitin and chitosan with different degrees of deacetylations are given in Figure 3.16 and IR spectra of chitosan films crosslinked with gluteraldehyde are given in Figure 3.17.

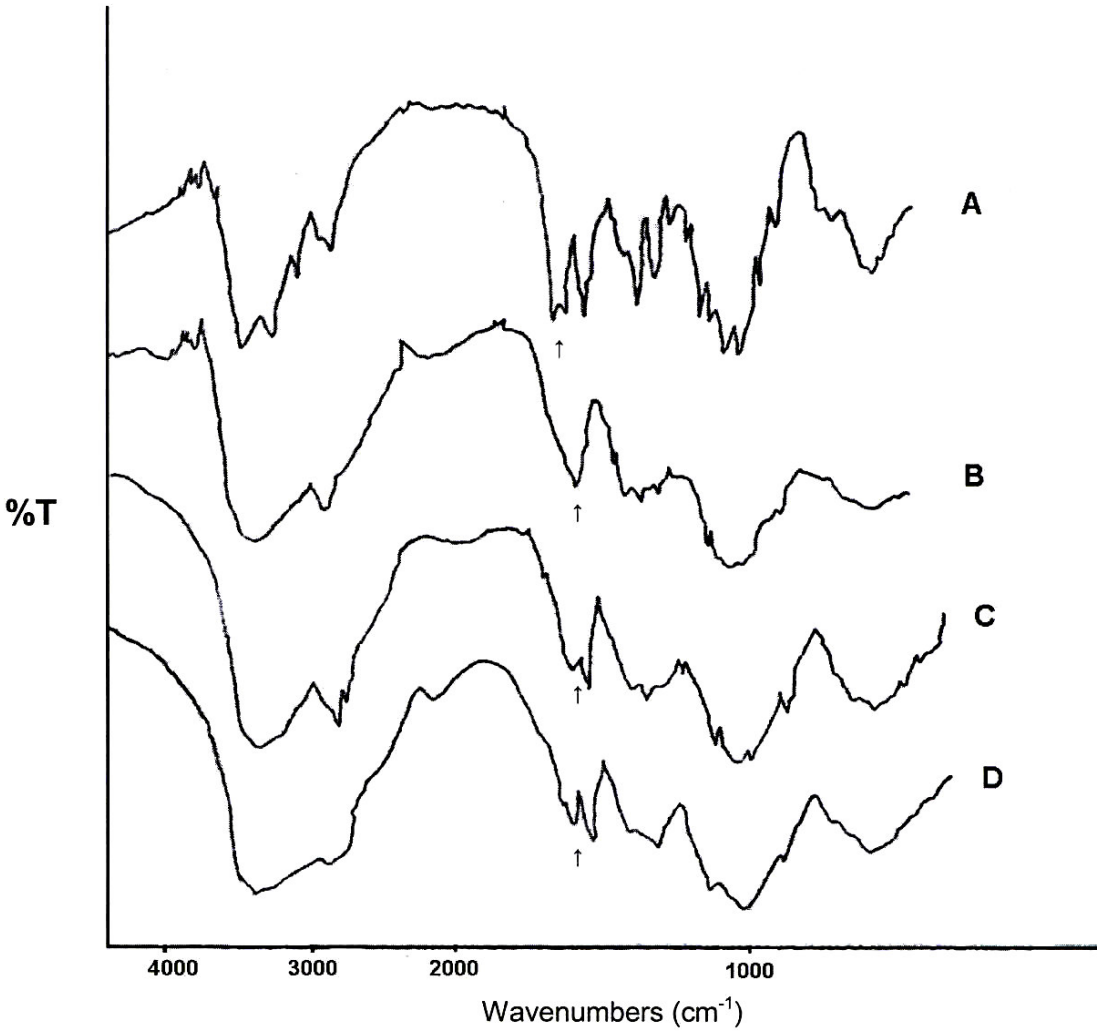


Figure 3.16 IR spectra of chitosan samples (A) chitin, (B) 75% DDA, (C) 85% DDA, (D) 90% DDA

IR spectra of chitin shows absorption bands at about 897 and 1153 cm^{-1} corresponding to the saccharide structure of chitin. The peaks at about 1300 cm^{-1} and 1600 cm^{-1} designates the C-N stretching and N-H bending, respectively. The peak at about 1650 cm^{-1} indicates amide (C=O) band which is characteristic for N-acetylated chitin. The bands at about 2900-3000 cm^{-1} belong to C-H stretching vibrations and Ar-H stretching (H linked to aromatic ring). The bands at about 3500 cm^{-1} indicates OH linked to polymer and N-H of secondary amines of chitin.

IR spectra of 75%, 85% and 90% deacetylated chitosan show a stronger amino characteristic peak at about 1600 cm^{-1} when compared with chitin and the band belonging to the C=O group at 1650 cm^{-1} weaken due to the deacetylation process. C=O band which is the characteristic band for chitin structure disappears at the spectrum belonging to 75% DDA.

Figure 3.17 shows the IR spectra of chitosan films crosslinked with gluteraldehyde. IR spectra show that the peak for the amino group in chitosan became weaker due to the Schiff reaction between amino and aldehyde groups. A peak forms at about 1630 cm^{-1} indicating the formation of C=N due to immine reaction between amino groups of chitosan and aldehyde groups of GA. The two strong peaks at about 2900 cm^{-1} indicates the aldehyde C-H stretchings belonging to GA. OH of chitosan shows an absorption peak at about 3400 cm^{-1}. These bands indicate the presence of both molecules in the film structure and thus proves that the crosslinking reaction occurs between them.

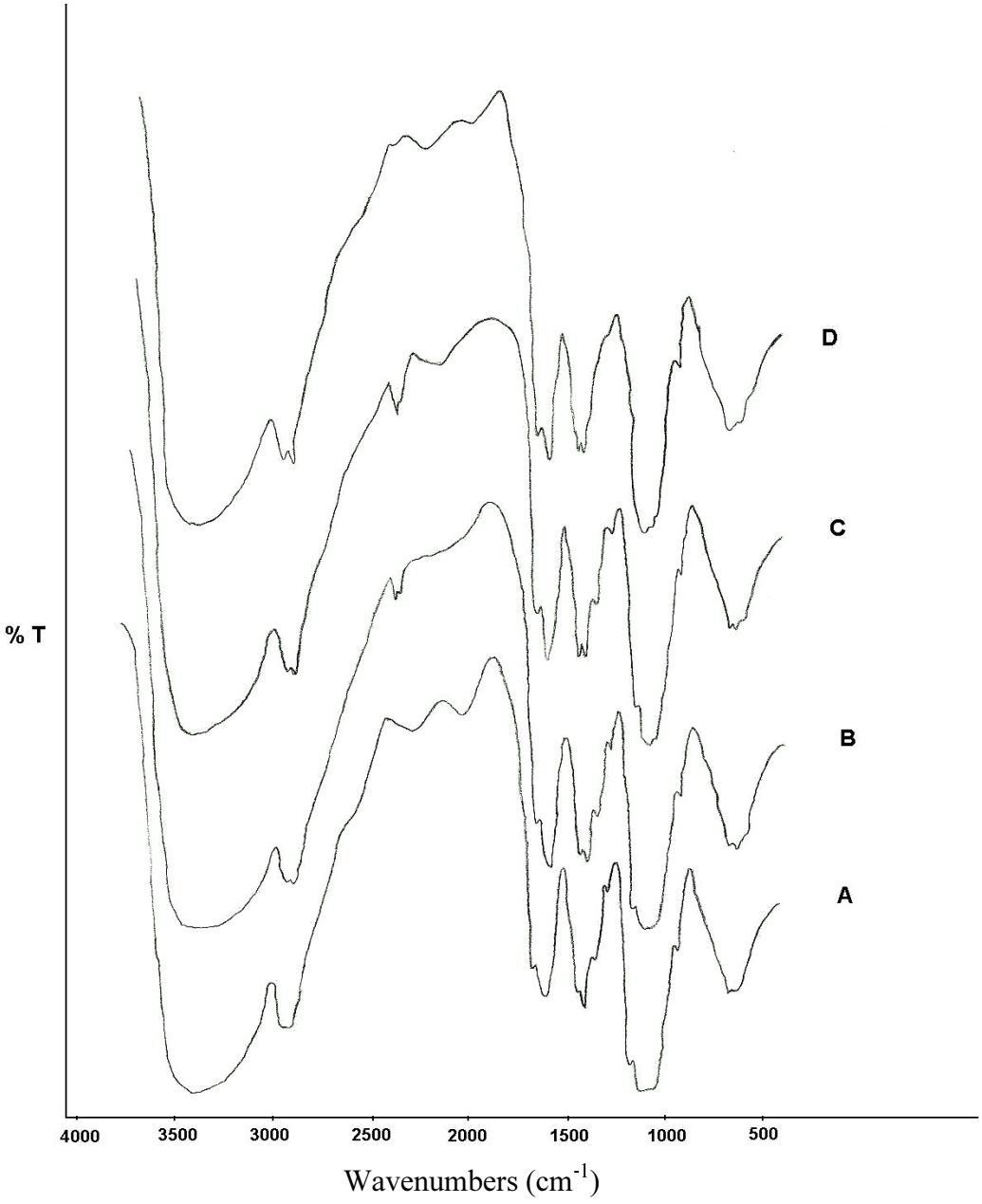


Figure 3.17 IR spectra of chitosan films with (A) no crosslinker, (B) 0.01% GA, (C) 0.1% GA, (D) 1% GA

3.3.2 Differential Scanning Calorimetry Analysis

DSC thermograms of chitin, chitosan and films crosslinked with GA are shown in Figures 3.18 and 3.19.

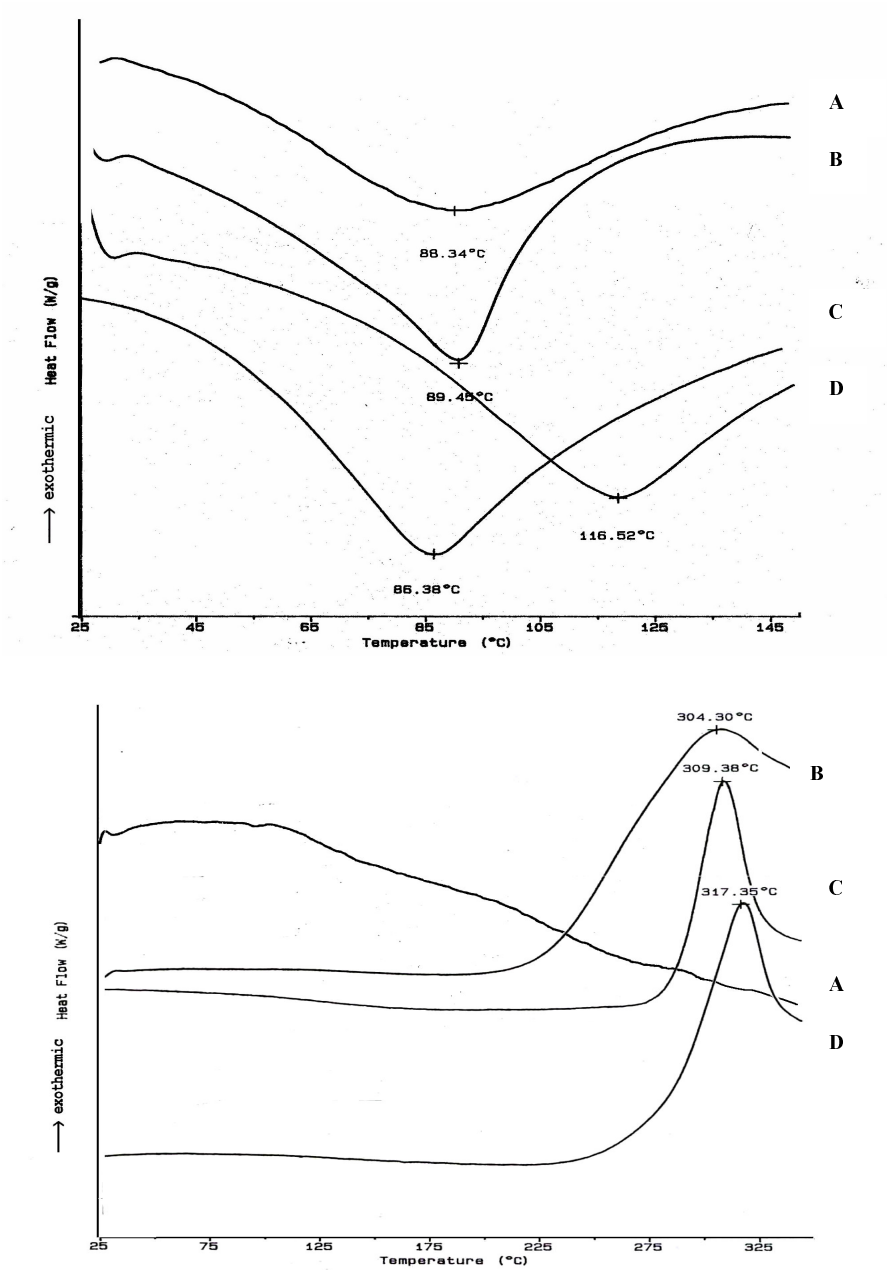
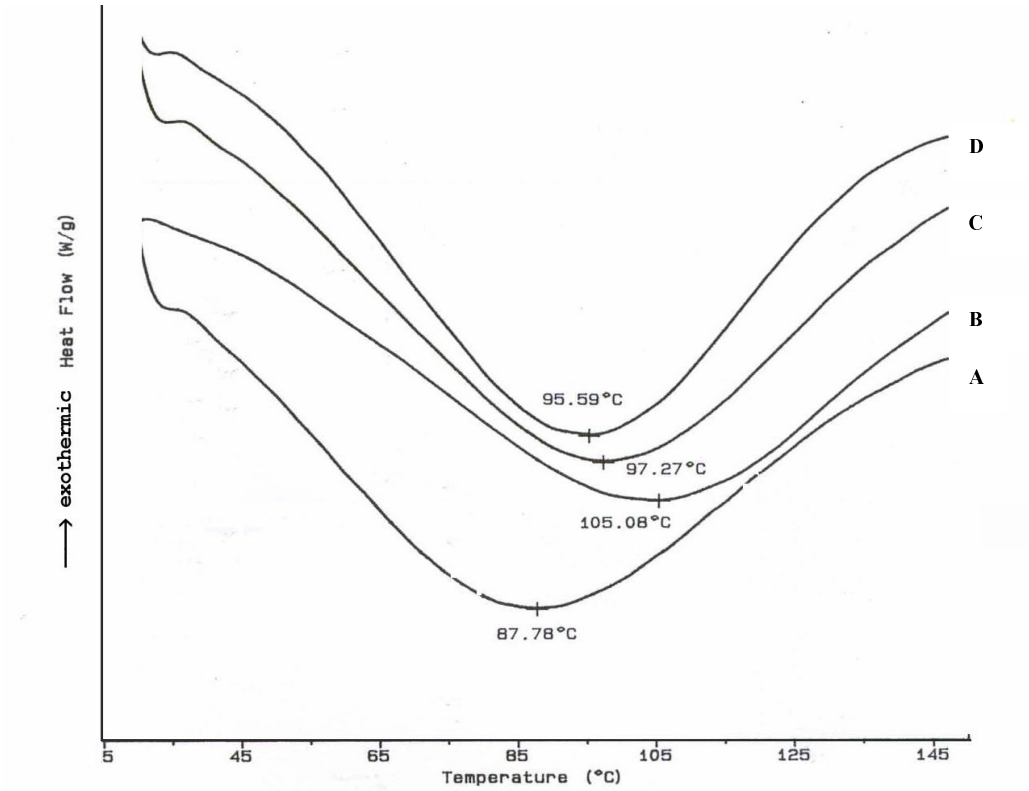
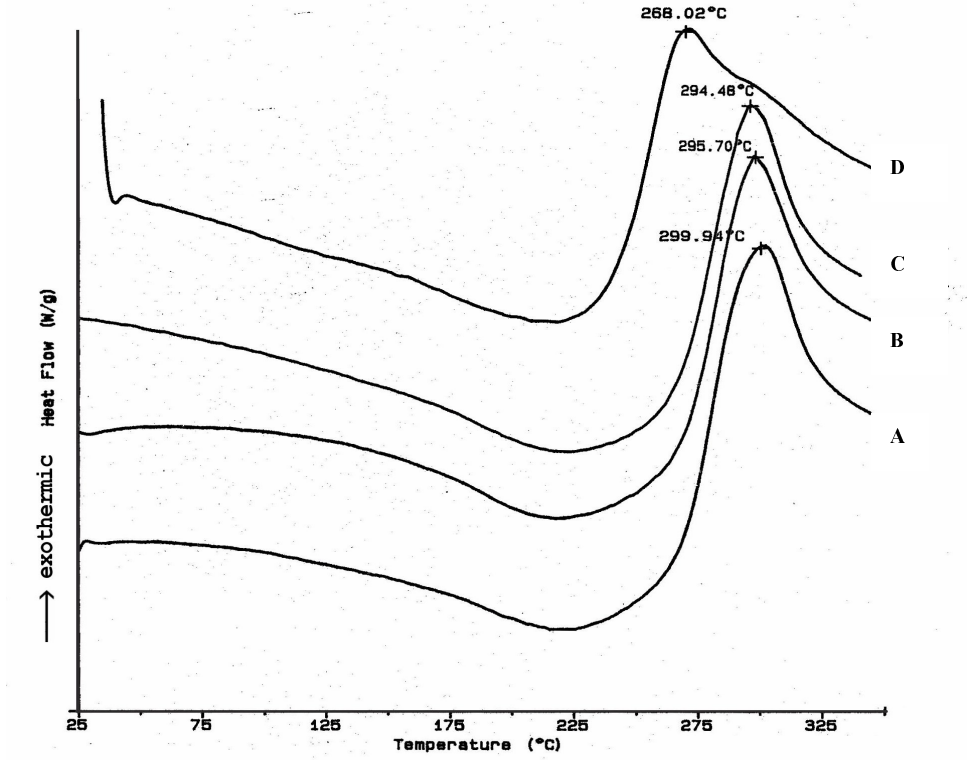


Figure 3.18 DSC thermograms of (A) chitin, (B) chitosan (DDA=75%), (C) chitosan (DDA=85%), (D) chitosan (DDA=90%), in the (a) first heating, (b) second heating



(a)



(b)

Figure 3.19 DSC thermograms of chitosan films with (A) no crosslinker, (B) 0.01% GA, (C) 0.1% GA, (D) 1% GA, in the (a) first heating, (b)second heating

As seen from the figures 3.18 and 3.19, major thermal processes occurred between 150ºC and 350ºC. Peaks observed in the first heatings (Figures 3.18a and 3.19a) could be most probably caused by the loss of water.

For chitosan samples, as the DDA increases, crystallization temperature shifted from 304.30ºC to 317.35ºC (Figure 3.18b). In the case of chitosan films, crystallization temperature shifted from 299.94ºC to 268.02ºC, as the crosslinker concentration increases (Figure 3.19b). These peaks are corresponding to reorganization of the molecules and intermolecular attractions. During this reorganization, small molecules are lost and the color turns black. The reorganization temperature of CF is 299.94ºC, CF/0.01 is 295.70ºC, CF/0.1 is 294.48ºC and CF/1 is 268.02ºC. So it can be concluded that high concentration of the crosslinker caused deficiency in the crystalline structure of the polymer.

3.3.3 Mechanical Test Results

Chitosan (DDA=85%) films, prepared at different crosslinking degrees, were analyzed for their tensile properties. The obtained results for the films; CF(no crooslinker), CF/0.01 (0.01% GA), CF/0.1 (0.1% GA) and CF/1 (1% GA) are as follows:

Ultimate Tensile Strength (UTS)

The mean ultimate tensile strength (UTS) value of chitosan films with no crosslinker (CF) was found as 94.89 MPa. Crosslinking increased the UTS value of CF/0.01

samples to 102.71 MPa. But for the film with 0.1% GA (CF/0.1) the UTS value decreased to 83.29 MPa. The decrease was much more for the samples with 1% GA (CF/1) and measured as 50.59 MPa. The differences between the groups, in all comparisons, were found to be statistically significant ($p<0.05$). UTS results of the samples are given in Figure 3.20 and Table 3.6.

Modulus of Elasticity (E)

Mean Elastic modulus (E) value of CF (non-crosslinked) samples was found as 3.7 GPa. Although it was increased to 3.8 GPa for CF/0.01 samples, this difference is not found to be significant statistically ($p>0.05$). When the crosslinker concentration was increased to 0.1% (CF/0.1) and to 1% (CF/1) samples, E values decreased to 3.4 GPa and 3.3 GPa, respectively. These differences are statistically significant ($p<0.05$).

Strain At Break (SAB%)

Mean strain at break (SAB%) of CF samples was found as 4.29% (Figure 3.20). For CF/0.01 samples it was decreased to 4.26%, but this difference is not statistically significant ($p>0.05$). SAB values for CF/0.1 and CF/1 were 3.72% and 2.06%, respectively. These differences are found to be statistically significant.

Table 3.6 Mechanical properties of chitosan films

Sample	Crosslinker Concentration	UTS (MPa)	E (MPa)	SAB (%)
CF	0.0%	94.89+11.36	3703.22+267.23	4.29+0.55
CF/0.01	0.01%	102.71+12.81	3790.14+279.17	4.26+0.51
CF/0.1	0.1%	83.29+15.75	3447.06+295.42	3.72+0.56
CF/1	1.0%	50.59+14.29	3328.72+388.04	2.06+0.37

Tensile strength of polymers increases with the crosslinking degree, thus crosslinking the polymers improves their mechanical properties. In our study, it was found that the samples prepared with 0.01% GA (CF/0.01) were stronger than the samples that have no crosslinker (CF). This is in accordance with literature [60]. When compared to crystalline parts, non-crystalline parts of polymer structure are loose and more suitable for added chemicals to penetrate in. Therefore addition of crosslinker first crosslinks the non-crystalline parts of the polymer chains, thus the structure becomes stiffer. When the crosslinker concentration was increased to 0.1% and to 1%, the values of all mechanical parameters decreased. This situation can be explained by two reasons:

- The unreacted excess crosslinker in the matrix acts as plasticizer in the crystalline structure.
- In literature it was indicated that aldehyde polymers from homopolymerization of GA exist in most commercial products and form during the reaction [61]. Thus, some of GA can be included in more complex graft polymers on the chitosan, as illustrated in Figure 3.21.

Crosslinking the samples with 0.01% GA caused a slight increase in the modulus of elasticity because of linkages between the crosslinker and the polymer chain, especially in the amorphous regions. But, when the crosslinker concentration was increased more, E values decreased, as seen in the UTS values. Addition of crosslinker also affected the extensibility of the films. After an optimum value, as the UTS of samples decreased with increased crosslinker, the SAB values decreased because more crosslinked samples could not bear the stress values of less crosslinked samples.

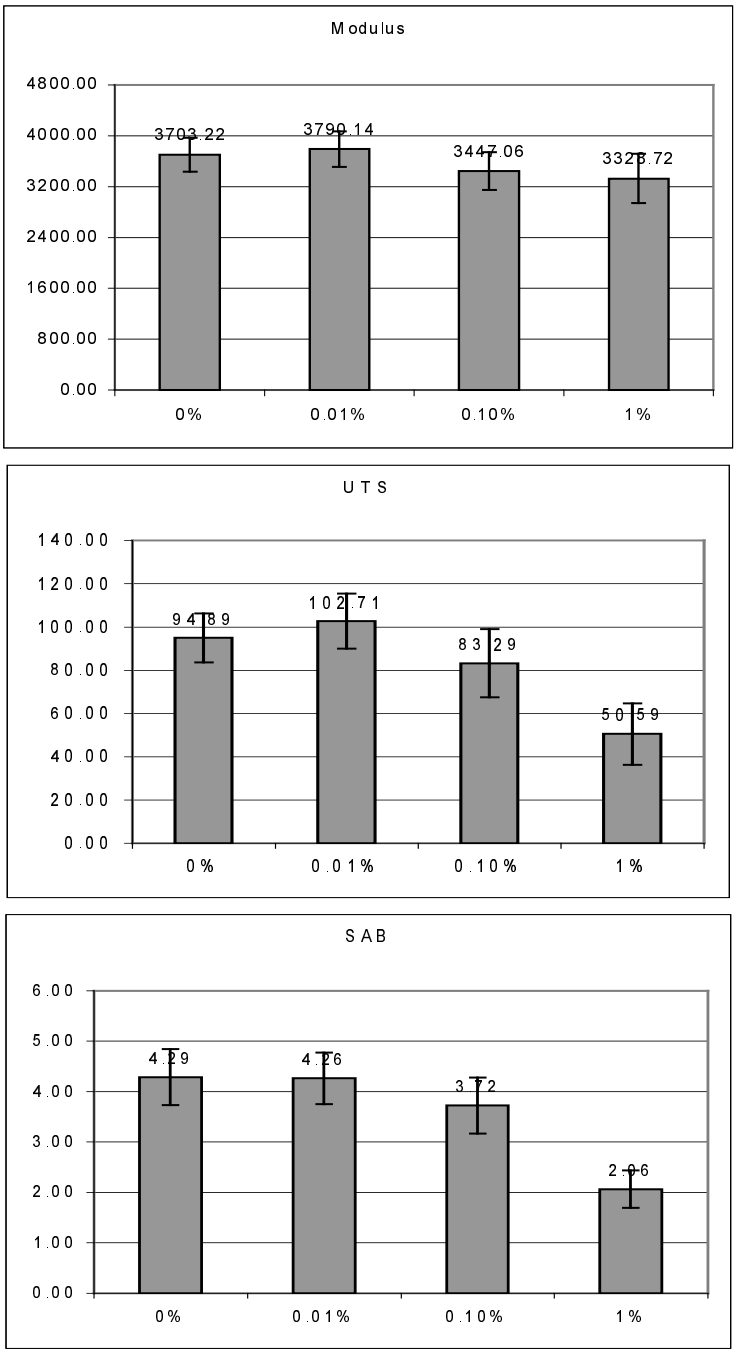


Figure 3.20 Mechanical properties of chitosan films

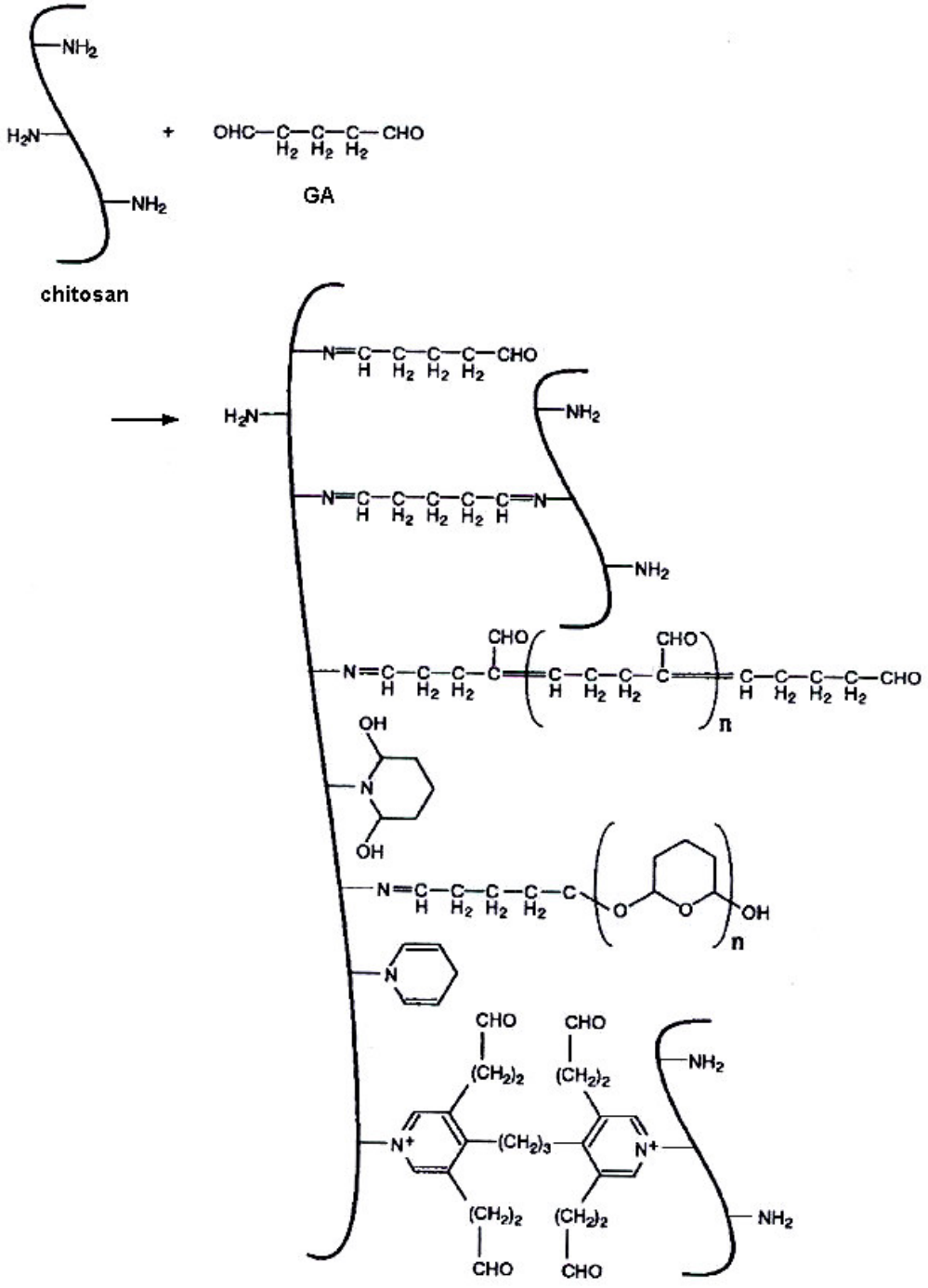


Figure 3.21 Chemical reactions between chitosan and glutaraldehyde

CHAPTER IV

CONCLUSION

Severe anemia remains the major underlying problem for patients with thalassemia, and combined red blood cell transfusion and iron-chelation therapy will remain the primary treatment for the foreseeable future. The single development that would most improve the lives of transfused patients with thalassemia would be the development of effective iron chelating agents that avoid the daily, prolonged intravenous or subcutaneous infusions of Desferal that are now needed to prevent disability and death from transfusional iron overload. Studies with the hydroxypyridinone, Deferiprone, have demonstrated the feasibility of effective iron-chelation therapy with an orally administered drug, but the risk of neutropenia with this drug may restrict its clinical usefulness. Continued research efforts to develop safe and effective orally active iron-chelating agents or other alternative delivery methods for the treatment of thalassemia are urgently needed.

Chitosan, a deacetylated derivative of chitin, with the nontoxicity, biocompatibility and biodegradability properties, has a variety of promising pharmaceutical uses and is considered as a novel carrier material in drug delivery systems. In recent years,

studies have studies have shown that gluteraldehyde crosslinked chitosan microspheres are long acting biodegradable carriers suitable for controlled delivery of many drugs.

In this study, chitin (Sigma) was purified and deacetylated by refluxing with concentrated NaOH for 88 h and converted to chitosan which has 75% degree of deacetylation (DDA). On the other hand, chitosan obtained from Sigma (DDA=85%) was further deacetylated and for the resultant chitosan DDA was measured as 90%. IR analysis confirmed the structural changes in the chitin that turned into chitosan. Chitosan microspheres were prepared by emulsion polymerization, using two different chitosans, one with 75% DDA, and the other with 85% DDA, and changing the crosslinker (gluteraldehyde, GA) concentration. Particle size, SEM, and in vitro drug release analysis were performed. In the second stage of the study, chitosan (DDA=85%) films were prepared with various crosslinking degrees, by film casting with aqueous acidic solutions of chitosan and IR, DSC, and mechanical analysis were performed. Crosslinking of the films was also achieved by gluteraldehyde.

For the microspheres;

- Increase in the concentration of crosslinker caused a decrease in the average mean diameters of the microspheres from 104.57μm to 48.53μm for microspheres with 85% DDA and 83.57μm to 30.75μm for microspheres with 75% DDA. Degree of deacetylation is another factor affecting the sizes. It was observed that as DDA increases, viscosity of the chitosan solution increases, causing formation of bigger microspheres.

- The amount of released Deferiprone was analyzed spectrophotometrically at 278 nm. It was observed that, release trend of Deferiprone slightly depends on the DDA of chitosan. As the DDA, the release became slower. contrary to expectations, as the crosslinking degree increased, the release rate of the drug also increased, which can be explained by the deformations in the crystalline structure of chitosan, which in turn affects the size and the morphology of the microspheres. Encapsulation efficiencies were 36.2%, 34.0% and 16.6% for CMD85/10, CMD85/1 and CMD85/0.1, respectively. And for the microspheres prepared with chitosan having 75%DDA, Deferiprone encapsulation efficiencies were found as 40.1% for CMD75/10, 36.3% for CMD75/1 and 29.1% for CMD75/0.01. Low encapsulation efficiencies can be explained by the high solubility of the drug in aqueous medium.

For the films;

- IR analysis confirmed the formation of covalent bond in the films between chitosan and gluteraldehyde.
- It had seen from the DSC analysis that, major thermal processes occurred between 150ºC and 350ºC. As the crosslinker concentration increased, crystallization temperature shifted from 299.94ºC to 268.02ºC which may be due to the deficiency in the crystalline structure of the polymer caused by increasing concentration of the crosslinker.
- In the case of mechanical analysis, the results showed that crosslinked chitosan films had the required strength for biomedical applicability. It was found that UTS values differed from 50.59 MPa to 102.71 MPa, mean elastic modulus

values differed from 3328.72 MPa to 3790.14 MPa and SAB values differed from 2.06% to 4.29%. All the differences were found to be statistically significant.

The present study demonstrated that, by enhancing the DDA and preparation composition, the chitosan based systems can be modulated to have the desired drug release rate or the desired mechanical strength needed for biomedical applications.

REFERENCES

1. Cooley's Anemia: Progress in Biology and Medicine - a report by the National Heart, Lung and Blood Institute of USA; National Heart, Lung and Blood Institute of USA: New York, 1995.
2. http://www.cooleysanemia.org
3. Faa G, Crisponi G. Iron chelating agents in clinical practice, Coordination Chemistry Reviews Vol. 184, 291-310, 1999.
4. Hider RC, Choudhury R, Rai BL, Dehkordi LS, Singh S. Design of orally active iron chelators, Acta Haematologica Vol. 95, 6-12, 1996.
5. Hider RC, Kontoghiorghes GJ, Silver J. UK Patent GB2118176, 1982.
6. Porter JB. Evaluation of New Iron Chelators for Clinical Use, Acta Haematologica, Vol. 95, 13-25, 1996.
7. Burke A, Yılmaz E, Hasırcı N. Evaluation of chitosan as a potential medical iron (III) ion adsorbent, Turk J Med Sci, Vol.30, 341-348,2000.
8. Burke A, Yılmaz E, Hasırcı N, Yılmaz O. Iron (III) ion removal from solution through adsorption on chitosan, Journal of Applied Polymer Science, Vol. 84, 1185-1192, 2002.
9. Kydonieus AF. 'Fundamental Concepts of Controlled Release' in Controlled Release Technologies: Methods, Theory, and Applications, Vol. I, CRC Press, US, 1-19, 1980.

10. Narayani R, Panduranga Rao K. Gelatin microsphere cocktails of different sizes for the controlled release of anticancer drugs, Int. J of Pharmaceutics, Vol.143, 255-258, 1996
11. Byung H. Woo, Janusz W. Kostanski, Sisay Gebrekidan, Bhas A. Dani, BC Thanoo, Patrick P. DeLuca. Preparation, characterization and in vivo evaluation of 120-day poly(D,L-lactide) leuprolide microspheres, J Controlled Release, Vol.75, 307-315, 2001.
12. Liggins RT, D'Amours S, Demetrick JS, Machan LS, Burt HM. Paclitaxel loaded poly(L-lactic acid) microspheres for the prevention of intraperitoneal carcinomatosis after a surgical repair and tumor cell spill, Biomaterials, Vol.21, 1959-1969, 2000.
13. Bezemer JM, Radersma R, Grijpma DW, Dijkstra PJ, van Blitterswijk CA, Feijen J. Microspheres for protein delivery prepared from amphiphilic multiblock copolymers 2. Modulation of release rate, J Controlled Release, Vol.67, 249-260, 2000.
14. Akbuğa J. Effect of microsphere size and formulation factors on drug release from controlled-release furosemide microspheres, Drug Development and Industrial Pharmacy, Vol.17, 593-607, 1991.
15. Berkland C, King M, Cox A, Kim KK, Pack DW. Precise control of PLG microsphere size provides enhanced control of drug release rate, J Controlled Release, Vol. 82, 137-147, 2002.
16. Langer R, Novel drug delivery systems, Chemistry in Britain, March, 232-236, 1990.

17. Jameela SR, Jayakrishnan A. Glutaraldehyde crosslinked chitosan microspheres as along acting biodegradable drug delivery vehicle: studies on the in vitro release of mitoxantrone and in vivo degradation of microspheres in rat muscle, Biomaterials, Vol.16, 769-775, 1995.
18. He P, Davis SS, Illum L. Chitosan microspheres prepared by spray drying, Int J Pharmaceutics, Vol.187, 53-65, 1999.
19. Seong-Bae Park, Jin-Oh You, Ham-Yong Park, Seung Joo Haam, Woo-Sik Kim. A novel pH-sensitive membrane from chitosan – TEOS IPN; preparation and its drug permeation characteristics, Biomaterials, Vol.22, 323-330, 2001.
20. Puttipipatkhachorn S, Nunthanid J, Yamamoto K, Peck GE. Drug physical state and drug-polymer interaction on drug release from chitosan matrix films, J Controlled Release, Vol.75, 143-153, 2001.
21. Muzzarelli RAA. Chitin, Pergamon Press Ltd, 1, 1977.
22. F.HOPPE-SEYLER, *Ber.***27,** 3329 (1894)
23. T.ARAKI, *Z.Physiol.Chem.***20,** 498 (1895)
24. O.VON FURTH and M.RUSSO, *Chem. Zentr.* **77,** 133(1906)
25. RIGBY GW. U.S.Patent 2,040,879 (1936)
26. Muzzarelli RAA. Natural Chelating Polymers, 144-147, 1973.
27. Muzzarelli RAA. Chitin, Pergamon Press Ltd, 87-90, 1977.
28. Muzzarelli RAA. Chitin, Pergamon Press Ltd, 208, 1977.
29. Skajak-Braek G, Anthonsen T, Sanford P. Chitin and Chitosan, Ed. Elsevier, London, 487, 1989.

30. Muzzarelli R, Biagini G, Pugnaloni A, Filippini O, Baldassarre V. Reconstruction of parodontal tissue with chitosan, Biomaterials, Vol.10, 598-603, 1989.
31. Hyun-Jung Kim, Hyun-Chul Lee, Jong-Suk Oh, Boo-Ahn Shin, Chang-Seok Oh, Ro-Dong Park, Kap-Seung Yang, Chong-Su Cho. Polyelectrolyte complex composed of chitosan and sodium alginate for wound dressing application, J Biomaterials Science Polymer Ed, Vol.10, 543-556, 1999.
32. Ueno H, Yamada H, Tanaka I, Kaba N, Matsuura M, Okumura M, Kadosawa T, Fujinaga T. Accelerating effects of chitosan for healing at early phase of experimental open wound in dogs, Biomaterials, Vol.20, 1407-1414, 1999.
33. Obara K, Ishihara M, Ishizuka T, Fujita M, Ozeki Y, Maehara T, Satio Y, Yura H, Matsui T, Hattori H, Kikuchi M, Kurita A. Photocrosslinkable chitosan hydrogel containing fibroblast growth factor-2 stimulates wound healing-impaired *db/db* mice, Biomaterials, Vol.24, 3437-3444, 2003.
34. Lin-Shu Liu, Shu-Qui Liu, Steven Y. Ng, Michael Froix, Tadao Ohno, Jorge Heller. Controlled release of interleukin-2 for tumour immunotheraphy using alginate/chitosan porous microspheres, J Controlled Release, Vol.43, 65-1414, 1997.
35. Thanou M, Verhoef JC, Junginger HE. Oral drug absorption enhancement by chitosan and its derivatives, Adv Drug Delivery Reviews, Vol.52, 117-126, 2001.
36. Thanou M, Verhoef JC, Junginger HE. Chitosan and its derivatives as intestinal absorption enhancers, Adv Drug Delivery Reviews, Vol.50, S91-S101, 2001.
37. Skajak-Braek G, Anthonsen T, Sanford P. Chitin and Chitosan, Ed. Elsevier, London, 60, 1989.

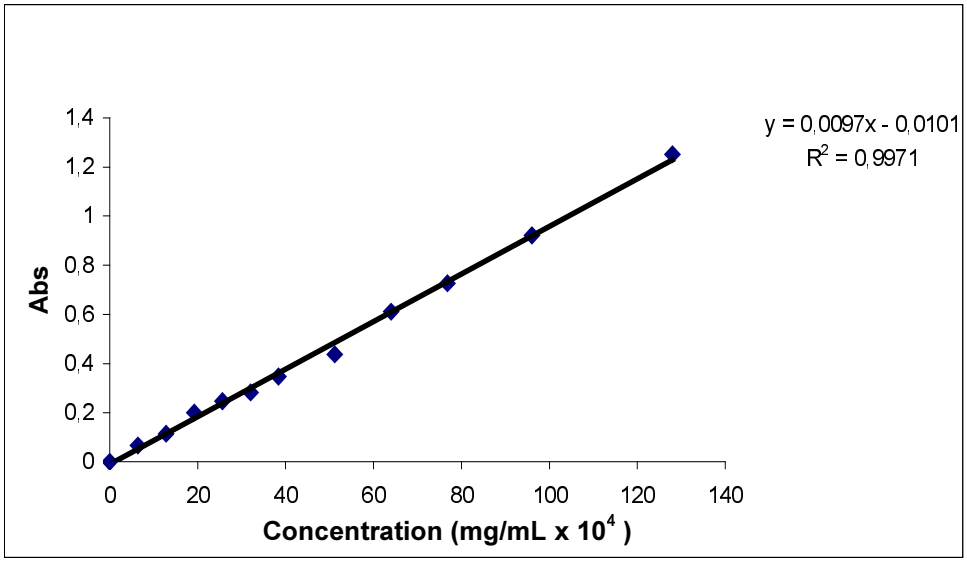
38. Skajak-Braek G, Anthonsen T, Sanford P. Chitin and Chitosan, Ed. Elsevier, London, 63, 1989.
39. Wang J, Qian Y. Microbial degradation of 4-chlorophenol by microorganisms entrapped in carrageenan-chitosan gels, Chemosphere, Vol.38, 3109-3117, 1999.
40. Methal Albarghouthi, Dib Abu Fara, Mohamad Saleem, Talal El-Thaher, Khalid Matalka, Adnan Badwan. Immobilization of antibodies on alginate-chitosan beads, International Journal of Pharmaceutics, Vol.206, 23-34, 2000.
41. Dodane V, Vilivalam VD. Pharmaceutical applications of chitosan, PSTT, Vol.1, No.6, 246-253, 1998.
42. Illum L. Chitosan and its use as a pharmaceutical excipient, Pharmaceutical Research, Vol.15, No.9, 1326-1331, 1998.
43. Lee JY, Nam SH, Im SY, Park YJ, Lee YM, Seol YJ, Chung CP, Lee SJ. Enhanced bone formation by controlled growth factor delivery from chitosan-based biomaterials, J Controlled Release, Vol.78, 187-197,2002.
44. Gallaher CM, Munion J, Hesslink R, Wise J, Gallaher DD. Cholesterol reduction by glucomannan and chitosan is mediated by changes in cholesterol absorption and bile acid and fat excretion in rats, Journal of Nutrition, Vol.130, 2753-2759, 2000.
45. Remuñán-López C, Bodmeier R. Mechanical, water uptake and permeability properties of crosslinked chitosan glutamate and alginate films, J Controlled Release, Vol.44, 215-225, 1997.
46. Huang YC, Yeh MK, Chiang CH. Formulation factors in preparing BTM-chitosan microspheres by spray drying method, International Journal of Pharmaceutics, Vol.242, 239-242, 2002.

47. Tozaki H, Odoriba T, Okada N, Fujita T, Terabe A, Suzuki T, Okabe S, Muranishi S, Yamamoto A. Chitosan capsules for colon-specific drug delivery: enhanced localization of 5-aminosalicylic acid in the large intestine accelerates healing of TNBS-induced colitis in rats, J Controlled Release, Vol.82, 51-61, 2002.
48. Jameela SR, Misra A, Jayakrishnan A. Cross-linked chitosan microspheres as carriers for prolonged delivery of macromolecular drugs, J Biomater Sci Polym Ed., Vol.6 (7), 621-632, 1994.
49. Calvo P, Vila-Jato JL, Alonso MJ. Evaluation of cationic polymer-coated nanocapsules as ocular drug carriers, International Journal of Pharmaceutics, Vol.153, 41-50, 1997.
50. Borchard G. Chitosans for gene delivery, Adv Drug Deliv Reviews, Vol.52, 145-150, 2001.
51. Illum L, Watts P, Fisher AN, Hinchcliffe M, Norbury H, Jabbal-Gill I, Nankervis R, Davis SS. Intranasal delivery of morphine, The J of Pharmacology and Experimental Therapeutics, Vol.301, 391-400, 2002.
52. Ouchi T, Banba T, Matsumoto T, Suzuki S, Suzuki M. Synthesis and antitumor activity of conjugates of 5-fluorouracil and chito-oligosaccharides involving a hexamethylene spacer group and carbamoyl bonds, Drug Des Deliv, Vol.6, 281-287, 1990.
53. Mima S, Miya M, Iwamoto R, Yoshikawa S. Highly deacetylated chitosan and its properties, J App Polymer Sciences, Vol.28, 1909-1917, 1983.

54. Knaul JZ, Kassai MR, Bui VT, Creber KAM. Characterization of deacetylated chitosan and chitosan molecular weight review, Canadian J Chemistry, Vol.76, 1699-1706, 1998.
55. Terayama H. Method of colloid titration (A new titration between polymer ions), J Polymer Science, Vol.8, 243-253, 1952.
56. Tolaimate A, Desbriéres J, Rhazi M, Alagui A, Vicendon M, Vottero P. On the influence of deacetylation process on the physicocemical characteristics of chitosan from squid chitin, Polymer, Vol.41, 2463-2469, 2000.
57. Damink O, Dijkstra PJ. Glutaraldehyde as a crosslinking agent for collagen-based biomaterials, J Materials Science: Materials in Medicine, Vol.6, 460-472, 1995.
58. Jaworska M,Sakurai K, Gaudon P, Guibal E. Influence of chitosan on polymer properties. 1. Crystallographic properties, Polymer International, Vol.52, 198-205, 2003.
59. Prashanth KVH, Kittur FS, Tharanathan RN. Solid state structure of chitosan prepared under different N-deacetylating conditions, Carbohydrate Polymers, Vol.54, 245-251, 2002.
60. Choi YS *et al*. Studies on gelatin-containing artificial skin: II. Preparation and characterization of crosslinked gelatin-hyalunorate sponge, J Biomedical and Material Research (Applied Biomaterials, Vol.48, 631-639, 1999.
61. Matsuda S, Iwata H, Se N, Ikada Y. Bioadhesion of gelatin films crosslinked with gluteraldehyde, J Biomed Mater Res, Vol.45, 20-27, 1999.

APPENDIX A

Calibration curve for Deferiprone (278 nm)



APPENDIX B

Range: 300 mm Beam: 14.30 mm Sampler: MS7 Obs: 11.8 %
Presentation: 3OHD Analysis: Polydisperse Residual: 0.303 %
Modifications: None

Conc. = 0.0035 %Vol Density = 1.000 g/cm^3 S.S.A. = 0.2714 m^2/g
Distribution: Volume D[4, 3] = 48.53 um D[3, 2] = 26.86 um
D[v, 0.1] = 25.87 um D[v, 0.5] = 46.50 um D[v, 0.9] = 74.37 um
Span = 1.057E+00 Uniformity = 3.320E-01

Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %
0.494	0.05	3.21	0.01	20.84	1.31	135.3	0.00
0.532	0.06	3.46	0.02	22.46	1.84	145.8	0.00
0.574	0.06	3.73	0.02	24.20	2.13	157.2	0.00
0.618	0.10	4.02	0.03	26.08	2.58	169.4	0.00
0.667	0.11	4.33	0.03	28.11	3.04	182.5	0.00
0.718	0.10	4.66	0.04	30.29	4.09	196.7	0.00
0.774	0.09	5.03	0.05	32.65	4.33	212.0	0.00
0.834	0.08	5.42	0.05	35.18	6.77	228.5	0.00
0.899	0.07	5.84	0.05	37.92	6.42	246.2	0.00
0.969	0.06	6.29	0.05	40.86	7.00	265.4	0.00
1.04	0.05	6.78	0.05	44.04	7.46	286.0	0.00
1.13	0.04	7.31	0.05	47.46	7.77	308.2	0.00
1.21	0.03	7.88	0.04	51.15	7.63	332.1	0.00
1.31	0.02	8.49	0.04	55.12	6.34	358.0	0.00
1.41	0.01	9.15	0.04	59.41	6.03	385.8	0.00
1.52	0.01	9.86	0.05	64.02	5.08	415.7	0.00
1.64	0.00	10.62	0.06	69.00	4.17	448.1	0.00
1.76	0.00	11.45	0.07	74.36	3.28	482.9	0.00
1.90	0.00	12.34	0.11	80.14	2.49	520.4	0.00
2.05	0.00	13.30	0.16	86.36	1.80	560.8	0.00
2.21	0.00	14.33	0.24	93.07	1.29	604.4	0.00
2.38	0.00	15.45	0.33	100.3	0.88	651.4	0.00
2.56	0.00	16.65	0.45	108.1	0.60	702.0	0.00
2.76	0.00	17.94	0.60	116.5	0.03	756.5	0.00
2.98	0.01	19.33	0.78	125.6	0.00	815.3	0.00
3.21	0.01	20.84	1.02	135.3	0.00	878.7	0.00

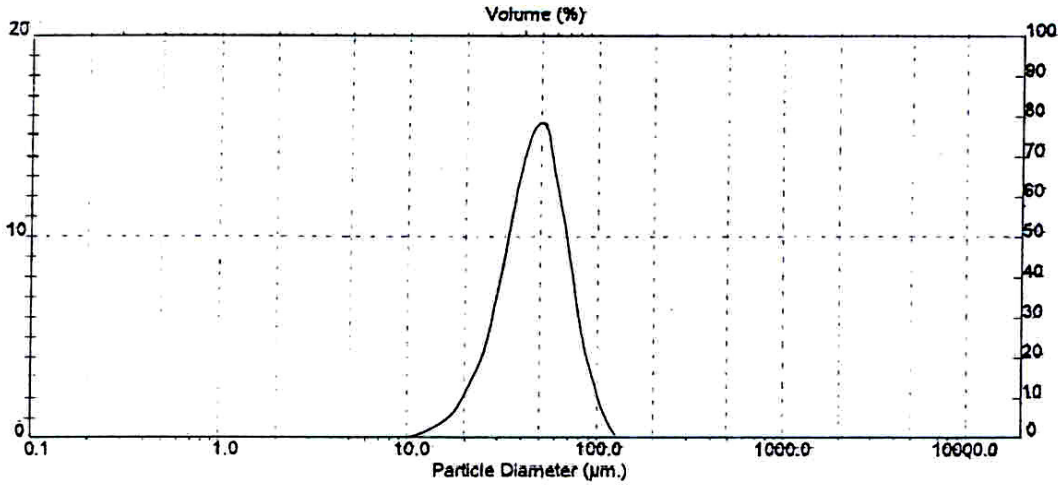


Figure B.1 The histogram table and plot of CM85/10

Range: 300 mm Beam: 14.30 mm Sampler: MS7 Obs': 10.6 %
Presentation: 3OHD Analysis: Polydisperse Residual: 0.198 %
Modifications: None

Conc. = 0.0105 %Vol Density = 1.000 g/cm^3 S.S.A.= 0.1565 m^2/g
Distribution: Volume D[4, 3] = 82.16 um D[3, 2] = 38.35 um
D(v, 0.1) = 33.17 um D(v, 0.5) = 72.00 um D(v, 0.9) = 132.28 um
Span = 1.377E+00 Uniformity = 4.842E-01

Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %
0.49	0.05	3.60	0.02	26.20	2.65	190.80	0.89
0.58	0.09	4.19	0.03	30.53	3.84	222.28	0.14
0.67	0.12	4.88	0.04	35.56	5.27	258.95	0.00
0.78	0.12	5.69	0.05	41.43	6.93	301.68	0.00
0.91	0.12	6.63	0.07	48.27	8.63	351.46	0.00
1.06	0.10	7.72	0.08	56.23	10.27	409.45	0.03
1.24	0.08	9.00	0.10	65.51	11.51	477.01	0.19
1.44	0.06	10.48	0.14	76.32	12.15	555.71	0.26
1.68	0.04	12.21	0.22	88.91	10.82	647.41	0.25
1.95	0.02	14.22	0.37	103.58	8.54	754.23	0.15
2.28	0.01	16.57	0.64	120.67	6.08	878.87	
2.65	0.01	19.31	1.08	140.58	3.85		
3.09	0.01	22.49	1.75	163.77	2.11		
3.60		26.20		190.80			

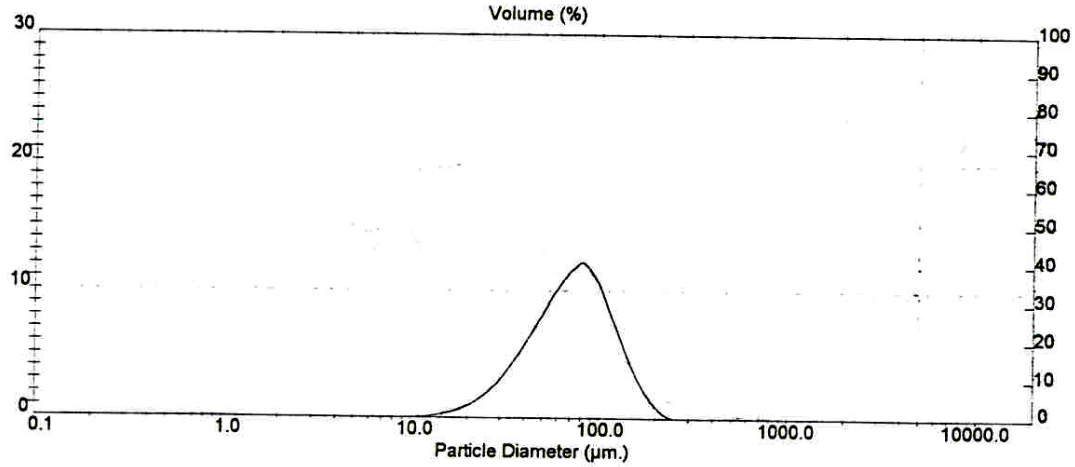


Figure B.2 The histogram table and plot of CM85/1

Range: 300 mm Beam: 14.30 mm Sampler: MS7 Obs: 9.4 %
Presentation: 3OHD Analysis: Polydisperse Residual: 0.268 %
Modifications: None

Conc. = 0.0097 %Vol Density = 1.000 g/cm^3 S.S.A.= 0.1523 m^2/g
Distribution: Volume D[4, 3] = 98.76 um D[3, 2] = 39.39 um
D(v, 0.1) = 43.54 um D(v, 0.5) = 72.67 um D(v, 0.9) = 121.20 um
Span = 1.069E+00 Uniformity = 6.199E-01

Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %
0.49	0.06	3.90	0.01	26.20	0.99	190.80	0.00
0.58	0.11	4.19	0.01	30.53	1.80	222.28	0.00
0.67	0.14	4.88	0.02	35.56	3.29	258.95	0.00
0.78	0.14	5.69	0.04	41.43	5.94	301.66	0.00
0.91	0.14	6.63	0.06	48.27	9.64	351.46	0.24
1.06	0.12	7.72	0.07	56.23	14.39	409.45	0.65
1.24	0.10	9.00	0.09	65.51	16.84	477.01	0.97
1.44	0.07	10.48	0.08	76.32	16.11	555.71	1.12
1.68	0.04	12.21	0.07	88.91	11.43	647.41	1.08
1.95	0.02	14.22	0.06	103.58	6.85	754.23	0.67
2.28	0.01	16.57	0.11	120.67	3.59	878.67	
2.65	0.01	19.31	0.26	140.58	1.50		
3.09	0.00	22.49	0.54	163.77	0.32		
3.60		26.20		190.80			

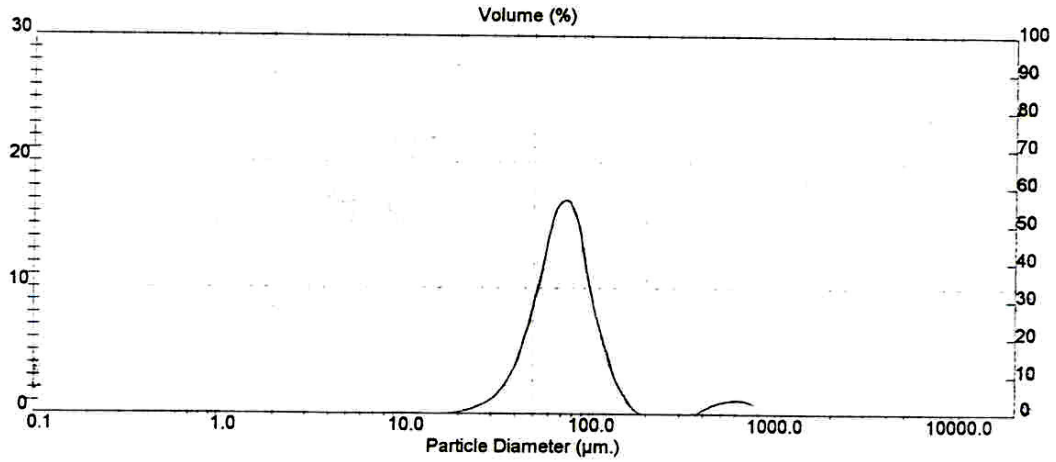


Figure B.3 The histogram table and plot of CM85/0.1

Range: 300 mm
Presentation: 3OHD
Modifications: None

Beam: 14.30 mm Sampler: MS7
Analysis: Polydisperse

Obs: 9.9 %
Residual: 0.773 %

Conc. = 0.0098 %Vol
Distribution: Volume
D(v, 0.1) = 33.09 um
Span = 2.423E+00

Density = 1.000 g/cm^3
D[4, 3] = 104.57 um
D(v, 0.5) = 75.55 um
Uniformity = 7.553E-01

S.S.A.= 0.1543 m^2/g
D[3, 2] = 38.88 um
D(v, 0.9) = 216.16 um

Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %
0.494	0.02	3.21	0.02	20.84	0.56	135.3	2.44
0.532	0.02	3.46	0.02	22.46	0.73	145.8	2.20
0.574	0.03	3.73	0.02	24.20	0.92	157.2	1.98
0.618	0.04	4.02	0.02	26.08	1.14	169.4	1.79
0.667	0.05	4.33	0.03	28.11	1.41	182.5	1.62
0.718	0.05	4.66	0.03	30.29	1.71	196.7	1.48
0.774	0.06	5.03	0.04	32.65	2.03	212.0	1.35
0.834	0.06	5.42	0.04	35.18	2.39	228.5	1.24
0.899	0.06	5.84	0.04	37.92	2.77	246.2	1.13
0.969	0.06	6.29	0.05	40.86	3.14	265.4	1.04
1.04	0.06	6.78	0.05	44.04	3.50	286.0	0.95
1.13	0.06	7.31	0.05	47.46	3.82	308.2	0.86
1.21	0.06	7.88	0.05	51.15	4.09	332.1	0.77
1.31	0.05	8.49	0.05	55.12	4.30	358.0	0.69
1.41	0.05	9.15	0.05	59.41	4.43	385.8	0.60
1.52	0.05	9.86	0.05	64.02	4.48	415.7	0.51
1.64	0.04	10.62	0.05	69.00	4.45	448.1	0.42
1.76	0.04	11.45	0.06	74.36	4.35	482.9	0.33
1.90	0.03	12.34	0.07	80.14	4.20	520.4	0.25
2.05	0.03	13.30	0.09	86.36	4.01	560.8	0.16
2.21	0.03	14.33	0.13	93.07	3.80	604.4	0.08
2.38	0.02	15.45	0.17	100.3	3.59	651.4	0.00
2.56	0.02	16.65	0.24	108.1	3.34	702.0	0.00
2.76	0.02	17.94	0.32	116.5	3.03	756.5	0.00
2.98	0.02	19.33	0.43	125.6	2.71	815.3	0.00
3.21	0.02	20.84		135.3		878.7	

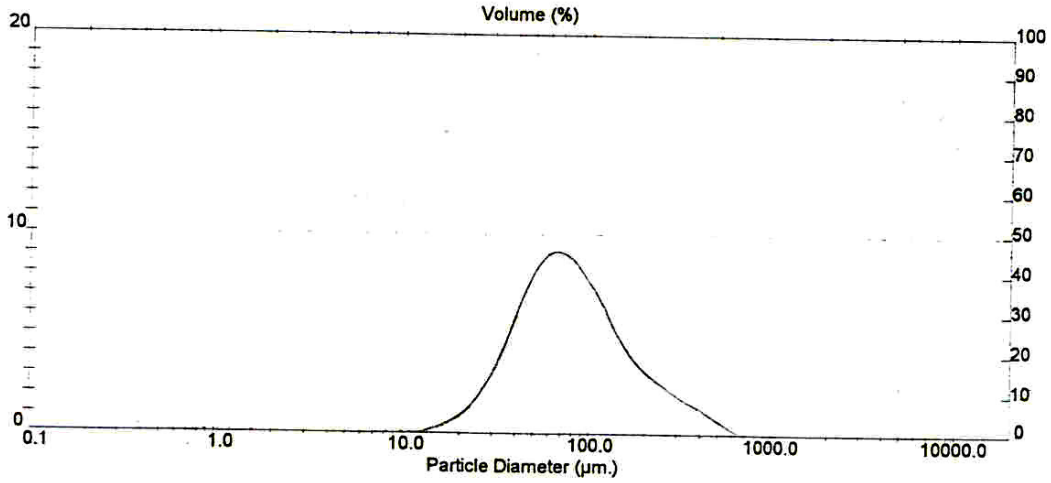


Figure B.4 The histogram table and plot of CM85/0.01

Range: 300 mm Beam: 14.30 mm Sampler: MS7 Obs: 11.6 %
Presentation: 3OHD Analysis: Polydisperse Residual: 1.331 %
Modifications: None

Conc. = 0.0075 %Vol Density = 1.000 g/cm^3 S.S.A. = 0.1104 m^2/g
Distribution: Volume D[4, 3] = 30.75 um D[3, 2] = 26.00 um
D(v, 0.1) = 16.47 um D(v, 0.5) = 28.96 um D(v, 0.9) = 47.34 um
Span = 1.086E+00 Uniformity = 3.350E-01

Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %
0.494	0.00	3.21	0.00	20.84	5.43	135.3	0.00
0.532	0.00	3.46	0.00	22.46	5.99	145.8	0.00
0.574	0.00	3.73	0.00	24.20	6.40	157.2	0.00
0.618	0.00	4.02	0.00	26.08	6.69	169.4	0.00
0.667	0.00	4.33	0.00	28.11	6.90	182.5	0.00
0.718	0.00	4.66	0.00	30.29	7.19	196.7	0.00
0.774	0.00	5.03	0.00	32.65	7.03	212.0	0.00
0.834	0.00	5.42	0.00	35.18	6.41	228.5	0.00
0.899	0.00	5.84	0.01	37.92	5.66	246.2	0.00
0.969	0.00	6.29	0.02	40.88	4.88	265.4	0.00
1.04	0.00	6.73	0.04	44.04	4.12	286.0	0.00
1.13	0.00	7.31	0.06	47.46	3.39	308.2	0.00
1.21	0.00	7.88	0.11	51.15	2.69	332.1	0.00
1.31	0.00	8.49	0.16	55.12	2.03	358.0	0.00
1.41	0.00	9.15	0.28	59.41	1.38	385.8	0.00
1.52	0.00	9.86	0.42	64.02	0.74	415.7	0.00
1.64	0.00	10.62	0.62	69.00	0.25	448.1	0.00
1.76	0.00	11.45	0.89	74.36	0.02	482.9	0.00
1.90	0.00	12.34	1.22	80.14	0.00	520.4	0.00
2.05	0.00	13.30	1.65	86.36	0.00	560.8	0.00
2.21	0.00	14.33	2.16	93.07	0.00	604.4	0.00
2.38	0.00	15.45	2.75	100.3	0.00	651.4	0.00
2.56	0.00	16.65	3.42	108.1	0.00	702.0	0.00
2.76	0.00	17.94	4.10	116.5	0.00	756.5	0.00
2.98	0.00	19.33	4.79	125.6	0.00	815.3	0.00
3.21	0.00	20.84		135.3		878.7	

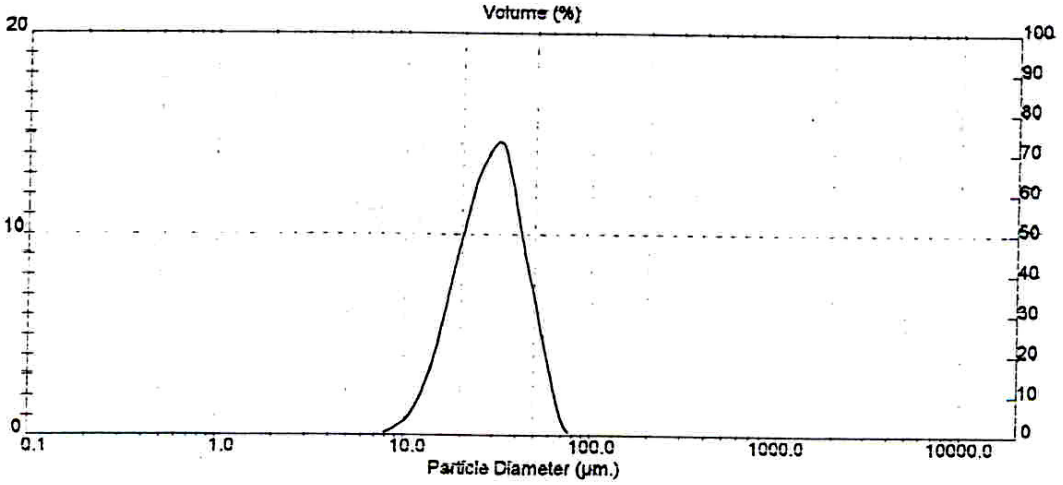


Figure B.5 The histogram table and plot of CM75/10

Range: 300 mm Beam: 14.30 mm Sampler: MS7 Obs: 10.6 %
Presentation: 3OHD Analysis: Polydisperse Residual: 0.313 %
Modifications: None

Conc. = 0.0097 %Vol Density = 1.000 g/cm^3 S.S.A. = 0.1660 m^2/g
Distribution: Volume D[4, 3] = 66.09 um D[3, 2] = 36.15 um
D(v, 0.1) = 28.07 um D(v, 0.5) = 57.09 um D(v, 0.9) = 118.58 um
Span = 1.620E+00 Uniformity = 5.100E-01

Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %
0.49		3.60		26.20		190.80	
0.58	0.04	4.19	0.04	30.53	4.87	222.28	1.01
0.67	0.07	4.88	0.06	35.56	6.59	258.95	0.36
0.78	0.09	5.69	0.08	41.43	8.08	301.68	0.00
0.91	0.09	6.63	0.09	48.27	9.35	351.46	0.00
1.06	0.08	7.72	0.11	56.23	10.05	409.45	0.00
1.24	0.06	9.00	0.13	65.51	10.24	477.01	0.00
1.44	0.04	10.48	0.18	76.32	10.09	555.71	0.00
1.68	0.03	12.21	0.30	88.91	8.73	647.41	0.00
1.95	0.01	14.22	0.52	103.58	7.10	754.23	0.00
2.28	0.01	16.57	0.89	120.67	5.39	878.67	0.00
2.65	0.01	19.31	1.46	140.58	3.85		
3.09	0.01	22.49	2.29	163.77	2.59		
3.60	0.02	26.20	3.43	190.80	1.65		

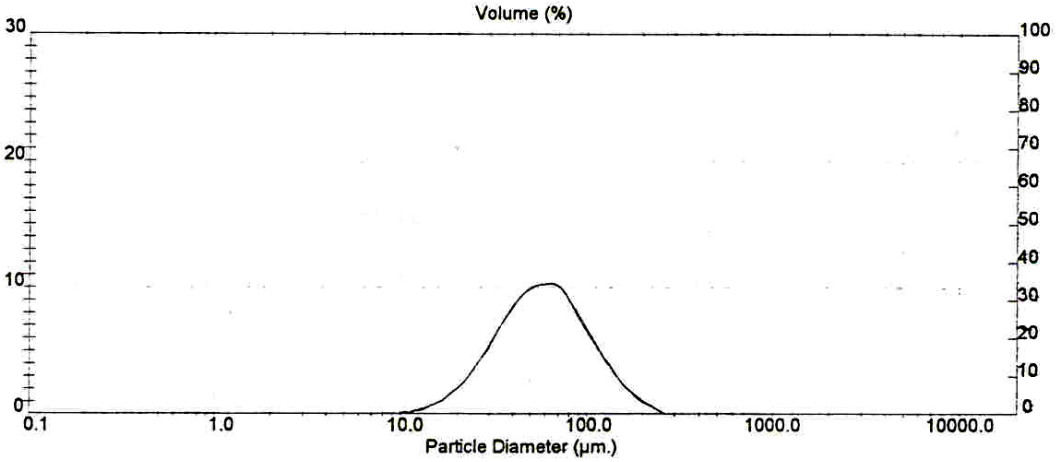


Figure B.6 The histogram table and plot of CM75/1

Range: 300 mm Beam: 14.30 mm Sampler: MS7 Obs': 9.9 %
Presentation: 3OHD Analysis: Polydisperse Residual: 1.340 %
Modifications: None

Conc. = 0.0077 %Vol Density = 1.000 g/cm^3 S.S.A.= 0.1840 m^2/g
Distribution: Volume D[4, 3] = 83.57 um D[3, 2] = 32.61 um
D(v, 0.1) = 28.27 um D(v, 0.5) = 76.59 um D(v, 0.9) = 150.63 um
Span = 1.598E+00 Uniformity = 4.958E-01

Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %	Size (um)	Volume In %
0.484	0.01	3.21	0.11	20.84	0.61	135.3	3.40
0.532	0.01	3.46	0.11	22.46	0.73	145.8	2.94
0.574	0.02	3.73	0.11	24.20	0.88	157.2	2.50
0.618	0.02	4.02	0.12	26.08	1.05	169.4	2.06
0.667	0.03	4.33	0.12	28.11	1.25	182.5	1.64
0.718	0.03	4.66	0.12	30.29	1.48	196.7	1.20
0.774	0.04	5.03	0.13	32.65	1.73	212.0	0.70
0.834	0.04	5.42	0.13	35.18	2.01	228.5	0.26
0.899	0.05	5.84	0.14	37.92	2.31	246.2	0.03
0.969	0.05	6.29	0.14	40.86	2.63	265.4	0.00
1.04	0.06	6.78	0.15	44.04	2.96	286.0	0.00
1.13	0.06	7.31	0.15	47.46	3.28	308.2	0.00
1.21	0.07	7.88	0.16	51.15	3.59	332.1	0.00
1.31	0.07	8.49	0.17	55.12	3.88	358.0	0.00
1.41	0.08	9.15	0.17	59.41	4.15	385.8	0.00
1.52	0.08	9.86	0.18	64.02	4.39	415.7	0.00
1.64	0.09	10.62	0.19	69.00	4.58	448.1	0.00
1.76	0.09	11.45	0.21	74.36	4.72	482.9	0.00
1.90	0.10	12.34	0.23	80.14	4.86	520.4	0.00
2.05	0.10	13.30	0.25	86.36	5.02	560.8	0.00
2.21	0.10	14.33	0.28	93.07	5.06	604.4	0.00
2.38	0.10	15.45	0.32	100.3	4.87	651.4	0.00
2.56	0.11	16.65	0.37	108.1	4.56	702.0	0.00
2.76	0.11	17.94	0.43	116.5	4.21	756.5	0.00
2.98	0.11	19.33	0.51	125.6	3.83	815.3	0.00
3.21		20.84		135.3		878.7	

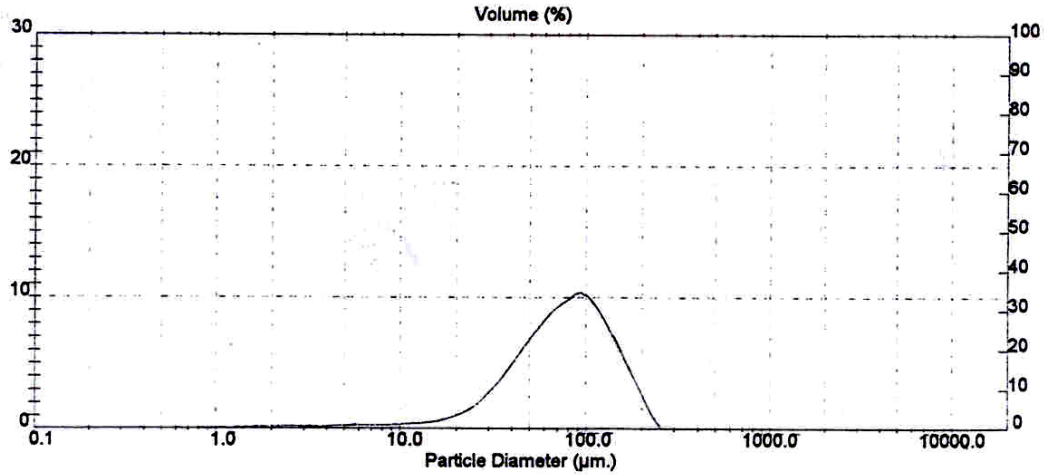


Figure B.7 The histogram table and plot of CM75/0.01