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A Thesis

entitled

A New Interfacial Cross Linking Technique: Preparation, Characterization and

Evaluation of Calcium alginate Nanoparticles as a Protein Delivery System

by

Priti Singh

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Masters of Science Degree in Pharmaceutical Sciences

with Industrial Pharmacy Option

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May 2013

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An Abstract of

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Nanoparticles prove to be excellent carriers for site specific and/or time controlled delivery of small or large molecular weight drugs and other bioactive agents. The use of biopolymers as a natural carrier system not only makes the formulation easy but provides an additional advantage of being non toxic and easily degradable within the body, thereby making them safer for use. Alginate which is a polysaccharide polymer is particularly versatile in encapsulation of unstable and water soluble molecules such as proteins. In the presence of a divalent ion, alginates form bead like structures which can be used as carriers in drug delivery. The purpose of this research was to develop a new technique for synthesizing calcium alginate nanoparticles and to evaluate its potential as a protein delivery system. Two template w/o microemulsions were prepared using DOSS as surfactant and cyclohexane as the organic phase respectively. Various proportions of these mixtures were tested for their ability to form stable microemulsions using ternary phase diagrams. Based on the results, suitable mixtures containing 0.5% to 1% of aqueous sodium alginate dispersion in 0.2 M DOSS/cyclohexane and 0.5% to 1%

aqueous calcium chloride in 0.2 M DOSS/cyclohexane were selected for nanoparticle preparation. Nanoparticle characterization studies indicated formation of calcium alginate nanoparticles in the size range 200-500nm.. Bovine Serum Albumin (BSA) loaded nanoparticles were prepared in the same way and characterized using dynamic light scattering, transmission electron microscopy, zeta potential, and differential scanning calorimetry. *In vitro* drug release studies showed that 90% of entrapped BSA was released within first 8 hrs followed by a slow release pattern. All entrapped protein was released within 30 hours. The presence of protein within the matrix system was investigated using FITC –BSA as a model via confocal laser scanning microscopy (CLSM). Zeta potential and CLSM results support the idea that BSA was partially adsorbed on the nanoparticle surface and the rest was encapsulated within the matrix. As an effort to increase the efficacy of these nanoparticles for protein delivery, chitosan was investigated as a cationic polymer to coat them. Zeta potential analysis and CLSM of nanoparticles subjected to coating with chitosan demonstrated deposition of the cationic polymer on the surface of the nanoparticle. Calcium alginate nanoparticles were successfully prepared via a novel interfacial cross linking method developed in our laboratory. Results obtained from various physico-chemical properties indicate that the nanoparticles possess characteristics suitable for use as a protein drug delivery system.

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Chapter 1

Introduction

Nanoparticles may be purposely engineered and constructed from various systems [1]. In drug delivery, they are defined as submicron ($<1\mu\text{m}$) colloidal particles. This definition comprises monolithic nanospheres in which drug is absorbed, dissolved, or dispersed throughout the matrix and nanocapsules, in which the drug is confined to an aqueous or oily core, surrounded by a shell-like wall [2, 3]. These drug carriers may or may not be biodegradable [4-6]. When compared to microparticles, nanoparticles differ significantly in two aspects, namely surface area and quantum effects. These factors can enhance properties such as reactivity, electrical characteristics and *in vivo* behavior [7]. Also they are better suited for intravenous delivery, because the smallest capillary in the body are 5-6 μm in diameter. Therefore the size of particles being distributed to the bloodstream must be significantly smaller than 5 μm , without forming aggregates, to ensure that the particles do not cause embolism [8]. Depending on the process of preparation of nanoparticles, nanospheres and nanocapsules can be fabricated to possess different physical, chemical, and biological properties and drug release characteristics for the delivery or encapsulation of the therapeutic agent [9].

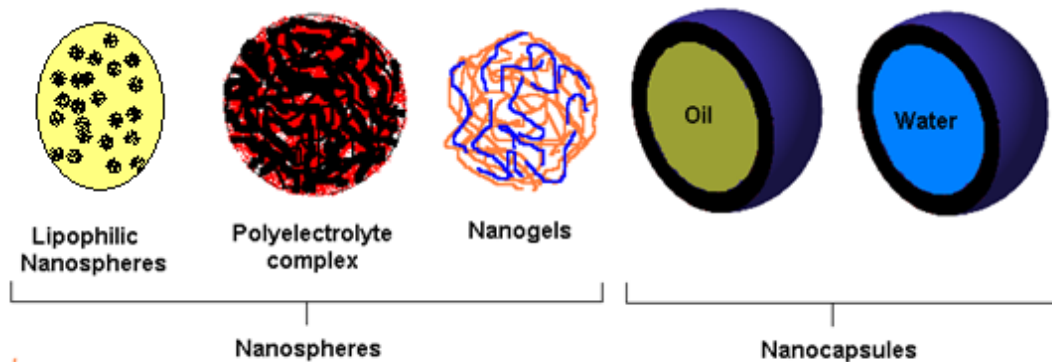


Figure-1-1 Polymeric Nanospheres and Nanocapsules [10]

1.1 Nanocapsules

Nanocapsules are vesicular systems in which the drug is confined to a cavity consisting of an inner liquid core surrounded by a polymeric membrane [4, 6]. Hence drug localization is an important parameter in the characterization of nanocapsule preparation. They can contain the drug in the liquid or solid form or as a molecular dispersion [11]. The nanocapsule core can be aqueous, or composed of a lipophilic solvent, commonly oil. In order to achieve good drug loading, the core materials are ideally good solvents for the drug [12]. The reservoir can be lipophilic or hydrophobic depending on the method of preparation [11].

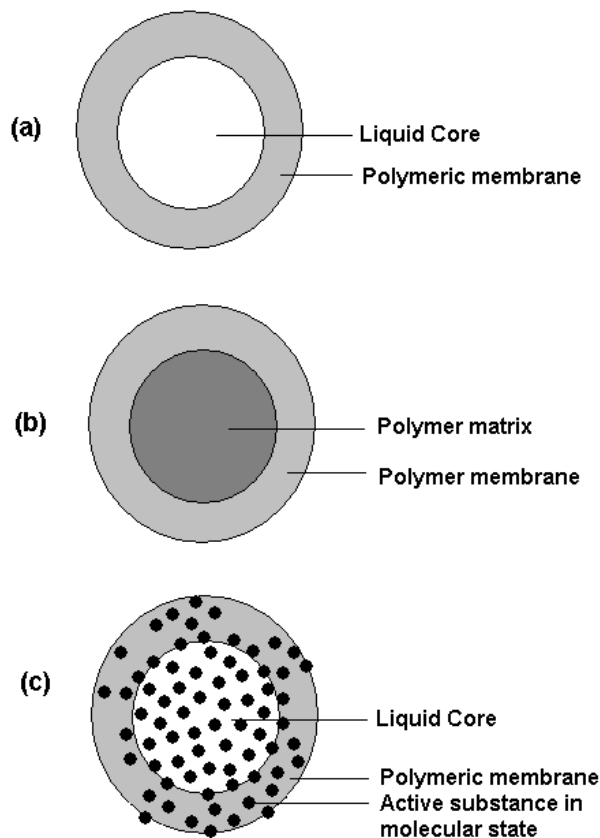


Figure 1-2)-Nanocapsule containing(a)-Liquid core (b)Polymer matrix(c)Active substance in the molecular dispersion enclosed within the polymeric membrane [11]

Advantages of nanocapsules include, 1) burst effect can be avoided when the drug is confined within the central cavity of the nanocapsule, 2) the drug is not in direct contact with the tissue and therefore irritation at the site of administration can be avoided due to the polymeric shell, 3) the drug may be protected from degradation factors like pH and light [6, 11, 13], 4) another advantage of nanocapsules over nanospheres include low polymer content and a high loading capacity for lipophilic drugs. Nanocapsules have been formed by interfacial polymerization of monomers or from prefabricated polymers. In the former method the molar mass of the coating polymer will depend on the

preparation conditions and even on the drugs used, whereas in the later, it is determined by the outset for e.g.: at times low viscosity polymer helps in achieving desired smaller particle sizes [12]. Most of the techniques described for making nanocapsules in literature involve the preparation of emulsions. Oil in water (O/W) emulsions lead to formation of nanocapsules with an oily core whereas, water in oil (W/O) leads to formation of nanocapsules with an aqueous core.

1.1.1 Preparation of Nanocapsules

There are several methods by which nanocapsules can be prepared, a few of which are listed below;

1) Nanoprecipitation Method

This is a simple, reproducible method that comprises of an interfacial deposition process. This process needs both solvent (organic) and non-solvent (aqueous) phases. In this technique a water miscible organic phase such as an alcohol or a ketone containing oil is mixed with an aqueous phase containing hydrophilic surfactant. Preformed polymers (synthetic, semi synthetic and naturally occurring polymers), soluble in both oily and aqueous phase are dispersed in the solvent or organic phase. Following the addition of aqueous phase, polymer diffuses with the organic solvent towards the aqueous phase and is stranded at the interface between oil and water. Acetone is always used as the polymer containing medium in all cases, whereas water or buffer solutions are used as nonsolvent phase. Rapid diffusion of the organic solvent in the aqueous phase, induces interfacial nanoprecipitation of the polymer around the droplets of the oily phase. The polymers used are usually biodegradable polyesters especially

PCL(poly- ϵ -caprolactone), PLA(poly lactic acid)and eudragit [11].Arabic gum, gelatin, ethylcellulose, or hydroxypropylmethylcellulose phthalate have also been successfully used [12]. Synthetic polymers have higher purity and better reproducibility than natural polymers [11, 14]. Also some polymers are PEG copolymerized in order to reduce nanocapsule recognition by the mononuclear phagocyte system [11, 15]. Nanocapsules produced by this method are in the size range of 100-500nm.The particle size usually depends on factors like amount of surfactant, ratio of organic solvent to water, concentration of oil in the organic solution, and the speed of diffusion of organic phase in the aqueous phase. Lower the interfacial tension and the viscosity of the oil; the smaller the nanocapsules produced. In an alternative solvent displacement method,an O/W emulsion was formed wherein the organic phase contained the polymer, the oil and the drug while the non-solvent phase contained the stabilizing agent. One advantage of this technique was that the smallest particles produced were below 100nm [particle size range (80-900 nm)], but a disadvantage is that large amount of water has to be removed at the end of the process during the nanoparticle isolation and purification stage.

2) Emulsion Diffusion Method

This technique allows preparation of nanocapsules containing both lipophilic and hydrophilic active substances [11, 16]. This is a two-step process, based on formation of an emulsion, succeeded by dilution which causes deposition of polymer around the droplets, which thereby brings about the formation of nanocapsules. Emulsion diffusion involves a partially water- soluble solvent, previously saturated with water containing a

polymer, an oil as the organic phase , and an aqueous phase previously saturated with an aqueous stabilizer [17].

In this method the organic phase is emulsified under vigorous agitation in the aqueous phase. When water is added to the system , the solvent diffuses into the external phase resulting in formation of nanocapsules. Water is used as the solvent, compounds like poloxamer, ionic emulsifiers, poly vinyl alcohol (PVA) are used as stabilizing agents. PVA has several advantages and is the preferred stabilizing agent [18, 19]. Ethyl acetate is the first choice for solvents, though propylene carbonate, benzyl alcohol, and dichloromethane can also be used [11].

Nanocapsule of the size range 150-200nm are produced. The particle formation is a dynamic process associated with the diffusion of the solvent from the droplet to the external phase caused by addition of water to the emulsion and resulting in the transformation of each droplet into a particle of a smaller size [11, 20].

3) Double emulsification Method

This method contains a complex heterodisperse system, broadly classified into two subtypes: (1)water in oil in water(W/O/W) emulsion, (2)oil in water in oil(O/W/O) emulsion [21, 22].The dispersed phase itself is an emulsion and the inner dispersed globule is separated from the outer liquid phase by a layer of another phase. Double emulsions are usually prepared in a two step emulsification process using two surfactants: one of them is hydrophilic, while the other one hydrophobic in nature. The hydrophobic surfactant stabilizes the interface of w/o internal emulsion, whereas the hydrophilic surfactant stabilizes the external interface of the oil globules for w/o/w emulsions.

In a typical preparation of particles by double emulsification, the primary emulsion is formed by ultrasonication, where the w/o surfactant stabilizes the interface of the w/o internal emulsion. The subsequent emulsion is also formed by ultrasound and the dispersion is stabilized by addition of a stabilizing agent. Removal of the solvents is done by vacuuming or evaporation, leaving hardened nanocapsules in the aqueous medium [11].

In this method, the surfactants play a dual role: as a film former and as a barrier to drug release at the internal surface, and as a stabilizer [14]. The inner aqueous phase is composed of mainly only active substance, in some cases water. Ethyl acetate, methylene chloride, Poly Caprolactone(PCL) ,Poly Lactic Acid(PLA), and Poly (Lactic co-Glycolic acid) PLGA have been used as solvents.

4) Emulsion Coacervation Method

This method is generally used for hydrophilic biodegradable polymers eg; Sodium alginate, chitosan and gelatin. It is also called as ionic gelation method. This technique involves preparation of o/w emulsion of an organic phase with an aqueous phase, either by stirring or by ultrasound. The organic phase comprises of oil, active substance, and solvent if necessary. The polysaccharides are dissolved in water or weak acidic medium (chitosan) which also contains the stabilizing agent .These solutions are then added drop wise under constant stirring to the solutions containing counter ions. Due to complexation between oppositely charged species, polysaccharides undergo gelation and precipitate to form spherical particles. The beads are removed by filtration, then washed and dried [23]. By altering pH or temperature, one can achieve physical intermolecular or covalent cross

linking that helps in stabilization of nanocapsules. This physical cross-linking by electrostatic interaction instead of chemical cross-linking avoids the potential toxicity of reagents [24]. The critical stage in this process is coacervate phase formation. In some cases electrolytes have been used for polymer desolvation, this process is then called salting out method.

5) Polymer Coating Method

In this technique a thin layer of polymer is deposited on the nanoparticle surface. This can be done by adsorbing the polymer onto preformed uncoated nanocapsules. Another method proposed for synthesis of nanocapsules includes, firstly the preparation of nanoemulsion template, followed by coating it by polymer deposition on the W/O nanoemulsion surface. The polymer is added in the continuous phase and their precipitation onto the nanoemulsion droplets is mainly triggered by solvent evaporation. Polymers which can be used in this process are poly(methyl methacrylate)(PMMA), and poly methacrylate(PMA). Nanocapsule formation is based on engulfment in a three phase system. Salmon calcitonin has been encapsulated via this method using chitosan and PEG chitosan [25].

Drug encapsulation efficiency was found to be maximum (approximately 80%) in nanoprecipitation and emulsion diffusion methods. The factors that govern this outcome in both the methods are active chemical nature of the drug and its polarity. Hydrophilic drugs reach a maximum of 10% incorporation whereas lipophilic compounds have a major encapsulation efficiency of more than 70% [26, 27]. Double emulsification technique had encapsulation ranges from 65%-75%, this outcome being affected by both

the polymers used and the surfactant used. Polymer coating method has encapsulation efficiency between the ranges of nanoprecipitation and double emulsification method depending on the method used for nanocapsule template formation. For emulsion coacervation method the particle surface shows presence of holes which might cause the drug to leak and hence it has the lowest encapsulation efficiency. Also this technique requires an extensive purification process which serves as a major disadvantage when compared to other alternatives. It can be seen there is no ideal method for synthesis of nanocapsules, and the choice of a specific method depends on the physicochemical characteristics of the drug, particularly its solubility and objective of administration.

1.1.2 Applications

Nanocapsules when used as drug carriers can mask unpleasant taste, provide controlled release properties and protect the drug from degradation [28-30]. Nanocapsules have been proposed for administration by various routes such as oral, rectal, ocular, and parenteral administration. Triclosan, ethionamide and Indomethacin are a few drugs which have been loaded into nanocapsules for drug delivery [31]. The nanoprecipitation method is one of the most frequently used methods and is known for its low cost, simple procedure, reproducibility and high encapsulation efficiency [11].

1.2 Nanospheres

Nanospheres are matrix systems in which the drug is physically and uniformly distributed, therefore it is present throughout the whole particle [32]. These particles can be amorphous or crystalline in nature. Nanospheres derived from tyrosine serve as potential delivery systems for a wide range of lipophilic drugs. They form strong and

stable complexes with lipophilic substances, including the anticancer drug paclitaxel [33]. They are reported to have skin enhancement properties. Nanospheres are ideal candidates for sustained intracellular delivery of genes and other therapeutic agents, because when the particle size range is under a 100nm, eukaryotic cell can engulf these particles. Particle size of nanospheres is important because opsonization and subsequent recognition and phagocytosis by macrophages depends on it. Some advantages of these drug carrier systems include: demonstration of controlled release property, longer duration in bloodstream when they avoid rapid clearance by phagocytosis, and site specific targeting when ligands are attached to the surface of spheres [33].

1.2.1 Preparation of Nanospheres

There are various methods for preparing, four of which are listed below,

1) Polymerization method (emulsification polymerization)

In this method polymeric compounds such as polymethylmethacrylate and polyethylcyanoacrylate are used. Droplets of water-insoluble monomers are emulsified in an aqueous phase. Anionic polymerization takes place in micelles after monomer molecules have diffused through the water phase. This process is initiated by water itself. Drugs can be incorporated into the system after dissolution in the polymerization medium either before the introduction of the monomer or after its polymerization. This technique depends on the pH of the medium as it governs the polymerization rate and adsorption of drug when it is in its ionized state. Advantages of this technique include reproducibility regarding size and drug adsorption rate [34].

2) Preparation of nanospheres from preformed polymers

Grundy et al and Krause et al developed a method for formation of nanospheres, that involves dissolution of polyester in an organic volatile solvent such as chloroform that is miscible with water. This organic solvent is then dispersed in aqueous phase to form an O/W emulsion. Colloidal particles are formed as a result of continuous emulsification under mixing which also prevents coalescence of organic droplets and allows spontaneous evaporation of the solvent at room temperature. Any residual organic solvent can be removed under low pressure [34]. In this technique not only synthetic, but also natural biocompatible polymers such as chitosan, alginate, and gelatin can be used [35]. However an important limitation of this method is the difficulty to obtain the desired, uniform size and homogeneity of the droplets containing the polymer.

3) Solvent displacement method

This is a low energy method. Biodegradable polymers and polyesters can be used in the preparation of nanospheres by this method with the help of PEG [36, 37]. In this technique the polymer is dissolved in an organic water miscible solvent. This is then added into the aqueous phase in the presence or absence of a surfactant. Organic solvent diffuses rapidly from the oil phase to the aqueous phase precipitating polymers leading to the formation of nanospheres [35].

1.2.2 Applications of Nanospheres

Nanospheres can be used in targeted drug delivery to tumors, delivery to the brain, gene delivery, epithelial cells, and as oral drug delivery system etc. Nanospheres can deliver the highest dose of drugs to tumor targets through permeability enhancing and retention

effect. Interaction of nanospheres with specific receptor mediated transport system in BBB facilitate their easy passage across the brain e.g. Polysorbate 80/LDL.

1.3 Other types of Nanoparticles

Depending on the composition and shape, nanoparticles can also be classified as:-

1) Inorganic nanoparticles- are mainly ceramic nanoparticles, typically composed of inorganic compounds such as silica or alumina. The nanomaterial core is made up of metals, metal oxides and metal sulphides.

2) Polymeric Nanoparticles- are mainly made up of biodegradable and biocompatible polymers made from gelatin, chitosan, PLGA, PLA, PMMA, poly(butyl)cyanoacrylate etc..

3) Solid Lipid Nanoparticles- are mainly lipid based submicron colloidal carriers, having a large amount of surfactants

4) Liposomes-are concentric bilayered vesicles which are surrounded by a phospholipid membrane.

5) Nanocrystals-are aggregates of molecules that can be combined in the crystalline form of the drug surrounded by a thin coating of the surfactants

6) Nanotubes- are self assembling sheet of atoms arranged in tubes.

7) Dendrimers –are polymer based macromolecules formed from monomeric or oligomer units, such that each layer of branching unit doubles or triples the number of peripheral groups [37].

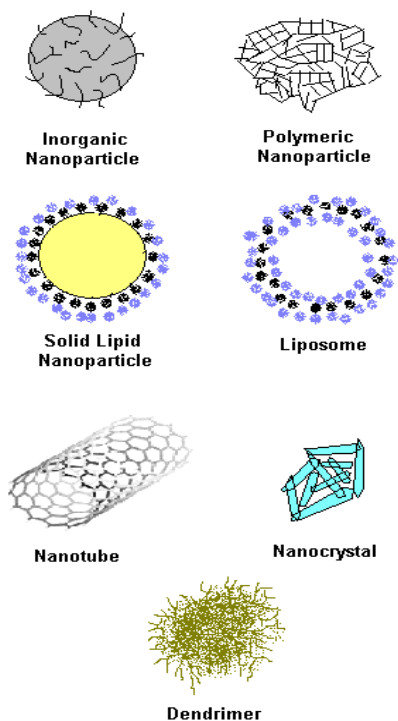


Figure 1-3-Variety types of nanoparticles used in research and drug delivery [37]

1.4 Polymeric nanoparticles

In recent years, biodegradable polymeric nanoparticles have gained considerable importance as potential drug delivery systems in view of their applications in drug targeting to particular organs/tissues, as carriers of DNA in gene therapy, and their ability to deliver proteins, peptides and genes. In spite of development of various synthetic and semi synthetic polymers, natural polymers still enjoy their popularity in drug delivery, Some common examples include gums (Acacia, Guar etc), chitosan, gelatin, sodium alginate, albumin [38].

1.4.1 Physical Properties of Natural polymers

Chitosan-It is a nontoxic, biodegradable polymer obtained from hydrolysis of chitin, a natural polysaccharide that is a chief constituent of crustacean exoskeleton. Unmodified chitosan is soluble in acidic media and has significant muco-adhesive properties. The size of chitosan nanoparticles depends on its molecular weight, concentration and surface charge.

Gelatin- it is obtained by either alkaline or acidic hydrolysis of collagen. It has a triple helical structure with a high content of glycine, proline, and hydroxy proline residues. Gelatin obtained from alkaline treatment of collagen has more carboxyl groups and a lower isoelectric point than that derived from acidic hydrolysis.

Sodium alginate-Alginates are primarily derived from algae *Macrocystis pyrifera* and *Laminaria hyperborea*. These are linear, unbranched polymers containing β -(1-4) linked mannuronic acid and α -(1-4)- linked guluronic acid residues that are arranged in blocks. These blocks are commonly called as G blocks or M blocks, can also alternate with each other. Alginates are hydrophilic anionic polymers and vary in molecular weight, depending primarily on G and M blocks. Ionotropic gelation results when the anionic alginate reacts with cationic ions or molecules. The inability of alginate polymer to re-swell in an acidic environment helps incorporation of acidic drugs into the bead and thus protect from gastric juice [39].

Albumin- Human serum albumin is the most abundant plasma protein, positively charged, multifunctional, and is involved in transportation, ligand binding and enzymatic

activities. Albumin is a globular protein containing approximately 585 amino acids and possesses an α - helical tertiary structure.

1.5 Characterization of Nanoparticles

Fine tuning of different nanoparticle properties are needed in order make them suitable for specific applications. In all these applications, particle size determination is of utmost importance, since it not only determines the efficacy of these products but also affect adverse effects [40]. Many tools based on different physical principles are currently available to measure particles smaller than 1 μm . One of these techniques is Light Scattering (LS) also called as photon correlation spectroscopy. It is a rapid method for determining the mean size, size distribution and polydispersity index (PI) of a sample. This technique is well adapted for routine measures. Electron microscopes use electromagnetic radiations of shorter wavelength and provides magnification that can disclose details with a resolution of up to about 0.1 nm [40]. Scanning Electron Microscopy (SEM) allows for a resolution between 3 and 5 nm and even 1 nm in some advanced microscopes [41]. Images of the sample are taken hence is it possible to study the external morphology of the particles along with determination of size. Nanoparticles prepared using different methods can produce particles of various morphologies. The internal morphology of substances as well as particle size can be measured using Transmission Electron Microscopy (TEM). TEM and high resolution TEM are more powerful than SEM in providing details at the atomic level and can yield information regarding the crystal structure, quality and grain size. Potentiometric techniques are used

to characterize phenomenon of counter condensation, the nature of interactions with oppositely charged surfactant molecules as well as stoichiometry of polyelectrolyte complexes. Zeta Potential measurements are useful to detect the absorption of polyelectrolytes on the particle surface.

1.6 Techniques used for modification of surface properties of nanoparticles

Surface properties of nanoparticles can be modified to module and achieve specific physical, optical, electronic, chemical, and biomedical properties by coating the particles with a thin layer of film of suitable polymer. This can be done by various conventional methods which include both wet and dry approaches.

Dry methods include:-

- 1) Physical vapor deposition
- 2) Plasma Treatment
- 3) Chemical Vapor Deposition
- 4) Pyrolysis of organic or non polymeric materials

Wet Methods include:-

- 1) Sol Gel process
- 2) Emulsification and solvent evaporation technique [42]

1.7 Therapeutic Applications of Nanoparticles

Potential use of nanoparticles arises from the reality that they have a high surface to mass ratio, unique quantum properties, and their ability to adsorb and carry biologically active compounds. The fact that nanoparticles exist in the same size domain as proteins makes them suitable for bio tagging or labeling. Hence they can be used as biomarkers for

diagnostic purposes. Antibody conjugated, hydrophilic, magnetic nanocrystals as smart nanoprobes are used for detection of breast cancer via Magnetic Resonance Imaging (MRI). The idea behind using nanoparticles for tumor targeting is based on the ability of these particles to deliver a concentrate dose of the drug in the vicinity of the tumor targets via enhanced permeability and retention effect or active targeting by ligands on the surface of nanoparticles. Kreuter et al. have described the application of covalently bonded apolipoprotein A-I and apolipoprotein B-100 to albumin nanoparticles, enabling these to deliver the drug to brain. Liposomes have been extensively used as drug delivery devices to target various tissues. It has been found that certain drugs including anti tumor and anti bacterial drugs entrapped in liposomes could alter their distribution and pharmacokinetics [43-45]. A PEGylated form of liposomally encapsulated doxorubicin is routinely used for the treatment of metastatic cancer [46]. Injectable nanovectors are used for cancer therapy, especially when multiple-drug therapy is used. They include fusion proteins, immunotoxins/polymers, dendrimers, polymer drug conjugates, polymeric micelles, polymersomes and liposomes. Poly (butyl cyanoacrylate) nanoparticles coated with polysorbate80, patented by Galeperina (Russia) show efficient brain targeting as drug delivery system crossing the blood brain barrier for delivery of doxorubicin. Lipid nanoparticles are used for topical delivery of compounds for treatment of psoriasis. Results show that these particles minimize the permeation differentiation between normal and hyper proliferative skin compared with that of free drug in aqueous control [46]. Lee et al used poly(lactide)-tocopheryl-polyethylene glycol succinate (PLA-TPGS) co polymers to deliver proteins, using BSA as a model drug. Chet al showed that

the size of nanoparticles affects the biodistribution of targeted and non-targeted particles in an organ specific manner [46].

Nanoparticles made from chitosan serve as promising alternatives for delivery of proteins, peptides and nucleic acid by the mucosal route, including oral and nasal administration and for topical delivery of drugs in the eye. This system is stable in the mucosal environment, including harsh conditions of the gastrointestinal tract. Oral absorption of salmon calcitonin was improved when it was associated with chitosan nanoparticles. Several polysaccharides can form complex with nucleic acids. Alginate nanoparticles are known to improve the stability of oligonucleotides, indicating the carrier system may be suitable for *in vivo* delivery of antisense oligonucleotide. Alginate nanoparticles are known to improve the efficiency of antituberculosics, thereby serving as potential candidates for intermittent therapy of tuberculosis [47]. Some commercially available nanospheres such as melamine (polymethylenemelamine) can be stored indefinitely and are easily resuspended in water.

1.8 Other uses of Nanoparticles

Surface recognition of biomacromolecules by nanoparticles provides a potential route for controlling cellular and extracellular processes of numerous biological processes such as transcription regulation, enzymatic inhibition and sensing. Due to their small particle size nanoparticles are utilized in cosmetics as well, an example includes titanium dioxide in sunscreen which exists as a transparent solution. Molecular forms of food can be manipulated with nanotechnology to provide enhanced functionality, lower costs and more sustainability.

Chapter 2

Instrumentation

2.1 Introduction

In this study nanoparticles were characterized using optical microscopy, dynamic light scattering, zeta potential, scanning electron microscopy, transmission electron microscopy, differential scanning calorimetry, gel electrophoresis and confocal laser scanning microscopy.

2.2 Dynamic Light Scattering

2.2.1 Introduction

Dynamic Light Scattering (DLS) also called as quasi elastic light scattering (QELS) or photon correlation spectroscopy (PCS) has been established as a popular tool for determining particle size. The DLS instrument can detect particles in the size range from 3 nanometer to several microns [48, 49]. Depending on the type of instrument used this technique may be used for measurements of sub micron particle sizes, diffusion coefficients, viscosities, and polymer molecular weights [50].

2.2.2 Principle

The DLS instrument works on the principle that when a beam of light passes through a colloidal dispersion, the particles or droplets scatter some of the incident light in all directions. If the light is monochromatic, time dependent fluctuations in the intensity can be seen. This is because the particles in a liquid medium undergo brownian motion which causes fluctuations in the local concentration of particles that produces local inhomogeneities of the refractive index leading to variations in the intensity of the scattered light.

Particles radius can be calculated using Stokes-Einstein relation,

$$D = kT / 6\pi\eta R$$

where,

D= diffusion coefficient of particles

k= Boltzmann's Constant (1.38×10^{-16} erg K⁻¹)

T= temperature in Kelvin

η = shear viscosity of the solvent (eg $\eta = 1.002 \times 10^{-2}$ poise for water at 20°C)

From the above equation we see that the rate, at which particles move in the medium as measured by D, is inversely related to the particle radius R.

2.2.3 Instrumentation

The diagram of a DLS module is shown below, the scattering angle for which is set at 90°. For this technique light from a laser is focused onto a glass tube containing a dilute

suspension of particles, each of these particles is illuminated by the incident laser beam which in turn scatters light in all directions. The intensity of incident light scattered by a single, isolated particle depends on its molecular weight, overall size, shape and the difference in refractive indices of particle and surrounding solvent [50].

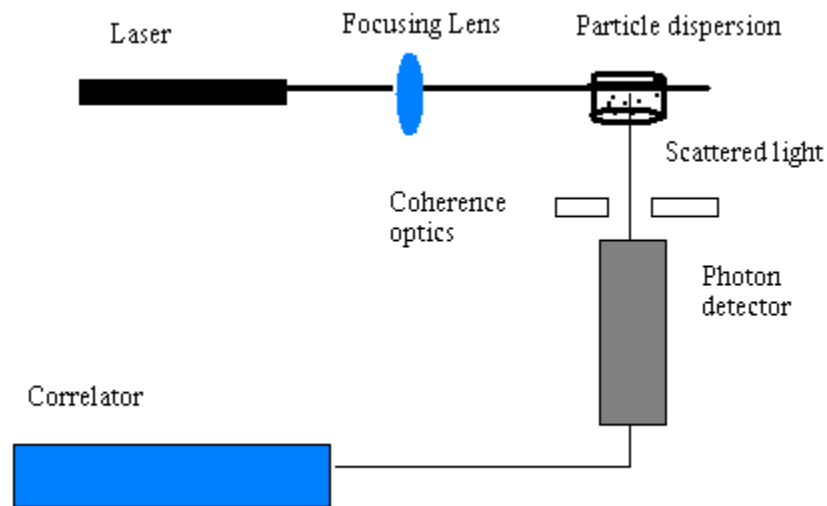


Figure-2-1 Schematic diagram of Dynamic light scattering [51]

Some advantages of this technique include precision of $\pm 1\%$, short duration of testing, reliable and repeatable analysis, and no need of sample preparation except dilution [52-54]

2.3 Zeta Potential

2.3.1 Introduction

Zeta potential measures the electrophoretic mobility and stability of charged particles in a liquid medium. It is a predictor of the repulsive forces being exerted by the suspended particles and is directly related to the stability of the colloidal system [51].

2.3.2 Basic Principle and Instrumentation

When a particle is present in the aqueous phase it acquires surface charge mainly by either ionization of surface groups or by adsorption of ions. These surface charges thereby modify the distribution of other surrounding ions resulting in the formation of a layer around the particle that is different from the bulk solution. Hence when the particle moves in the dispersed phase, this layer moves as a part of the particle [55-58]. Zeta potential can be defined as the potential difference between the dispersion medium and this stationary layer of fluid attached to the particle [59-64]. It is measured by applying an electric field across the dispersion. Particles with a particular zeta potential will migrate towards an electrode of opposite charge with a velocity proportional to the magnitude of charge [51].

Factors that affect zeta potential are:

1) pH

In an aqueous media pH of the solution is an important factor determining the zeta potential of the sample. It is seen that in an alkaline suspension, particles tend to possess a negative charge. If acid is added to this suspension, then a point is reached where the

charge will be neutralized. This point is called as the isoelectric point and is very important from a practical consideration. The system has least stability at this point. Further addition of acid will build up a further negative charge.

2) Conductivity

Ions present in the solution affect the thickness of the double layer referred to in the earlier section. Ions with more ionic strength and higher valency compress the double layer to a larger extent.

3) Concentration of a formulation component

This information helps in developing product with maximum stability. For e.g. while increasing the electrolyte concentration, surface charge is compensated at a lower distance from the particle surface, thereby reducing the zeta potential [55].

2.4 Scanning Electron Microscopy (SEM)

2.4.1. Introduction

It is one of the most versatile techniques applied to study particle size and surface morphology [65]. Scanning electron microscopy helps in observation and characterization of heterogeneous organic and inorganic materials of nanometer (nm) or micrometer (μm) scale [66]. The broad usage of this technique stems from its ability to obtain three dimensional images of the surface of a wide variety of materials.

2.4.2 Basic principles and Instrumentation

In SEM the area to be investigated is irradiated with a finely focused electron beam which helps in taking images of the field of view of the sample. The types of signals

produced from interaction of the incident electron beam with the sample include secondary electrons, backscattered electrons, characteristic x-rays and other photons of different energy. Signals of interest in imaging are secondary electrons and backscattered electrons because their properties vary primarily due to differences in surface topography of the sample [66]. These electron beams allow data to be collected over a selected area or can be swept throughout the surface of the specimen. The signals which are derived from the electron-sample interaction reveal information about the sample including external morphology, chemical composition, crystalline structure and orientation of the materials [58, 67, 68].

The microscope mainly comprises of two main parts 1) electron column and 2) electron detector.

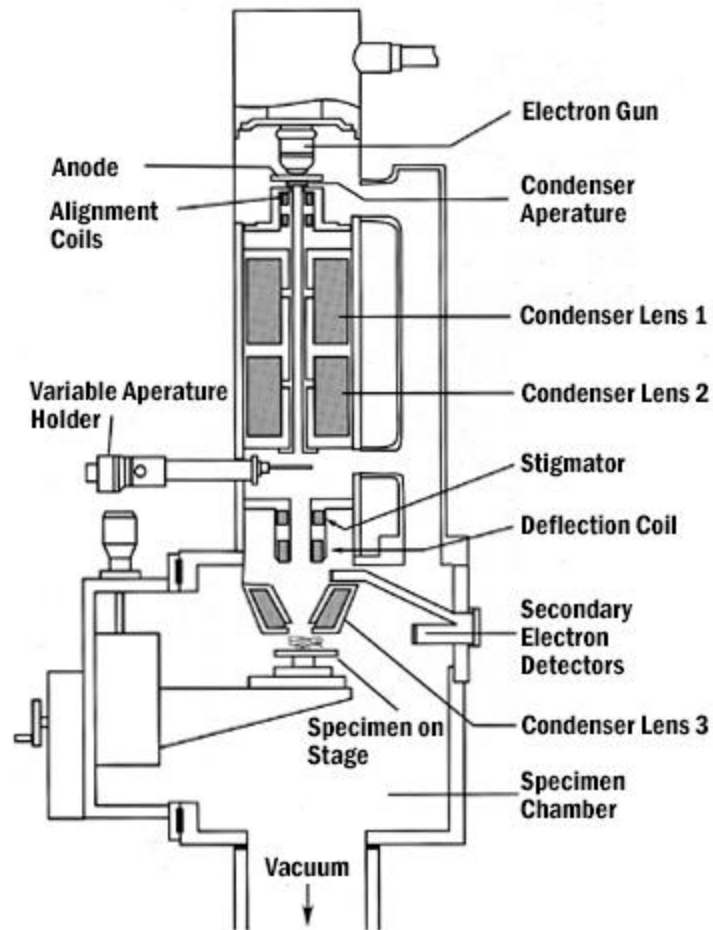


Figure -2-2 Schematic diagram of Scanning electron microscopy [69]

An electron beam is generated within the electron column, collimated by the condenser lenses and focused by objective lenses. The resultant secondary and backscattered electrons from the specimen are collected by the detector which forms the specimen image in the microscope [70]. Advantages of this technique is that it provides a variety of modes of imaging, excellent spatial resolution, modest requirement of sample preparation and condition, and straightforward interpretation of obtained images [71].

2.5. Transmission Electron Microscopy (TEM)

2.5.1. Introduction

Transmission electron microscopy utilizes electrons instead of light to magnify objects at very fine resolutions. TEM allows magnification of 100,000x and resolution limits in the nanometer range which is much beyond the limits of light microscope. One can see objects as small as 5000 angstroms. It is used for analysis in metallurgy [72], biological sciences, virology [73], cancer research, and semi conductor research [74].

2.5.2. Basic principle and instrumentation

In conventional TEM the sample is irradiated with an electron beam of uniform current density [70]. The electron beam interacts with the specimen as it passes through it. Only those electrons that pass the sample unobstructed, hit the screen on the other side .At this point electrons are converted to light and an image is formed [71].

TEM has three components 1) Electron gun and the condenser system, wherein electron beams are produced and focused on the object, 2) Image forming assembly, consisting of the lens system and stage which help in focusing the electrons passing through the sample to form a magnified image, and 3) Image recording system, this converts the image formed by the earlier assembly into some form such that it is perceptible to the human eye. In addition to the above components a set of vacuum system, pumps and power supplies are necessary [71].

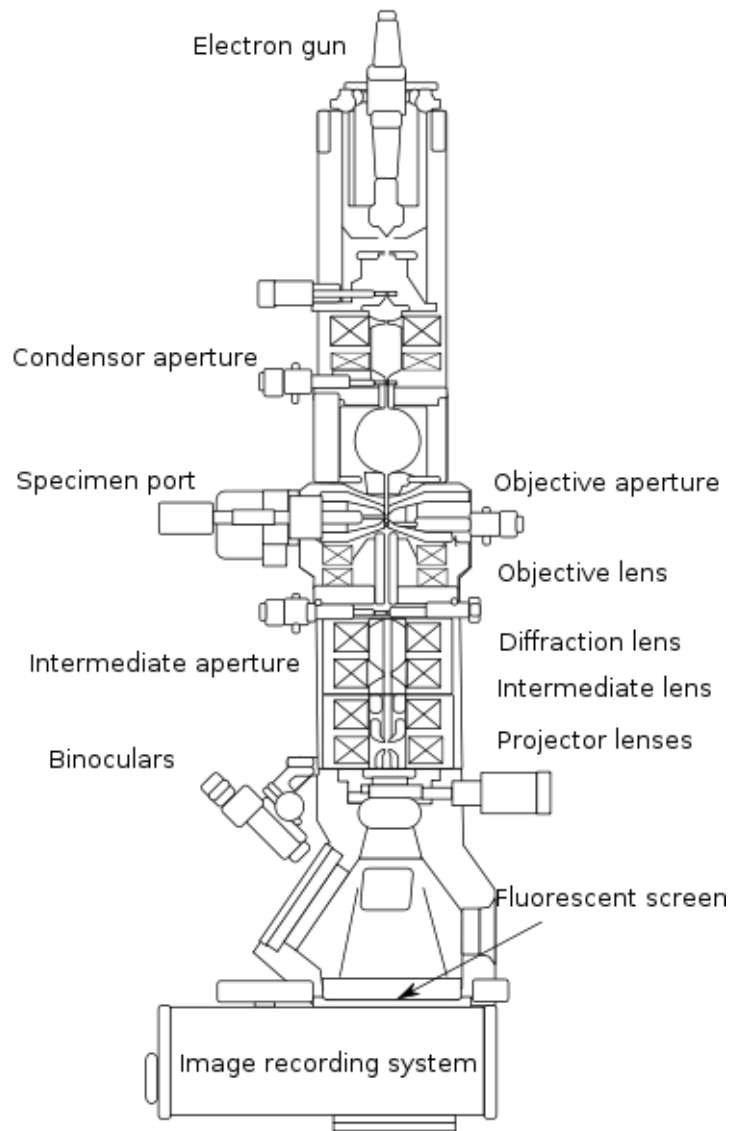


Figure-2-3 Schematic diagram of Transmission electron microscopy [75]

Electrons strongly interact with atoms via elastic and inelastic scattering. The specimen must therefore be very thin, typically of the order of 5-100nm. The image produced can be recorded by direct photographic exposure of the sample inside vacuum or digitally via a fluorescent screen coupled by a fiber optic plate to a CCD camera.

The dark areas of the image correspond to areas where fewer electrons were able to pass through and lighter areas indicate where more electrons did pass through. This variation allows the user to see differences in structures and gradients [63].

The advantages associated with this technique include greater magnification, enhanced depth of field, and finer magnification control.

2.6 Differential Scanning Calorimetry (DSC)

2.6.1 Introduction

Calorimetry has long been established as an important tool for relatively high temperature studies of pure materials and compounds to accelerate any degradation or degradations [72]. Using thermal analysis, it is possible to understand what is occurring in a material, even if there is no visual evidence that a change has occurred [76]. In biological sciences DSC has been used for studying drug-membrane interactions [76], protein denaturation, protein -DNA interaction, and stability studies [77].

2.6.2 Basic Principle and instrumentation

DSC is widely used to study thermal transitions i.e. solid-solid transitions, solid –liquid transitions, and various other transitions and reactions. It directly measures changes in heat flow that occurs within the sample molecule during controlled increase or decrease in temperature, making it an important analytical tool for studying materials in their native states [72].

The two basic types of DSC's are-

- 1) Heat flux DSC
- 2) Power Compensation DSC

The characteristic feature of all DSC measuring systems is a twin pan design which is isolated from the ambient environment in a chamber. One pan contains the sample to be analyzed and the other pan is empty and is used as a reference. Both these pans are placed on a plate which is connected to a thermocouple. The instrumentation is designed to ensure that the pans are heated at the same rate. However the heat flow i.e. the rate at which thermal energy is supplied to pans does differ, owing to the fact that there is material in one on them while the other one is empty. In proteins DSC measures the enthalpy (ΔH) of unfolding due to heat denaturation. A computer keeps track of the starting temperature, heating rate, and heat flow and also records the difference in heat flow between the sample and reference pan.

Owing to new materials and sensors, calorimetric instruments allows precise measurement to be carried out with high sensitivity and reproducibility. Also their operation is relatively simple and automated.

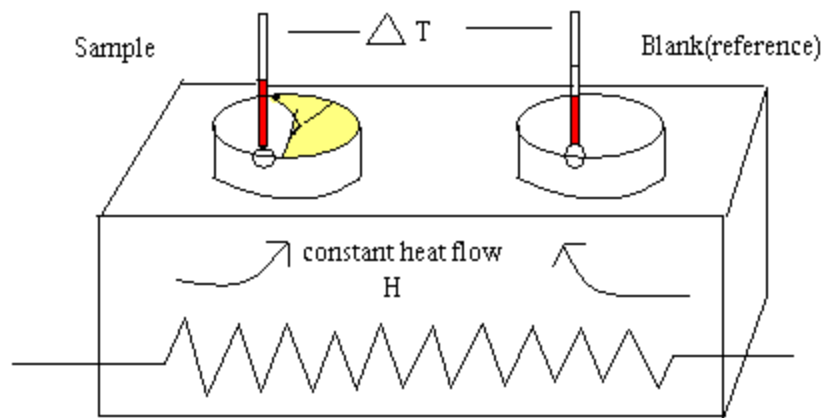


Figure2-4: Schematic representation of a Differential Scanning Calorimeter [78].

The heat flow may be measured exothermally or endothermally and is plotted against temperature. In the figure below a typical DSC isotherm demonstrating various thermal events are shown. Glass transition temperature is characterized by a dip in the curve. The point of recrystallization and melting point give rise to an exothermic and endothermic peaks respectively [77].

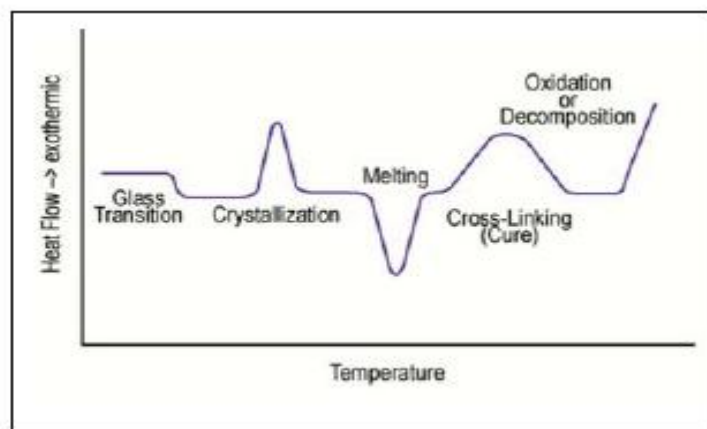


Figure 2-5: Representation of a DSC curve [78]

DSC has been used to determine the characteristic relaxation times below and above the glass transition temperature for various compounds such as ketoconazole, indomethacin etc [76]. Some other applications include identification of materials. When similar DSC profiles are obtained for two samples it may suggest similarity between the tested materials [77].

2.7 Sodium Dodecyl Sulphate –Poly Acryl amide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a method of separating proteins within a sample for analysis and molecular weight determination [79]. Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine relative abundance of proteins in a sample and to determine distribution of proteins among fractions

2.7.1 Basic principle and Instrumentation

This technique involves the use of a process based on separation of molecules in an electric field called electrophoresis. In this technique the molecules are rendered monomeric by boiling in the presence of reducing agents and a negatively charged surfactant SDS [79]. SDS is an anionic detergent that when dissolved ionizes into molecules that possess a net negative charge within a wide pH range. SDS converts the polypeptide into monomers and binds to them depending on their mass [80]. Acrylamide is polymerized with a crosslinker, bisacrylamide, to form a matrix through which the protein monomers must pass as they move in an electric field. The percentage of acrylamide used determines the size of monomers to be separated.

The charged ions or molecules migrate towards the electrode of opposite sign under the influence of an externally applied electric field. The movement of particles is retarded by interactions with the surrounding gel matrix, which act as molecular sieve. The opposing interactions of electrical force and molecular sieving results in differential rates of separation for the constituent samples [79]. Higher molecular weight samples are retained by the gel when compared to smaller ones. Coomassie blue-SDS PAGE helps in direct visualization of separated proteins and peptides during electrophoresis by staining the sample with the dye Coomassie blue. The need for fixation is avoided and recovery is almost quantitative [81].

In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis.

2.8 Confocal Laser Scanning Microscope

Confocal Laser Scanning Microscopy is a technique used for obtaining high resolution images with depth selectivity [82]. The most important feature of this instrument is its capability of isolating and collecting a plane of focus from within the sample. This technique can be used for surface profiling of opaque samples as well as interior imaging of transparent specimens [82].

2.8.1 Basic principle and Instrumentation

In a conventional microscope images are obtained as far as light can penetrate, whereas in confocal laser microscope images from only one depth level at a time are obtained.

The microscope consists of a point source of light that is used to probe a single point on the specimen. A scanning optical microscope does not form two-dimensional optical images of the sample as is seen in a conventional microscope. Instead a conical illuminating beam of light focused to a point by a conventional objective lens is scanned through the sample [83]. The strength of the reflected light from the single point on the specimen is then measured via a pinhole detector [84]. A non imaging light detector such as a photomultiplier tube (PMT) is used to measure the interaction with each point in the object of the illuminating probe as it is scanned through a specimen. Usually a computer is used to control the sequential scanning of the sample and to assemble the image for display onto a video monitor [83].

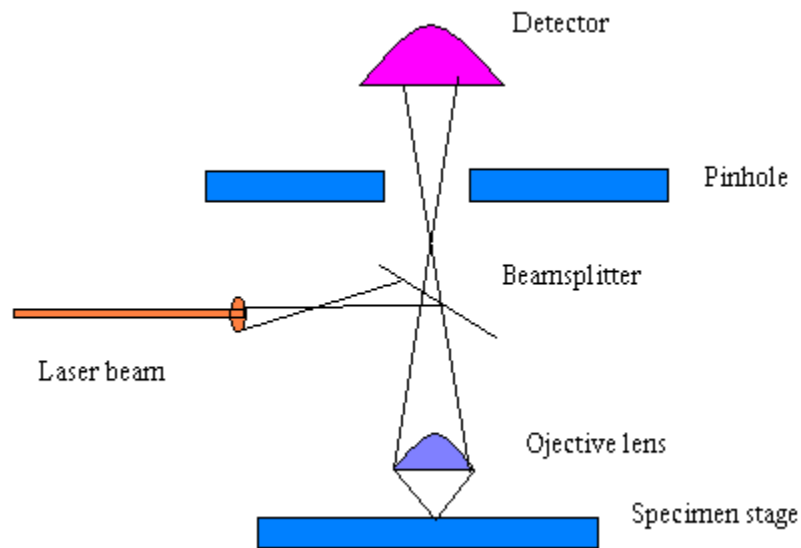


Figure 2-6: Principle of Confocal Scanning Laser Microscope [85]

When set up correctly confocal microscope will always give a better image than can be obtained with an epifluorescence microscope. It is helpful in constructing three dimensional fluorescent images from a series of sections, for the use of quantitative fluorescence indicators or for mapping of fluorescent markers of cell surface on non planar surfaces [86]. This technique also provides exceptional capabilities for spatially resolved photochemistry, particularly photolytic release of caged effector molecules [86]. A unique advantage of this technique is its capability to obtain three dimensional localization of fluorescently labeled compounds along (x,y,z) spatial dimensions [87].

Chapter 3

Materials and Method

3.1 Materials

3.1.1 Sodium Alginate

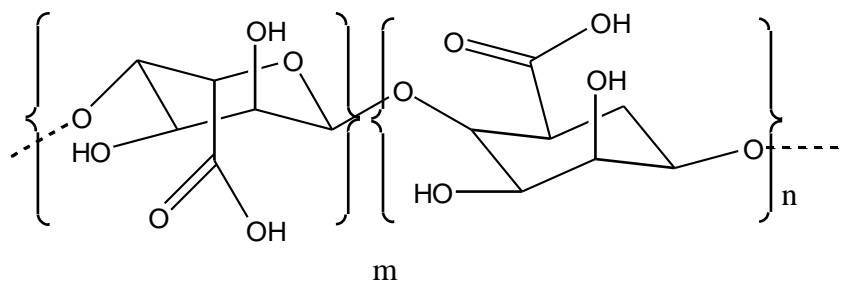


Figure 3-1 Chemical Structure of Alginic Acid.

Source: Fisher Scientific, Pittsburgh, PA; Lot No: 89H0178; CAS No-9005-38-3

Molecular Formula: $(C_6H_7O_6)_n Na$

Molecular Weight: N/A

Physical state: Odorless, Yellowish white, fine powder.

Viscosity of 2% solution (25°C): Approximately 250cps

Solubility: Sodium alginate dissolves in water, forming a viscous, colloidal solution [88]. It is insoluble in alcohol or in hydroalcoholic solutions in which the alcohol content is greater than approximately 30% by weight [89].

Sodium alginate, the purified carbohydrate product obtained from *Macrocystis pyrifera* (kelp) a brown algae [90] by use of dilute alkali consist chiefly of the sodium salt of alginic acid, a polyuronic acid composed of beta -D- mannuronic acid residues linked so that the carboxyl group of each unit is free while the aldehyde group is shielded by a glycosidic linkage [89].

Uses, Toxicity

The use of Alginates is mainly based on its three properties; to form a gel, to form a thick solution and lastly its ability to form films. Sodium alginate fibers are use to make bandages for large wounds. It is also used as an emulsifying agent and thickening agent in food industry. They are also used as an additive in the treatment of skin diseases [91].

3.1.2 Calcium Chloride

Chemical Structure



Source: Fisher Scientific Mfg. Corp, Lot No. 124 H06086; CAS # 10035-04-8

Molecular Formula: CaCl₂

Molecular Weight: 111.00 g/mol

Physical State: White or light grey, solid, odorless, hygroscopic powder with a characteristic salty taste [92].

Melting point: 176 °C

Solubility: 59.5 grams/100ml of water [93], freely soluble in water with heat liberation, soluble in alcohol.

Calcium chloride is slightly basic in nature (pH 8-9 in aqueous solution). The compound is stable under normal conditions of storage, but does pick up moisture from air and goes in the solution form [89].

Therapeutic Use

Calcium chloride is a firming agent and also used in electrolyte depletion. It is used to treat hypocalcemia, hyperkalemia, and as a treatment adjunct in cardiac arrest and in magnesium poisoning [94].

3.1.3 Dioctyl Sodium Sulphosuccinate (DOSS)

Chemical Name: Sodium 1,4-bis(2-ethylhexyl) sulfosuccinate, Dioctyl sodium sulphosuccinate (DOSS).

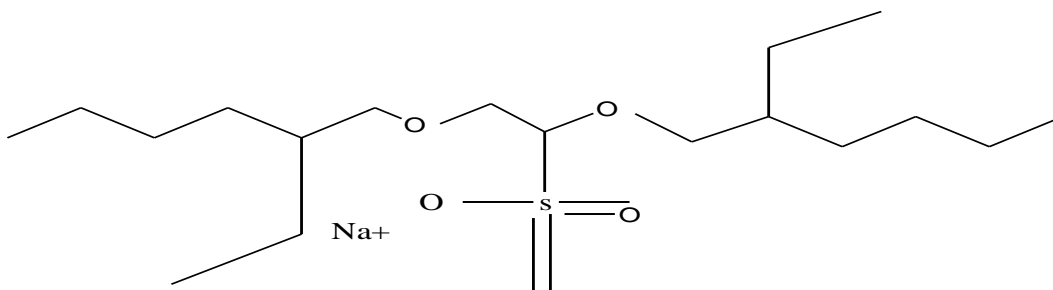


Figure3-2 Chemical Structure of DOSS

Source: Sigma-Aldrich, St Louis, MO. Lot # 092162 CAS # 577-11-7

Molecular formula: C₂₀H₃₇NaO₇S

Molecular weight: 444.56 g/mol

Physical Appearance: DOSS/Aerosol AOT is a white wax like, plastic solid with a characteristic odor suggestive of octyl alcohol [95].

Solubility: 1 g slowly in about 70ml water, freely soluble in alcohol or glycerin.

Melting point: 173-179°C

Uses, Toxicity and Contraindications

DOSS is mainly used as a surface active agent, often used to soften stools in impaction associated with megacolon, anal fissures, and postoperative anal atresia [96]. It is also useful for constipation in geriatric, pediatric, and obstetric patients. As a Pharmaceutical aid, it is used as an emulsifying, wetting and dispersing agent in the formulations for external use [97].

DOSS is contraindicated in patients with appendicitis, acute abdomen or ileus. Possibly the side effects include mild stomach pain and diarrhea. Several allergic reactions can occur with drug [98].

3.1.4 Bovine Serum Albumin

Source: Sigma Aldrich, St Louis, MO. Lot # 096555 CAS # 9048-46-8

Molecular weight: Approx 68,000 Da.

Physical Appearance: BSA appears as pale white flakes, and has a characteristic odor.

Solubility: It is readily soluble in water; its solution is clear or slightly hazy to amber in color.

Albumins occur as a group of acidic proteins that are found plentifully in the body tissues of mammals and in some plant seeds. The solution stability of albumin is good. BSA is a single polypeptide chain consisting of 583 amino acid residues and carbohydrates. It contains 17 interchain disulphide bridges and 1 sulfhydryl group. If stored at 2-5°C it is stable for at least 2 yrs.

Uses, Toxicity

They are often used as standards for protein and calibration studies [99]. It is used as a carrier protein and stabilizing agent in enzymatic reactions. The function of bovine serum albumin is associated with its lipid binding property [89]. It is also often used in cell regeneration studies [100]. Large amounts of circulating proteins such as albumin in the body can give rise to serum sickness which is an immune complex mediated glomerular injury [101].

3.1.5 Cyclohexane

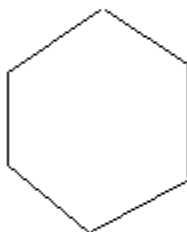


Figure 3-3 Chemical Structure of Cyclohexane

Other Names: Hexahydrobenzene; Hexamethylene; Hexanaphthalene.

Source: Fisher Scientific, Pittsburgh, PA. Lot # 096744; CAS# 110-82-7

Molecular Formula: C_6H_{12}

Molecular Weight: 84.16 g/mol

Physical Appearance: It is a clear, colorless liquid with a characteristic faint chloroform, like odor [102].

Solubility: It is insoluble in water.

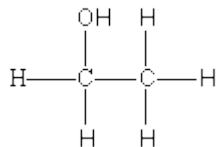
Density: 0.78g/ml

Boiling point: 80.74°C

Uses and Toxicity,

It is used as a non polar solvent in the chemical industry. It is also used as a starting compound in the synthesis of caprolactum and polyamides [103]. It is also used to calibrate DSC when operating in the sub-ambient mode [104]. Cyclohexane is mildly irritating to the eyes and cause unconsciousness when inhaled in large quantities.

3.1.6 Ethanol



Ethanol

Figure 3-4 Chemical Structure of Ethanol

Other Names: Absolute ethanol; Aethanol (German); Alcohol; Algrain; Alkohol.

Source: Fisher scientific, Pittsburgh, PA. Lot # KJC18F CAS # 64-17-5

Molecular weight: 46.07 g/mol

Molecular Formula: C₂H₆O

Physical Appearance: It appears as a volatile, colorless liquid that has a slight odor [105]. **Boiling point** - 78°C

Density: 0.789g cm⁻³

Viscosity: 1.002 mPa

Solubility: Ethanol is miscible in water and many organic solvents such as acetic acid, acetone, benzene, diethyl ether, chloroform, hexane and various other aliphatic hydrocarbons and chlorides [105].

Uses and Toxicity

Ethanol is used as a solvent in a variety of pharmaceutical preparations. In addition properties of ethanol stabilize tertiary and quaternary protein structure[106]. However large amounts of ethanol can cause liver damage, fetal disorders, tremors, dependency and other CNS disorders [107, 108].

3.1.7 Albumin, bovine-fluorescein isothiocyanate

Source: Sigma Aldrich, St Louis, MO. Lot # 080M7400

Molecular Weight: Approximately 66,000 Da.

Molecular Formula: N/A

Physical Properties: It appears as orange to dark orange colored powder, and has slight odor. FITC- BSA is light sensitive and should be stored in dry and dark conditions at 2-8°C.

Solubility: 10mg/ml in 0.01M Tris buffer, pH 7.0.Solubility in water is less than 8% when measured by Karl Fischer method.

Uses and toxicity: FITC conjugates are used to target receptors, to detect protease activity and to measure intracellular pH [109]. They are known to cause irritation to the eyes and respiratory tract.

3.1.8 Chitosan

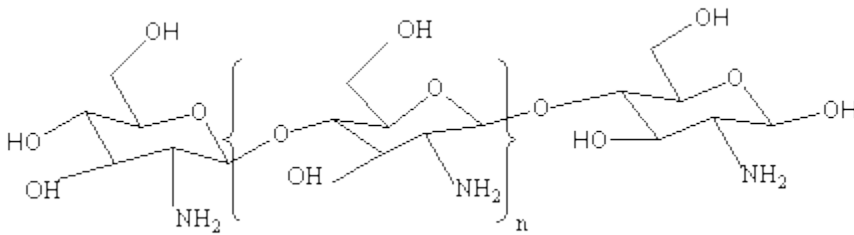


Figure 3-5 Chemical Structure of Chitosan

Other names: Poliglusam, Deacetylchitin, Poly-(D) glucosamine

Source: Polysciences, Inc, Warrington, PA Lot #617922 CAS# 9012-76-4

Molecular Formula: $(C_6H_{11}NO_4)_n$

Molecular Weight: Approx 15,000 Daltons

Physical Properties: Chitosan is a white to pale yellow flake or powder, with no odor and should be stored at room temperature.

Solubility: Soluble in water at low pH and soluble in dilute organic and inorganic acids.

Uses and Toxicity: Chitosan is prepared from chitin. It is essentially a natural water-soluble derivative of cellulose with unique properties. It is used as a flocculant, clarifier,

thickener, fiber source, anticancer agent, wound healing agent, and antimicrobial agent [110].

3.2 Methods

3.2.1 Formulation of Nanoparticles.

The Calcium alginate nanoparticles prepared in this research project was synthesized via a new method developed in our laboratory. The method involves use of microemulsified reactors. The use of microemulsions as templates arises from the idea that, by appropriate design and control of developmental parameters, one can use microemulsions as nanodispersions to produce particles in the nanorange [111]. A novel emulsion interfacial cross linking technique was developed in the laboratory from components regarded safe for use in humans [112]. In brief for this technique two template microemulsions were prepared. Both the systems were w/o microemulsions wherein the oil phase was chosen to be a volatile organic phase. After 24hrs of standing both the microemulsions were mixed giving rise to a resultant third microemulsion which was further processed to extract nanoparticles.

3.2.1. A) Preparation of Nanoparticles

Substances used were:-

- 1) Organic Phase- Cyclohexane, Ethyl Acetate
- 2) Aqueous Phase-
 - a) 0.5% Sodium alginate solution, 0.5 % Calcium chloride solution

b) 1% Sodium alginate Solution, 1% Calcium chloride solution

3) Surfactant- DOSS

Two solutions were made, one containing sodium alginate powder in reverse osmosis water (RO water) as dispersed phase while the other solution containing calcium chloride solution as the dispersed phase. Each of the above mentioned and the following chemicals were measured on a PB303-S/FACT balance (Mettler Toledo). Sodium alginate/calcium chloride solutions were emulsified in 0.2M DOSS/organic phase solution. The mixtures were vortexed for 3 min at 3000rpm and observed for visual clarity. On observation of turbidity the emulsions were homogenized for 15 min which were then allowed to reach equilibrium for 24 hrs. Further addition of water was stopped when the emulsion retained its turbidity even after 24hrs of stability. The same procedure was followed for preparation of calcium chloride/DOSS/organic phase microemulsion. Each of the template microemulsions were vortexed for 3 min at 3000rpm, followed by homogenization for 15mins. The homogenized samples were allowed to equilibrate for 24hrs. The microemulsions were further filtered through a 450 micron filter obtained from Fisher scientific. The calcium chloride microemulsion was then carefully poured into sodium alginate microemulsion. The resultant microemulsion mixture was stirred for 30 min in a homogenizer. The mixture was then poured in a glass beaker with a magnetic stirrer at 125 rpm and the solvent was allowed to evaporate. Following evaporation of cyclohexane, ethanol was added to the solid mass as a separating solvent. The mixture was allowed to mix till all the solid matter comprising of DOSS and nanoparticles was completely dispersed. In order to separate the nanoparticles the solutions were poured

into 15ml plastic capped centrifuge tubes which were then set for centrifugation at 5000rpm at 23°C for 15min. After each cycle the supernatant was discarded and fresh ethanol was added. The ethanol washing/separation process was repeated 3 times. Finally the settled nanoparticles were harvested on a glass petri dish and the remaining alcohol was allowed to evaporate at room temperature.

3.2.1. B) Bovine Serum Albumin (BSA) Formulations.

BSA was incorporated into the nanoparticles as follows. A total of 5 grams of BSA was added to 100ml of 0.5% sodium alginate solution. The solution was stirred for 24 hrs on a magnetic stirrer to ensure complete mixing. An aliquot of this solution was added to 0.2M cyclohexane/DOSS solution. Rest of the procedure was identical to that reported earlier in the nanoparticle preparation section.

3.2.1. C) Preparation of FITC labeled BSA nanoparticles

Calcium alginate nanoparticles were loaded with FITC- BSA to evaluate the drug entrapment pattern. An accurately weighed quantity of FITC-BSA was dispersed in aqueous 0.5% sodium alginate solution and the nanoparticle preparation technique reported earlier in the nanoparticle preparation section was used to synthesize FITC-BSA loaded Calcium alginate nanoparticles

3.2.1 D) Preparation of Chitosan coated Ca-Alginate nanoparticles

Chitosan is a biodegradable that has mucoadhesive properties and is suitable for peroral delivery of peptides [113]. Moreover its addition increases the mechanical strength of

alginate particles [114]. Since chitosan is insoluble in water, glacial acetic acid was used to dissolve it. This solution was diluted with water such that, the final concentration of chitosan is 0.4%. This stock solution was used as the aqueous phase. Microemulsions containing chitosan solution in cyclohexane and DOSS were prepared in the same fashion as mentioned in the previous sections. After 24 hrs of standing, the microemulsion containing Calcium alginate nanoparticles formulated via the procedure reported in earlier sections was added to this chitosan microemulsion. The system was allowed to equilibrate for 15 min in a homogenizer. The homogenized mixtures was then set aside for 15 minutes. The mixture was then filtered through 0.45 μ filter and the nanoparticles were isolated and purified using ethanol by using the procedure reported in earlier sections.

3.2.2 Optical Microscopy

For preliminary evaluation the samples were observed under optical microscope to assess the particle size. An aliquot of the reactor microemulsions, solutions used in the preparation of nanoparticles, and the microemulsion mixture produced after the addition of calcium chloride microemulsion to the sodium alginate containing microemulsion were placed on a slide and observed using a 60X objective on a Nikon model TiU microscope. Each sample was observed and pictures were taken using the photometric Coolsnap EZ 20 MHz monochrome camera attached to the microscope.

3.2.3 Dynamic Light Scattering

The particle size of the microemulsions was determined by using photon correlation spectroscopy (PCS) or dynamic light scattering technique(DLS). Both aqueous 0.5% Na-Alginate/ DOSS/cyclohexane and aqueous 0.5% CaCl₂/ DOSS/cyclohexane microemulsions were placed in 6X50 mm culture tubes (VWR Scientific product) and analyzed in a Nicomp 380 ZLS (Particle Sizing System, Santa Barbara, CA) and particle size determined. Each sample was run in triplicates and the average was reported. The run time was set at 10 min with a channel width of 100μ sec, temperature 23°C and scattering angle 90°.

3.2.4 Zeta Potential

The same DLS instrument was used to measure Zeta Potential but in the electrophoretic light scattering mode (ELS). The harvested nanoparticles were suspended in water and placed in a standard glass cuvette for measurement. The scattering angle set was at -14.06 at a temperature of 23°C. Each sample was run in triplicates and the average of 3 readings was reported.

3.2.5 Scanning Electron Microscopy (SEM)

The particle surface morphology, shape and size were examined using Hitachi S-4800 UHR scanning electron microscope. A concentrated solution of nanoparticles in ethanol was placed on double sided adhesive copper tape. These were placed on stainless steel stubs and the samples were made electrically conductive by sputtering with gold in vacuum for 55 sec at 20mA.

3.2.6 Transmission Electron Microscopy (TEM)

The polymer matrix of individual particles and their shape was visualized using a Hitachi HD-2300A Scanning transmission electron microscope. A dilute drop of nanoparticles in ethanol was placed on a holey copper grid with a mesh size of 300 .The images were obtained when a beam of electrons was transmitted through an ultra thin sample, interacting with the sample as it passes through.

3.2.7 Differential Scanning Calorimetry (DSC)

In order to analyze the thermal behavior of calcium alginate and bovine serum albumin loaded calcium alginate nanoparticles, a differential scanning calorimeter was performed. Samples were placed in aluminum pans which were sealed and subjected to a heating cycle from 10-250°C at the rate of 10°C/min against an empty pan under nitrogen atmosphere. Thermal differences in the sample and reference cell were taken into account by subtracting the scan of an empty pan from each of the sample to obtain a proper baseline. The DSC curves were analyzed using Pyris software supplied with the DSC.

3.2.8 *In Vitro*-Drug Release Study

The drug release profile was studied using Lowry's Method of total protein estimation [115]. Series of dilutions of 0.3gm/ml of bovine serum albumin were prepared in phosphate buffer of pH 7.4.From the above solution five different concentrations of 0.03,0.06,0.09,0.12,0.15mg/ml of BSA in buffer were prepared in glass tubes. For the assay three different solutions were prepared. Reagent A consists of 2gms of sodium

potassium tartarate (tetrahydrate), 100gm sodium carbonate, 500 ml 1N NaOH, made to one liter with water. Reagent B consists of 2gms of sodium potassium tartarate (tetrahydrate), 1gm copper sulphate (pentahydrate), 90 ml H₂O, 10ml 1N NaOH. Reagent C comprised of 1 ml Folin Ciocalteu reagent diluted with 15ml of water. To 1 ml of each of the dilutions prepared and the sample, 0.90 ml of reagent A was added, mixed thoroughly for 1min. Each of these tubes were then incubated for 10 min in a water bath maintained at 50°C, then cooled to room temperature. After cooling, to each tubes 0.1ml reagent B was added, mixed and incubated for 10 min at room temperature, followed by addition of 3ml reagent C to each tube, mixed and again incubated for 10 min at 50°C in a water bath and cooled to room temperature. 120µl of each of the solution were placed in a 96-well plate and all measurements were obtained at 650 nm in a 190 spectra max UV and the results were analyzed using 7500 SDS software. Phosphate buffer was used as blank. The absorbance's were measured and plotted as a function of albumin concentration (mg/ml) to obtain a calibration curve.

3.2.9 SDS –PAGE

The structural integrity of BSA was investigated by sodium dodecyl sulfate-polyacrylamide gel using the method described by Laemmli [116]. In this study 50µg/ml of BSA in water was the standard. A reference standard containing a mixture of proteins of various molecular weights was also run in the PAGE experiments. *In vitro* release experiment was performed by using BSA loaded nanoparticles for 8 hours and an aliquot of the same buffer containing released BSA from this experiment was used as the sample in PAGE. Resolving gel (10%) was prepared using 30% acrylamide, 1% bisacrylamide,

Tris-Cl (pH-8.6), Tris-Cl (pH6.8), 20% SDS, and water. Sample buffer was prepared using Tris-Cl (pH 6.8), SDS, mercaptoethanol, glycerol, and bromothymol blue. To 80µl of the protein containing samples 20µl of sample buffer was added in a centrifuge tube. The mixture was heated at 96°C for 4 mins. The tubes were then immediately placed in ice bath for 2 min followed by centrifugation for a minute at 10,000 rpm. 70 µl of the supernatant were loaded in the well. The gels were checked for any leak and then placed in a case and subjected to a voltage of 75V, 0.03A for 20 min. Electrophoresis was performed in Biorad vertical electrophoresis cells using Powerpac HC version 1.07. Voltage was increased to 100V till the samples completely ran through the gel. Once the proteins ran the whole length of the gel, it was carefully removed from the case and placed on a glass petri plate. Coomassie blue dye was added to the petri dish till the gel was totally immersed. For uniform contact of the dye the petri plate was placed on an orbital shaker. After 15 min the dye solution was removed and a destaining solution comprising of 45% methanol and 10% acetic acid was added to the gel in order to remove the excess dye for 20 min. Multiple washings were made with the destaining solution until the gel background was clear and bands were visible distinctly.

3.2.10 Confocal Laser Scanning Microscopy (CLSM)

a) The aim of the first study was to examine the localization of BSA in the nanoparticle polymer matrix. This study was done in a Leica TCS SP5-multiphoton laser scanning system. A drop of FITC-BSA loaded nanoparticles suspension in ethanol was placed on a glass cover slip. The samples were placed on thin cover slips. A drop of oil was placed on the glass surface of the cover slip directly behind the sample for better imaging. An

excitation wavelength of 488nm and emission wavelength of 514nm was used. All the images were obtained at 63X magnification. Images of drug loaded nanoparticles were obtained and processed using Leica software. The particles were observed individually. The software allowed visualization of the sample along various angles. Also the internal morphology and periphery of the particles could be studied in detail.

b) For the second study chitosan coated particles were observed by CLSM. The purpose of this study was to detect, the presence of chitosan coating on the surface of sodium alginate nanoparticles. Since chitosan was moderately auto fluorescing no external fluorescent marker was necessary. One drop of dilute chitosan coated calcium alginate nanoparticle suspension in ethanol was placed on glass cover slip and images were obtained at 63X magnification. The excitation wavelength was set at 500nm and emission wavelength at 520nm.

Chapter 4

Results and Discussion

4.1 Preparation of calcium alginate nanoparticles

Calcium alginate nanoparticles were synthesized by a new interfacial cross linking technique. In this technique w/o based microemulsions were used as templates for synthesis of solid uniform nanoparticles. The idea underlying development of this method is based on the fact that alginates in presence of a divalent metal ion form a gel like structure.

In molecular terms alginates are a family of unbranched binary copolymers, consisting of homopolymeric regions of mannuronic acid (M) and guluronic acid (G), termed MM and GG interspersed with regions of alternating MG. In solutions alginates behave like flexible coils, however upon interaction with divalent metal ions such as calcium , they form an ordered structure

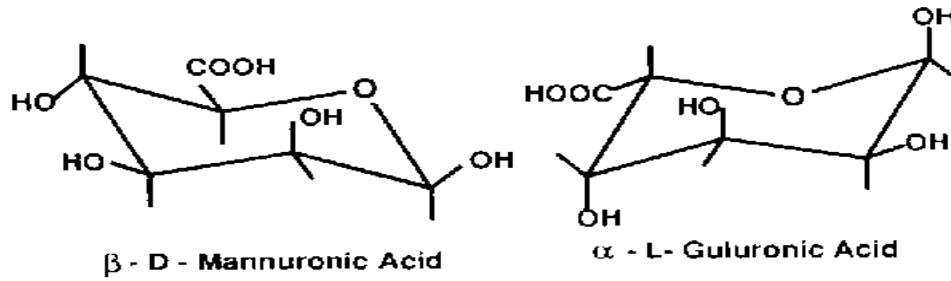


Figure 4-1 Conformational structures of mannuronic acid and guluronic acid respectively .

Because an M block is formed from equatorial groups at C-1 and C-4, it is a relatively straight polymer, like a flat ribbon. However G block is formed from axial groups at both C-1 and C-4 so the resulting chain is buckled. The gel strength therefore depends on the guluronic acid content and also on the average number of G units in the G block. This allows the calcium ion to fit into the spaces thus forming properly arranged structures. The resultant structure is called as an egg box model.

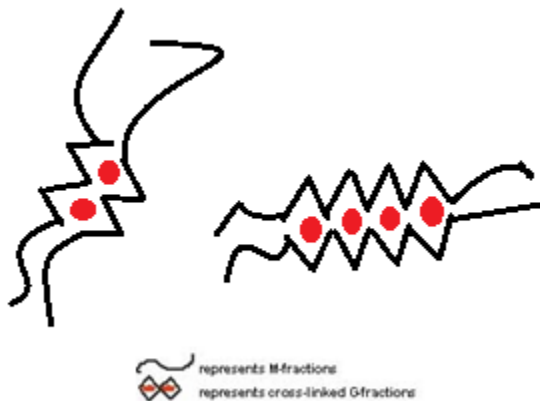


Figure 4-2- Egg Box model for formation of calcium alginate particle in M rich fraction and G rich Fraction respectively.

4.2 Formulation of W/O Microemulsion

As described in the above section, on exposure to a divalent ion calcium alginate beads are formed. Microemulsion systems were used as a media for formation of these nanoparticles. Two separate microemulsions containing sodium alginate and calcium chloride respectively were prepared. Upon mixing the two, a third template microemulsion containing calcium alginate was formed.

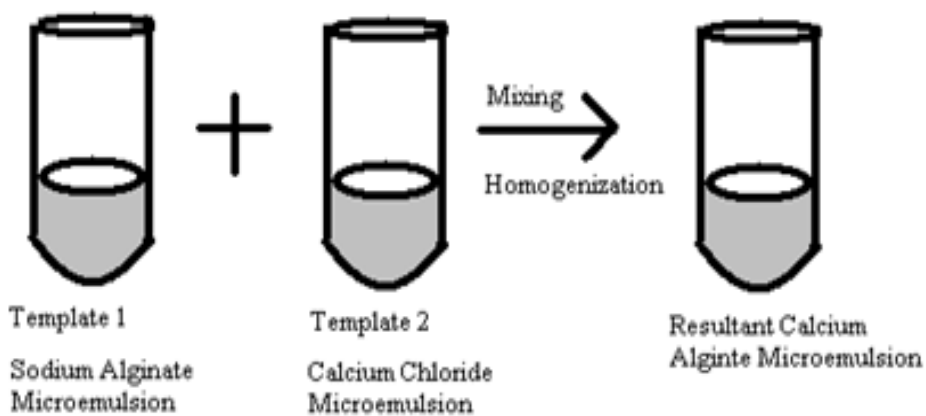


Figure 4-3: Schematic representation for preparation of calcium alginate nanoparticles by interfacial cross linking method.

After careful screening of excipients two different organic phases were chosen for further characterization. The primary criteria's behind screening these organic phases were,

- a) They should be volatile so that it is easy to recover solid nanoparticles at the culmination of the preparation process;
- b) They should form stable microemulsions with sodium alginate and calcium chloride and
- c) They should be safe to use.

DOSS is an FDA approved surfactant, used as a laxative in medications and an emulsifying agent. It is one of the most investigated anionic surfactant and forms microemulsions without the need of a co surfactant [112]. It is structurally balanced with respect to its hydrophobic and hydrophilic nature due to its double tailed pattern .

4.3 Ternary Phase Diagrams

Ternary phase diagrams are three dimensional, but are illustrated in two dimensions for ease of drawing and reading. For initial screening cyclohexane and ethyl acetate were chosen as suitable compounds for their use as external phases. Different concentrations of DOSS and cyclohexane/ ethyl acetate were prepared and tested as mentioned below;

Table 4.1 Surfactant-oil combinations used to prepare microemulsions

Type I	Type II
a)0.1M DOSS/Cyclohexane	a)0.1M DOSS/Ethylacetate
b)0.2M DOSS/Cyclohexane	b)0.2M DOSS/Ethylacetate
c)0.3M DOSS/Cyclohexane;	c)0.3M DOSS/Ethylacetate
d)0.4M DOSS/Cyclohexane	d)0.4M DOSS/Ethylacetate

To each of the DOSS/ Organic phase mixture, water was added drop wise using titration method and visually inspected. The resultant emulsions were transparent, slightly turbid or a milky emulsion depending on the composition. The trial compositions were plotted

on a ternary phase diagram. The area bounded by the points in the phase diagram, correspond to the concentration range of component mixtures that produced visually clear and transparent microemulsions. Both ethyl acetate and cyclohexane formed clear microemulsions with water in the presence of DOSS. 0.25% - 1% Sodium alginate solutions were used in place of water to test the efficiency of the organic phases to form stable microemulsions with them. The same procedure was repeated for calcium chloride.

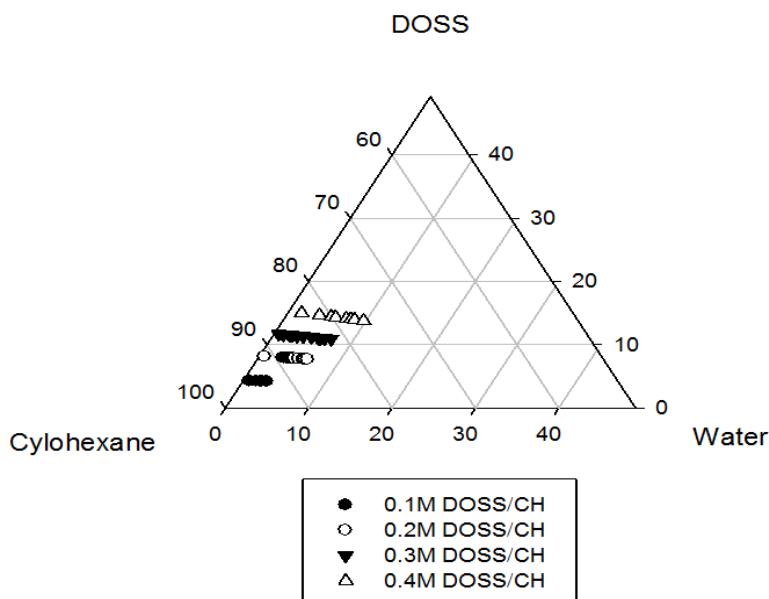


Figure 4-4: Ternary phase diagram showing incorporation of water by various concentrations of DOSS/cyclohexane.

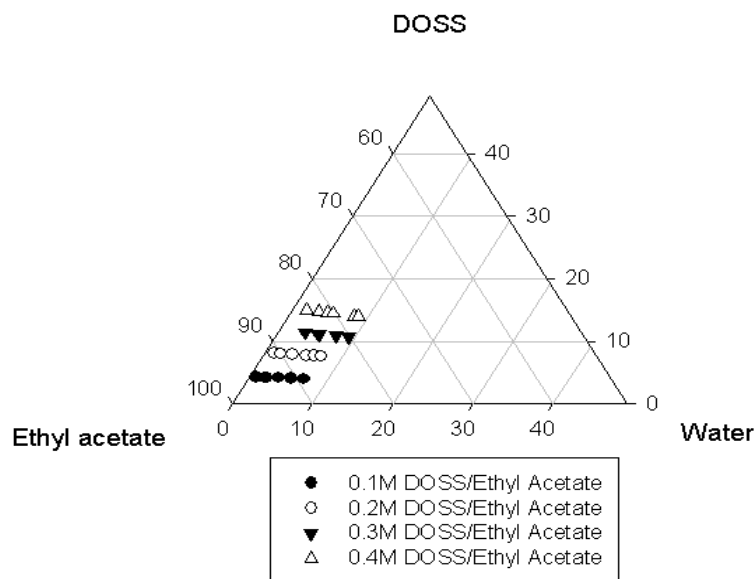


Figure 4-5: Ternary phase diagram indicating incorporation of water by various concentrations of DOSS/ethyl acetate.

All the points indicated in the graph gave rise to stable microemulsions. When figure 4.4 and figure 4.5 are compared it is clear that ethyl acetate produced clear and transparent microemulsions over a wider composition range than cyclohexane. However, when aqueous sodium alginate was added to DOSS-ethyl acetate mixtures precipitation occurred. This may be attributed to the acidic ethyl acetate, precipitating the alginate out of the dispersion. Hence future formulations were prepared using cyclohexane as the oil phase.

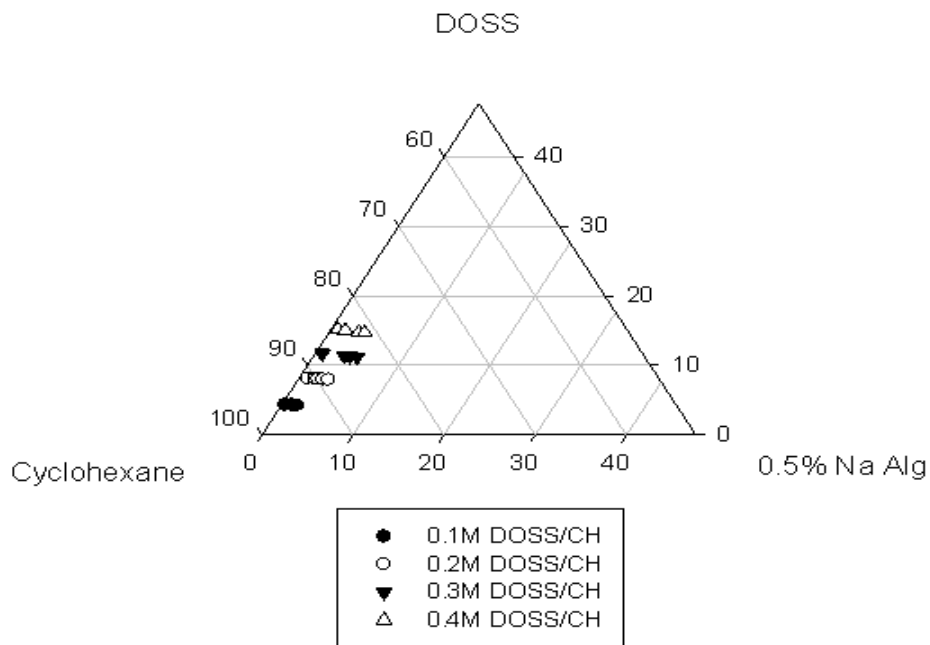


Figure 4-6: Ternary phase diagrams showing incorporation of 0.5% sodium alginate by various concentrations of DOSS/cyclohexane

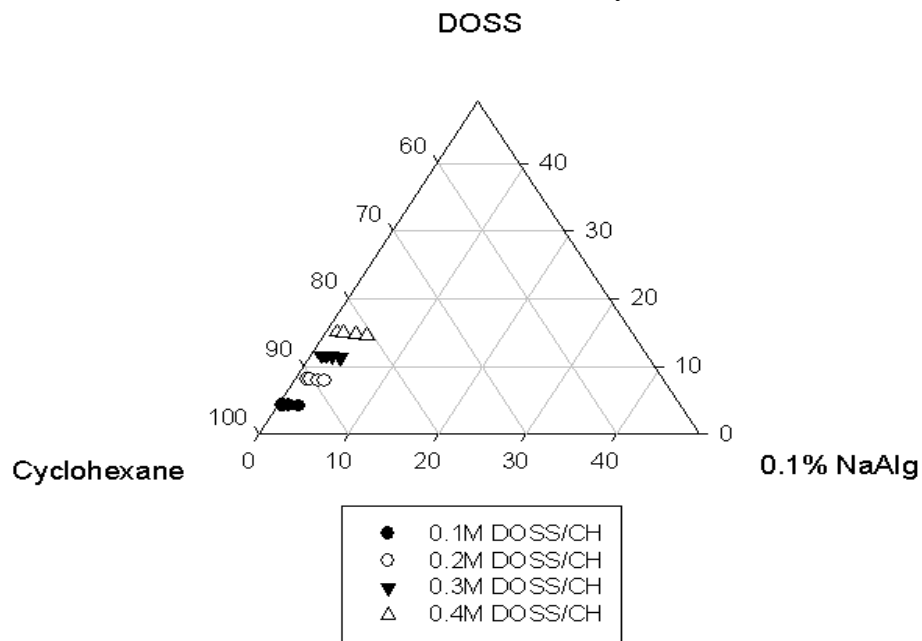


Figure 4-7: Ternary phase diagram showing incorporation of 1% sodium alginate by various concentrations of DOSS/cyclohexane

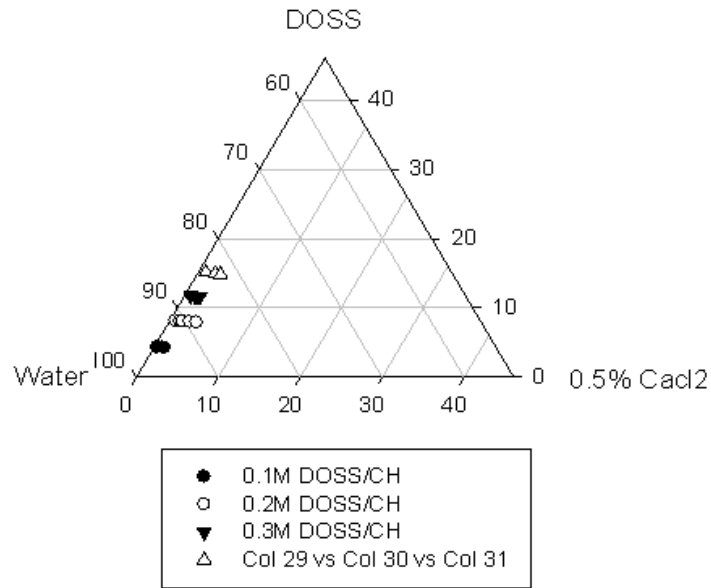


Figure 4-8: Ternary phase diagrams showing incorporation of 0.5% calcium chloride by various concentrations of DOSS/cyclohexane

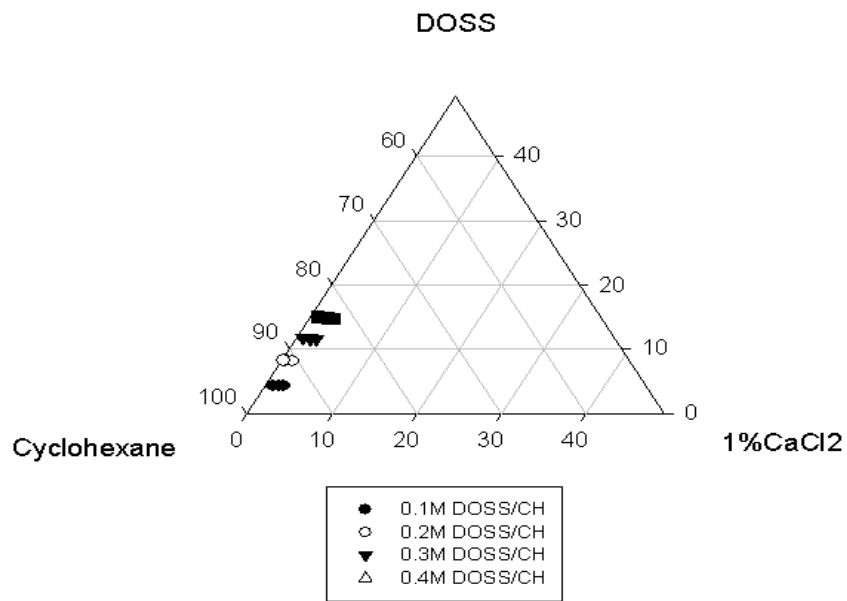


Figure 4-9: Ternary phase diagram showing incorporation of 1% calcium chloride by various concentrations of DOSS/Cyclohexane.

Cyclohexane demonstrated formation of clear microemulsion over a wide range of compositions of DOSS, aqueous sodium alginate, and aqueous calcium chloride. Based

on the stability of the formulations and amount of DOSS present, 0.2M

DOSS/cyclohexane was used for further characterization. Small amounts of DOSS were desirable in the final product to facilitate ease of recovery of nanoparticles.

BSA is used as a protein and a molecular weight standard [117]. The amount of aqueous BSA incorporated as a clear microemulsion was determined by a similar titration method. Figures 4.6 to 4.9 show incorporation of 0.5% sodium alginate, 1% sodium alginate, 0.5% calcium chloride and 1% calcium chloride respectively. It can be seen that not much difference was seen in the amount of aqueous phase incorporated for different concentrations and for different chemicals. When 5% BSA solution in sodium alginate was used clear and stable microemulsions were obtained.

4.4 Characterization of calcium alginate nanoparticles

4.4.1 Particles size Analysis

For preliminary analysis, optical microscopy was used to investigate the presence of nanoparticles. A Nikon model TiU microscope with integrated camera was used for the same. Images were taken without the use of polarized filters. Images indicate that particles prepared using concentrations greater than 1% NaAlg and greater than 1% CaCl₂ formed aggregates. Whereas nanoparticles prepared using concentrations less than 0.5% NaAlg and less than 0.5% CaCl₂ formed smaller and lesser aggregated particles.

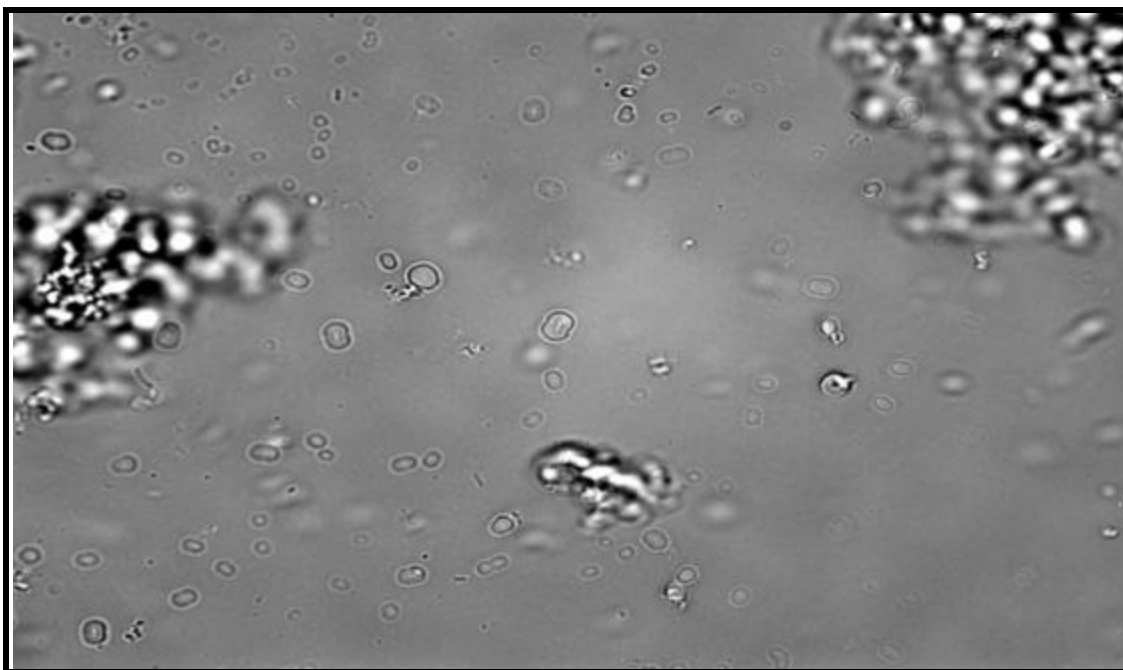


Figure 4-10: Optical microscopy image showing formation of aggregated particle by using 1% NaAlg and 1% CaCl

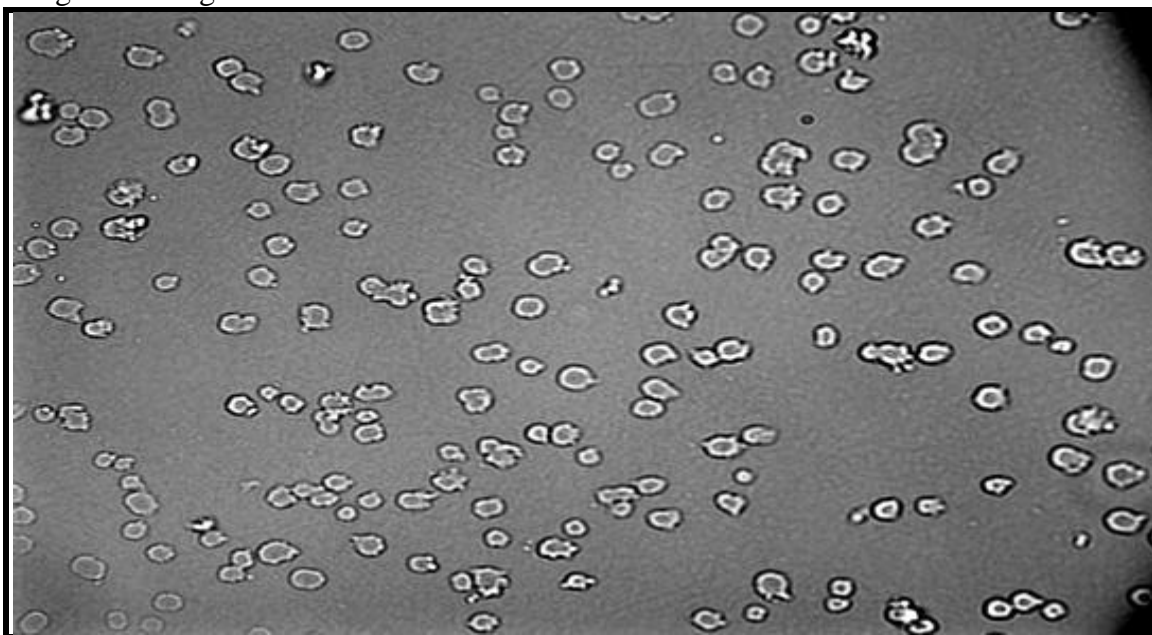


Figure 4-11: Optical Microscopy image showing formation of individual nanoparticles using 0.5%NaAlg and 0.5% CaCl₂

Based on results obtained from optical microscopy at least 0.5%NaAlg and 0.5% CaCl₂ in DOSS/CH were used for future experiments.

The particle size distribution determines the in vivo distribution, biological fate, toxicity and targeting ability of a nanoparticle system [118]. Smaller the particle size, greater is the surface area for drug absorption, hence most of the drugs associated would be at or near the surface [118, 119]. The particle size was measured using dynamic light scattering (DLS). For the particle size of reactor emulsions were found to be less than 10nm. The mean particle size of calcium alginate nanoparticles was found to be 421 ± 52.11 nm. The particles demonstrated a Nicomp® bimodal distribution model.

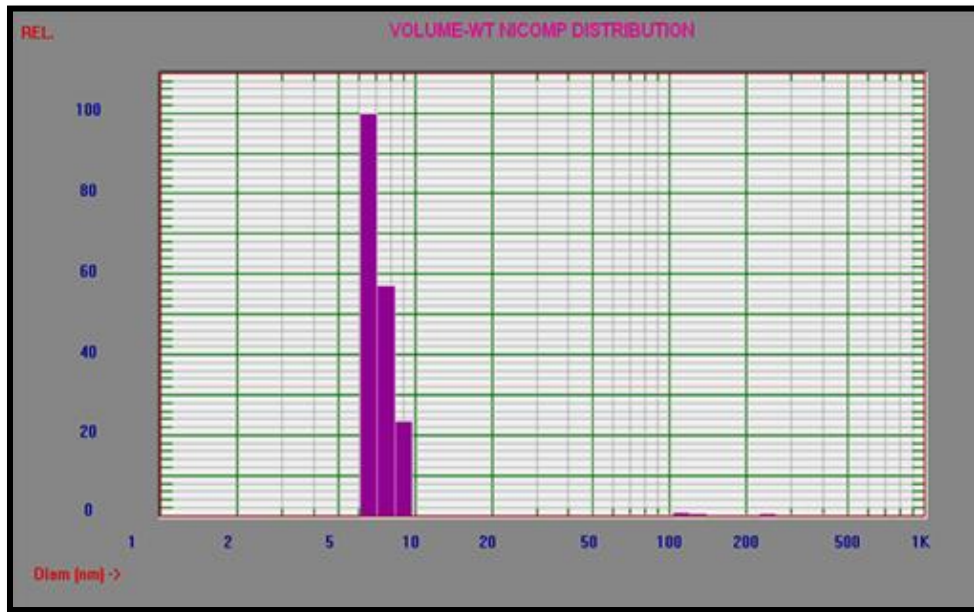


Figure 4-12: Particles Size of Na-Alg reactor emulsion (Volume weighted)

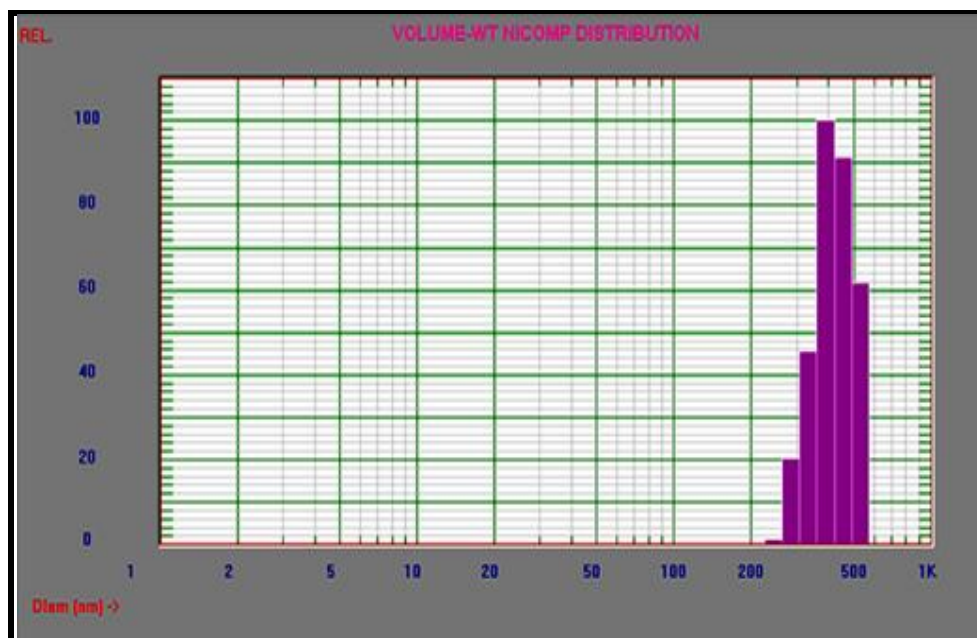


Figure 4-13: Particle size of Ca-Alg Nanoparticles (Volume weighted).

4.4.2 Zeta Potential Measurement

When nanoparticles are administered intravenously their surface charge determines its capability to interact with cell surface and other biological components. This in turn affects the in vivo fate of the nanoparticles [118, 120]. Zeta potential can also be used to determine whether a charged active material is encapsulated within the system or adsorbed onto the surface or both [118]. The DLS instrument was used in its electrophoretic light scattering mode to measure zeta potential. An average zeta potential of $-20.96 \pm 1.4 \text{ mV}$ was obtained ($n=3$). It has been observed in previous studies that in such type of system only the negative charges present in the core of the nanoparticle matrix are involved in electrostatic interaction with weakly basic drugs. Thus the negative charge could be the contribution of the sulphonate group of DOSS and carboxyl groups of alginates [121]. Nanoparticles are generally taken up by M cells and absorptive endocytes. It is likely that these negatively charged particles can be rapidly opsonized and

massively cleared by the fixed macrophages when exposed to these cells. Sometimes surface modifications of these nanoparticulate systems with hydrophilic polymers are the most common way to control the opsonization process and to improve the biological half-life of the system [6].

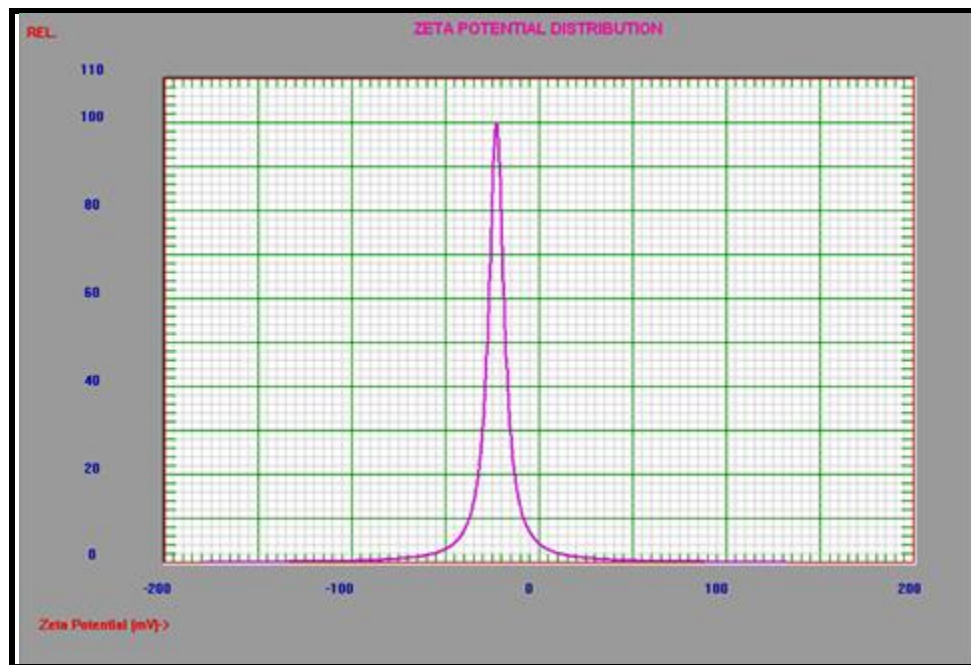


Figure 4-14: Zeta potential of calcium alginate nanoparticles.

4.4.3 Surface morphology of calcium alginate nanoparticles via SEM

The surface morphology of nanoparticles may have a profound influence on the surface properties that determine their interaction with the environment and biological system [122]. For sample preparation a drop of dilute suspension of the solid nanoparticles in ethanol was placed on an aluminum stub and visualized in the electron microscope. The SEM images confirmed the presence of spherical particles in the size range of 150-500

nm (figures 4.15a and b). This result was in concordance with DLS data. Subsequent examination of nanoparticle samples using TEM indicated formation of spherical and uniform nanoparticles with smooth periphery of size up to 500 nm as seen in figures 4.16a and b

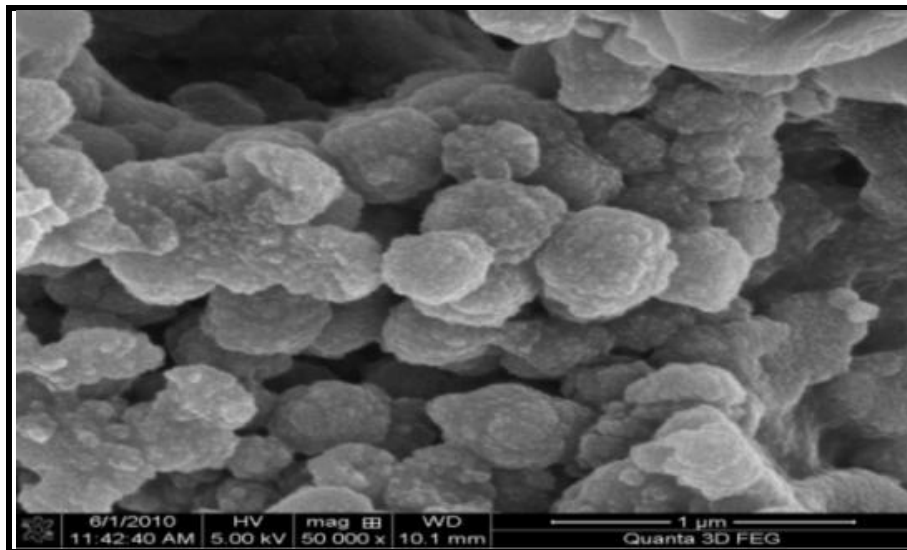


Figure 4.15: a) Scanning Electron Microscopy images of calcium alginate nanoparticles

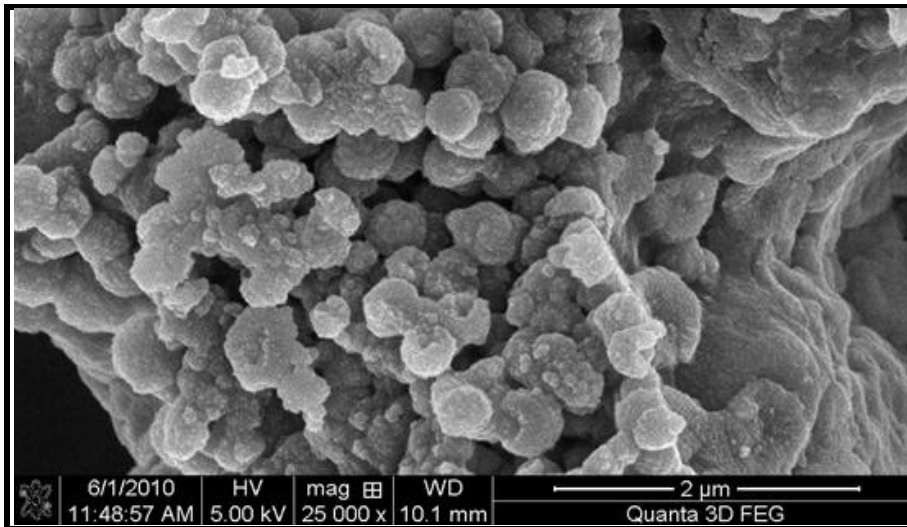


Figure 4-15: b) Scanning Electron Microscopy images of calcium alginate nanoparticles

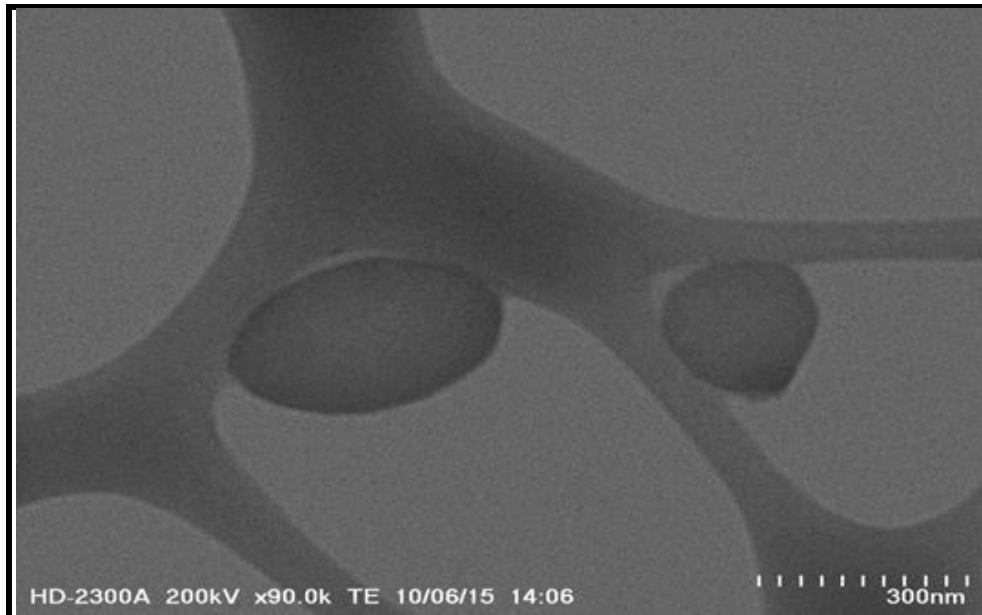


Figure 4-16: a) Transmission Electron Microscopy images of calcium alginate Nanoparticles

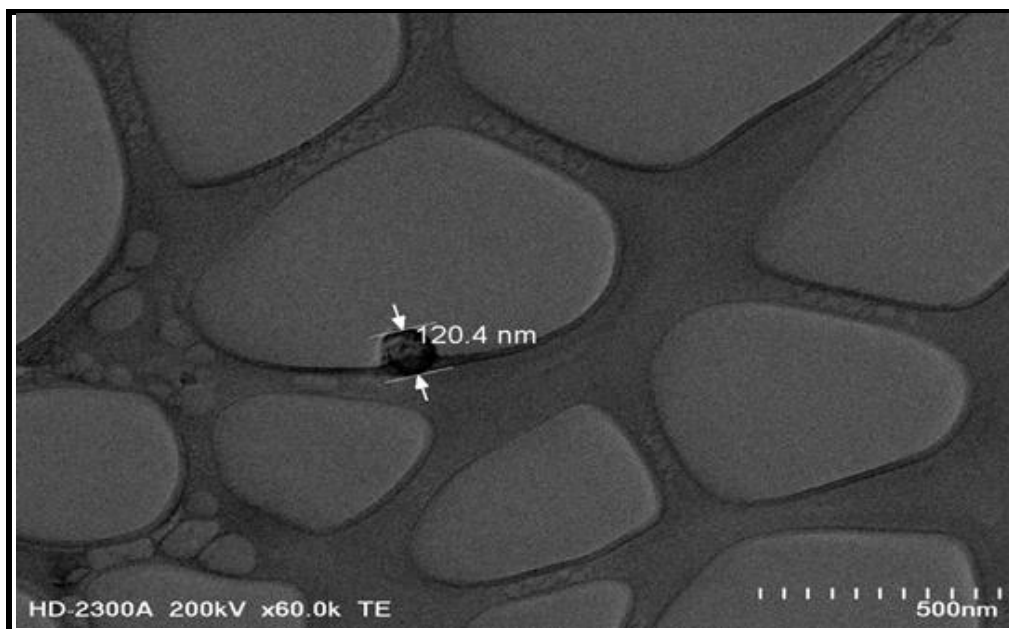


Figure 4-16: b) Transmission Electron Microscopy images of calcium alginate nanoparticles

4.5 Characterization of calcium alginate nanoparticles loaded with BSA

The objective of the study was to evaluate the efficacy of this new method of preparation of nanoparticles for protein delivery. Prior to studying the association of BSA with alginate, preparation conditions for BSA loaded calcium alginate were investigated. Compositions ranging from 2% to 30% of BSA in aqueous sodium alginate solution were used to determine the optimum loading concentration. It was found that 5% or less of BSA in aqueous sodium alginate was suitable for use in further studies.

4.5.1 Particle size analysis of BSA-calcium alginate

Results indicated that BSA loaded particles showed sizes ranging from 200-550nm. Thus BSA does not affect the particle size of nanoparticles at the concentrations used.

4.5.2 Zeta Potential of BSA loaded alginate nanoparticles

BSA is an amphiphilic protein due to presence of NH_2 and COOH groups in its molecular structure. Its net charge changes with the pH of the medium. BSA exhibits a net positive charge below its isoelectric point of 4.7, and a net negative charge above its isoelectric point [123]. Bovine Serum albumin at $\text{pH}=7$ imparts a negative charge of -18mv to the surface [123, 124]. Due to the anionic nature of the calcium alginate nanoparticles and BSA, zeta potential of the nanoparticles loaded with BSA was found to decrease from -20mv to -40 ± 15 mv..

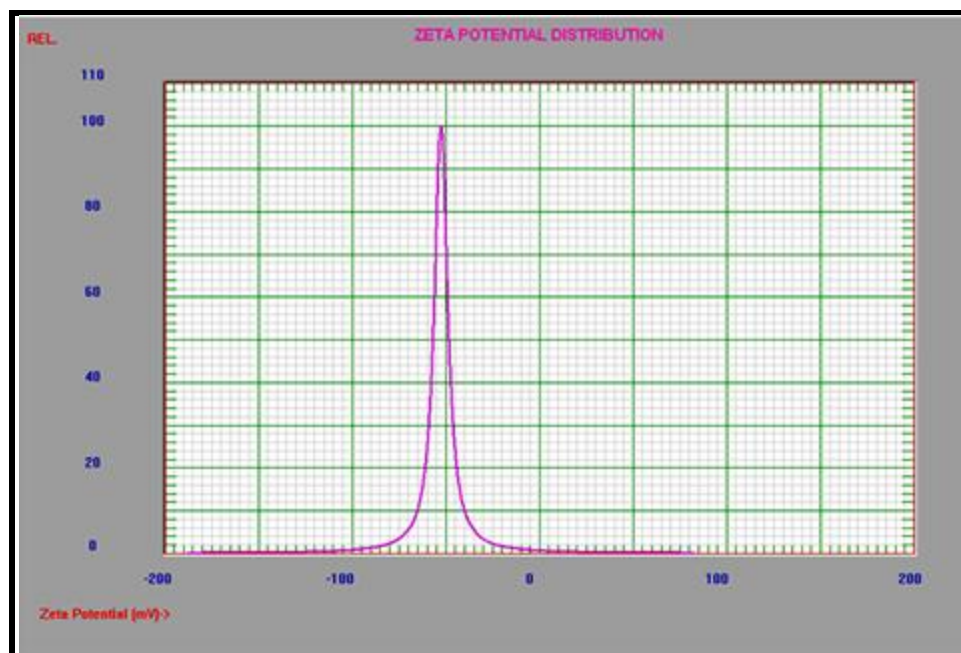


Figure 4-17: Zeta potential of BSA loaded calcium alginate nanoparticles.

4.5.3 Morphology of BSA loaded nanoparticles using TEM

TEM images of protein loaded calcium alginate nanoparticles were obtained. Images show that spherical particles within the size range of 200-550nm were present further validating the size of these particles.

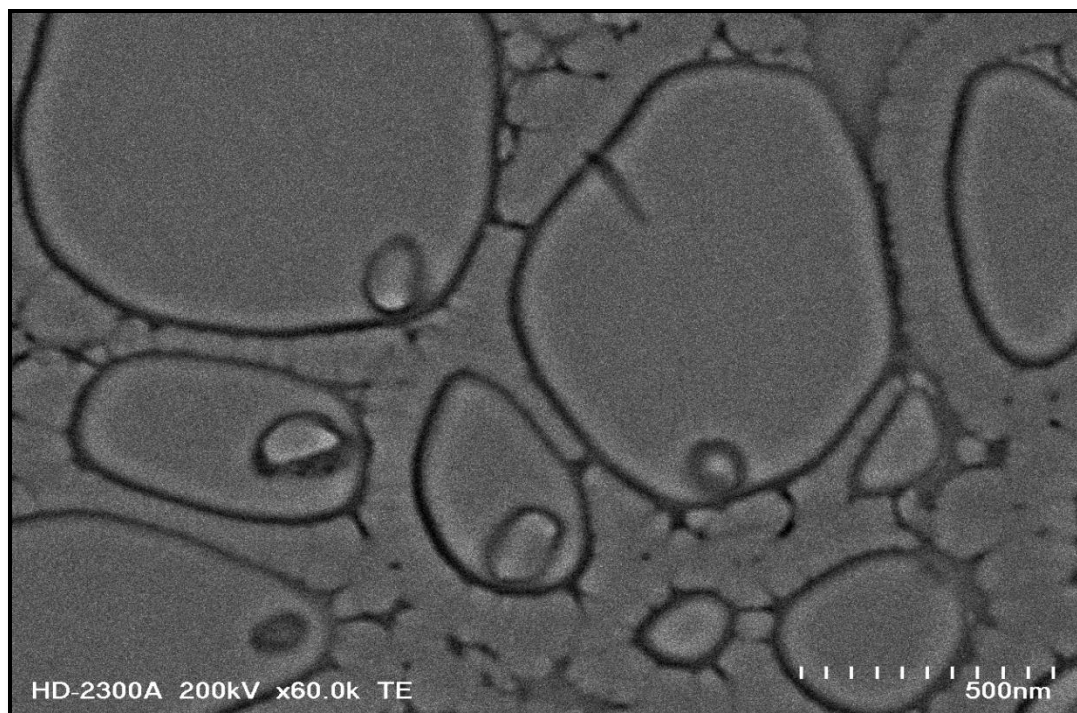


Figure 4-18: TEM of BSA loaded calcium alginate nanoparticles.

4.5.4 DSC of BSA loaded calcium alginate nanoparticles.

The purpose of this experiment was to determine the degree of interaction between the polymer matrix and incorporated BSA. Conformation of proteins can change reversibly or irreversibly depending on the degree of interaction between the protein and the polymer. The interaction between proteins and polymers will have an effect on the thermal behavior of the conjugate. Hence it was necessary to study the thermal behavior of BSA in the BSA loaded nanoparticle formulation. DSC thermograms of denaturation of BSA is reported to be concentration dependent [125]. The DSC scans were performed from 10°C-250°C with the temperature ramped at the rate of 10°C per minute. For each combination at least three runs were performed. In the experiment DSC curves for three different combinations were obtained. Plain BSA was used to compare the thermal

differences if any were there. An upward peak in the thermogram represents an endothermic reaction.

-Curve labeled a shows a peak at 120°C indicating evaporation of water followed by a peak at 200-220°C which is characteristic of protein denaturation,

-Curve labeled b shows a broad endothermic band at 130°C due to water loss, the slight shift in the thermal event related to BSA is possibly due to the presence of entrapped protein within the polymer matrix-Curve labeled c shows a broad peak at 110°C due to water loss and glass transition followed by decomposition at 180°C. In calcium alginate the glass transition temperature occurs around 110-130°C which is masked by water evaporation. In fact glass transitions in samples containing free water is not detectable [126].

The upward shift of the peaks during the experimental run is due to sample degradation of the sample in the pan.

A qualitative comparison of DSC curves shows no significant differences from normal thermal behavior expected from the material tested affirming that no significant interactions were prevalent.

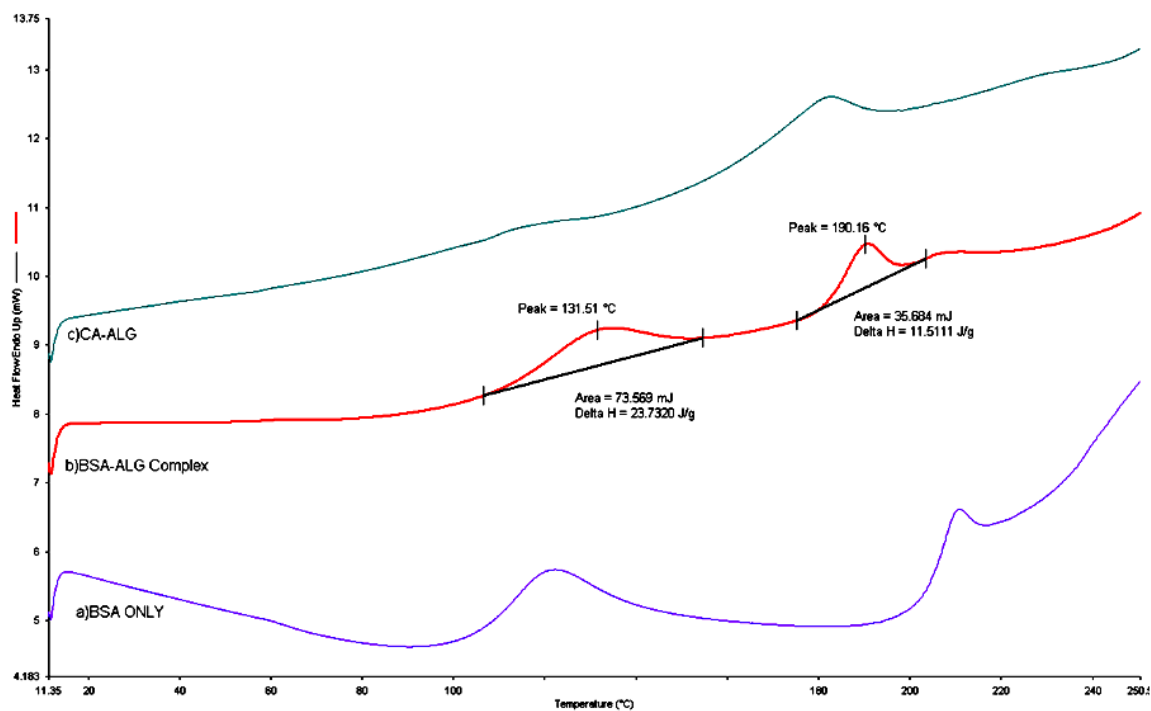


Figure 4-19: DSC thermograms for BSA, BSA loaded calcium-alginate and blank calcium alginate nanoparticles

4.5.5 BSA release profile

The inability of alginate particles to swell in acidic medium helps to protect the entrapped drug against the acidic environment. In vitro release profile of BSA was studied using Hartree Lowry method for protein estimation using phosphate buffer of pH 7.4 as medium, at 37°C. The calibration curve obtained for albumin standard solutions is given below. The equation for the line was calculated to be $y=0.0021x-0.0406$; $R^2=0.9983$.

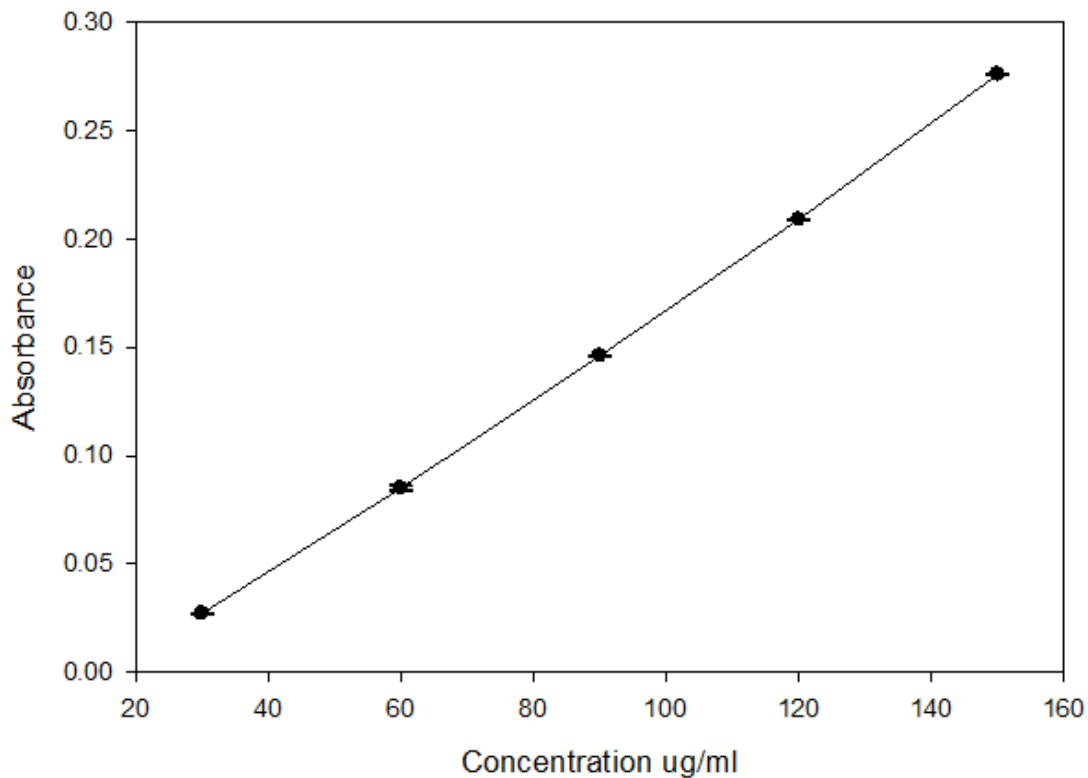


Figure 4-20: Calibration curve of BSA in phosphate buffer (PBS) pH=7.4

Absorbance of BSA from *in vitro* release study samples were substituted in the equation obtained from calibration curve to determine the release profile. Aliquots of the release medium were obtained at 0, 0.25, 0.5, 1, 2, 3, 4, 8, 18, 24 and 30 h and analyzed. The release profile was characterized by an initial burst effect followed by a continuous slow release pattern. Approximately 90% of entrapped drug was released during the initial 8 h, followed by a continuous slow release giving rise to a plateau. It can be assumed that the presence of protein adsorbed on the nanoparticle surface causes the initial burst release to occur, followed by slower release of the entrapped BSA within the core.

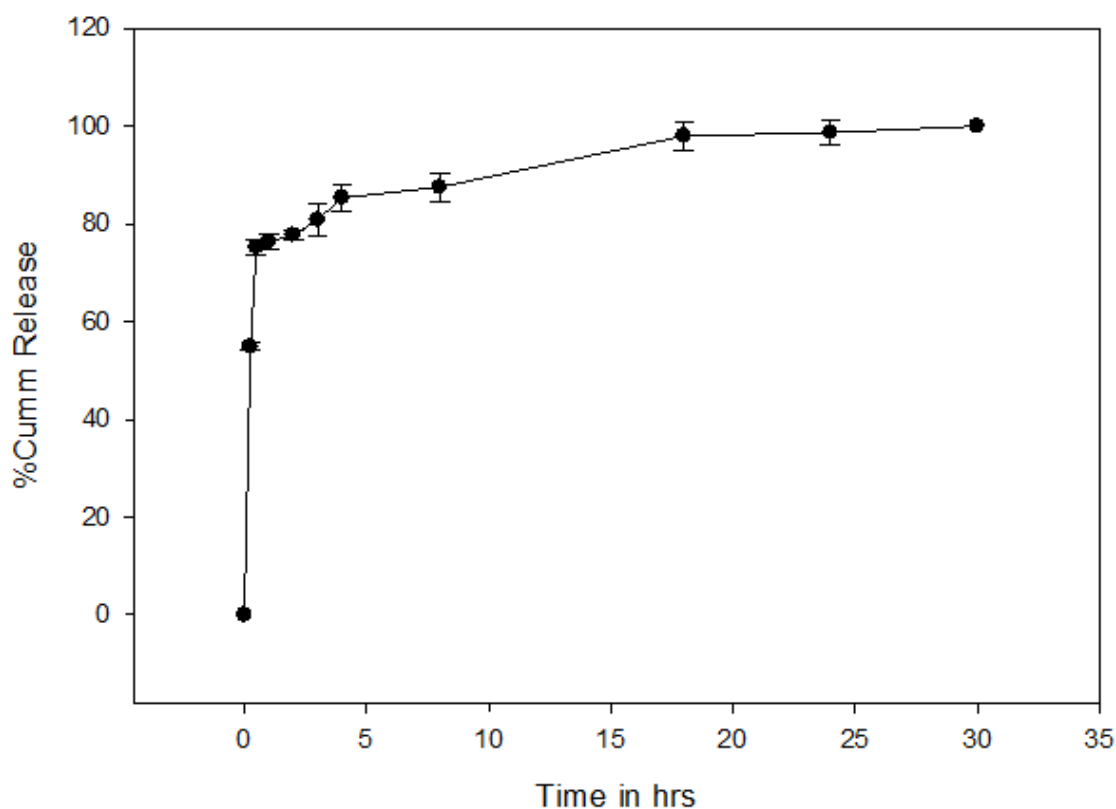


Figure 4-21: Cumulative release of BSA from calcium alginate matrix

4.5.6 SDS –PAGE

Structural integrity of BSA released from alginate nanoparticles into the phosphate buffer medium was analyzed using SDS-PAGE followed by Coomassie brilliant blue staining. Preliminary analysis showed that molecular weight marker and BSA standard shown in lane 1 and lane 2 exhibits clear bands near 72 kDa. The released samples of BSA showed identical band pattern when compared to the molecular weight marker and standard. A single band in the gel provides evidence that the entrapped proteins in the nanoparticles did not suffer a significant covalent aggregation and fragmentation during the process [127].

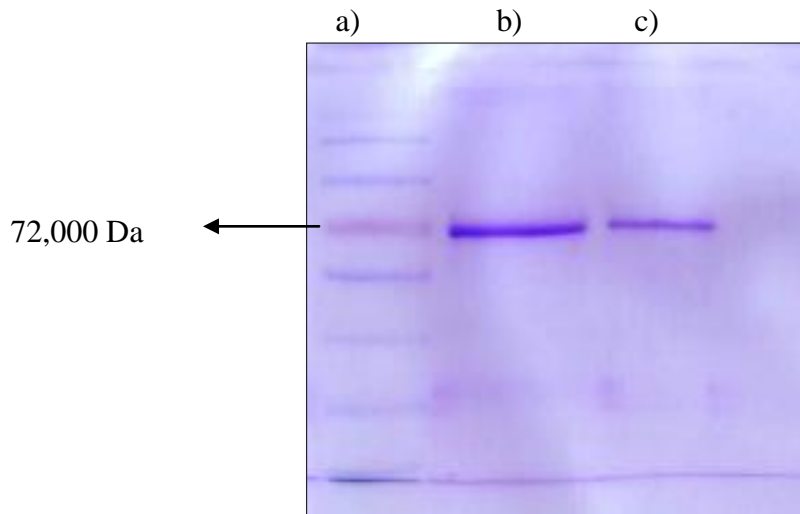


Figure 4-22- SDS- PAGE gel of BSA, Lane a) Molecular weight marker, Lane b) BSA Standard, Lane 3) BSA from nanoparticles

4.5.7 Confocal Laser Scanning Microscopy (CLSM) of BSA loaded particles

FITC labeled BSA was used as model fluorescent drug to visualize the presence of BSA in the nanoparticle. CLSM permits the user to depict and unambiguously identify all fluorescently labeled compounds at light microscopic resolution [87]. CLSM images showed circular illuminated particles indicating that the protein was uniformly and homogeneously dispersed in the nanoparticle matrix in the interior as well as on the surface. The images as shown in figures 4.22 a and b provided the first visual evidence for the presence of BSA on the nanoparticle surface.

The topographical or 3D-surface view of these, showed the presence of spherical particles as well. The Z stack feature allows capturing images through the particle in horizontal as well as vertical manner. This helps in understanding the internal morphology of the particles thoroughly. The image in figure 4.24 indicates illumination

through the horizontal as well as vertical axes. This helps to confirm that FITC –BSA was incorporated throughout the core of the nanoparticle matrix.

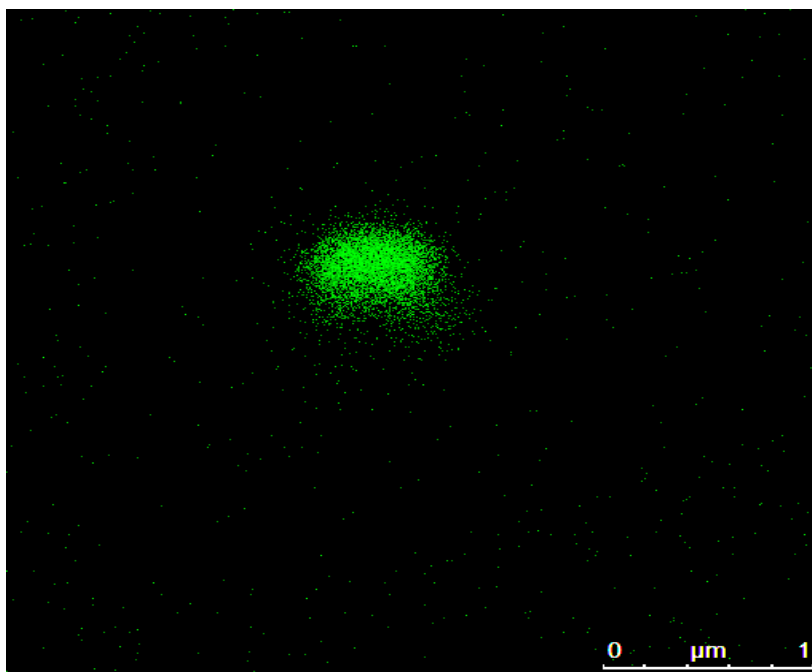


Figure 4-23 a): Image of FITC –BSA Calcium alginate matrix

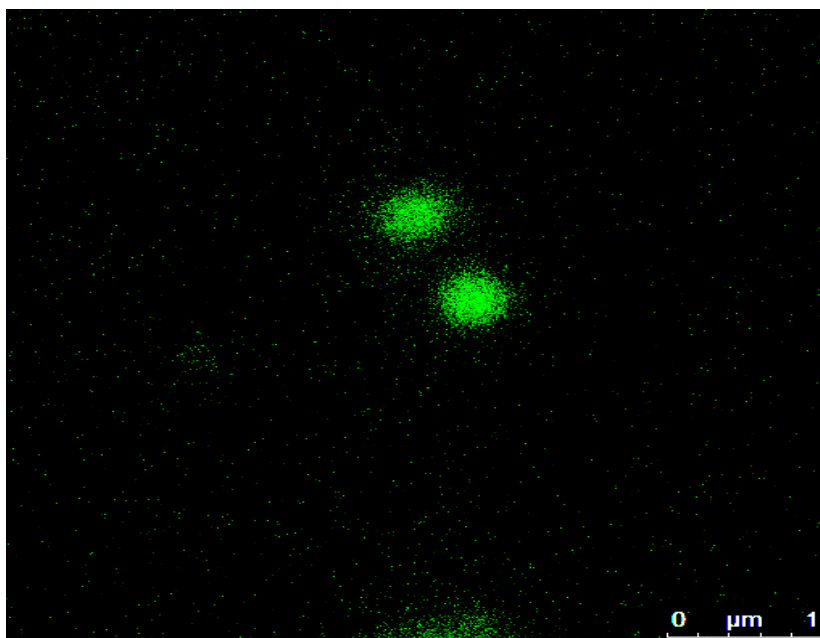


Figure 4-23 b): Image of FITC –BSA Calcium alginate matrix

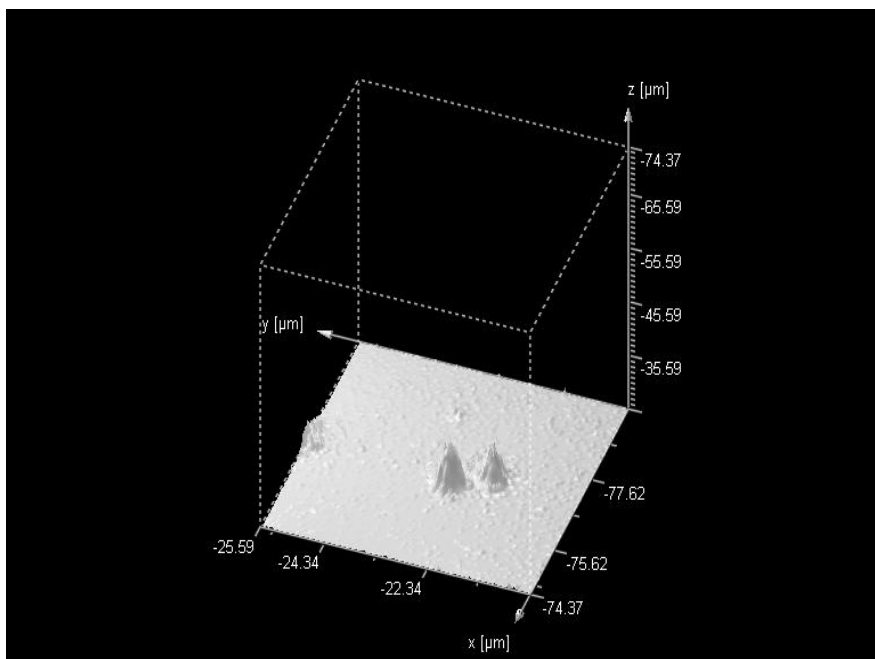


Figure 4-24: Surface topography of FITC BSA particles in figure 4.23 (b)

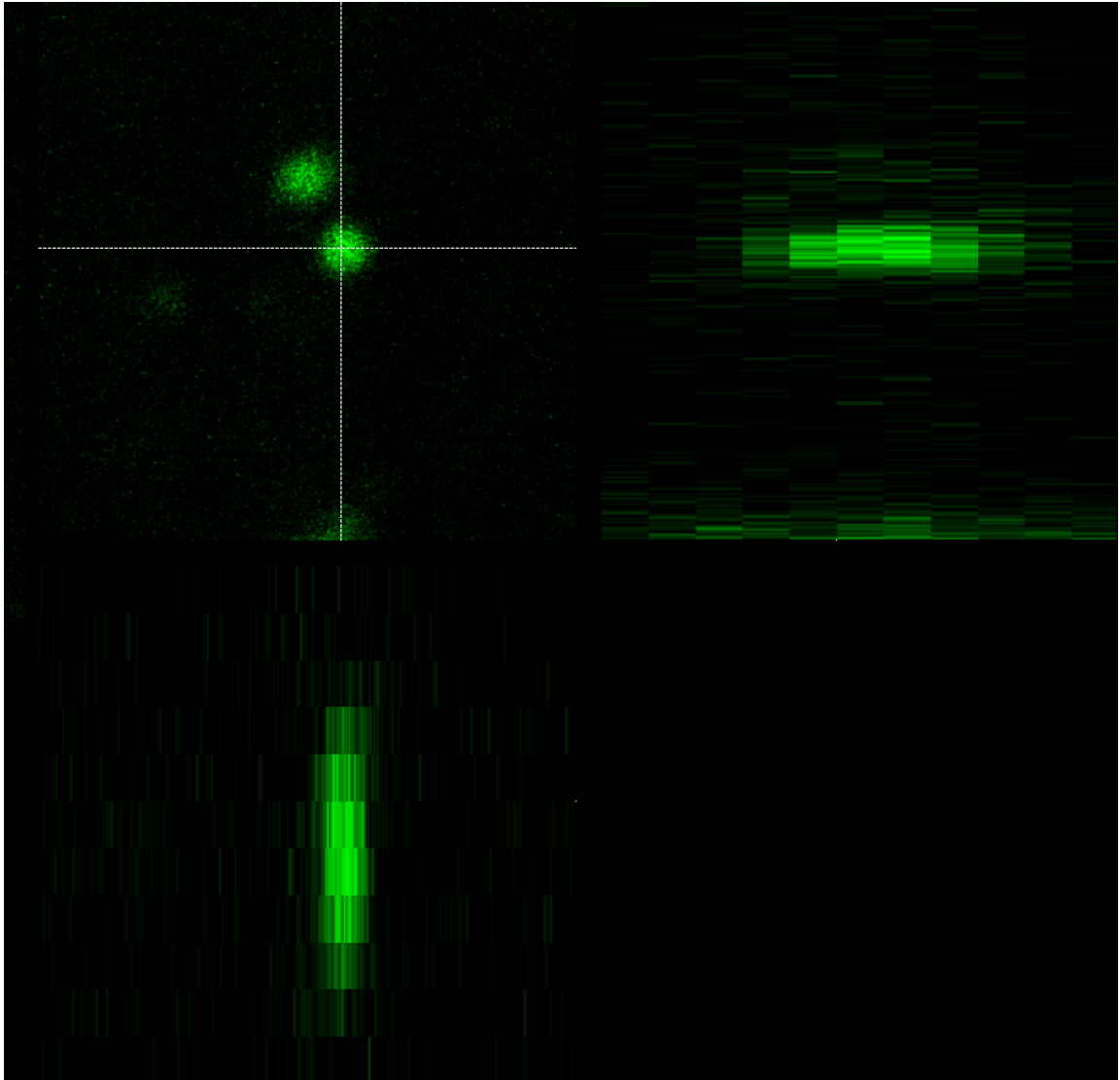


Figure 4-25: Z stack images showing presence of FITC BSA throughout the X and Y axis of an individual particle.

4.6 Characterization of chitosan coated Ca-Alg nanoparticles

Chitosan in the presence of alginate forms a polyelectrolyte complex. It has been reported that by coating alginate beads with chitosan, the ease of solubility of chitosan in low pH is prevented by the alginate network since alginate is insoluble in low pH conditions. The possible dissolution of alginate at higher pH is controlled by chitosan which is stable at higher pH [128]. Hence protein delivery can be obtained in a more controlled manner by coating calcium alginate with chitosan.

This study was performed to determine whether the coating method could successfully deposit chitosan on the calcium alginate nanoparticle surface. The advantages of the resultant system are; chitosan endows a positive surface charge on the nanoparticle which prolongs the nanoparticle residence time and its contact with the epithelium [113]. Zeta potential analysis and CLSM were performed to obtain surface charge and images of the chitosan coated nanoparticles.

4.6.1 Zeta potential of chitosan coated Ca-alg nanoparticles

Zeta Potential results showed an increase in surface charge from -23 to -0.4 mV. Since chitosan is a cationic polymer, the increase in zeta potential suggests the presence of chitosan on the surface.

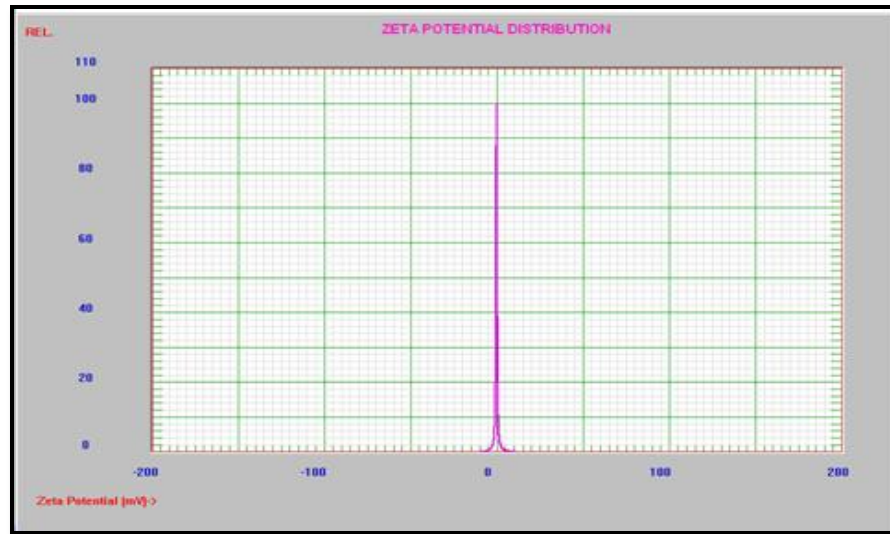


Figure 4-26: Zeta potential of chitosan coated nanoparticles

4.6.2 CLSM of Chitosan coated Ca-Alg nanoparticles

Chitosan being moderately auto fluorescent eliminates the need for addition or labeling with a fluorescent dye. Images showed presence of an irregular shell around circular particles. Photographs were obtained in different views. Particles were ranging from 700 - 900 nm in size

Image a shows presence of a hollow core and a darker capsule surrounding it. This view provides images depending on densities. No fluorescence is detected here.

Image b shows the same field of vision but with illumination of the fluorescent compound. It can be seen that chitosan (greenish tint) is present around the core.

Image c shows only the fluorescent component. Portions of chitosan covering the matrix can be seen here. The core cannot be seen in this case.

These images support the view that chitosan forms a layer around the particles

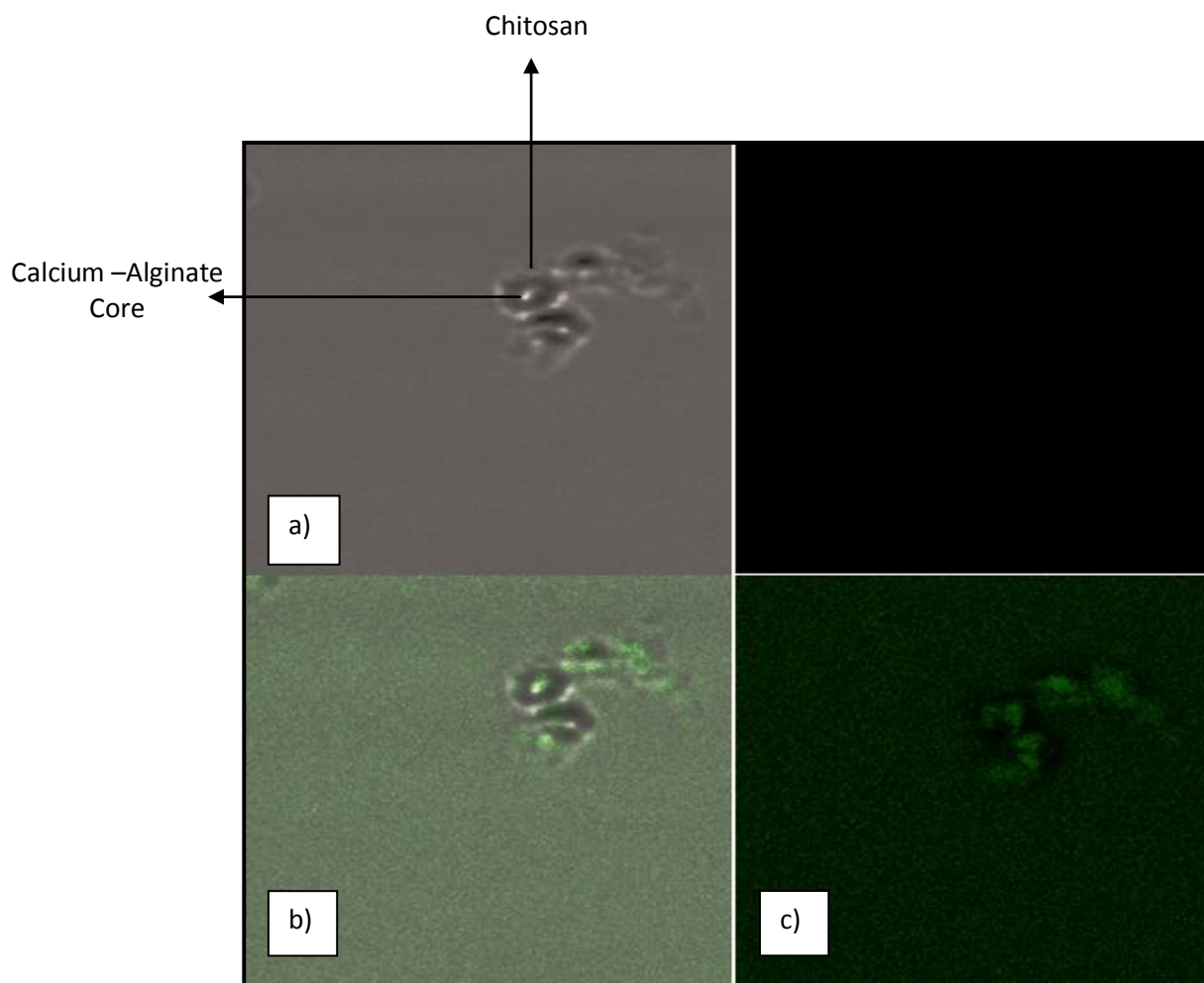


Figure 4-27: Confocal Laser Microscopy images of chitosan coated nanoparticles

Chapter 5

Conclusion

The research demonstrated that calcium alginate nanoparticles were successfully prepared by the new interfacial cross linking technique developed in our laboratory. The inherent tendency of alginates to form gels in presence of a divalent ion, enables preparation of nanosized particles using emulsions as templates. Careful screening of excipients and preparation conditions led to formation of stabilized microemulsions which were then further processed to form solid nanoparticles. These nanoparticles were characterized using optical microscopy, DLS, SEM, TEM, DSC and SDS-PAGE studies for their physicochemical properties that are desirable in drug applications. Nanoparticles in the size range of 200-500 nm were obtained. SEM and TEM studies indicate the spherical shape of these particles.

The use of BSA verified the efficiency of these nanoparticles to incorporate biomacromolecules of therapeutic interest. In vitro drug release studies showed that 90% of entrapped BSA was released within first 8 h followed by a slow release pattern. The initially observed burst release of BSA from the core matrix indicates its adsorption onto the surface along with encapsulation. Further decrease in zeta potential from -20 to -40 mV supports this view. Use of FITC –BSA helped to establish the presence of the protein on the surface and the interior of the nanoparticle matrix.

Preliminary experiments investigating the use of chitosan as a cationic polymer to envelope the calcium alginate particles yielded positive outcomes. The rise in zeta potential from -23 to -0.04 mV and CLSM studies showed the deposition of chitosan on the surface of the nanoparticles. Future work in this project includes studies that will further the understanding of the nanoparticle preparation process by delineating parameters influencing particle size, incorporation of a protein of therapeutic interest, and coating the alginate nanoparticle using chitosan.

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