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ABSTRACT

PREPARATION OF NANOVEHICLES FOR TARGETED DRUG DELIVERY

By
Anagha Avinash Bhakay

Application of nanotechnology in pharmaceutical research has opened new frontiers for drug development and its efficient delivery. In recent years, the focus of the research has been on the engineering of nano/micro-particulate structures for efficient and targeted delivery of drugs for selective and enhanced absorption. This work throws light on the preparation of biodegradable and non-toxic nanoparticulate systems for targeted drug delivery. The drug vehicles were made by two different processes namely electrostatic interactions and desolvation process. The drug carriers made by these processes were found to be in the size range of 150-200nm which is necessary for cellular uptake of the drug carriers. The albumin nanocarriers were modified by antibody and showed binding to the Ramos lymphoma cells in-vitro.

Formation of nano-sized Active Pharmaceutical Ingredient (API) particles is another application of nanotechnology, where small size leads to improved solubility. Hence the API size reduction by milling was carried out to increase the bioavailability of the APIs after their incorporation in different dosage forms. The stable aqueous suspensions of the API were obtained using polymers and surfactants which could be mixed with excipients and formulated into thin film strips.

PREPARATION OF NANOVEHICLES FOR TARGETED DRUG DELIVERY

by
Anagha Avinash Bhakay

A Thesis
Submitted to the Faculty of
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CHAPTER 1

INTRODUCTION

1.1 Background

Drug delivery is the method or process of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals. They are the means of administering drugs to the body in a safe, efficient, reproducible and convenient manner. They modify the drug release profile, absorption, distribution and elimination for the benefit of improving product efficacy, safety, patient convenience and compliance. They also protect therapeutic agents from degradation in the biological system.[1]

Most common routes of drug delivery include the oral (through the mouth), topical (skin), transmucosal (nasal, buccal/sublingual, vaginal, ocular and rectal), inhalational and parenteral (intravenous, intramuscular, subcutaneous).[2] Oral dosage forms like tablets, capsules, syrups, suspensions are the most preferred formulations. However many medications such as proteins, antibodies, vaccines, gene based drugs cannot be delivered orally because they might be susceptible to enzymatic degradation or cannot be absorbed efficiently into the systemic circulation due to their molecular size, charge issues and chemical nature. Apart from advances in formulation development, protect therapeutic agents from degradation in the biological system.[1] efforts are being made in the development of new drugs using combinatorial chemistry, drug modeling to prepare drugs that selectively bind to certain receptors in the body.[3] Also despite of advances in the development of new drugs, a drug may never reach the target organ, or it may be difficult to achieve the necessary level of drug in the body. Large doses can result

in serious side effects and can harm normal cells and organs, and for this reason there is a need to develop controlled release formulations and targeted drug delivery systems.[4]

During the past three decades, formulations that control the rate and period of drug delivery (i.e., time-release medications) and target specific areas of the body for treatment have become increasingly common and complex. Because of the ever-evolving understanding of the human body and the explosion of new and potential treatments resulting from discoveries of bioactive molecules and gene therapies, pharmaceutical research hangs on the precipice of yet another great advancement. However, this next leap poses questions and challenges to not only the development of new treatments but also the mechanisms with which to administer them.

The current methods of drug delivery exhibit specific problems like drug potencies and its therapeutic effects which are limited or otherwise reduced because of the partial degradation that occurs before they reach a desired target in the body. Also certain time-release medications, after ingestion, deliver treatment continuously, rather than providing relief of symptoms and protection from adverse events. However, this improvement cannot happen until methods are developed to safely shepherd drugs through specific areas of the body, such as the stomach, where low pH can destroy a medication, or through an area where healthy bone and tissue might be adversely affected.[5] Hence current efforts in the area of drug delivery include the development of targeted delivery in which the therapeutic molecule is only active in the target area of the body, for example, in cancerous tissues. Drug delivery to the targeted areas of the body prevents' side effects caused by chemotherapeutic agents and thereby protects the healthy tissue. Dose of the drug administered can also be reduced since drug release occurs at the

required site, causing reduction in the drug loss by absorption, distribution, metabolism and excretion (ADME) and thus increasing the bioavailability.

1.2 Problem Definition

There are different types of delivery systems like polymeric micelles, liposomes, nanosomes and dendrimers which are being used for targeted delivery.[6] Most of these systems make use of polymers, strong organic solvents and complex reaction systems. The basic problem faced in such systems is the toxicity of organic solvents which compromises the biocompatibility of the final formulation. To circumvent these problems water-soluble, biodegradable, polymeric nanoparticles, derived from polysaccharides proteins, polyelectrolytes were developed as drug carriers.[7] These systems find application in treatment of diseases like cancer.

All the dosage forms incorporate active pharmaceutical ingredients (API). The pharmacokinetic and pharmacodynamic of API depends on its chemical nature, particle size, partition coefficient, lypophilic/hydrophilic, amorphous/crystalline nature and the presence of functional groups. Almost 40-70 % of APIs are hydrophobic and have low water solubility which limits their bioavailability. Therefore, particle size also plays a crucial role in the solubility and stability of the dosage forms. Micronization of such APIs increases interfacial surface area and hence increases the solubility and bioavailability. Also lower the particle size, easier it is for the API to enter the cells and cause a therapeutic effect. Particle size reduction of APIs thus becomes one of the most important unit operations in the process of manufacturing of pharmaceutical dosage forms. An attempt has been made to resolve these issues in this work.

1.3 Objective

The objective of the study was preparation, characterization, crosslinking and conjugation of biodegradable nanocarriers for targeted drug delivery. The aim was to obtain stable suspensions with nanoparticle size < 200 nm for cellular uptake. The conjugated antibody was to carry the nanocarriers to specific cells for the drug release from the carrier and cause a therapeutic effect. The particle size of APIs had to be reduced to < 1 μm and overcome the problem of particle agglomeration for their incorporation in thin film strips.

1.4 Scope and Organization of Thesis

The work revolves completely around particle formation processes for drug delivery. It includes the preparation of drug delivery vehicles for parenteral administration and micronization of APIs for the development of oral/inhalational dosage forms. The parenteral formulation systems can be prepared in the form of microemulsions and colloidal systems for intravenous administration; biodegradable fibers and fibrin-gels for intramuscular/subcutaneous administration. For the oral dosage forms the particles after micronization can be made available as dry powders, suspensions, emulsions to be incorporated in tablets, gels and syrups. The particles have to be in dry powder form for their incorporation in metered dose/powder inhalers and as liposomes for nebulisers. In the current work the drug delivery vehicles were made in the form of nanoparticles for parenteral route of administration and as suspensions for the thin film strips.

The processes of particle formation, the current methods in use and the contributions made by this work have been discussed in the following chapters. This study begins with an overview of different drug delivery systems. The second chapter

reviews the polyelectrolyte and albumin nanoparticles, their method of preparation and targeted drug delivery. Also it reviews the different processes of particle size reduction of APIs and the process of milling in particular. The chapter 3 consists of experimental work that includes parametric study, particle characterization and in-vitro studies. It is followed by the results and discussions and conclusions in chapters 4 and 5, respectively.

CHAPTER 2

NANOPARTICLES FOR DRUG DELIVERY- A REVIEW

2.1 Introduction

Research into the rational delivery and administration of pharmaceutical, therapeutic and diagnostic agents is at the forefront of the pharmaceutical industry. The range of bioactive substances emerging as potential drug candidates, together with those currently under research and development, continue to provide major challenges for efficient drug delivery and targeting. The important task of the drug delivery system is to provide a defined dose, at a chosen rate, at a selected time, to a targeted biological site. Key issues in the design of delivery systems include use of materials that are not toxic, compatible with other excipients, target specific, achieve regulatory approval and clearance and also handle the constraints imposed by the nature of the various routes of administration.

A large number of delivery systems have been made using polymers, polysaccharides, proteins. Therapeutic and diagnostic agents can be encapsulated, covalently attached or adsorbed on to such nanocarriers. The nanoparticulate carriers can be surface modified with synthetic polymers and appropriate ligands; they can be targeted to specific cells and locations within the body after parenteral routes of administration.[7] These approaches can overcome drug toxicity issues. Some nanocarriers are being designed with the focus on multifunctionality that is these carriers can target cell receptors and simultaneously deliver drugs and biological sensors. But some carriers have poor drug loading capacity. If the drug loading capacity of the carrier is low, the drug used should be highly potent and also have a small particle size.[7] There are various

approaches like liquid anti-solvent, desolvation process, rapid expansion of supercritical solutions, jet milling, spray drying and ball milling used for particle size reduction of active pharmaceutical ingredients.[8] The scale-up of some of these approaches becomes difficult. Hence, milling continues to be the most commonly used method. The drug substances are milled in millers and the smaller particles are then stabilized with the use of surfactants, polymers and stabilizers. These processes form particles in size ranges that are suitable for incorporation in different delivery platforms. The reduced particle size has high surface area and hence, a strategy for faster drug absorption and therapeutic effect.

2.2 Limitations and Opportunities for Targeting

The fate of drugs after administration depends on several processes: distribution, metabolism and excretion when given by the parenteral route whereas absorption, distribution, metabolism and excretion when administered by the oral delivery. When injected intravenously the particles are cleared rapidly from the circulation by the liver cells (Kupffer cells) and the spleen macrophages. Opsonization, which is surface deposition of blood opsonic factors such as fibronectin, immunoglobulin and complement proteins, often aid particle recognition by these macrophages.[9] Larger particles (200 nm and above) efficiently activate the human complement system and hence are cleared faster from the blood by Kupffer cells.[10] The binding of blood proteins and opsonins to nanoparticles depends on the surface properties such as the presence of certain functional groups and surface charge density. [9, 11, 12]

It is necessary to overcome these limitations for efficient therapeutic delivery. The distribution of drug at the required tissue is important for which there is a need of locally acting carrier since the drug exposure to the biological environment might inactivate it by the mechanisms described above. Suppression of opsonization events is also important for enhancing the passive retention of nanoparticles at sites and compartments other than macrophages in contact with the blood and is a reflection of long circulatory profile of such surface manipulated nanoparticles.[9] Apart from surface engineering, now there are novel materials like synthetic polymers, polymer-conjugates and block copolymers that can form sterically stabilized nanostructures with macrophage evading properties.[13]

Drug incorporation into delivery systems offers many advantages like enhancing the therapeutic potential of drugs, alteration of pharmacokinetics, biodistribution and sustained release, drug stabilization, reduction of systemic drug toxicity, decreased dosing frequency, patient compliance and effective accumulation in the targeted site. Also the carrier system should be non-toxic, biocompatible and compatible with the incorporated drug, preserve the drug activity and deliver the drug at the required site with reproducible pharmacodynamics.

2.3 Nanoparticles as Delivery Vehicles

Nanoparticles are structures that range from 1 to 1000nm and their preferred size is < 300 nm for cellular uptake. They can be used as carriers for drugs, DNA, peptides and genes.[14] Polymer-based nanoparticle systems can be made from different processes like emulsion, dispersion and inverse microemulsion polymerization and may involve use of

toxic organic solvents in the preparation process which compromises the biocompatibility of the formulation.

Water soluble, biodegradable, polymeric, polyelectrolyte nanoparticles were developed to overcome the use of toxic solvents. They degrade at a slow rate, do not alter normal cell function and use water as the solvent. Polyelectrolyte complex dispersions are formed by strong electrostatic interaction between at least two oppositely charged polyelectrolytes. Protein-based nanoparticles can be prepared by emulsion formation, desolvation or coacervation processes.[15] Both the systems are biocompatible, non-toxic and have a high drug loading capacity and hence can be used as carriers for targeted delivery.

2.4 Polyelectrolyte Complex Nanoparticles [7, 16]

The polyions of opposite charges interact electrostatically with each other to form polyelectrolyte complex (PEC) nanoparticles. Hydrogen bonding, hydrophobic interactions and van der Waals forces complement PEC formation.[17] Two major steps involved in PEC formation:

- Kinetic diffusion process of mutual entanglement between polymers which depends on molar size differences
- Thermodynamic rearrangement of already formed aggregates due to conformational changes and disentanglement

Different types of aqueous PECs that have been prepared: [18]

- Soluble PEC, i.e., macroscopically homogenous systems containing small PEC aggregates
- Turbid colloidal, PEC systems in the transition range to phase separation

- Two phase system of supernatant liquid and precipitated PEC, which are readily separated as a solid

Two structural models for PECs that are described in the literature are dependant on the polyion groups, stoichiometry and molecular weight:

- The ladder-like structure where complex formation takes place on a molecular level via conformational adaptation
- The scrambled egg model where a large number of chains are incorporated into the particle architecture [19]

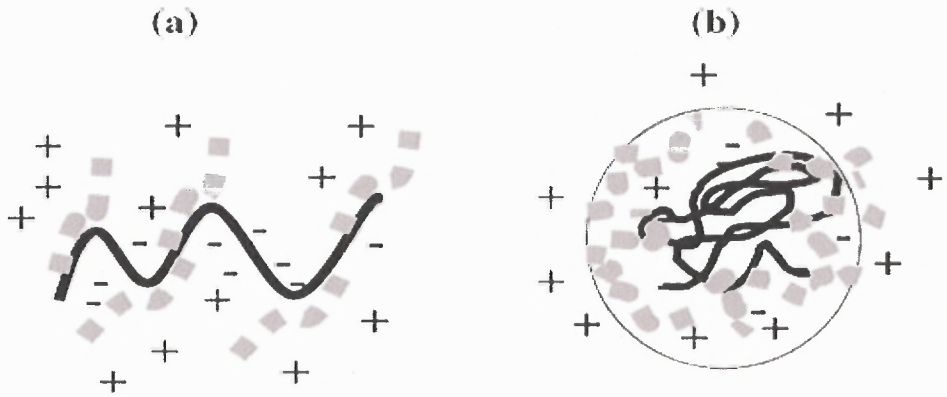


Figure 2.1 schematic representations of (a) ladder-like structure and (b) scrambled egg structure. Black represents polyanion and gray represents polycation. [16]

The ladder-like structure consists of hydrophilic single stranded and hydrophobic double stranded segments. These phenomena result from mixing of polyelectrolytes having weak ionic groups and large differences in molecular dimensions and can result into water-soluble and insoluble PECs. The scrambled egg structures are a product of polyions with strong ionic groups and comparable molar masses yielding insoluble and highly aggregated complexes. The stoichiometry is adjusted under dilute conditions. The colloidal PECs contain a neutral core surrounded by excess binding polyelectrolytes which stabilize the particles against agglomeration. [20] The surplus cation bound to a neutral anionic core leads to stability by repulsion between similarly charged particles

and vice versa in case of surplus anion bound to a neutral cationic core. The steps involved in the formation of both ladder-like and scrambled egg assemblies are the same but they only result in desired structures under certain conditions.

2.4.1 Biological Interactions of PECs

The size of PECs is critical in cell binding and internalization. It has been demonstrated that 100 nm NPs exhibited a 2.5-fold greater uptake relative to 1 μm particles and they had a 6 fold greater uptake relative to 10 μm particles.[21] In addition to the size, surface charge on the PEC nanoparticles also controls their binding. The surface charge also controls the particle stability in aqueous liquid suspension by the electrostatic forces exerted by the particle surfaces.

Cellular surfaces are dominated by negatively charged sulfated proteoglycans, molecules that play pivotal roles in cellular proliferation, migration, and motility. Cell surface proteoglycans consist of a core protein anchored to the membrane and linked to one or more glycosaminoglycan side chains (heparan, dermatan, keratan or chondroitin sulfates) to produce a structure that extends away from the cell surface. Glycosaminoglycans are highly anionic, and the interactions between proteoglycans and NP shells, if positively charged, tend to be largely ionic. Hence the polycationic PEC nanoparticles have advantage of cell binding and also particle stability.[7] Once inside the cell, degradation of polymers may occur, but targeting specific intracellular organelles is possible depending on the surface charge and attached ligands.[22]

2.4.2 Modes of PEC Internalization

The PEC nanoparticles are taken up by the cells by endocytosis. Endocytosis results in internalization of the cell's plasma membrane to form vesicles that capture macromolecules and particles present in the extracellular fluid and/or bound to membrane-associated receptors. The internalized cargo is then directed to appropriate intracellular compartment. Endocytosis takes place by fluid-phase pinocytosis, adsorption and receptor mediated. The bulk uptake of solutes in the exact proportion to their concentration in the extracellular fluid occurs in fluid-phase pinocytosis whereas during adsorptive and receptor mediated endocytosis macromolecules are bound to the cell surface and concentrated before internalization.[7, 16]

It has been described in the literature that PEC internalization is through macropinocytosis. In the absence of a targeting ligand cationic PECs bind cells through electrostatic interactions where cells serve as the anionic sink to mediate the attachment.[23]

2.5 Protein Nanoparticles

Protein-based drug delivery can potentially result in high intratumor drug concentration, ease of administration and shorter infusion times.[15] Protein nanoparticles can be made from bovine serum albumin, human serum albumin etc.

Albumin nanoparticles are versatile protein carriers for drug targeting and improving the pharmacokinetic profile of peptide or protein based drugs. Albumin is the most abundant plasma protein (35–50 g/L human serum) with a molecular weight of 66.5 kDa. It is synthesized in the liver where it is produced at a rate of approximately 0.7 mg/h

for every gram of liver (i.e. 10–15 g daily); Human serum albumin (HSA) exhibits an average half-life of 19 days. The functions and binding properties of HSA are multifold: a) it binds a great number of therapeutic drugs such as penicillin, sulfonamides, indole compounds, and benzodiazepines; b) it is the major protein responsible for the colloid osmotic pressure of the blood; c) when HSA is broken down, the amino acids provide nutrition to peripheral tissue.[24]

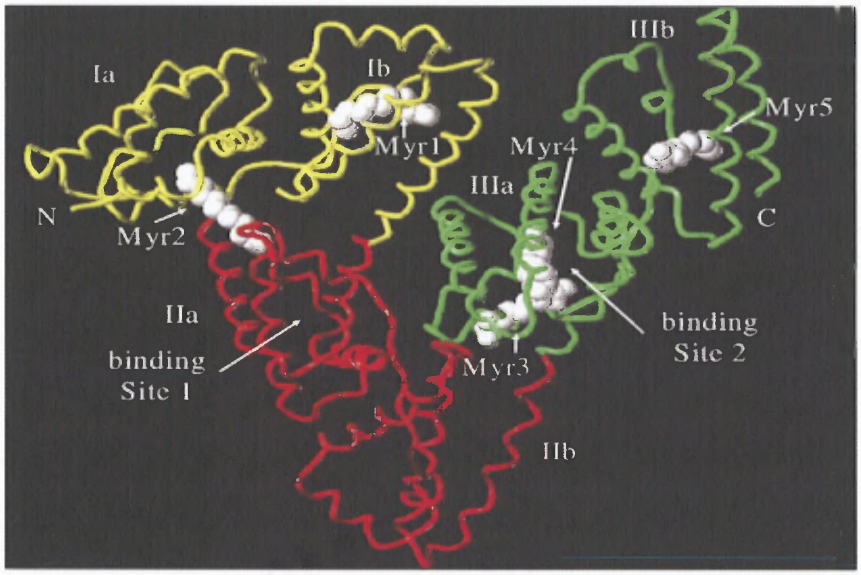


Figure 2.2 X-ray structure of human serum albumin.

The three-dimensional structure of HSA can be described as an ellipsoid consisting of three flexible spheres in a row (domains I, II, III) and is illustrated schematically. HSA is one of the smallest proteins present in blood plasma. Both size and abundance help the transport of large number of metabolic compounds and therapeutic drugs. The binding sites for metabolic substrates and diagnostic as well as therapeutic drugs have been shown in the Figure 2.2.

Albumin is an acidic, very soluble protein that is extremely robust: it is stable in the pH range of 4–9. These properties as well as its preferential uptake in tumor and

inflamed tissue, its ready availability, its biodegradability, its non-antigenicity and its lack of toxicity and immunogenicity make it an ideal candidate for drug delivery.[24]

2.5.1 Mode of Internalization of Albumin NPs

As given in the literature the HSA nanoparticles are mostly distributed into the primary human macrophages into non-nucleic intracellular compartments. Albumin accumulates in malignant and inflamed tissue due to a leaky capillary combined with an absent or defective lymphatic drainage system. It has been demonstrated that tumors are able to trap plasma proteins and utilize their degradation products for proliferation. Albumin is found to be a major energy and nutrition source for tumor growth.[24]

Albumin is thought to facilitate endothelial transcytosis of unbound and albumin-bound plasma constituents into extravascular space. This process is initiated by binding of albumin to a cell surface, 60-kDa glycoprotein (gp60) receptor (albondin), which in turn results in binding of gp60 with an intracellular protein (caveolin-1) and subsequent invagination of the cell membrane to form transcytotic vesicles, referred to as caveolae.[15] Albumin reversibly binds to a number of hydrophobic substances and allows their transport in the body and release at the cell surface. Albumin NPs can thus be used as drug delivery vehicles.

2.6 Targeted Drug Delivery

The nanoparticles act as delivery vehicles for therapeutic agents to prevent the side effects of the drug, protect the healthy tissue, promote sustained release and reduce the dose of the drug. Targeting therapeutics to specific sites in the body will increase the

efficacy of drugs while reducing the dose required along with negative side effects. The delivery of drugs to hard to reach places, such as the brain or cancerous tissues, may also be possible.

The polymeric nanoparticles are conjugated to different ligands like poly-ethylene glycol, peptides, and antibodies for targeting the drug vehicles to selected extracellular and intracellular targets. Among these targeting moieties antibodies have the advantage of high specificity but they are expensive, time-consuming to produce and have problems with stability and storage. Non-antibody ligands suffer from lower selectivity and affinity but they are inexpensive and easy to handle.[7]

Antibodies are well-established systems to target the colloidal carriers to specific cell types. This principal is based on defined receptor ligand interaction which enables the surface binding and cellular internalization of drug carriers conjugated to the antibody. Also the antibody has to be chemically modified to enable the conjugation with drug carrier. The surface of the drug carrier also needs to be modified for conjugation. The drug carriers are crosslinked and the crosslinker is then conjugated with the chemically modified antibody. Thus the antibody carries the drug carrier to the specific sites. A variety of antibodies can be used depending on the receptors present on the cells.[25] Cancer cells often over express receptors for peptides which provide an opportunity for the active and specific targeting of the drug carriers to cancerous tissues. Thus the nanoparticle system loaded with an anticancer drug and conjugated with an antibody can be used for cancer therapy while reducing the side effects and increasing the effectivity of the drug.

2.7 API Micronization

The drug can be loaded into the core of nanoparticles or adsorbed onto their surface. The particle size of the API needs to be reduced to increase the surface area of absorption and enhance the effect of drug. Two different approaches namely top-down and bottom-up approach can be used to obtain particles in the nano-size range for drug delivery. In the top down approach the larger particles are broken down to nano-size range. In the bottom-up approach the particles are built up from molecules.[8]

2.7.1 Top-Down Approach

Comminuting or grinding or milling is the oldest mechanical unit operation for size reduction of APIs and for producing large quantities of particulate materials. The material is subjected to stress which results in breakage of particles. With decreasing particle size, materials exhibit increasing plastic behavior making it more difficult to break the small particles. A limit in grindability can be reached where further grinding does not decrease the particle size.[26] Most of the pharmaceutical size reduction operations utilize the high-shear wet milling for the production of NPs. The API being milled needs to be stable during the entire process of milling. Also care needs to be taken because of the contamination due to milling.

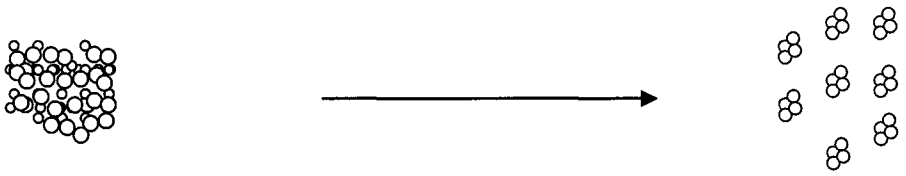


Figure 2.3 Schematic diagram of top-down approach.

2.7.2 Bottom-up Approach

In this process the drug is dissolved in a solvent, the nanoparticles are then precipitated by removing the solvent rapidly or by mixing an antisolvent to the solution, reducing its solubilizing strength. Initially the nuclei are formed which grow because of condensation and coagulation giving the final particles. If the rate of desolubilization is slow then the sticky nuclei particles are formed that have a higher tendency to agglomerate giving large-size final particles. Based on these requirements two general methods for nanoparticle production are used namely supercritical fluid process[27] and emulsification-diffusion process. The key aspect of getting nanoparticles of the desired size is to control the particle coagulation.[8]

Figure 2.4 Schematic diagram of bottom-up approach.

2.8 API Micronization by Milling

In the case of bottom-up technologies one starts with the molecules in solution and moves via association of these molecules to the formation of solid particles. The disadvantage of this technology is the use of solvents creates additional costs and increases the number of manufacturing steps. Also the prerequisite of this process is the drug has to be soluble in one solvent and this solvent has to be miscible with the nonsolvent. Many of the drug compounds are poorly soluble in aqueous and simultaneously in non-aqueous media, thus

excluding this formulation approach.[28] Hence the top-down technology is preferred. They can be categorized into pearl/ball milling and high pressure homogenization.

Micronization of drug powders by colloid mills or jet mills are of limited use since they lead to particles in the size range of 0.1 and 20 um. It has been shown that drug nanosuspensions can be obtained by running a pearl mill over a sufficiently long period of time. These mills consist of a milling container filled with milling pearls or balls. The container can be static and the milling material is moved by means of a stirrer; alternatively the complete container is moved in a complex movement leading to movement of milling pearls. A problem associated with this technology is the erosion from milling material during the milling process. The extent of erosion depends on the solid concentration of the macrosuspension to be processed, the hardness of the drug and the required milling time and milling material.[8] The erosion of the material from the container also may occur. Normally the product containers are made of steel (SS 316) to solve the problem of container material erosion and maintain the required quality specifications of the formulation.

The nano/micro suspensions are stabilized by the addition of stabilizers and surfactants.[29] The coarse drug powder is dispersed by high speed stirring in a surfactant/stabilizer solution to yield a microsuspension. The choice of surfactants and stabilizers depends on the properties of drug (affinity to surfactant/stabilizer), physical principles (electrostatic/steric stabilization) and the route of administration. Electrolytes reduce the zeta potential and thus impair the physical stability of the suspension. The optimal approach is use a combination of steric stabilizer for steric stability and ionic surfactant for electrostatic stabilization. For oral administration there is a wide choice of

charged surfactants, however for parenteral route it is limited e.g. for intravenous injection poloxamer 188, lecithin, tween 80, sodium glycocholate, polyvinyl pyrrolidone are accepted. Production of nanosuspensions of the drug for parenteral route has to be done in sterile conditions and the product has to be pyrogen free. Also in case of pearl mills the product needs to be separated from the milling pearls at the end of the milling process.[8] Apart from pearl/ball mills, other grinding mills like Micros can be used. The milling material is attached to a central shaft which rotates vigorously and helps breaking of particles. Milling material consists of ceramic rings attached to sub shafts. The separation step of milling material from the product is reduced in this case.

2.9 Remarks based on Literature Review

2.9.1 Preparation of Nanoparticles for Targeted Delivery

The polyelectrolyte complex nanoparticles are made from water-soluble and biodegradable polymers. They are biocompatible and stable. The structure of PEC nanoparticles is available in the literature but there is a need to understand the mechanism of formation of the particles. The process of formation of the nanoparticles needs to be optimized to get the particles in a reproducible manner. The nanoparticles can be targeted by conjugation with various ligands like peptides and polyethylene glycol. The amine groups on the PEC NPs can also be modified by crosslinking with SMCC by an amide linkage and then conjugated with a thiolated antibody.

The albumin nanoparticles are non-antigenic, have a number of binding sites and made of amino acids which indicate the presence of a large number of amine groups that are required for conjugation. Albumin nanoparticles can be prepared reproducibly by the

desolvation process as available in the literature. They have been efficiently conjugated with antibody for drug delivery. A different antibody could be conjugated with the albumin nanoparticles to target different cells in the body to study the cell binding and internalization of the nanoparticles.

2.9.2 Preparation of Nanosuspensions of APIs by Milling

It has been discussed in the literature that there is a need for nanosize APIs for their quick absorption and efficient therapeutic effect. Milling has been the most commonly used method for API size reduction. The ball/pearl mills are commonly used for wet milling. However wet milling can be done in Micros since it has an advantage that the milling material is not mixed with the product as is the case with ball/pearl mills. Micros thus reduces one step of separation of milling material from the product in the process of manufacturing.

As given in the literature the API suspensions can be stabilized with a variety of surfactants and stabilizers to prevent particle agglomeration and coagulation and yield stable suspensions in a reproducible manner. The particle size of a large number of APIs have been reduced by wet milling. However particle size reduction of drugs like griseofulvin (an antifungal agent), by the process of wet milling using Micros still needs to be tried.

CHAPTER 3

EXPERIMENTAL SECTION

3.1 Nanoparticles for Parenteral Drug Delivery

Two different nanovehicles were prepared namely polyelectrolyte complex nanoparticles and albumin nanoparticles. In this chapter materials and methods used in this work have been discussed.

3.1.1 Polyelectrolyte Complex Nanoparticles

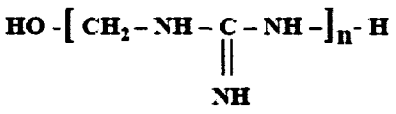
Materials

Polyanion: Sodium alginate was obtained from Vanderbilt University at Nashville, Tennessee and chondroitin sulfate from Sigma Aldrich.

Polycation: Spermine tetrahydrochloride was obtained from Sigma Aldrich and Polymethylene (-co-guanidine) hydrochloride (PMCG) was ordered from Polymeric Products Inc.

Pluronic F-68 and Calcium chloride were obtained from Sigma Aldrich.

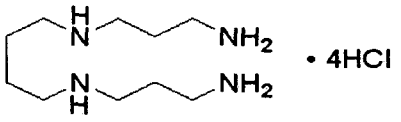
poly(methylene-co-guanidine) hydrochloride



+ 1 charge

5000 kDa

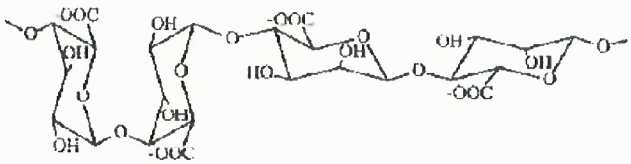
spermine tetrahydrochloride



+ 4 charge

348 kDa

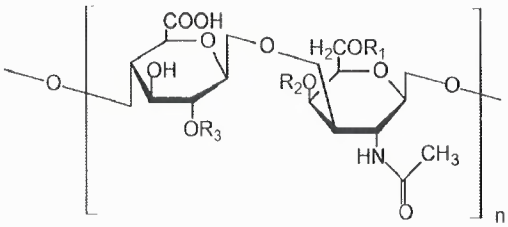
sodium alginate



- 1 charge

12000 kDa

chondroitin sulfate

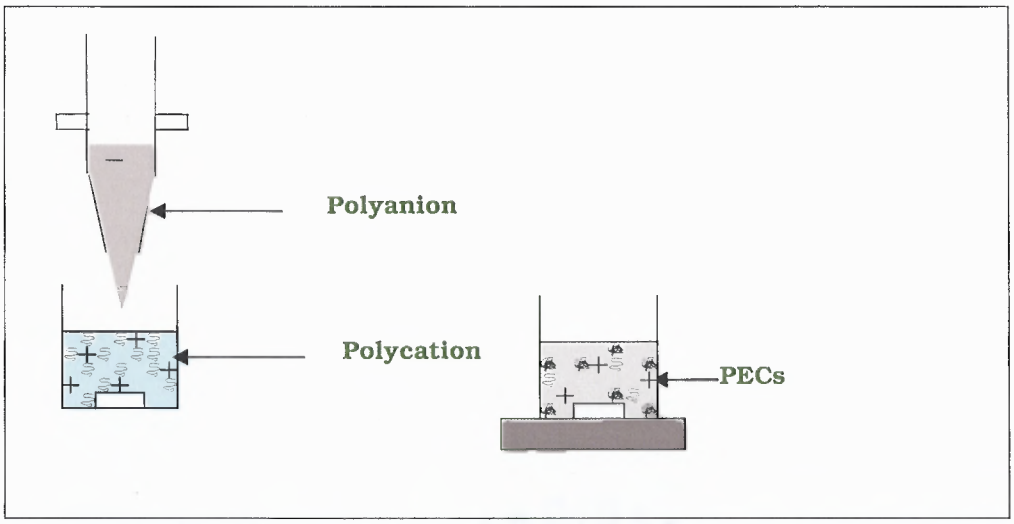


- 2 charge

15000 kDa

Figure 3.1 Structures of polymethylene (co-guanidine) hydrochloride, spermine tetrahydrochloride, sodium alginate and chondroitin sulfate.

Experimental Section



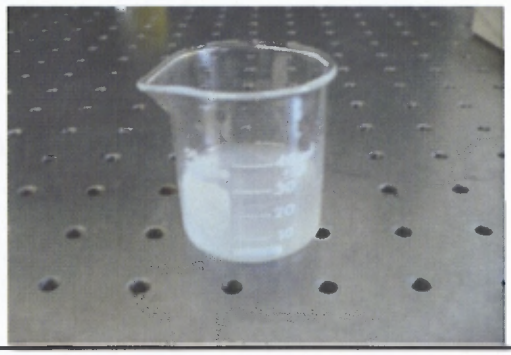


Figure 3.2 Experimental set-up (top), polycation (bottom left) and polyelectrolyte nanoparticles (bottom right).

Method of Preparation

The solution of polyanions sodium alginate (0.5mg/ml) and chondroitin sulfate (0.5mg/ml) in distilled water were taken in the burette. The solution of polycations PMCG (0.5mg/ml) and spermine tetrahydrochloride (0.5mg/ml) were taken in a glass beaker. The Pluronic F-68 (1mg/ml) and Calcium Chloride (0.5mg/ml) were dissolved in the cationic solution. The cationic solution was kept under continuous magnetic stirring at room temperature. The polyanion solution was added to the polycationic solution at a rate of 1ml/min. The polyelectrolyte nanoparticles were obtained instantaneously. The nanoparticles were separated from the solution by centrifugation at a speed of 10,000 rpm (13900g) (in Sorvall centrifuge) at 4° C for 10 minutes. After centrifugation the nanoparticles were resuspended to its original volume with 10mM sodium acetate buffer pH 4.2. The nanoparticles were washed three times to remove the excess polycations and stabilizer.[23] The particles were characterized by particle size measurements using N4 plus, particle images by Scanning Electron Microscopy (SEM) and the amine group determination by Fluorescence Microplate Reader.[30]

3.1.2 Albumin Nanoparticles

Materials

Human serum albumin (Fraction V powder), sodium chloride, ethanol (USP) and 8% aqueous solution of glutaraldehyde were obtained from Sigma Aldrich.

Method of Preparation

The experimental set-up was the same as the polyelectrolyte complex nanoparticles. The 200 mg Human Serum Albumin was dissolved in 2 ml 10 mM sodium chloride solution. The pH of the solution was adjusted to 9.0 using 10mM sodium hydroxide solution.[32] The albumin solution was kept under continuous magnetic stirring. 8 ml ethanol solution was added through the burette at a rate of 1ml/min to the albumin solution at room temperature. After complete addition of ethanol the particles were crosslinked by the addition of 235 μl 8% aqueous glutaraldehyde solution. The particles were then separated from the solution by centrifugation at 11,500 rpm (16,100 g) (in Sorvall centrifuge) at 15°C for 8 minutes.[31] The albumin nanoparticles were washed three times and after every wash they were resuspended to the original volume with 10mM sodium chloride solution pH 9.0.[32] The particles size measurements were done by N4 plus, images were taken by Scanning Electron Microscopy and amine group determination by Fluorescence Microplate Reader.

3.1.3 Particle Size Measurement

The nanoparticle solution (3 ml) was taken in a cuvette and particle size was measured at angles of 23°, 43.6°, 60° and 90° using N4 Plus by the Photon Correlation Spectroscopy (PCS). A drop of the NP solution was placed on the SEM stub for SEM micrographs. The stub was coated with carbon to prevent degradation of sample due to charging.

3.1.4 Amine Group Determination

The amine groups available on the surface of nanoparticles were measured by the reaction with fluorescamine. 10 μl of the sample was added to 140 μl 40mM PBS buffer pH 7.4 taken in the well of fluorescence microplate reader. 40 μl of fluorescamine (3mg/ml) solution was pipetted into this well. The fluorescence was measured at an excitation wavelength of 400 nm and emission of 460 nm.[30] A calibration curve was plotted for solutions with known amount of amine groups and the amine groups of the unknown solutions were calculated from the calibration curve.

3.1.5 Crosslinking and Conjugation

After the surface available amines were determined, the crosslinking and conjugation were done at the Center for Molecular Medicine and Immunology (CMMI), Belleville, NJ. The nanoparticle solution was divided into two parts A (1 ml) and B (7 ml). SMCC was not added to solution A and it was retained as a control sample. 128 μl of SMCC solution (6mg/ml in water) was added to solution B and was magnetically stirred for 45 minutes for crosslinking. The excess SMCC was removed by three steps of centrifugation at 16,000 g at 15°C for 8 min (in Beckman Coulter JA 17 rotor) and after every wash the sample was resuspended to the original volume with 10mM sodium chloride solution pH 7.4. After the last wash the particles were resuspended in acetate buffer (pH 6.0) for antibody conjugation. Solution A also underwent centrifugation since it was retained as control. The solution B was then split into two parts (3.5 ml each), one part received the 800 μl (1.53 mg/ml) antibody and the other was retained as control. The Fab'-SH was stirred with NP-sufo-SMCC for 45 minutes.[25] The Fab'-NP conjugate was purified by

three centrifugation steps (16,000g, 8 min) and redispersion of pellet to original volume in sodium chloride pH 7.4. The particle size of these samples was measured by N4 plus and the in-vitro studies were performed by Fluorescence Assisted Cell Sorting (FACScan) [33] at CMMI.

3.2 API Size Reduction for Oral Drug Delivery

Materials

Griseofulvin, Hydroxypropylmethyl cellulose (HPMC), Polymer JR, Polyvinyl Pyrrolidone (PVP), Sodium Alginate (SA) and Pluronic F-68 were obtained from Sigma Aldrich.

Griseofulvin with molecular formula $C_{17}H_{17}O_6$ is an antibiotic with antifungal properties. It is derived from a species of Penicillium. It is used in the treatment of ringworm and other fungal infections of the skin or nails.

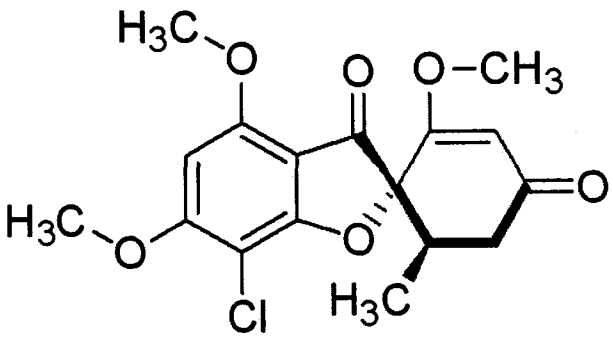
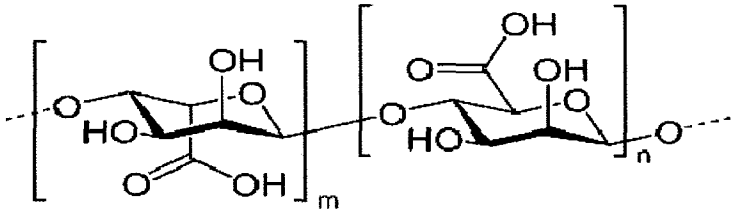
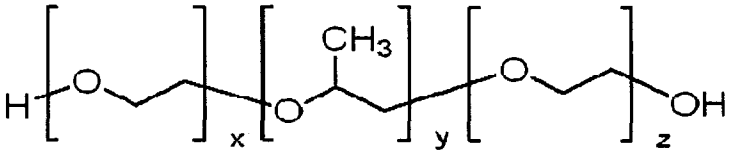


Figure 3.3 Structure of Griseofulvin.

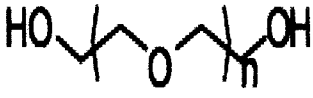
The polymers and surfactants were used for stabilization of the particle size. The structures of the polymers are given below.



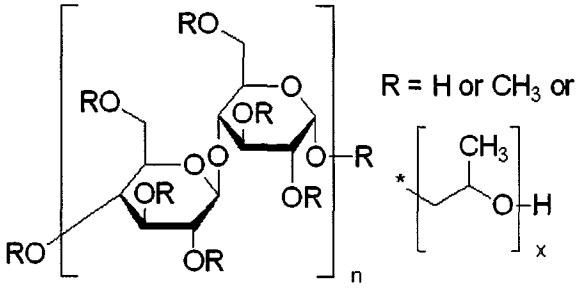
Sodium alginate



Pluronic

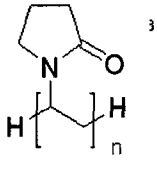


PEG

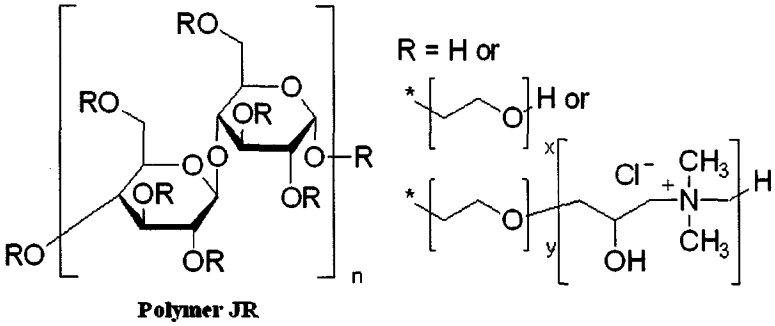


HPMC

R = H or CH_3 or



PVP



Polymer JR

R = H or

Figure 3.4 Structures of sodium alginate, PEG, Pluronic, HPMC, PVP and Polymer JR.

Experimental Set-up

Figure 3.5 Construction of micros.

The vessel of Micros is made of stainless steel and has a capacity of 400 ml. The grinding parts consist of ceramic rings arranged on sub-shafts and the sub-shafts in turn revolve around a central main shaft (planetary motion) which has an impeller at the bottom. The grinding takes place due to the shear forces between the rings and the wall of the vessel and in-between the rings. Cooling water is passed continuously through the jacket to maintain a constant temperature.

Method

The polymer/surfactant solutions at concentrations of 1, 5, 10 wt% of the drug were prepared. The polymers used were sodium alginate, polymer JR, polyethylene glycol, pluronic F-68 and polyvinyl pyrrolidone. 2.0 Wt % of griseofulvin was suspended in the above solutions. The 350 ml sample was loaded in the Micros vessel and cooling water

was passed through the jacket to maintain a constant temperature at 24°C. The suspension was milled at 1500 rpm for 75 minutes. The suspension was then unloaded from Micros and taken for particle size measurement.

Particle Size Measurement

The particle size was measured by light scattering (Beckman Coulter LS 230). The Polarization Intensity Diffraction Scattering (PIDS) was performed. The SEM images were taken by placing a drop of the sample on the SEM stub and the sample was coated with carbon to prevent the charging of the sample.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Polyelectrolyte Complex Nanoparticles

The particle size of the polyelectrolyte complex nanoparticles depends on the pH, charge ratio, stoichiometry of the ions, [19] molecular weight, stirring speed, concentration of stabilizer[34] and calcium chloride. Hence the process of preparation of polyelectrolyte complex nanoparticles was optimized by variations in the above parameters to get stable nanoparticles.

4.1.1 Effect of Charge Ratio

The charge ratios were varied knowing the molecular weights and charge on each polymer unit.

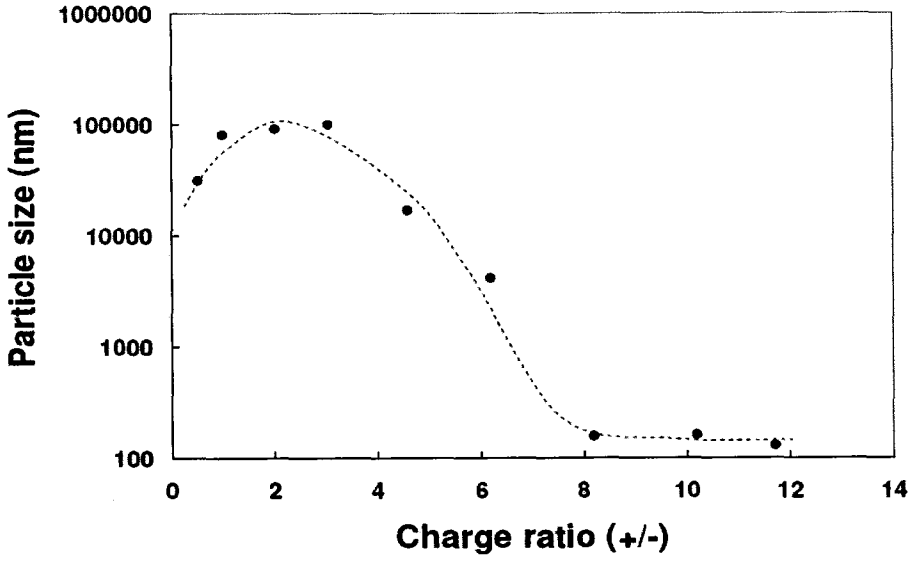


Figure 4.1 Effect of charge ratio on particle size of the PEC nanoparticles.

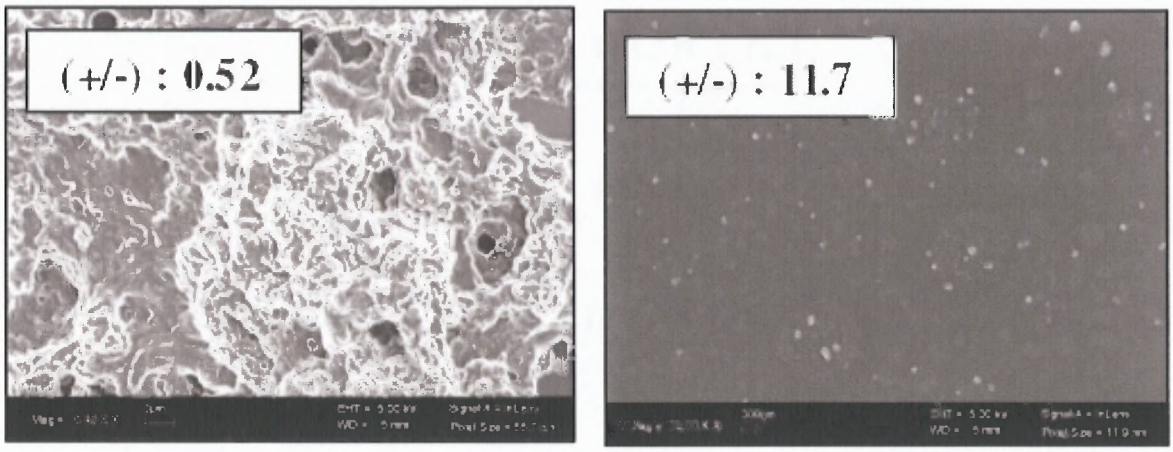


Figure 4.2 SEM Micrographs of PEC nanoparticles at charge ratios 0.52 and 11.7.

The particle size of the PEC nanoparticles increased with increase in charge ratio. The excess positive charge on the particles promoted the repulsion between the particles and hence stabilized the particles. At low charge ratios there was no excess charge on the particles and hence they agglomerated as seen in the SEM micrographs.

4.1.2 Effect of pH

The effect of pH on the particle size was studied in two ways:

- Change in pH of polycation solution (Before Centrifugation)
- Change in pH after particle separation (After Centrifugation)

4.1.2.1 Before Centrifugation. The pH of the polycationic solution was varied by the addition of Hydrochloric acid for acidic pH and Sodium hydroxide for basic pH.

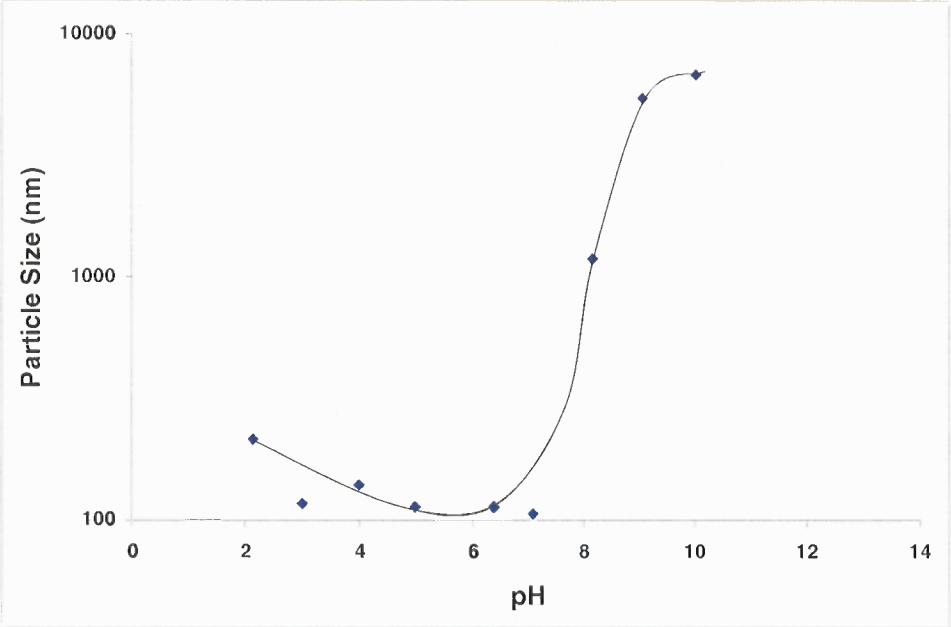


Figure 4.3 Variation in particle size with change in pH before centrifugation.

$$RNH_3^+ \leftrightarrow RNH_2 + H^+$$

$$HCl \leftrightarrow H^+ + Cl^-$$

$$NaOH \leftrightarrow Na^+ + OH^-$$

At acidic pH excess of protons were present in the solution. As per the Le Chateliers principle the equilibrium shifted to the left, the nanoparticles do not dissociate and the excess positive charge was retained on the particles leading to small particle size. At basic pH excess hydroxide ions were present. To maintain equilibrium the nanoparticles dissociated loosing their excess positive charge and hence increase in particle size was observed.

4.1.2.2 After Centrifugation. The particles were separated by centrifugation and resuspended in sodium acetate buffer for acidic pH and phosphate buffer for basic pH.

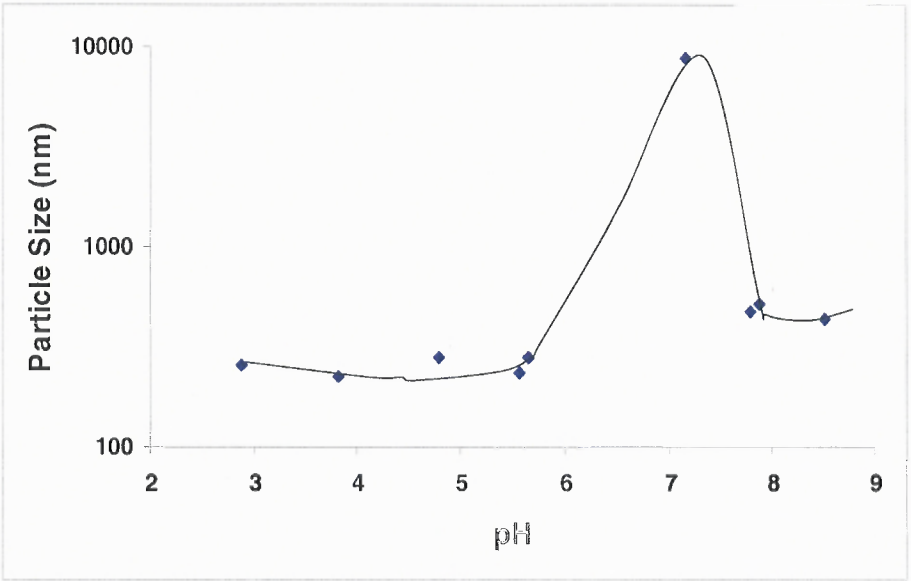


Figure 4.4 Change in particle size with variation in pH after centrifugation.

Figure 4.3 showed a clear tendency towards aggregation as pH approached neutrality corresponding to change in surface charge. As the pH increased from 3 to 7, the particle size increased rapidly due to decrease in the repulsive surface charges. Also the particle size stabilized after increasing the pH above 7.6. This type of behavior on either side of neutrality indicated that PEC nanoparticles exhibit Zwitterionic behavior. At low pH the carboxyl and amino groups were in the protonated state indicating excess positive surface charge. As the pH increases to 7, the amino and carboxyl groups dissociated depleting the surface charge leading to the coalescence of the particles.[16] As pH was increased above 7.6, the carboxyl anions were formed, thus retaining an excess negative charge and leading to a stable particle size.

4.1.3 Effect of Stabilizer Concentration

The stoichiometry of polycations and polyanions and the pH was kept constant. The concentration of Pluronic F-68 was increased from 0 to 10mg/ml to study its effect on particle size.

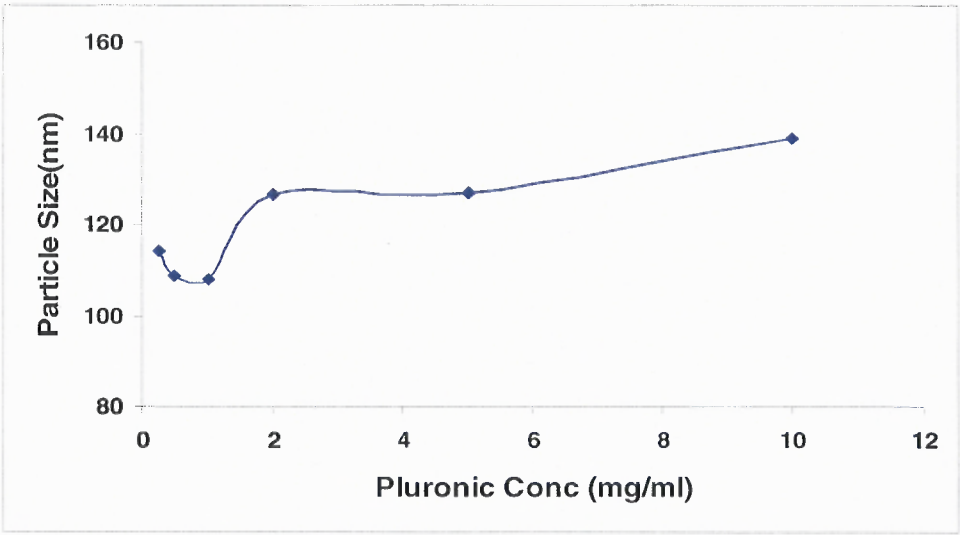


Figure 4.5 Change in particle size of PEC NPs with increase in stabilizer concentration.

A slight dip in particle size was observed with increase in pluronic concentration from 0 to 1 mg/ml and the particle size increased after pluronic concentration of 2 mg/ml. The surfactant stabilized the PEC NPs by forming a coat on the particle surface, thus preventing particle agglomeration. However the critical micellar concentration of Pluronic F-68 is 1.8 mg/ml. Beyond the CMC the surfactant formed reverse micelles and lost its tendency to protect the particles from coalescence. Hence the increase in particle size was observed.

4.1.4 Effect of Electrolyte Concentration

The concentrations of polyanion, polycation and stabilizer and the pH were kept constant. The concentration of Calcium Chloride was varied to study its effect on particle size.

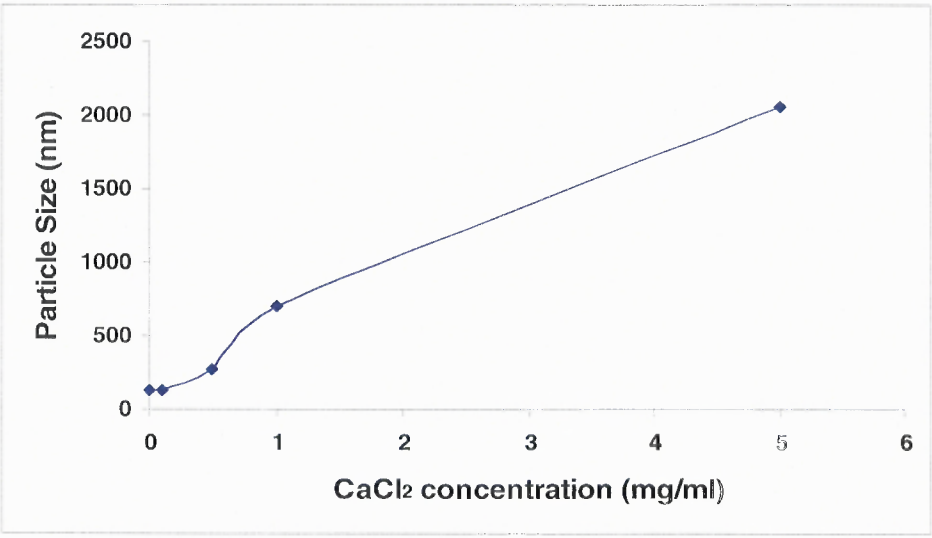


Figure 4.6 Change in particle size with variation in electrolyte concentration.

The particle size was found to increase with increase in the electrolyte concentration. The chloride ions reacted with the amine groups on the particle surface or were adsorbed on the surface. It reduced the surface charge on the particles and hence the particles coalesce leading to larger particle size.[35] The calcium chloride was used to enhance the electrostatic interaction between the polyelectrolytes and it played a role of maintaining an electrolyte balance inside the body.

4.1.5 Effect of Mixing Speed

The polycation solution was kept under continuous magnetic stirring during the particle formation process. The stirring speed was varied to optimize the speed that can be used to get nanoparticles in a reproducible manner. The concentration of polyelectrolytes, stabilizer, salt and pH were kept constant to study the effect of mixing speed.

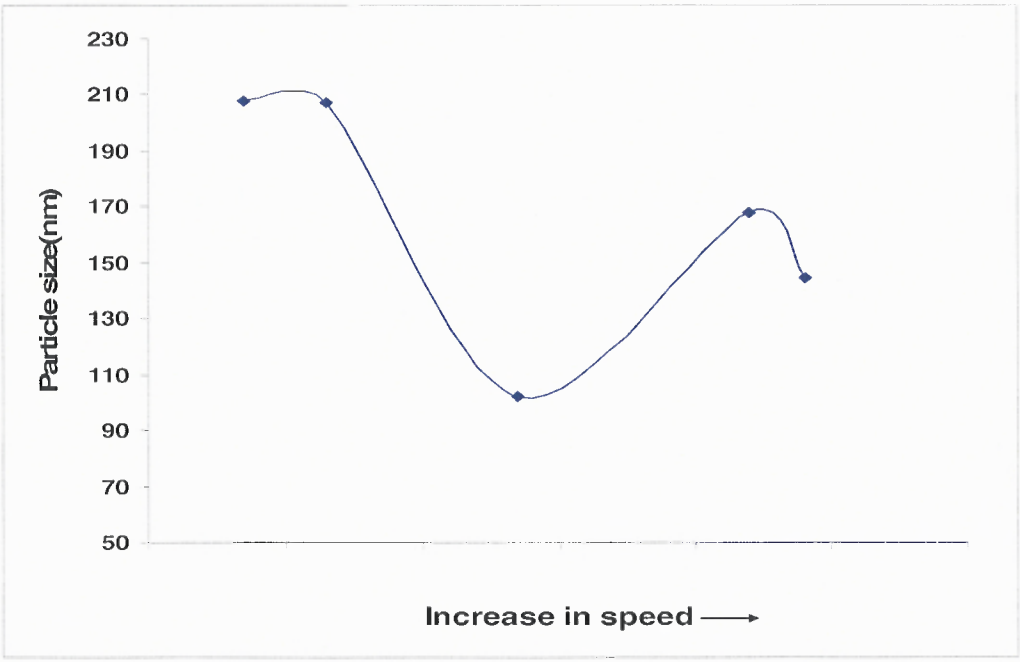


Figure 4.7 Variation of particle size with stirring speed.

Figure 4.7 indicated a decrease in particle size initially with an increase in the mixing speed (using a VWR stirrer). However, the particle size increases at higher speeds. At low speed, the mixing was not sufficient and the particles tend to coalesce to form larger particles. At higher speeds, the mixing was vigorous and the frequency of particle collisions increased resulting in an increase in particle size. Thus it was observed that the optimum speed was crucial for sufficient mixing and lower particle size.

4.1.6 Effect of Centrifugation on Particle Size

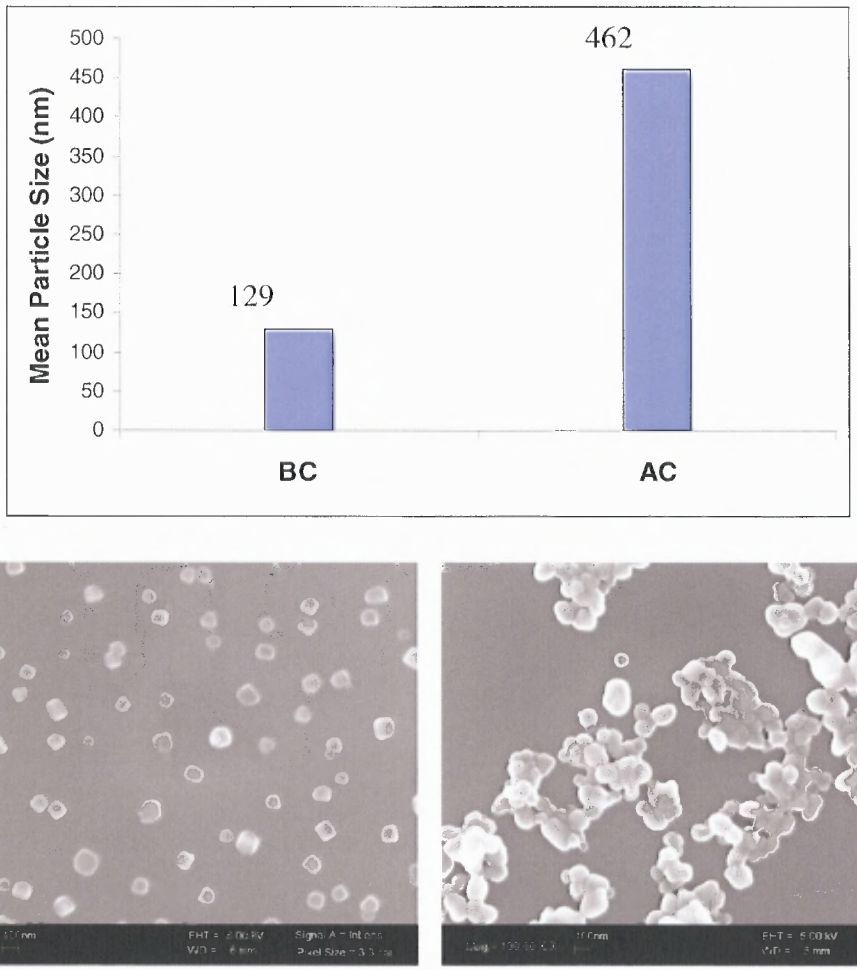


Figure 4.8 Bar graph showing increase in PEC particle size after centrifugation (top) and SEM micrographs of PEC particles before (bottom left) and after centrifugation (bottom right).

The particle size of PEC NPs increased after centrifugation due to agglomeration between the particles caused by the increased collisions during centrifugation and removal of excess positive charge. The size increased to 450-500 nm and hence the particles could not be used for cellular uptake studies.

The optimized parameters were as given below:

- Charge ratio of polycation : polyanion was 25: 1
- Pluronic F-68 concentration: 1 mg/ml
- Use of calcium chloride was avoided since it causes an increase in particle size.
- Resuspension buffer was 10 mM sodium acetate buffer pH 4.2
- Mixing speed was fixed at 2800 rpm

4.1.6 Amine Group Determination

The amount of amine groups available for crosslinking was determined by reaction with fluorescamine.

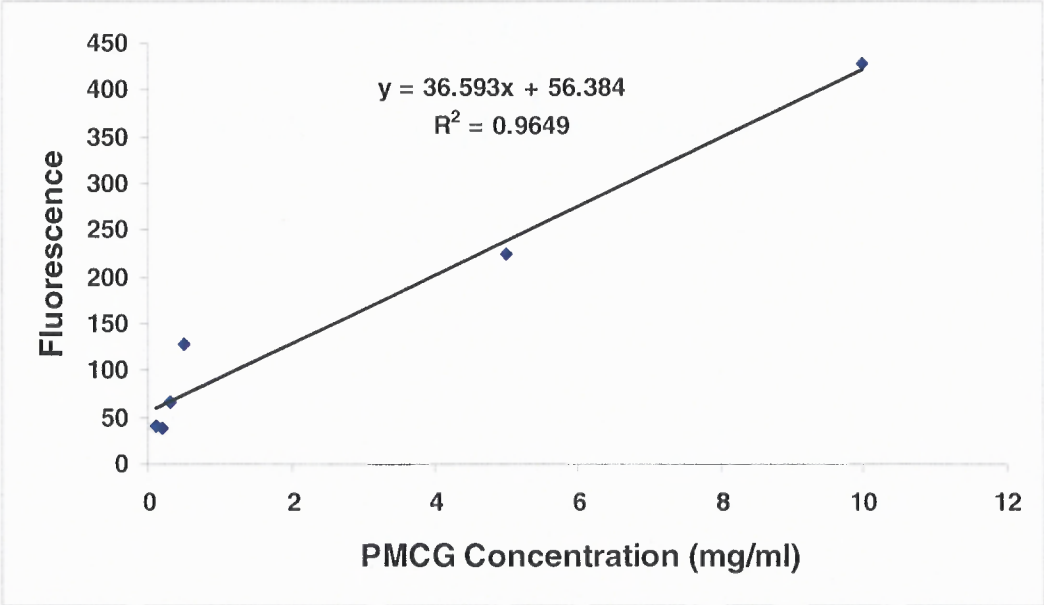


Figure 4.9 Calibration curve for amine group determination.

PMCG was dissolved in water to make a series of solutions at concentrations of 0.1, 0.2, 0.3, 0.5, 5 and 10 mg/ml. 10 μl of each sample was pipetted into 140 μl of the PBS buffer pH 7.4 taken in the fluorescence microplate reader. 50 μl of the fluorescamine

solution 3 mg/ml in acetone was pipetted into each of the sample and the fluorescence was determined. The moles of amine in the polyelectrolyte nanoparticles were calculated from the number of amine groups on PMCG.

The number of surface amines on the PEC NPs was found to be 9.75×10^{-11} moles. From the fluorescence readings it was clear that most of the PMCG remains unreacted since the supernatant solution obtained after centrifugation gave a higher reading. The amines available on the surface of PEC NPs were insufficient for crosslinking. So the PEC NPs could not be conjugated for targeted delivery. Hence a different system of nanoparticles was chosen for drug delivery.

4.2 Albumin Nanoparticles

The optimized procedure for making the albumin nanoparticles was obtained from literature. The particle size and amine groups of the albumin nanoparticles were determined.

The amine count of albumin nanoparticles was 1.678×10^{-9} moles which was feasible for crosslinking with SMCC. The complex of albumin nanoparticles and SMCC was conjugated with antibody for drug delivery as shown in the schematic diagram.

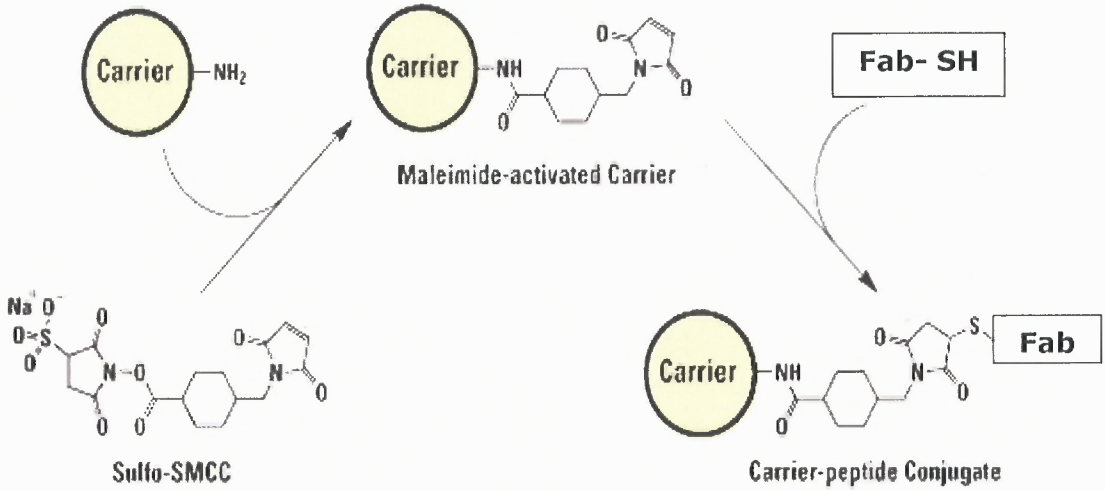


Figure 4.10 Schematic diagram of antibody conjugation.

The particle size of the albumin nanoparticles at different stages are given below:

Table 4.1 Mean Particle Size of Albumin Nanoparticles at Different Stages

Type	Particle size (nm)	Standard deviation (nm)	Polydispersity index
After preparation	81	36.1	0.921
Without SMCC and antibody	172.4	64.0	0.351
Without SMCC, with antibody	220	80.4	0.421
With SMCC, without antibody	169.8	62.7	0.351
With SMCC and antibody	229.8	89.4	0.432

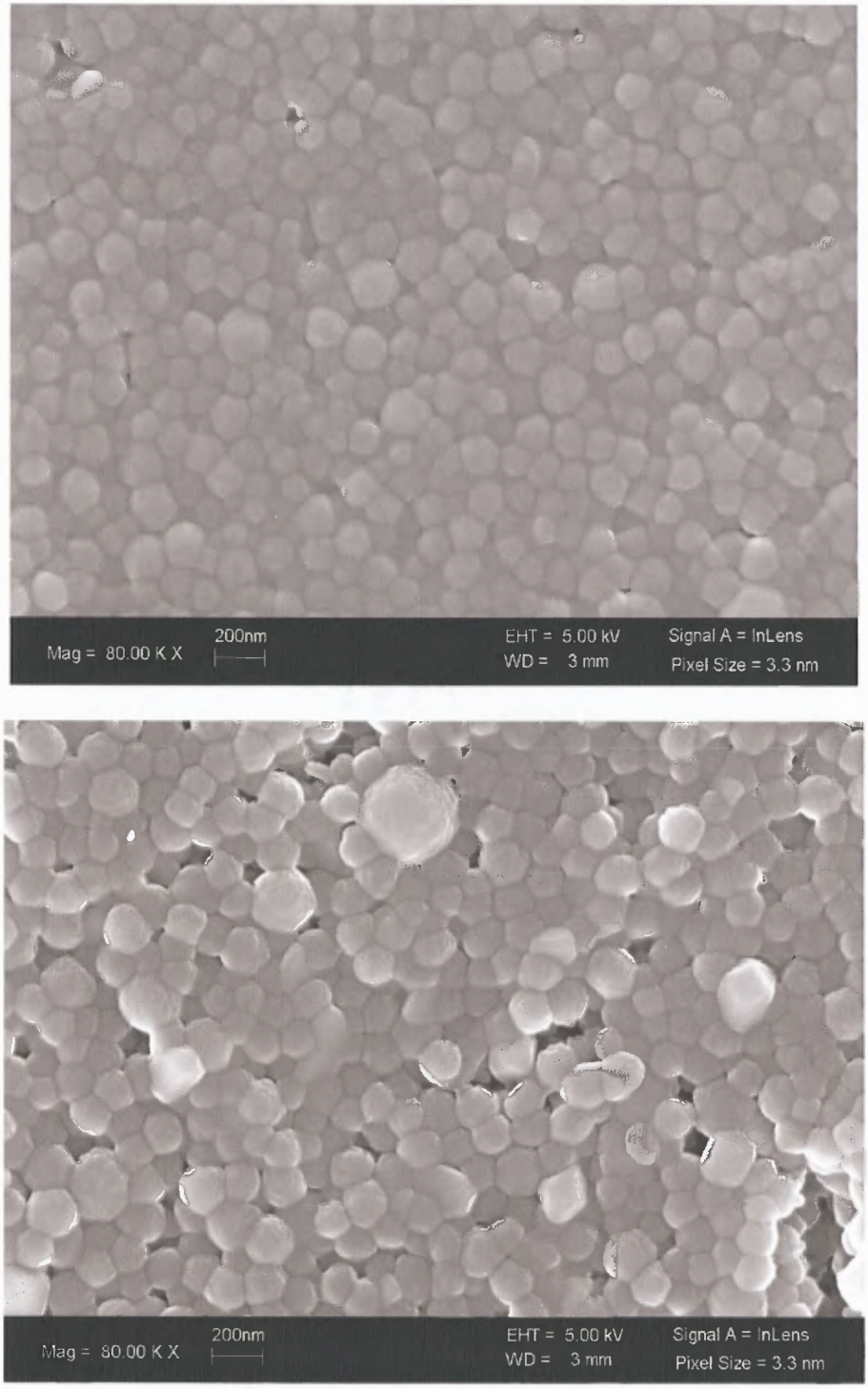


Figure 4.11 SEM micrographs of albumin nanoparticles after preparation (top) and after conjugation (bottom).

The particle size of the nanoparticles was acceptable for cellular uptake. The in-vitro studies of the nanoparticles were done by Fluorescence Assisted Cell Sorting (FACScan). The FACScan images shown below clearly indicate nanoparticle binding to the lymphoma cells. The Figure 4.12 serves as a control. In the Figures 4.13 and 4.14, the indirect staining method was used. In this method the antibody that binds to the surface of the cell is not fluoresceinated and it binds to a second step fluoresceinated FITC-Ab reagent specific for the determinants on the non-fluorescent first-step antibody. Hence the stain amplifies because several molecules of second-step FITC-Ab bind to one molecule of first-step reagent. The cell count decreased because the size of the Fab'-NP conjugate was larger and hence bound to fewer cells.

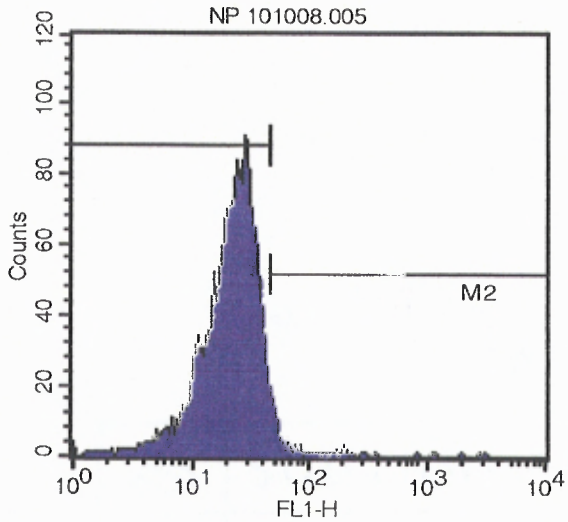


Figure 4.12 FACScan image showing FITC-Ab + Ramos cells.

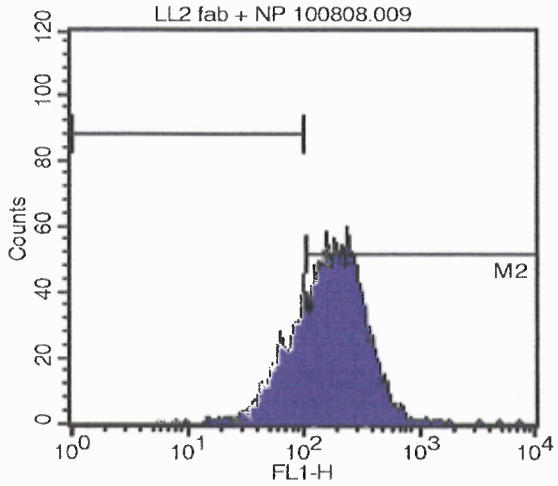


Figure 4.13 FACScan image of Ramos cells + Fab'-SH + FITC-Ab.

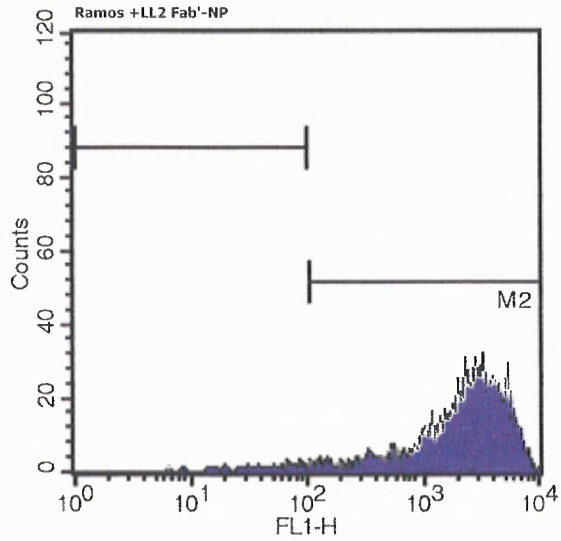
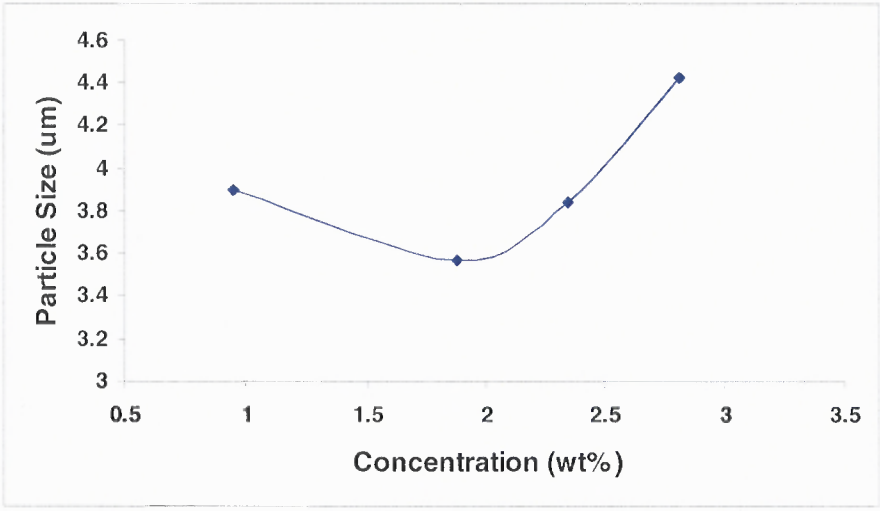


Figure 4.14 FACScan image of Ramos cells + Fab'-NP + FITC-Ab.

4.3 API Size Reduction

4.3.1 Optimization of Process Parameters*

The milling conditions that are the time of milling, optimum speed of milling and the concentration of the sample to be used were optimized in NJCEP laboratory. The optimized conditions have been shown below.



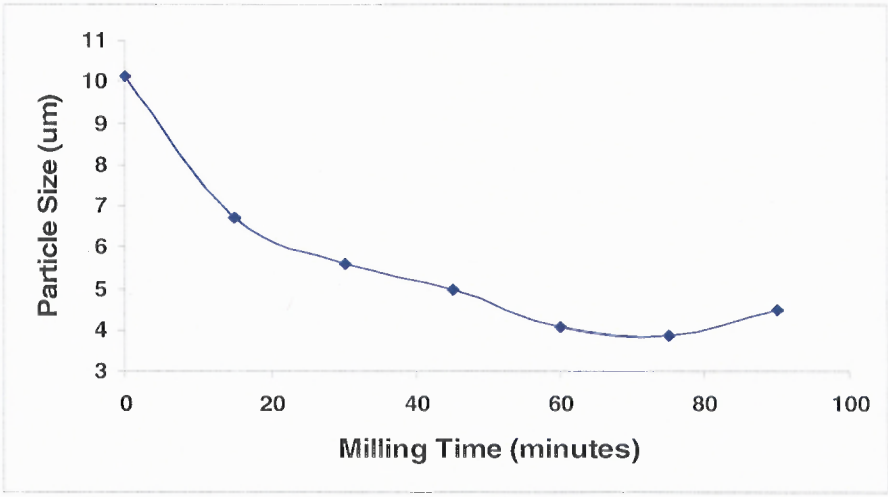


Figure 4.15 Plot showing the effect of concentration of the drug sample (top) and milling time (bottom) on the mean particle size of drug.

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* Results obtained by Marie Aloia (RET) under the guidance of Dr. Sameer Dalvi and Prof. Rajesh Dave

The particle size of griseofulvin decreased initially with increase in concentration because of the shear stress acting on the particles. However at higher concentration particle agglomeration dominated over the shear stress and hence there was an increase in particle size. Similarly when the time of milling was increased, the residence time for the growth of particles increased and hence the increase in size was observed.

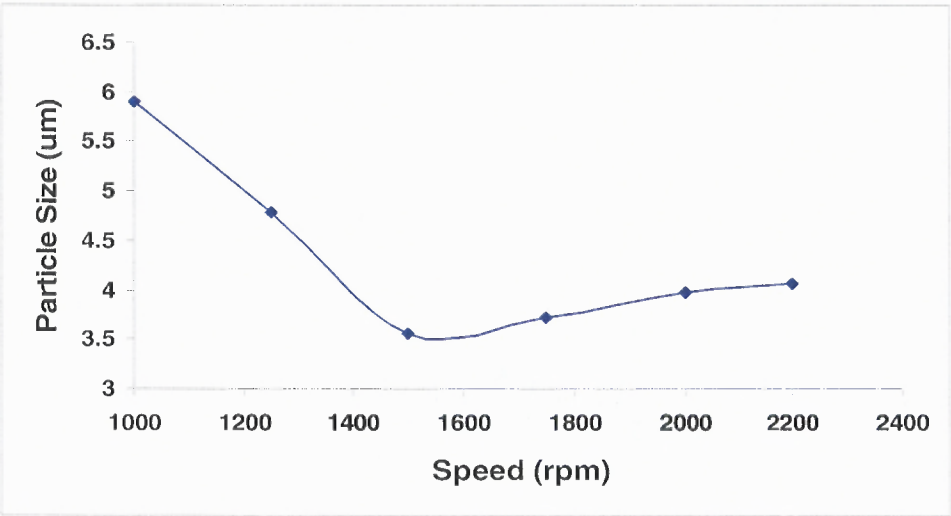


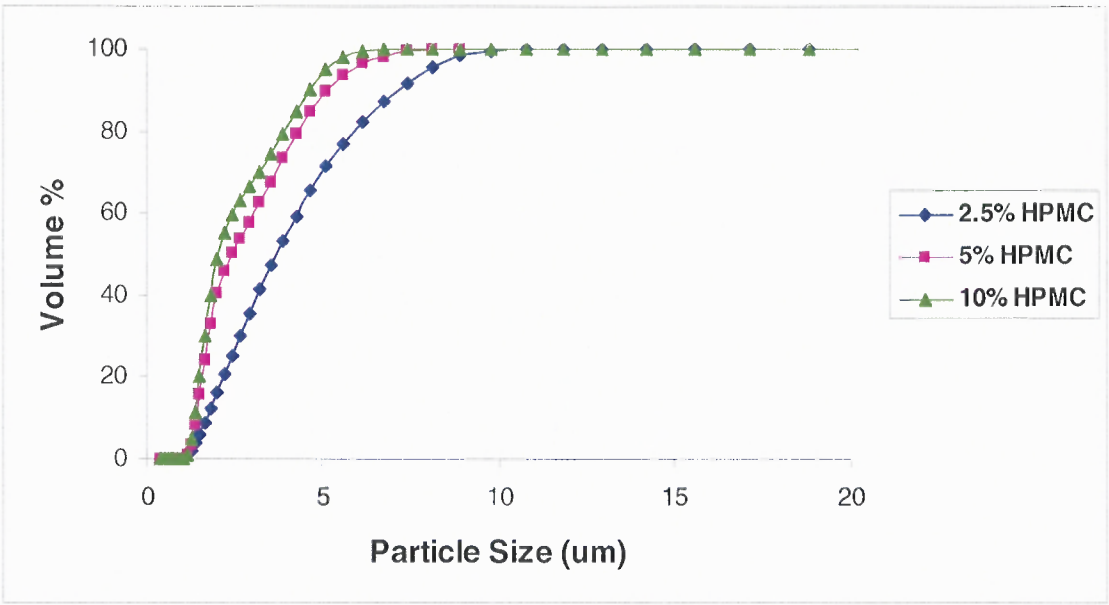
Figure 4.16 Plot showing the effect of milling speed on the mean particle size of the drug.

The particle size of griseofulvin decreased initially with an increase in the milling speed due to the increase in shear stress acting on the particles. At higher speeds the particle collisions increased that lead to a slight increase in the particle size due to agglomeration.

These optimized process parameters were used to stabilize the API suspensions by co-milling using different surfactants and polymers. The effects of these materials have been discussed below.

4.3.2 Co-milling with Polymers

Hydroxypropyl methyl cellulose (HPMC), sodium alginate, polymer JR, polyethylene glycol (PEG) and polyvinyl pyrrolidone (PVP) were used to control the particle size of the API suspensions. The concentrations of the polymers are with respect to the drug.



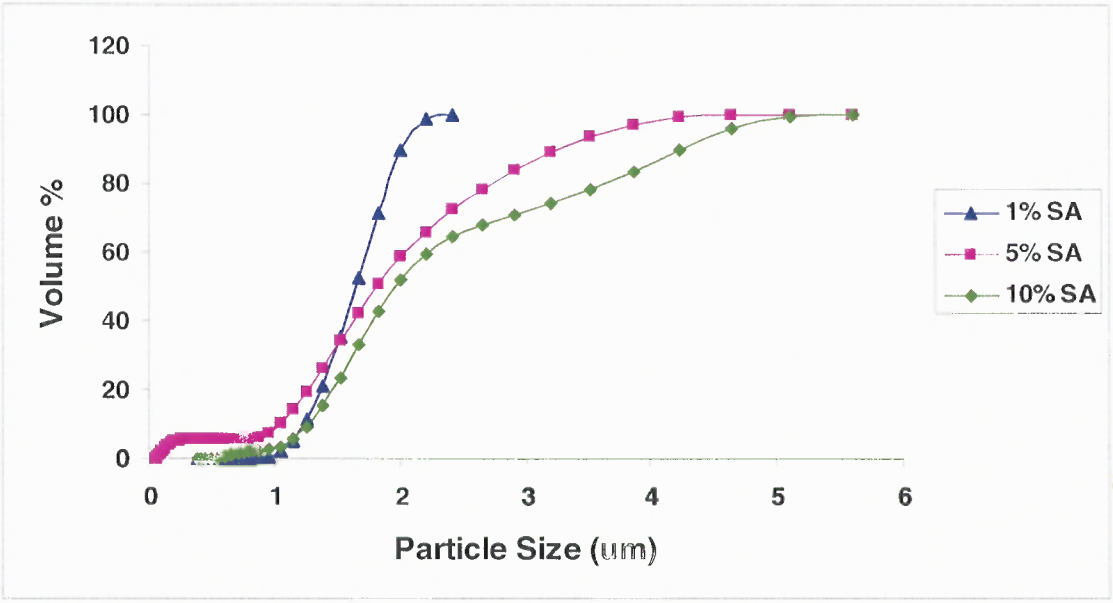


Figure 4.17 Plot showing the effect of varying concentrations of HPMC and sodium alginate on the particle size of griseofulvin.

The particle size was reduced by milling. In the absence of polymer/stabilizer the particle size was not stable since the particles agglomerated. HPMC and sodium alginate formed a coat around the drug particles and hence protected direct interaction between the drug particles, thereby preventing agglomeration.

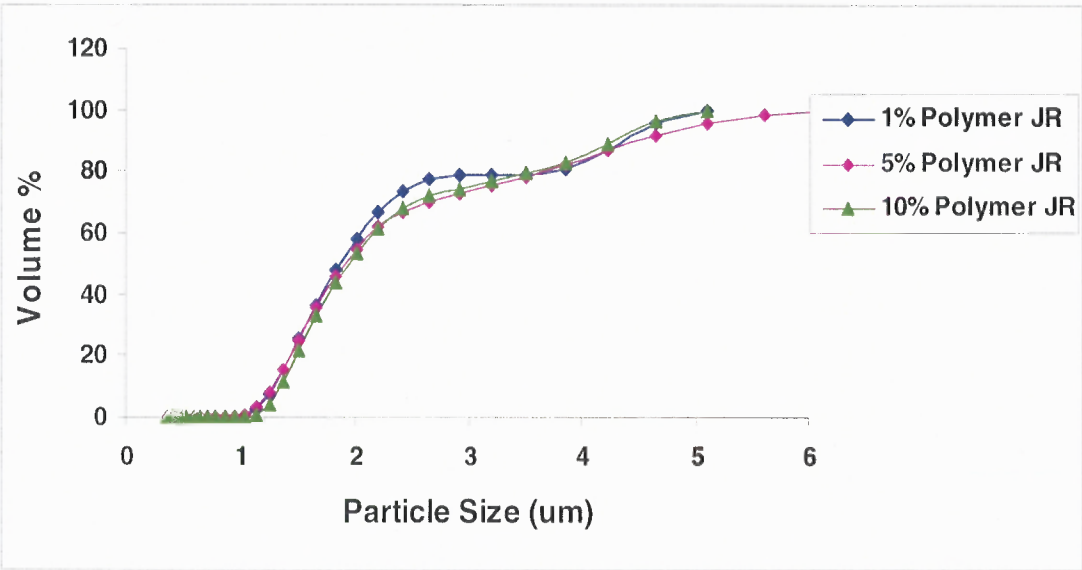


Figure 4.18 Plot of cumulative volume percent versus particle size for Polymer JR.

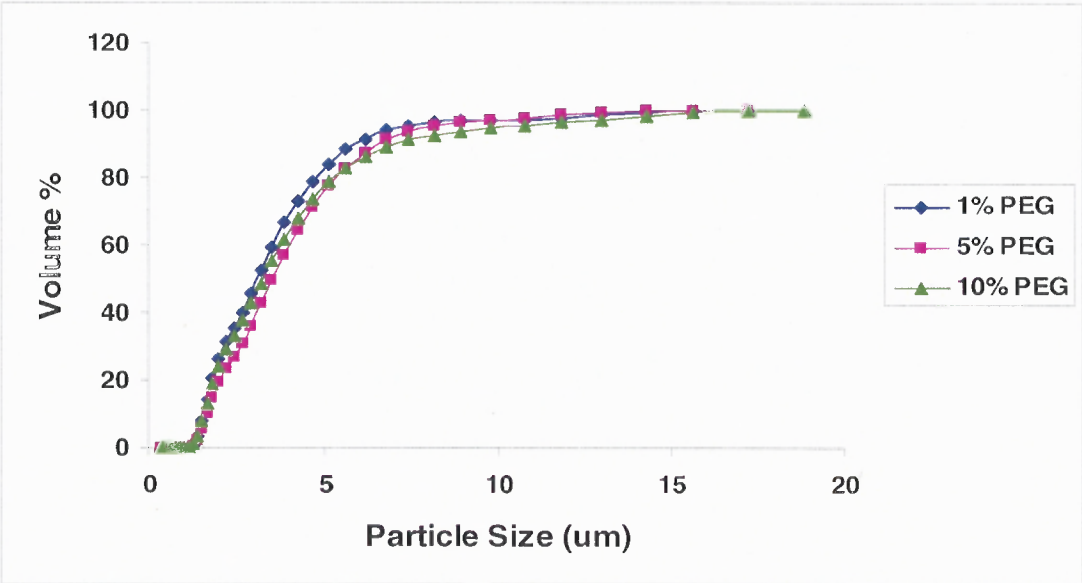


Figure 4.19 Plot of cumulative volume percent versus particle size for polyethylene glycol.

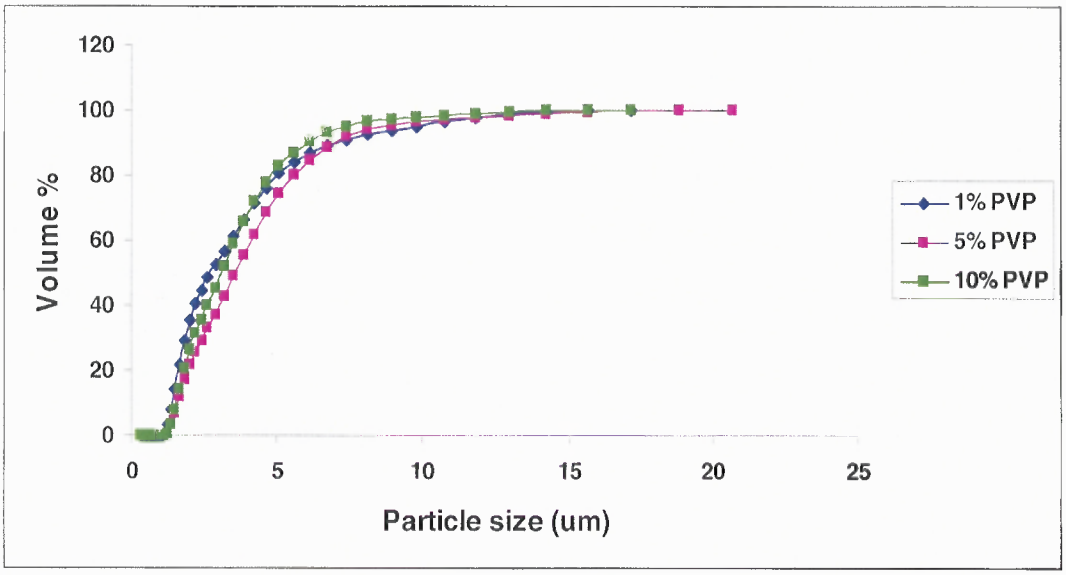


Figure 4.20 Plot of cumulative volume percent versus particle size for polyvinyl pyrrolidone.

Figures 4.18, 4.19 and 4.20 indicated that there was no change in particle size with the increasing polymer concentration. The polymer JR, PEG and PVP might not be wetting the drug particles and hence the particles could not be prevented from agglomeration.

4.3.3 Co-milling with Surfactants

Sodium dodecyl sulfate (SDS) and Pluronic F-68 were used to prevent particle agglomeration. The concentrations of the surfactants used have been calculated with respect to the drug concentration.

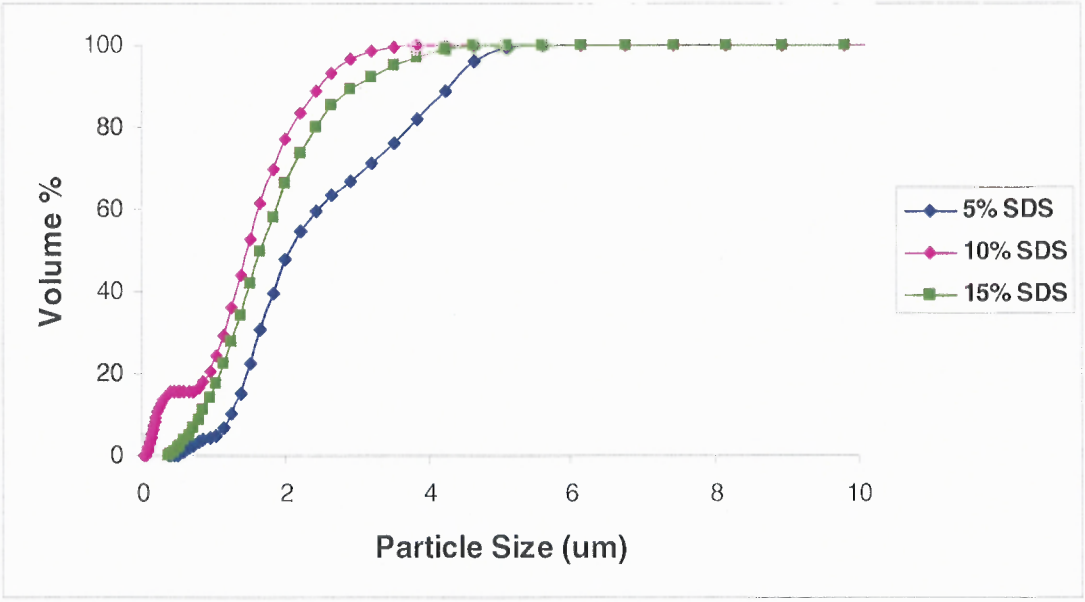


Figure 4.21 Plot showing the effect of SDS on particle size.

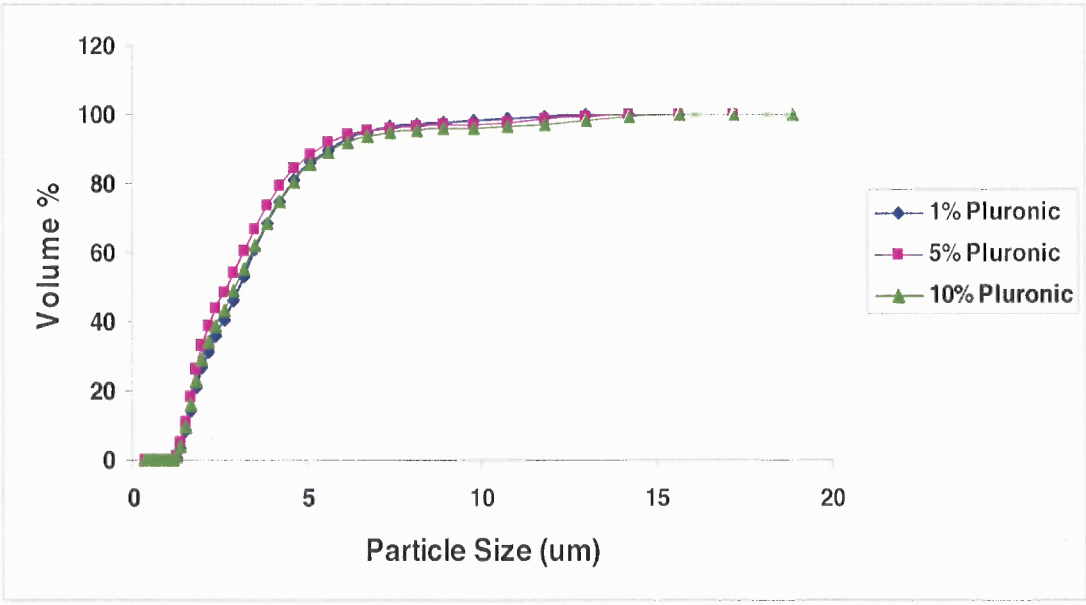


Figure 4.22 Plot of cumulative volume percent versus particle size for Pluronic F-68.

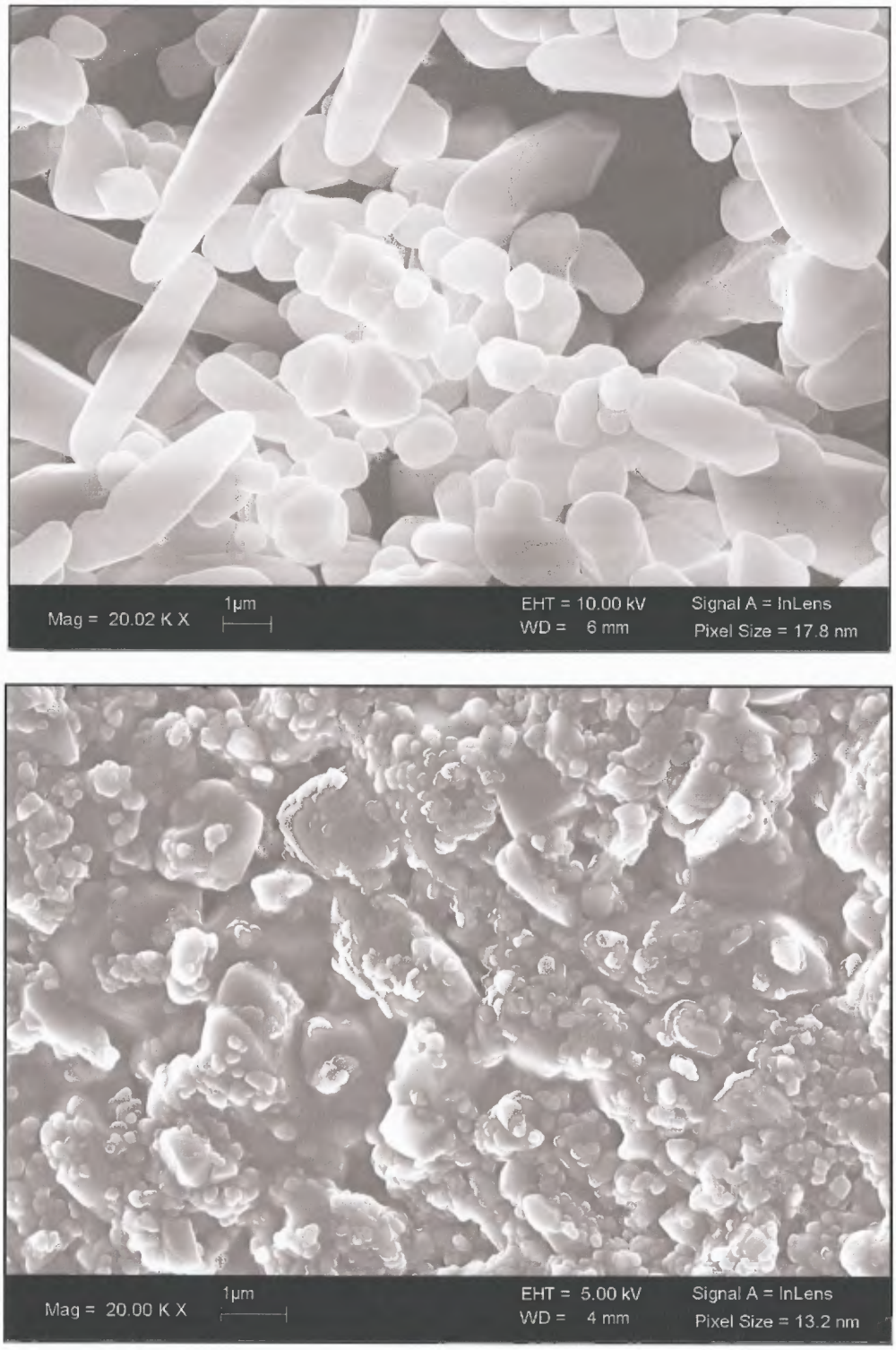


Figure 4.23 SEM Micrographs of the griseofulvin particles before milling (top) and after milling with sodium alginate (bottom).

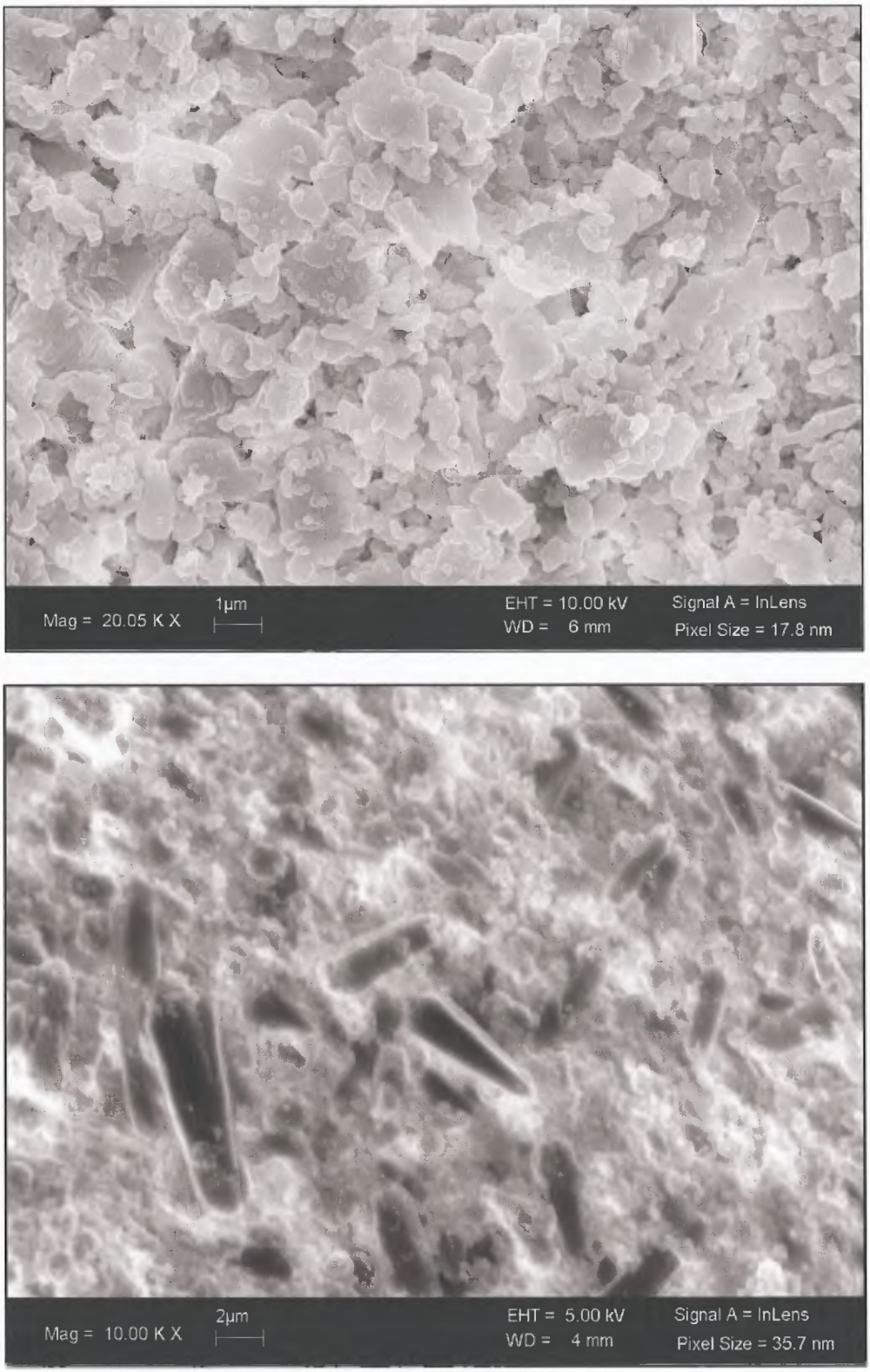


Figure 4.24 SEM Micrographs showing griseofulvin after milling with SDS (top) and Pluronic (bottom).

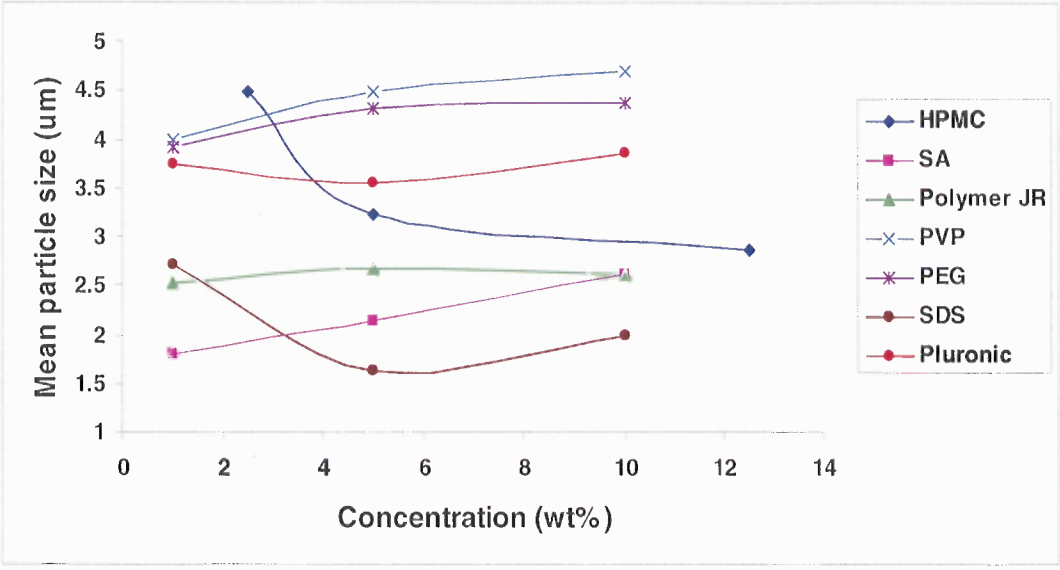


Figure 4.25 Plot showing the mean particle size of griseofulvin after co-milling with polymers/surfactants at different concentrations.

The results of the effect of different polymers and surfactants have been summarized in the table.

Table 4.2 Summary of the Results Obtained for Different Polymers and Surfactants

Polymer	Mean	SD	D 10	D 50	D 90	%<1μm
HPMC	2.860	1.411	1.494	2.256	5.088	0
Sodium Alginate	1.798	0.327	1.355	1.809	2.219	29.6
Polymer JR	2.525	1.236	1.428	2.049	4.816	0
PEG	3.905	2.415	1.717	3.410	6.464	0
PVP	3.996	2.923	1.568	3.029	7.735	0
SDS	1.621	0.854	0.225	1.626	2.727	19.2
Pluronic	3.752	2.091	1.713	3.366	6.168	0

4.3.4 Liquid Antisolvent followed by Micros Milling

Another approach for decreasing the particle size even further was tried. The griseofulvin particles were formed by the liquid antisolvent process and then milled in Micros. 6 g of griseofulvin was dissolved in 30 ml dichloromethane. The drug solution was pumped at the rate of 100ml/min into 1wt% of HPMC solution placed in a cold bath. The solution was added in the presence of ultrasonication. The suspension was then immediately transferred to the micros vessel and milled at 1500 rpm for 75 minutes. The particles size was measured by LS 230 and SEM micrographs. The plot of the stability of the particles has been shown below.

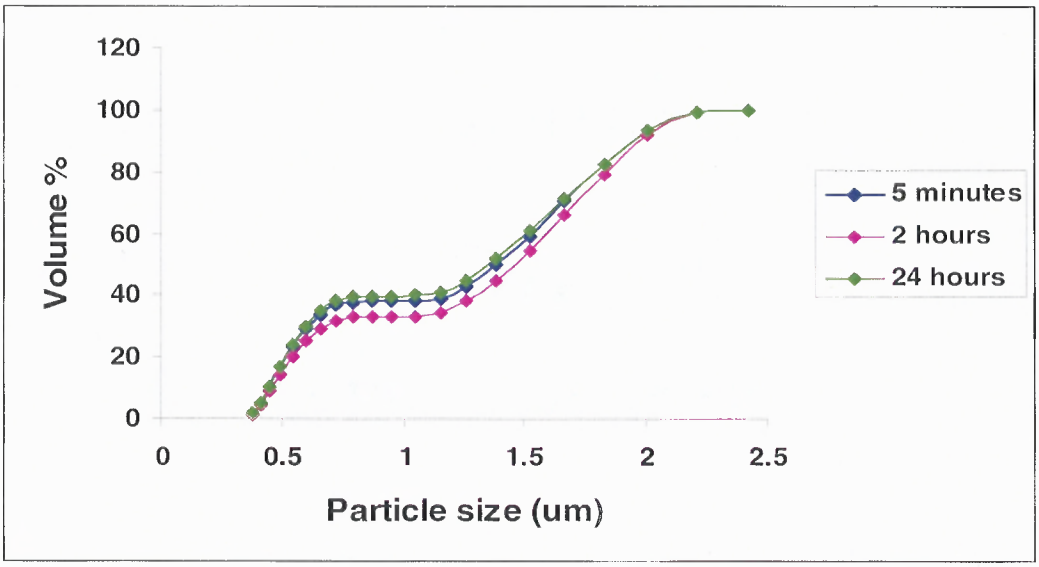


Figure 4.26 Plot of cumulative volume percent versus particle size showing the stability of particles formed by the liquid antisolvent process followed by milling process.

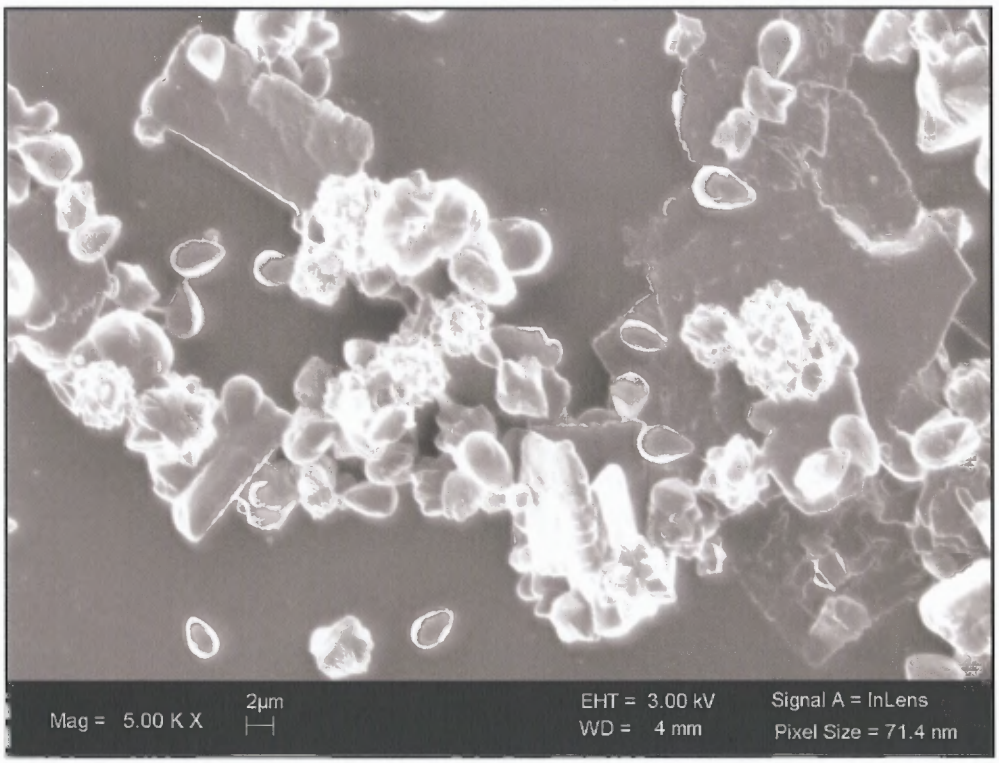


Figure 4.27 SEM Micrograph showing the griseofulvin particles obtained by the liquid antisolvent process follwed by milling.

The particle size was found to be stable over 24 hours. In this process the HPMC solution was an antisolvent to griseofulvin which caused the precipitation of the drug particles. HPMC prevented the particles from agglomeration by coating the griseofulvin particles. This method was more effective since the particles were protected by HPMC as soon as they were produced by precipitation.

CHAPTER 5

CONCLUSIONS

5.1 Targeted Drug Delivery

5.1.1 Polyelectrolyte Complex Nanoparticles

The process of preparation of the PEC nanoparticles was optimized. The nanoparticles could be produced in a reproducible manner. The effect of charge ratio, pH, stirring speed and centrifugation speed on particle size were studied for optimization of the process. The particle size increased with increase in charge ratio due to the presence of excess positive charge on the particles promoting the repulsion between the particles, thus causing a decrease in the particle size. The particle size was found to be fairly constant between 100-150 nm for charge ratios 15-25. The acidic pH was conducive for the particle formation process and maintaining the same pH after the separation of particles was necessary for particle size stabilization. The mixing speed of 2800 rpm was fixed since it gave the lowest particle size. The PEC NPs were found to be sensitive to high centrifugation speeds. The particle size increased to 450-500 nm at high speeds and hence the particles were not suitable for cellular uptake. In addition the number of amine groups available on the surface of NPs was found to be low indicating the low yield of particles. Hence they could not be crosslinked and conjugated for drug delivery.

5.1.2 Albumin Nanoparticles

The albumin nanoparticles were made to overcome the problems of crosslinking, conjugation and cellular uptake faced in the polyelectrolyte complex nanoparticles. The albumin nanoparticles were prepared by an already optimized procedure available in the literature. The amine groups on the surface of NPs were feasible for crosslinking.The particles size was 117 nm after centrifugation and 230 nm after antibody conjugation. It thus met the requirements for cellular uptake. The albumin nanoparticles could be internalized into the cells which was clearly evident from the in-vitro studies.

5.2 Wet Milling

The effect of polymers HPMC, sodium alginate, polymer JR, polyethylene glycol and polyvinyl pyrolidone on particle size of griseofulvin was studied by milling in Micros. Sodium alginate and HPMC gave the mean particle size of 1.798 and 1.984 μm respectively since they could wet the griseofulvin particles. None of the other polymers used, showed any significant effect on particle size reduction. Similarly SDS was found to be a better surfactant than Pluronic F-68. The use of liquid antisolvent process followed by milling was found to be the most effective method. The particle size was 1.344 μm and remained stable over 24 hours.

5.3 Future Work

For future work, it is proposed that more detailed in-vivo studies are carried out for the nanocarriers. The nanocarriers can also be directed to different cell lines by different antibodies used for conjugation. In addition, it is suggested that the albumin nanoparticles

are loaded with a drug and studied for the drug release profile. The Griseofulvin size reduction could be further explored by trying a combination of surfactants and polymers. In a long-term study, various other APIs should be investigated and final milled size should be correlated with material properties such as the melting point, crystal strength, and surface energy. That investigation should also include examination of additives and the selected APIs should be studied for size reduction, stability, change in morphology and activity of the API. Also the degree of polymer coating and drug release profile should be further studied.

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