DESIGN AND DEVELOPMENT OF AMIKACIN NANOPARTICLES AND ITS IN VITRO ACTIVITY AGAINST PREFORMED BIOFILMS

Thesis Submitted In Partial Fulfillment for the Requirements
Of the Degree of **MASTER OF PHARMACY**,
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BY

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Dedicated to my parents, project guide and all the well-wishers.

CERTIFICATE

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*This is to certify that research work entitled "**DESIGN AND DEVELOPMENT OF AMIKACIN NANOPARTICLES AND ITS IN VITRO ACTIVITY AGAINST PREFORMED BIOFILMS"** has been carried out by **MD SHARIQUE** having Examination Roll No: **M4PHA1609** and Registration No **129007 of 14-15** under my supervision in Department of Pharmaceutical Technology of this university. He has incorporated his findings into this thesis of the same title, being submitted by him, in partial fulfillment of the requirements for the degree of **Master of Pharmacy** of **Jadavpur University**. He has carried out this research work independently and with proper care and attention to my entire satisfaction.*

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Declaration of Originality and Compliance of Academic Ethics

I hereby declare that this original research work entitled ***"DESIGN ANDDEVELOPMENT OF AMIKACIN NANOPARTICLES AN ITS IN VITRO ACTIVITY AGAINST PREFORMED BIOFILMS"*** *is a record of original research work done by me, as part of my* ***Master of Pharmacy (Pharmaceutics)*** *studies at* ***Jadavpur University****. All information in thisdocument have been obtained and presented in accordance with academic rules and ethicalconduct. I also declare that as required by these rules and conduct, I have fully cited andreferenced all materials and results that are not original to this work.*

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PREFACE

This thesis is presented for the partial fulfillment for the degree of Master of Pharmacy. The present research work entitled **"Design and Development of Amikacin Nanoparticles and Its In Vitro Activity against Preformed Biofilms"** was designed and investigated in order to facilitate the nanoparticulate drug delivery system for aminoglycoside antibiotic amikacin sulfate.

The controlled release of amikacin loaded nanoparticles enhances the bioavailability and gives a better therapeutic effect against infections caused by bacterial biofilms. Amikacin in nanoparticle form will provide sustained release of the drug. The nanoparticles will also reduce the dose and dosage frequency.

Biofilms development inside the medical implants like artificial pacemakers, artificial hips and joints presents a serious threat. Conventional antibiotic drug delivery system in the form of orals and parentrals generally fails to penetrate the biofilms which may lead to serious life threatening infections.

Recent studies has shown that antibiotic in the form of nanoparticles has shown significantly better results against biofilm. The polymer chitosan has the property to adhere to biofilms which insures the direct release of drug at the site of biofilm, leading to better antibiotic action.

This thesis is divided into nine chapters describing fundamentals, methodologies, results and discussions. Chapter 1 describes the fundamentals related to nanoparticle drug delivery system their preparation, characterization, application and also about biofilms their properties and formation. Chapter 2 describes aims and objectives of the research work. Chapter 3 is the literature survey related to nanoparticles and biofilms. Chapter 4 describes the materials and methodologies used in this research work. Chapter 5 contains the tables and graphs related to the experimental results. Chapter 6 is the result discussion section which describes the outcomes obtained from the applied methods explained in Chapter 4. Chapter 7 and Chapter 8 describes the conclusion and future prospects of the research work. Chapter 9 is the reference section.

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Introduction

1.1 NANOPARTICLES

Nanoparticles are sub-nanosized colloidal structures composed of synthetic or semi-synthetic polymers ranging from 1-1000 nm.

Nanoparticles possess several special characteristics such as small particle size, large surface area and the capability of changing their surface properties offers numerous advantages as compared with other delivery systems. [1]

Nanoparticles as drug delivery systems are designed to improve the pharmacological and therapeutic properties of conventional drugs. The incorporation of drug molecules into nanoparticles can protect a drug against degradation as well as offers possibilities of targeting and controlled release. Due to small dimensions, nanoparticles are able to cross the blood-brain-barrier (BBB) and operate on cellular level. In comparison with the traditional form of drugs, nanoparticles are more effective and selective. They can reduce the toxicity and other adverse side effects in normal tissues by accumulating drugs in target sites. Thus the required doses of drugs are lower.

The active pharmaceutical ingredients (drug or other biologically active materials) are either dissolved, entrapped, encapsulated or attached to the surface of the polymers. [2]

The emergence of resistance to antibiotics acquired by microbial variants is a serious threat in combating against infectious diseases. Most research into bacterial pathogenesis has focused on acute infections, but these diseases have now been supplemented by a new category of chronic infections caused by bacteria growing in slime-enclosed aggregates known as biofilms. [3]

Because of high surface area to volume ratio and unique physico-chemical properties, Nanoparticles are promising antimicrobial agents of a new class. Nanoparticles themselves have been employed as potent antimicrobial agents for a variety of medical

applications. Of many different approaches to overcome antimicrobial resistance, using nanoparticles as antibiotics carriers seems to hold highest promise. Various nanoparticles have been investigated as efficient antibiotics delivery vehicles which also protect antimicrobial drugs from a resistant mechanism in a target microbe (e.g., degradation by β-lactamases).

Nanoparticles tagged with antibiotics have been shown to increase the concentration of antibiotics at the site of bacterium-antibiotic interaction, and to facilitate binding of antibiotics to bacteria. [4]

1.2 Types of Nanoparticles: [5]

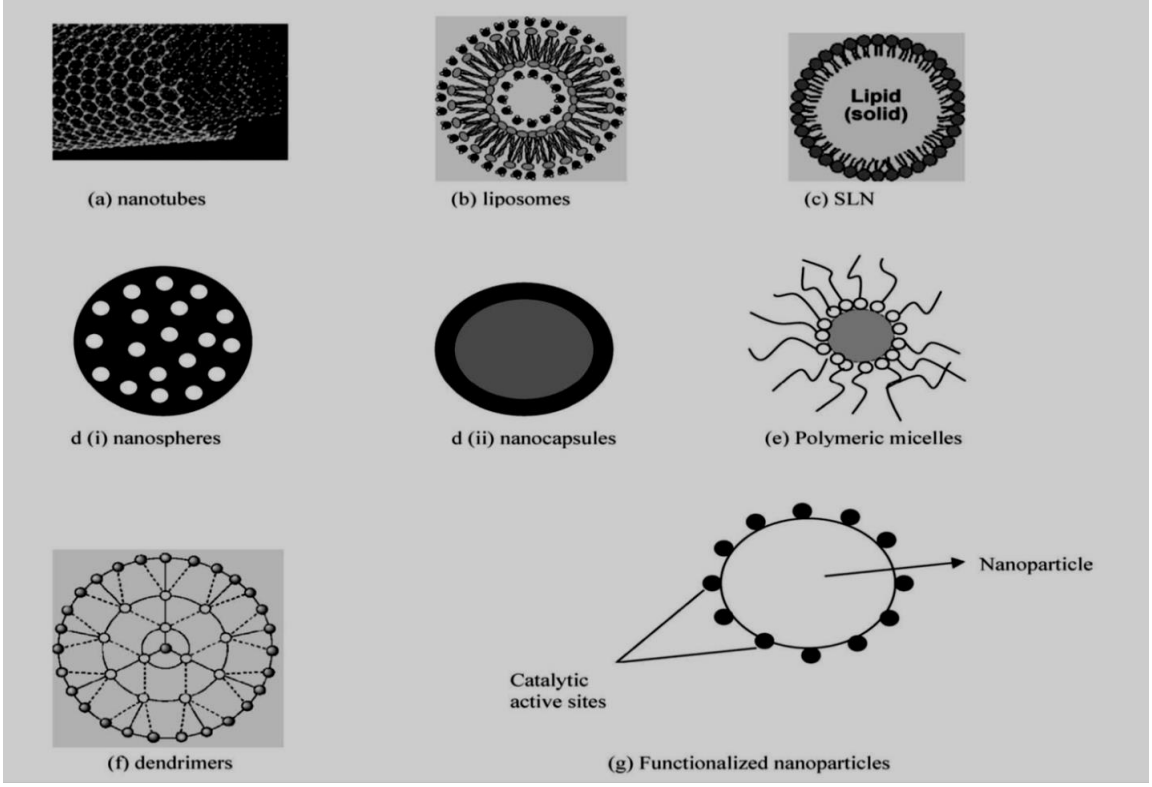


Fig.1 Types of nanoparticles

1.2.1 Solid Lipid Nanoparticles:

Solid lipid nanoparticles (SLN) have attracted considerable attention and are potential drug delivery system. SLNs are aqueous colloidal dispersion, the matrix of which comprises of Solid biodegradable lipids (i.e. lipids solid at room temperature and also at body temperature) and stabilized by surfactants. Many biocompatible/biodegradable lipids are solid at room temperature, can be obtained in high purity, are generally recognized as safe and are inexpensive. Some common lipids used to make SLNs includes Triglycerides (eg, Compitol 880 ATO and Dynasan 112), carnauba wax, bees wax, cetyl alcohol, emulsifying wax, cholesterol and cholesterol butyrate. [6]

The SLNs consist of a biocompatible lipid core and an amphiphilic surfactant as an outer shell. The lipid core of the SLNs, which has physical stability and biocompatibility, can incorporate both lipophilic and hydrophilic drug.

A clear advantage of the SLNs over polymeric nanoparticles is the fact that lipid matrix is made from physiological lipids which decreases the chance of acute and chronic toxicity. [7] The use of SLNs as drug carrier system is a very attractive possibility to achieve controlled drug release.

1.2.2 Polymeric nanoparticles (PNPs):

PNPs are defined as particulate dispersions or solid particles with size in the range of 10-1000nm. Composed of synthetic or semi-synthetic Polymers. Biodegradable polymeric nanoparticles Polylactic acid (PLA), polyglycolic acid (PGA), Polylactic - glycolic acid (PLGA), and Polymethyl methacrylate (PMMA) Phospholipids Hydrophobic core.

1.2.3 Ceramic Nanoparticles:

These are the nanoparticles made up of inorganic (ceramic) compounds silica, (Inorganic/metal) titania and alumina. Exist in size less than 50 nm, which helps them in evading deeper parts of the body.

1.2.4 Hydrogel nanoparticles:

Polymeric system involving the self-assembly and self-aggregation of natural polymer amphiphiles cholesterol pullulan, cholesterol dextran and agarose cholesterol groups provide cross linking points.

1.2.5 Copolymerized Peptide Nanoparticles:

Drug moiety is covalently bound to the carrier instead of being physically entrapped.

1.2.6 Nanocrystals and Nano suspensions:

Pure drug coated with surfactant, Aggregation of these particles in crystalline form .Drug powder dispersed in aqueous surfactant solution.

1.2.7 Functionalized Nanocarriers:

Biological materials like proteins, enzymes, peptides etc… are being utilized as a carriers for the drug delivery.

1.3 Merits of Nanoparticles:

1. Nanoparticle can be administered by parenteral, oral, nasal, ocular routes.

2. By attaching specific ligands on to their surfaces, nanoparticles can be used for directing the drugs to specific target cells.

3. Improves stability and therapeutics index and reduce toxic effects.

4. Both active and passive drug targeting can be achieved by manipulating the particle size and surface characteristics of nanoparticles.

1.4 Limitations of Nanoparticles:

1. Small size and large surface area can lead to particle aggregation, making physical handling of nanoparticles difficult in liquid and dry forms.

2. Small particle size and large surface area results in limited drug loading and burst release.

3. Physical handling of nanoparticles is difficult in liquid and dry forms.

4. Toxic metabolites may form.

1.5 SELECTON OF POLYMER: [8]

Polymers, building blocks of nanoparticles belong to natural and synthetic origins. The selection of matrix materials depends on many factors including:

(a) Size of nanoparticles required.

(b) Inherent properties of the drug, e.g., aqueous solubility and stability.

(c) Surface characteristics such as charge and permeability.

(d) Degree of biodegradability, biocompatibility and toxicity.

(e) Drug release profile desired.

(f) Antigenicity of the final product.

1.6 Polymers for the preparation Nanoparticles: [9]

1.6.1 Natural hydrophilic polymers:

Proteins: - Gelatin, albumin, lectins, legumin.

Polysaccharides: - alginate, dextran, chitosan, agarose.

1.6.2 Synthetic hydrophobic polymers:

Pre-polymerized polymers: - Poly (e-caprolactone) (PECL), Poly (Lactic acid) (PLA), Polystyrene.

Polymerized in process polymers: - Poly (isobutyl cyanoacrylates) (PICA), Poly (butyl cyano acrylates)

1.7 PREPARATION METHODS OF NANOPARTICLES: [10]

Table 1. Methods used for the production of chitosan-based nanoparticles and Composition of the carriers' matrix:

Emulsification and crosslinking	Chitosan, glutaraldehyde
Emulsion droplet Coalescence	Chitosan
Emulsion solvent diffusion	Chitosan
Reverse micellisation	Chitosan, glutaraldehyde
Ionic gelation	Chitosan, tripolyphosphate
Polyelectrolyte Complexation	Chitosan, alginate, Arabic gum, carboxymethyl cellulose, carrageenan, chondroitin sulfate, cyclodextrins, dextran sulfate, polyacrylic acid, poly-γ-glutamic acid, insulin, DNA.
Modified ionic gelation with radical polymerisation	Chitosan, acrylic acid, methacrylic acid, polyethylene glycol, polyether.
Desolvation	Chitosan

1.7.1 Ionic gelation method:

Materials applied in the production of such chitosan nanoparticles include polymers such as dextran sulfate, sodium alginate, carrageenan, arabic gum, glucomannan, carboxymethyl cellulose, chondroitin sulfate, pectin, heparin, hyaluronic acid, cyclodextrins, poly-γ-glutamic acid and poly(acrylic acid), as well as protein-based molecules, like insulin, or even DNA . Examples of these systems include chitosan, alginate, tripolyphosphate, or chitosan, glucomannan, tripolyphosphate, chitosan, hyaluronic acid/tripolyphosphate or chitosan/cyclodextrin/tripolyphosphate nanoparticles. Benefiting from chitosan gelation in the presence of anions, production of chitosan beads for oral administration.

A very mild adaptation of ionic gelation to prepare chitosan nanoparticles in a complete hydrophilic environment. That method involves an ionic interaction between the positively charged amino groups of chitosan and the polyanion tripolyphosphate (TPP), which acts as chitosan crosslinker. Nanoparticles formation takes place immediately after the addition of a TPP solution to a solution of chitosan, under mild stirring, at room temperature. Stirring should be maintained for approximately 10 minutes to allow particle stabilization and the obtained suspension centrifuged to separate the nanoparticles from unreacted chitosan and TPP. The resultant pellet of nanoparticles is then re suspended in water.

The formed nanoparticles were then used to administer different drugs (insulin, bovine serum albumin, polypeptidic mixtures, ovalbumin, tetanus toxoid, estradiol, carvacrol, heparin, cyclosporin-A, saponin and epidermal growth factor receptor) through various routes of administration (oral, nasal, pulmonary, ocular). Controlling the stirring pattern (speed and type of vial, most importantly) and the conditions of centrifugation (speed and duration), is of utmost importance, independently of the specific materials composing the nanoparticle matrix.

Concentration of acetic acid used to dissolve chitosan and the temperature at which the crosslinking process occurs, strongly affect the polydispersity of the obtained

nanoparticles. Performing the reaction below 4 ºC results in a polydispersity index below 0.05. This was attributed to the fact that reduced temperature increased hydrogen bonds between the polar groups of chitosan and the surrounding water, leading to the formation of a hydration layer around the nanoparticles, which decreased the probability of nanoparticle collision. In parallel, a concentration of 0.2 mg/mL of acetic acid was shown to be optimal for achieving a narrow particle size distribution, as it is the concentration required to provide sufficient protonation of chitosan for interaction with TPP.

The most important advantage claimed by those working with the methods of ionic gelation and polyelectrolyte complexation is the complete hydrophilic environment and the mild preparation conditions. In fact, the avoidance of organic solvents or high shear forces makes encapsulation of labile drugs an easier task and, possibly for this reason, those two methods are the most widely used to produce chitosan nanoparticles. Importantly, through physical cross-linking mediated by an electrostatic interaction, instead of a chemical cross-linking with glutaraldehyde, those methods of chitosan nanoparticle production have improved cell viability and drug integrity.

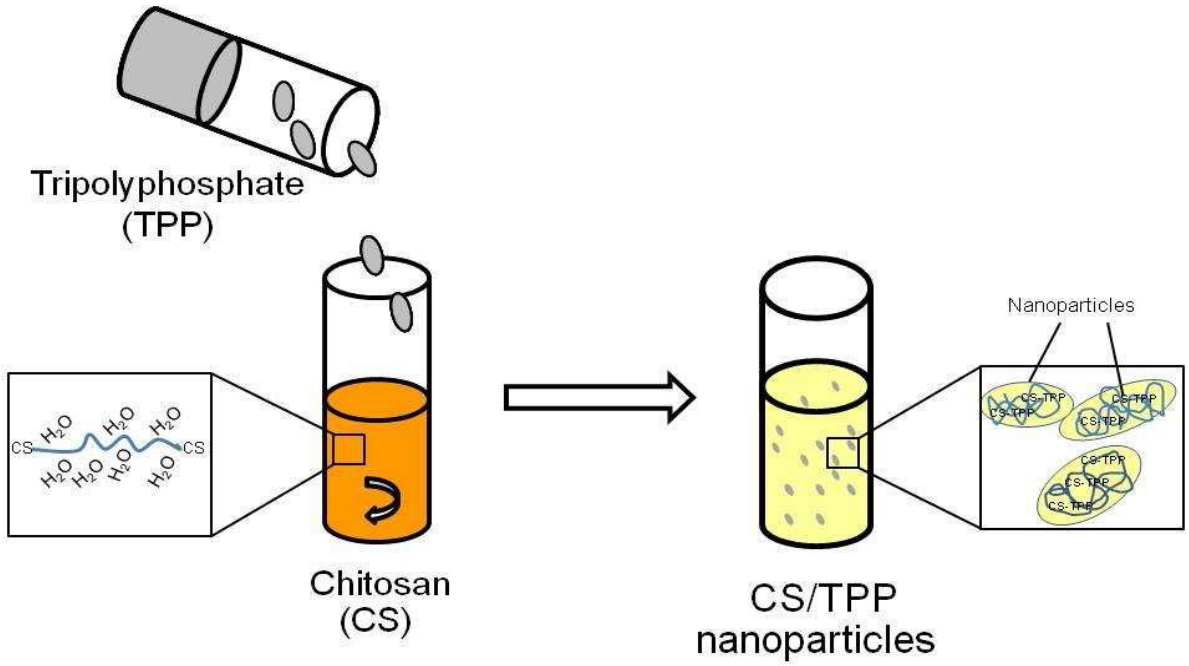


Fig.2 Schematic representation of the method of ionic gelation

1.7.2 Emulsification and cross-linking:

This method was the first to be used to form chitosan nanoparticles and involves the preparation of a w/o emulsion, with subsequent addition of a cross-linking agent that has the function of hardening the formed droplets. The reactive amino groups of chitosan undergo a covalent cross-linking with the aldehyde groups of glutaraldehyde, which is added after the emulsion formation and, consequently, after nanoparticle production. The final particle size was demonstrated to be highly dependent on stirring speed, as well as on the extent of cross-linking. Several drawbacks have been progressively pointed out for this method, including the need of tedious procedures and the application of harsh cross-linking agents. In fact, cross-linkers such as glutaraldehyde were found to cause overt toxicity and to compromise drug integrity, contributing to a progressive shift of interest towards less aggressive procedures. Consequently, the application of this method to obtain chitosan nanoparticles was restricted to a few works.

1.7.3 Emulsion droplet coalescence:

Chitosan is dissolved in the aqueous solution of gadolinium and a small aliquot (1 mL) of this is added to 10 mL of liquid paraffin containing sorbitan sesquiolate. The mixture is stirred with a high-speed homogeniser, thus forming an W/O emulsion. In parallel, another W/O emulsion is prepared by adding 1.5 mL NaOH to 10 mL of a similar outer phase. Both emulsions are then mixed using a high-speed homogeniser, leading to droplet coalescence. This results in the solidification of chitosan particles by action of sodium hydroxide, which acts as precipitating agent. Further set of washing and centrifugation steps is applied using toluene, ethanol and water.

This method exploits the fact that, when two emulsions with equal outer phase are mixed together, droplets of each collide randomly and coalesce, resulting in final droplets with

uniform content. The nanoparticles are formed within the emulsion-droplets. Decreasing chitosan deacetylation degree was shown to increase particle size and to reduce nanoparticle capacity for drug association, as a consequence of the diminished capacity of ion-pair formation and de-swelling.

1.7.4 Emulsion solvent diffusion:

The emulsion solvent diffusion method of preparing chitosan nanoparticles is an adaptation of the original procedure developed to produce PLGA-based nanoparticles, setting its basis on the partial miscibility of an organic solvent with water. The specific method for preparation of chitosan nanoparticles involves the addition of an organic phase (e.g. methylene chloride and acetone) containing the hydrophobic drug, to an aqueous solution containing chitosan and a stabiliser (e.g. poloxamer and lecithin), under stirring. This leads to the formation of an o/w emulsion which is then subjected to high pressure homogenisation. Methylene chloride is subsequently removed under reduced pressure at room temperature. At this stage, acetone diffuses to the aqueous phase, decreasing chitosan solubility and, thus, nanoparticles are formed upon polymer precipitation. An additional amount of water is usually added in order to permit the complete diffusion of acetone. Finally, nanoparticles are isolated by centrifugation.

This method demonstrated to be suitable for encapsulating hydrophobic drugs like cyclosporin-A, with high encapsulation efficiencies. Parameters such as chitosan molecular weight, homogenisation rate and reaction time (period of evaporation and diffusion), are expected to affect the final properties of the vehicles. The presence of acetone was also reported as essential, since its rapid diffusion disturbs the organic/aqueous phase interface, which spontaneously produces a larger area and, thus, leads to the formation of much smaller droplets. Particles produced without acetone presented sizes outside the submicron range (above 1.2 nm).

1.7.5 Reverse Micellisation:

The production of chitosan nanoparticles from reverse micelles is a strategy for tumor targeted delivery. Reverse micelles are w/o droplets and thus form in a w/o system, in contrast to conventional micelles that form in o/w environment. In this method of reverse micellisation, a w/o microemulsion is prepared using a lipophilic surfactant that is dissolved in an appropriate organic solvent, like *n*-hexane. Surfactants like sodium sulfosuccinate or cetyl trimethylammonium bromide. An aqueous phase comprising chitosan, the drug and glutaraldehyde is then added over the organic phase under continuous stirring. Nanoparticles are extracted following solvent evaporation.

Increase in the crosslinking rate results in the production of larger particles. As compared to other emulsion-based methods, the reverse micellisation method has the advantage of producing ultrafine nanoparticles of around 100 nm or even less, with a narrower size range, in contrast to the larger nanocarriers (> 200 nm) usually obtained by other emulsification techniques.

Disadvantages such as the difficult isolation of nanoparticles and the need for larger amounts of solvent, have been mentioned. Apart from the application in anticancer therapy, other authors have later used this method as a strategy for enzyme immobilization or to encapsulate oligonucleotides.

1.7.6 Desolation*:*

The method of desolvation is also frequently referred to as simple coacervation or phase separation and involves a macromolecular aggregation brought about by partial desolvation of fully solvated molecules. The use of desolvating agents to produce chitosan particles was reported for the first time for the preparation of micron-sized carriers but, nowadays, this procedure is frequently applied to the production of chitosan

nanoparticles. Substances such as sodium sulfate and non-solvents miscible with water, like acetone, have been proposed as precipitating agents, although the former has been used more frequently.

The preparation of chitosan nanoparticles by this method is very simple and mild as it involves the dropwise addition of the solvent competing agent of greater hydrophilicity (e.g. sodium sulfate) into a previously formed chitosan solution. As the salt enters in contact with the aqueous environment of chitosan solution, a progressive elimination of solvation water surrounding chitosan occurs as a consequence of the higher affinity of water for the salt. Eventually, this process leads to the polymer insolubilisation and its consequent precipitation. This effect is observed because water-salt interactions are more favorable than those occurring between the water and the polymer, inducing the partial desolvation of chitosan. This, in turn, leads to increased interactions between chitosan molecules, forming the nanocarriers. It is very frequent to include a stabilizer such as polysorbate 80 in the preparation medium, to stabilise the nanoparticle suspension.
Factors such as chitosan molecular weight, chitosan concentration, amount of desolvating agent and stirring rate have been found to strongly affect the final characteristics of nanoparticles.

1.8 Characterization of Nanoparticles:

1.8.1 Particle size:

Particle size and size distribution are the most important characteristics of nanoparticle systems. They determine the in vivo distribution, biological fate, toxicity and the targeting ability of nanoparticle systems. In addition, they can also influence the drug loading, drug release and stability of nanoparticles.
Many studies have demonstrated that nanoparticles of sub-micron size have a number of advantages over microparticles as a drug delivery system. [11] Generally nanoparticles

have relatively higher intracellular uptake compared to microparticles and available to a wider range of biological targets due to their small size andrelative mobility.

The fastest and most routine method of determining particle size is by photon-correlation spectroscopy or dynamic light scattering. Photon-correlation spectroscopy requires the viscosity of the medium to be known and determines the diameter of the particle by Brownian motion and light scattering properties. [12] The results obtained by photon-correlation spectroscopy are usually verified by scanning or transmission electron microscopy (SEM or TEM).

1.8.2 Surface properties of nanoparticles:

The zeta potential of a nanoparticle is commonly used to characterise the surface charge property of nanoparticles. It reflects the electrical potential of particles and is influenced by the composition of the particle and the medium in which it is dispersed. Nanoparticles with a zeta potential above (+/-) 30 mV have been shown to be stable in suspension, as the surface charge prevents aggregation of the particles. The zeta potential can also be used to determine whether a charged active material is encapsulated withinthe centre of the nanocapsule or adsorbed onto the surface. [13] Surface hydrophobicity/hydrophilicity determines the amount of adsorbed blood components. This in turn influences the in vivo fate of nanoparticles.

1.8.3 Drug Loading:

Ideally, a successful nanoparticulate system should have a high drug-loading capacity thereby reducing the quantity of matrix materials for administration. Drug loading and entrapment efficiency very much depend on the solid-state drug solubility in matrix material or polymer (solid dissolution or dispersion), which is related to the polymer composition, the molecular weight, the drug polymer interaction and the presence of end

functional groups (ester or carboxyl). [14] The macromolecule or protein shows greatest loading efficiency when it is loaded at or near its isoelectric point when it has minimum solubility and maximum adsorption. [15] For small molecules, studies show the use of ionic interaction between the drug and matrix materials can be a very effective way to increase the drug loading. [16]

1.8.4 Drug Release

To develop a successful nanoparticulate system, both drug release and polymer biodegradation are important consideration factors. In general, drug release rate depends
1) Solubility of drug.
2) Desorption of the surface bound adsorbed drug.
3) Drug diffusion through the nanoparticle matrix.
4) nanoparticle matrix erosion/degradation.

In the case of nanospheres, where the drug is uniformly distributed, the release occurs by diffusion or erosion of the matrix under sink conditions. If the diffusion of the drug is faster
than matrix erosion, the mechanism of release is largely controlled by a diffusion process. The rapid initial release or 'burst' is mainly attributed to weakly bound or adsorbed drug to the large surface of nanoparticles. [17]

Methods which can be used to study the in vitro release of the drug are:

1) Side-by-side diffusion cells with artificial or biological membranes.
2) Dialysis bag diffusion technique.
3) Reverse dialysis bag technique.
4) Agitation followed by ultracentrifugation/centrifugation.

5) Ultra-filtration or centrifugal ultra-filtration techniques.

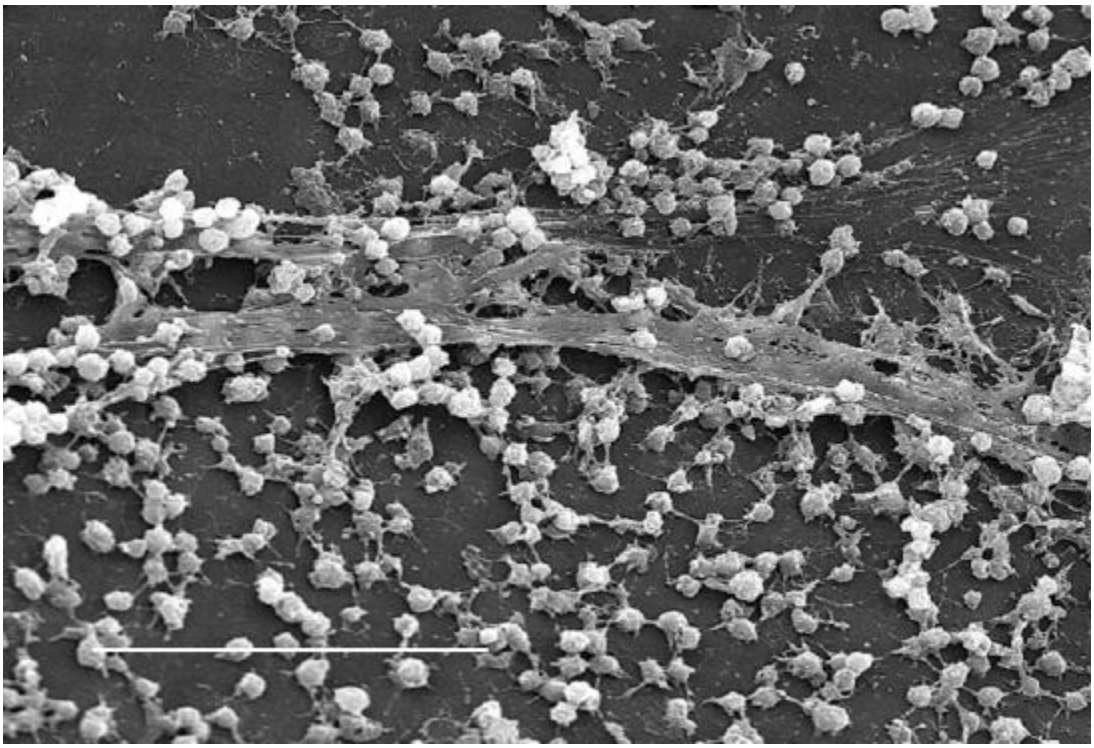
1.9 Applications of Nanoparticles:

Application	Purpose	Material
Cancer therapy	Targeting, Reduced toxicity, enhance uptake of anti-tumor agent	Polyalkylcyanoacrylate with anticancer agent
Intra cellular targeting	Target reticuloendothelial system for intracellular infection	Poly alkyl cyanoarylate
Vaccine adjuvant	Prolong systemic drug effect. Enhance immune response	Poly methyl metha acrylate nanoparticles with vaccines
DNA delivery	Enhanced bioavailability and significantly higher expression level .	DNA gelatin nanoparticles, DNA chitosan nanoparticles,
Ocular delivery	Improved retention of the drug and reduced washed out.	Poly alkyl cyanoacrylate nanoparticles , anti-inflammatory agent

1.10 BIOFILM:

A biofilm is an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material. Non cellular materials such as mineral crystals, corrosion particles, clay or silt particles, or blood components, depending on the environment in which the biofilm has developed, may also be found in the biofilm matrix. Biofilm-associated organisms also

differ from their planktonic (freely suspended) counterparts with respect to the genes that are transcribed. Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping, or natural aquatic systems. The variable nature of biofilms can be illustrated from scanning electron micrographs of biofilms from a medical device, respectively (Figure). The water system biofilm devices, industrial or potable water system piping, or natural aquatic systems. The variable nature of biofilms can be illustrated from scanning electron micrographs of biofilms from a medical device, respectively (Figure). The water system biofilm is highly complex, containing corrosion products, clay material, fresh water diatoms, and filamentous bacteria. The biofilm on the medical device, on the other hand, appears to be composed of a single, coccoid organism and the associated extracellular polymeric substance (EPS) matrix. [18]



Scanning electron micrograph of a staphylococcal biofilm on the inner surface of an indwelling medical device.

1.10.1 Formation of Biofilm:

Formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. These first colonists adhere to the surface initially through weak, reversible adhesion via van der Waals forces. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures such as pili. Hydrophobicity also plays an important role in determining the ability of bacteria to form biofilms, as those with increased hydrophobicity have reduced repulsion between the extracellular matrix and the bacterium.

Some species are not able to attach to a surface on their own but are sometimes able to anchor themselves to the matrix or directly to earlier colonists. It is during this colonization that the cells are able to communicate via quorum sensing using products such as AHL. Some bacteria are unable to form biofilms as successfully due to their limited motility. Non motile bacteria cannot recognize the surface or aggregate together as easily as motile bacteria. Once colonization has begun, the biofilm grows through a combination of cell division and recruitment. Polysaccharide matrices typically enclose bacterial biofilms. In addition to the polysaccharides, these matrices may also contain material from the surrounding environment, including but not limited to minerals, soil particles, and blood components, such as erythrocytes and fibrin. [19] The final stage of biofilm formation is known as dispersion, and is the stage in which the biofilm is established and may only change in shape and size.

The development of a biofilm may allow for an aggregate cell colony (or colonies) to be increasingly antibiotic resistant. Cell-cell communication or quorum sensing (QS) has been shown to be involved in the formation of biofilm in several bacterial species. [20]

A. baumannii is infamous for its ability to form biofilms both on inanimate objects as well as biotic surfaces. *A. baumannii* has been reported to commence secretion of exopolysacchrides once it has successfully adhered to a surface, be it hydrophilic or

hydrophobic like glass and plastic, respectively, or surfaces of living cells. Previous findings indicate that within the protective environment of the biofilm, the pathogen remains protected from starvation, desiccation and the action of antibiotics. As such, the ability to form biofilms alone may be linked to the increased virulence in some of the strains. Direct evidence to support this hypothesis is still lacking. However, reports have shown that multidrug resistant strains are efficient biofilm producers, indicating a direct relationship between biofilm formation and antibiotic resistance. In addition, reports have also shown that the biofilm-associated protein (BAP) in *A. baumannii*, involved in biofilm formation, is capable of stimulating humoral response in mice, which suggests that it may have a role in virulence. [21]

The ability of *A. baumannii* to form biofilms has been shown to be related to certain outer membrane surface associated proteins like OmpA and BAP as well as certain pili-associated adhesins. The presence of metal cations has also been reported to be required for biofilm formation as indicated by the reduced ability of *A.baumannii* to produce biofilms in presence of chelators like ethylenediaminetetraacetic acid (EDTA). The formation of CsuA/BABCDE-dependent pili appears to be essential for the adherence and biofilm formation on abiotic surfaces and the assembly of these pili seems to involve chaperone and usher-like proteins. However, this system does not seem to be involved in the adherence of *A. baumannii* to living cells, and the underlying mechanisms governing attachment to biotic surfaces still remain to be elucidated. Formation of biofilm by A. baumannii is under tight regulation both at the transcriptional and translational levels involving highly efficient and cross-linked two component regulatory systems. However, the identification of these regulatory systems has not yet been achieved. Quorum sensing has also been implicated in the regulation of biofilm formation. A. baumannii has been shown to be capable of producing quorum sensing molecules namely N-acylhomoserine lactones of various chain length with (3-hydroxydodecanoyl)-L-HSL reported as the primary signal molecule. However, only a single auto inducer synthase gene named abaI

has been identified till date. Quorum sensing is the main method of communication between the bacterial cells within the biofilm and may also serve as a mechanism to coordinate and regulate the multiple virulence factors in *A. baumannii*. There are reports indicating that quorum sensing might possibly be involved in host–pathogen interactions as well. Thus, biofilm formation and quorum sensing are important components in the wide arsenal of virulence determinants produced by *A. baumannii.* [22]

1.10.2 Extracellular polymeric substances (EPS):

Extracellular polymeric substances (EPS), are high-molecular weight compounds secreted by microorganisms into their environment. EPS establish the functional and structural integrity of biofilms, and are considered the fundamental component that determines the physiochemical properties of a biofilm. [23]

EPS are mostly composed of polysaccharides (exopolysaccharides) and proteins, but include other macro-molecules such as DNA, lipids and humic substances. EPS are the construction material of bacterial settlements and either remain attached to the cell's outer surface, or are secreted into its growth medium. These compounds are important in biofilm formation and cells attachment to surfaces. EPS constitutes 50% to 90% of a biofilm's total organic matter. [24]

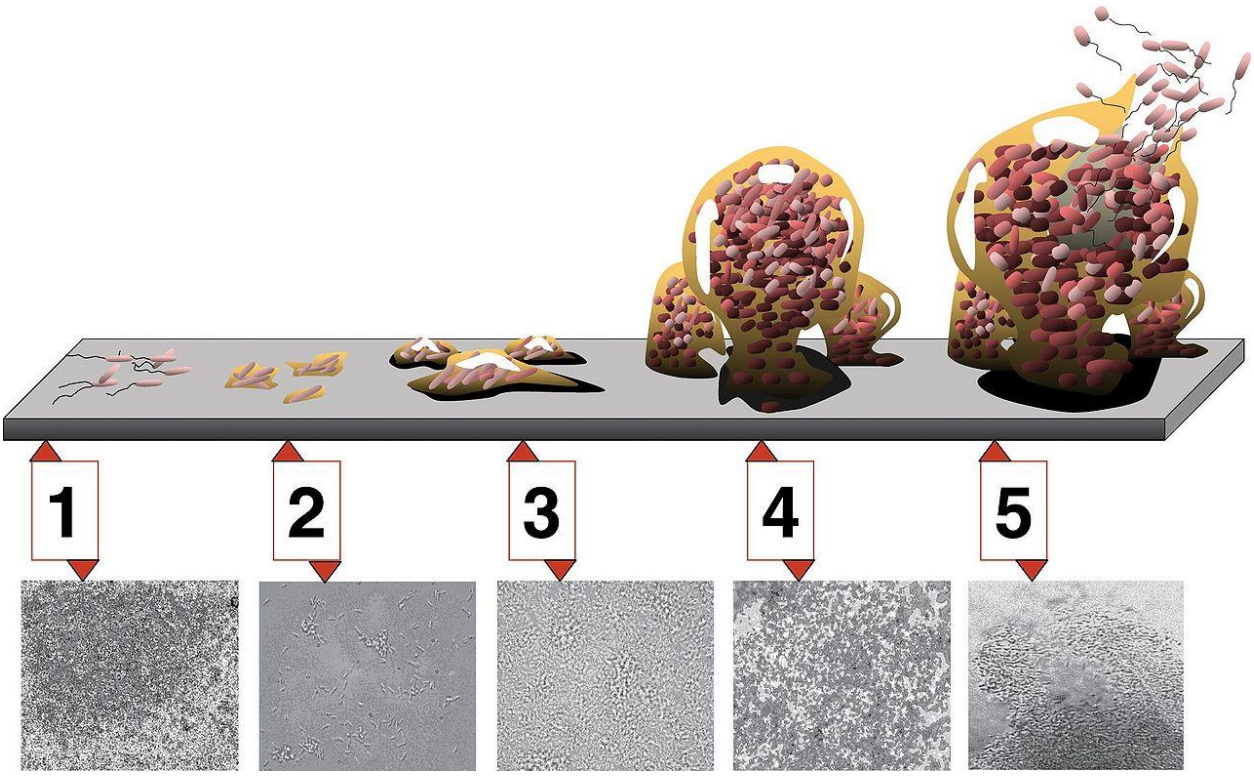
Exopolysaccharides are high-molecular-weight polymers that are composed of sugar residues and are secreted by a microorganism into the surrounding environment. Microorganisms synthesize a wide spectrum of multifunctional polysaccharides including intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides or exopolysaccharides (EPS). Exopolysaccharides generally consist of monosaccharides and some non-carbohydrate substituents (such as acetate, pyruvate, succinate, and phosphate). Owing to the wide diversity in composition, exopolysaccharides have found multifarious applications in various food and pharmaceutical industries. Many microbial EPS provide properties that are almost identical to the gums currently in use. With innovative approaches, efforts are underway to supersede the traditionally used

plant and algal gums by their microbial counterparts. Moreover, considerable progress has been made in discovering and developing new microbial EPS that possess novel industrial significance. [25]

1.10.3 Development of Biofilm:

There are five stages of biofilm development: [26]

1. Initial attachment
2. Irreversible attachment
3. Maturation I
4. Maturation II
5. Dispersion



Stages of development *P. aeruginosa* biofilm with photomicrographs.

Dispersion of Biofilm:

Dispersal of cells from the biofilm colony is an essential stage of the biofilm life cycle. Dispersal enables biofilms to spread and colonize new surfaces. Enzymes that degrade the biofilm extracellular matrix, such as dispersin B anddeoxyribonuclease, may play a role in biofilm dispersal. Biofilm matrix degrading enzymes may be useful as anti-biofilm agents. Recent evidence has shown that a fatty acid messenger, cis-2-decenoic acid, is capable of inducing dispersion and inhibiting growth of biofilm colonies. Secreted by Pseudomonas aeruginosa, this compound induces cyclo heteromorphic cells in several species of bacteria and the yeast Candida albicans. Nitric oxide has also been shown to trigger the dispersal of biofilms of several bacteria species at sub-toxic concentrations. Nitric oxide has the potential for the treatment of patients that suffer from chronic infections caused by biofilms. [27]

It is generally assumed that cells dispersed from biofilms immediately go into the planktonic growth phase. However, recent studies have shown that the physiology of dispersed cells from Pseudomonas aeruginosa biofilms is highly different from those of planktonic and biofilm cells. Hence, the dispersal process is a unique stage during the transition from biofilm to planktonic lifestyle in bacteria. Dispersed cells are found to be highly virulent against macrophages and *Caenorhabditis elegans*, but highly sensitive towards iron stress, as compared with planktonic cells. [28]

1.10.4 Properties of Biofilm:

Biofilms are usually found on solid substrates submerged in or exposed to an aqueous solution, although they can form as floating mats on liquid surfaces and also on the surface of leaves, particularly in high humidity climates. Given sufficient resources for growth, a biofilm will quickly grow to be macroscopic (visible to the naked eye). Biofilms can contain many different types of microorganism, e.g. bacteria, archaea,

protozoa, fungi and algae; each group performs specialized metabolic functions. However, some organisms will form single-species films under certain conditions. The social structure (cooperation, competition) within a biofilm highly depends on the different species present. [29]

1.10.5 Extracellular matrix:

The biofilm is held together and protected by a matrix of secreted polymeric compounds called EPS. EPS is an abbreviation for either extracellular polymeric substance orexopolysaccharide, although the latter one only refers to the polysaccharide moiety of EPS. In fact, the EPS matrix consists not only of polysaccharides but also of proteins (which may be the major component in environmental and waste water biofilms) and nucleic acids. [30]

A large proportion of the EPS is more or less strongly hydrated, however, hydrophobic EPS also occur; one example is cellulose which is produced by a range of microorganisms. This matrix encases the cells within it and facilitates communication among them through biochemical signals as well as gene exchange. [31]

The EPS matrix is an important key to the evolutionary success of biofilms. One reason is that it traps extracellular enzymes and keeps them in close proximity to the cells. Thus, the matrix represents an external digestion system and allows for stable synergistic microconsortia of different species. Some biofilms have been found to contain water channels that help distribute nutrients and signalling molecules. This matrix is strong enough that under certain conditions, biofilms can become fossilized (Stromatolites). Bacteria living in a biofilm usually have significantly different properties from free-floating bacteria of the same species, as the dense and protected environment of the film allows them to cooperate and interact in various ways. [32]

One benefit of this environment is increased resistance to detergents and antibiotics, as the dense extracellular matrix and the outer layer of cells protect the interior of the

community. In some cases antibiotic resistance can be increased a thousand fold. Lateral gene transfer is greatly facilitated in biofilms and leads to a more stable biofilm structure. Extracellular DNA is a major structural component of many different microbial biofilms. Enzymatic degradation of extracellular DNA can weaken the biofilm structure and release microbial cells from the surface. [33]

However, biofilms are not always less susceptible to antibiotics. For instance, the biofilm form of Pseudomonas aeruginosa has no greater resistance to antimicrobials than do stationary-phase planktonic cells, although when the biofilm is compared to logarithmic phase planktonic cells, the biofilm does have greater resistance to antimicrobials. This resistance to antibiotics in both stationary phase cells and biofilms may be due to the presence of persister cells. [34]

AIMS & OBJECTIVES

2. AIMS & OBJECTIVES

The present research work deals with the design and development of Amikacin Sulphate loaded chitosan nanoparticles and the *in-vitro* release of drug from the nanoparticles. The prepared nanoparticles will then be tested against preformed biofilms for its *in-vitro* antibacterial activity.

The present investigative work is carried out with the following objectives:

1. Analytical method development and preparation of the calibration curve of Amikacin Sulphate by High Performance Liquid Chromatography (HPLC).

2. Preparation of Amikacin Sulphate loaded chitosan nanoparticles by Ionic-Gelation method.

3. *In-vitro* drug release of the prepared nanoparticles using Franz diffusion cell.

4. *In-vitro* activity of Amikacin nanoparticle against preformed biofilm.

5. Characterization of the nanoparticles:

    a) Determination of particle size, Polydispersity Index and zeta potential by Dynamic Light Scattering (DLS).
    b) Drug entrapment efficiency.
    c) *In-vitro* drug release using Franz diffusion cell.
    d) Fourier transform infrared spectroscopy (FTIR).
    e) Differential Scanning Calorimetry (DSC).
    f) Morphological Scanning by Scanning Electron Microscopy (SEM).

LITERATURE REVIEW

3. LITERATUE REVIEW

- *Li-Ming Zhao et al*. reported that micro/nanostructured chitosans can be used as bioactive ingredients carriers. They have the potential for the development of novel encapsulation or immobilization carriers. Due to their favorable biological properties such as non-toxicity, biocompatibility, biodegradability and antibacterial ability, they are also promising candidates for drug delivery carriers and cell proliferation enhancers. [35]

- *Quan Gan et al*. in his study showed that the formation of high yield chitosan–TPP nanoparticles with predetermined nano-metric size and surface charge density can be simply manipulated and controlled by varying the key processing conditions of chitosan concentration, chitosan to TPP weight ratio, and solution pH value. Within the tested range of conditions, the increase in particle size and particle zeta potential showed a simple linear relationship with increasing chitosan to TPP weight ratio, but the zeta potential at fixed chitosan to TPP ratio showed a linear decrease with increasing chitosan concentration. Solution pH value and chitosan concentration also had profound influence on the stability of the nanoparticle system. [36]
- *Hui Liu et al*. in his study concluded that the particle size and zeta potential increase along with the pH increase below pH 3.5, but decrease again above pH 3.5. The nanoparticles show good storage stability at 48ºC, with no apparent agglomeration and severe size increase until 85 days. The applicability of the chitosan nanoparticles as drug carriers was demonstrated by loading and release of a model drug, ciprofloxacin. [37]

- *L. Zhang et al*. in his study concluded that many antimicrobial drugs are difficult to administer because of their low water-solubility, cytotoxicity to healthy tissues, and rapid degradation and clearance in the blood stream. Their antimicrobial

activities against intracellular microbes are also severely limited by poor membrane transport ability. Extensive studies have demonstrated that nanoparticles such as liposomes, polymeric nanoparticles, solid lipid nanoparticles and dendrimers are able to overcome these issues and facilitate antimicrobial delivery to microbial infection sites. While most of these nanoparticle based antimicrobial drug delivery systems are currently in preclinical development, several have been approved for clinical use. With the ongoing efforts in this field, there is no doubt that nanoparticle-based drug delivery systems will continue to improve treatment to bacterial infections, especially in life-threatening diseases such as staph infections and tuberculosis. [38]

- *Zhilong Shi et al.* demonstrated that the incorporation of nanoparticles of CS and quaternary ammonium CS derivative in bone cements can provide effective antibacterial action against S. aureus and S. epidermidis. These nanoparticles also enhance the antibacterial efficacy of gentamicin-loaded bone cements. The nanoparticles provide a high surface charge density for interacting with and disrupting bacterial cell membranes. [39]

- *Lifeng Qi et al*. has concluded that nanoparticles obtained in his study have small particle size and positive surface charges, which may improve their stability in the presence of biological cations and improve for their antibacterial activities due to the interaction with negatively charged biological membranes and site-specific targeting in vivo. These studies show that chitosan nanoparticles and copper-loaded nanoparticles could inhibit the growth of various microorganisms markedly and exhibit higher antibacterial activity than chitosan itself or doxycycline. The study revealed that the antibacterial action was probably via membrane disruption and leakage of cellular protein so as to kill the bacteria cells due to the change of

membrane penetrability. It is anticipated that chitosan nanoparticles could be applied broadly as antimicrobial agents in medicine for their high antibacterial activity and acceptable biocompatibilities. [40]

- *S. Wazed Ali et al*. describes the preparation of novel chitosan nanoparticles and its application on bioactive polyester fabric to impart enhanced antimicrobial activity at a very low concentration. The antimicrobial activity of chitosan gets much enhanced in nanoparticle form, as indicated by reduction in MIC from 0.5% to 0.01%. The synthesized nanoparticles were found thermally more stable than bulk chitosan. [41]

- *Xu and Du et al.* have studied different formulations of CS nanoparticles produced by the ionic gelation of TPP and CS. TEM indicated their diameter ranging between 20 and 200 nm with spherical shape. FTIR confirmed tripolyphosphoric groups of TPP linked with ammonium groups of CS in the nanoparticles. Factors that affect the delivery of bovine serum albumin (BSA) as a model protein have been studied. These include molecular weight and deacetylation degree of CS, concentrations of CS and BSA, as well as the presence of polyethylene glycol (PEG) in the encapsulation medium. Increasing molecular weight of CS from 10 to 210 kDa, BSA encapsulation efficiency was enhanced nearly twice. The total release of BSA in phosphate buffered saline pH 7.4 in 8 days was reduced from 73.9% to 17.6%. Increasing deacetylation degree from 75.5% to 92% promoted the encapsulation efficiency with a decrease in release rate. Encapsulation efficiency decreased greatly by increasing the initial concentration of BSA and CS. Higher loading capacity of BSA enhanced the BSA release from nanoparticles. However, adding PEG hindered the BSA encapsulation and increased the release rate. [42]

- *Ko et al.* prepared chitosan nanoparticles with TPP by the ionic cross-linking method. Particle sizes of TPP-Chitosan nanoparticles varied from 500 to 710 nm with drug encapsulation efficiencies more than 90%. Morphologies of TPP-CS nanoparticles have been examined by SEM. As the pH of TPP solution decreased and molecular weight of chitosan increased, nanoparticles acquired better spherical shape having smooth surface. Release of felodipine as a model drug was affected by the preparation method. Chitosan nanoparticles prepared at lower pH or higher concentration of TPP solution resulted in a slower release of felodipine. With a decreasing molecular weight and concentration of chitosan solution, the drug release increased. The release of drug from TPP-Chitosan nanoparticles decreased when the cross-linking time was increased. [43]

- *Hernandez-Lauzardo et al*., 2008 explains that chitosan owning a broad spectrum of antimicrobial activity, exhibits differing inhibitory efficiency against different fungi, Gram-positive and Gram-negative bacteria. Chitosan exerts an antifungal effect by suppressing sporulation and spore germination. [44]

- *Chen and Chou et al*. explained that for a given microbial species, age of the cell can influence antimicrobial efficiency. For example, S. aureus CCRC 12657 in late-exponential phase are the most susceptible to lactose chitosan derivative with no viability evident after 10 h of incubation. Meanwhile, a relatively less population reduction in viable cells of 3.75 and 3.96 log cfu/mL, respectively, was observed with cells in the mid-exponential phase and late-stationary phase. [45]

- *Kong et al.* reported that chitosan microspheres with a high DD (97.5%) lead to higher positive charge density, which confers stronger antibacterial activity than

moderate degree of substitution (83.7%) against Staphylococcus aureus at pH 5.5. [46]

- *Takahashia et al*. reported that a higher degree of substitution with more positive charge was especially successful in inhibiting the growth of S. aureus, suggesting antibacterial activity of chitosan towards *S. aureus* enhanced with increasing degree of substitution. [47]

- *Raafat et al.* explained that the cell wall of Gram-positive bacteria comprises peptidoglycan (PG) and teichoic acid (TA). TA is an essential polyanionic polymer of the cell wall of Gram-positive bacteria, traversing the wall to contact with the PG layer. They can be either covalently linked to N-acetylmuramic acid of the peptidoglycan layer (wall teichoic acids) or anchored into the outer leaflet of the cytoplasmic membrane via a glycolipid (lipoteichoic acids, LTA). [48]

- S. Hancock in his book explained that chitosan's antibacterial activity closely correlates with the cell surface characteristics. Bacterial surfaces are structurally complex and chemically heterogeneous, and cannot be considered as a smooth surface of a sphere. Many bacteria possess a variety of surface appendages such as pili, fimbriae or flagella, and even bacteria without these contain several types of polymers which can project from the surface, such as lipopolysaccharide, mycolic acids, lipoteichoic acids, capsular polysaccharides or proteins. [49]

- *Luis E. Chávez de Pazet al*. explains that nanoparticles prepared from chitosan with high molecular weight showed a low antimicrobial effect (20 to 25% of cells damaged), whereas those prepared from low molecular weight chitosan showed high antimicrobial effect (>95% of cells damaged). [50]

MATERIALS & METHODS

4.1 MATERIALS

4.1.1 Chemicals and Reagents

- Amikacin Sulphate was purchased from Himedia Pvt. Ltd, Mumbai.
- Chitosan was purchased from Loba Chemie Pvt. Ltd, Mumbai.
- Sodium Tripolyphosphate was purchased from Merck Pvt. Ltd, Mumbai.
- Glacial acetic acid was purchased from Pvt. Ltd, Mumbai.
- Sodium hydroxide pellets was purchased from Process Chemicals, Kolkata
- Potassium dihydrogen phosphate was purchased from Merck Pvt. Ltd, Mumbai.
- Disodium hydrogen phosphate was purchased from Process Chemicals, Kolkata.
- Sodium chloride was purchased from Process Chemicals, Kolkata.
- Potassium chloride was purchased from Process Chemicals, Kolkata.
- Milli-Q (deionized) water.

4.1.2 Analytical Instruments:

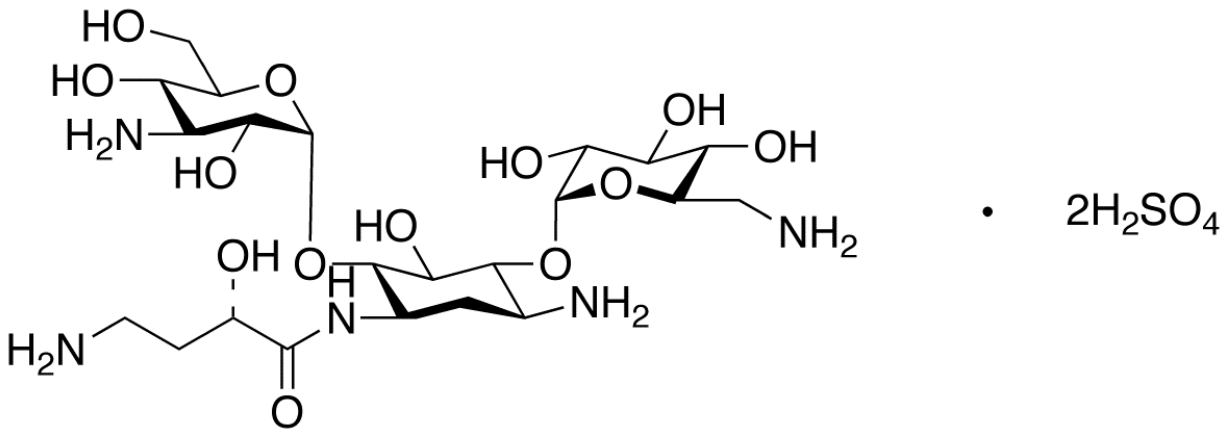
- Electronic Balance (Precisa XB 600M-C).
- Magnetic Stirrer (Spinot, Tarsons).
- pH Meter (Mettler Toledo).
- Zeta Sizer (Malvern ZS 90S)
- High Performance Liquid Chromatography (LC-20, Shimadzu, Japan)
- Ultracentrifuge with cooling (Thermo Scientific)
- Fourier Transform Infrared Spectrometer (IR Prestige-21, Shimadzu, Japan)
- Scanning Electron Microscope (JEOL JSM 6360 LV).

4.1.3 Drug Profile:

4.1.3.1 Amikacin Sulphate:

IUPAC Name : (2S)-4-Amino-N-[(2S,3S,4R,5S)-5-amino-2-[(2S,3R,4S,5S,6R)-4-amino-3,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-4-[(2R,3R,4S,5R,6R)-6-(aminomethyl)-3,4,5-trihydroxy-oxan-2-yl]oxy-3-hydroxy-cyclohexyl]-2-hydroxy-butanamide.

Structure:



Molecular Formula: C22H43N5O13

Molecular Weight: 781.75

Solubility: 50 mg/mL in water at 25°C

State: Solid, white powder.

Category: Antibiotic.

Mechanism of Action:

Amikacin primarily act by binding to the aminoacyl site of 16S ribosomal RNA within the 30S ribosomal subunit, leading to misreading of the genetic code and inhibition of

translocation. The initial steps required for peptide synthesis are uninterrupted, such as binding of mRNA and the association of the 50S ribosomal subunit, but elongation fails to occur due to disruption of the mechanisms for ensuring translational accuracy. The ensuing antimicrobial activity is usually bactericidal against susceptible aerobic gram-negative bacilli. [51]

4.1.3.2 Pharmacokinetic Properties

1. **Absorption**: Amikacin is poorly absorbed from the G.I tract. Absorption by i.m route is rapid and complete, however in critically ill patients i.m absorption can vary considerably. Peak serum concentrations of Amikacin is reached within 30-120 minutes after i.m. injection. Therapeutic or toxic concentrations may be obtained by i.p route. Amikacin is usually administered by parenteral routes: 30-60 minutes intravenous infusion or intramuscular injection.

2. **Distribution**: Amikacin is a polar drug and their distribution is mainly restricted to extracellular fluids. Protein binding of these antibiotics is less than 10%. Amikacin distribute well in synovial, peritoneal, ascitic and pleural fluids. High concentrations of Amikacin are obtained in renal tissue especially in renal cortex. Penetration of these drugs is poor in eye and central nervous system. Concentrations of Amikacin in biliar and bronchial secretions are variable. The distribution volume of Amikacin approximates to the extracellular fluid (20-25% of body weight). Modifications of the distribution coefficient of aminoglycosides occur in some kind of patients as those suffering from gram negative sepsis, dehydrated, febrile, critically ill, hematological, burned patients. [52]

3. **Elimination**: Amikacin is primarily excreted unchanged through the kidney by glomerular filtration. The 80-90% of the administered dose is excreted in the urine

resulting in high urinary concentrations. A small amount of Amikacin is excreted by bile. Serum half-life in patients with normal renal function is about 2-3 hours. Linear correlations are obtained between the clearance and the elimination constant of amikacin and creatinine clearance of the patient. [53]

4.1.3.3 Adverse Effects

1. **Nephrotoxicity**: A wide variation in the incidence. Usually reversible. Increase in serum creatinine and BUN.

2. **Otoxicity**: Cochlear and vestibular. Bilateral and permanent.

3. **Neuromuscular blockade**: Low incidence. Enhanced by concomitant administration of neuromuscular blocking drugs and anesthetics, patients with hypocalcemia or myasthenia gravis or when the i.p or rapid i.v injection are used.

4. **Other adverse effects**: Hypersensitivity reactions, superinfection, CNS effects and GI disturbances.

4.1.3.4 Uses:

Gram-negative: Amikacin is active against Pseudomonas species, Escherichia coli, Proteus species (indole-positive and indole-negative), Providencia species, Klebsiella-Enterobacter-Serratia species, Acinetobacter (formerly Mima-Herellea) species and Citrobacter freundii. When strains of the above organisms are found to be resistant to other aminoglycosides, including gentamicin, tobramycin and kanamycin, many are susceptible to amikacin in vitro.

Gram-positive

Amikacin sulfate is active against penicillinase and non-penicillinase-producing Staphylococcus species, including methicillin-resistant strains. However, aminoglycosides in general have a low order of activity against other Gram-positive organisms, viz., Streptococcus pyogenes, enterococci and Streptococcus pneumoniae (formerly Diplococcus pneumoniae).

Amikacin resists degradation by most aminoglycoside inactivating enzymes known to affect gentamicin, tobramycin and kanamycin.

In vitro studies have shown that amikacin sulfate combined with a beta-lactam antibiotic acts synergistically against many clinically significant Gram-negative organisms. [20]

4.1.3.5 Bacterial Minimum Inhibitory Concentration (MIC):

Amikacin is usually used as a last-resort medication against multidrug-resistant bacteria. The following represents susceptibility data on a few medically significant microorganisms. [21]

- *Pseudomonas aeruginosa* - 0.5 μg/mL - 32 μg/mL
- *Pseudomonas aeruginosa* (aminoglycoside-resistant) - 32 μg/mL - 64 μg/mL
- *Serratia marcescens* - ≤0.25 μg/mL - 8 μg/mL
- *Serratia marcescens* (multidrug-resistant) - 32 μg/mL

4.1.4 Polymer Profile

4.1.4.1 Chitosan:

Nonproprietary names

BP: Chitosan hydrochloride

Chemical name

Poly-b-(1, 4)-2-Amino-2-deoxy-D-glucose

Description

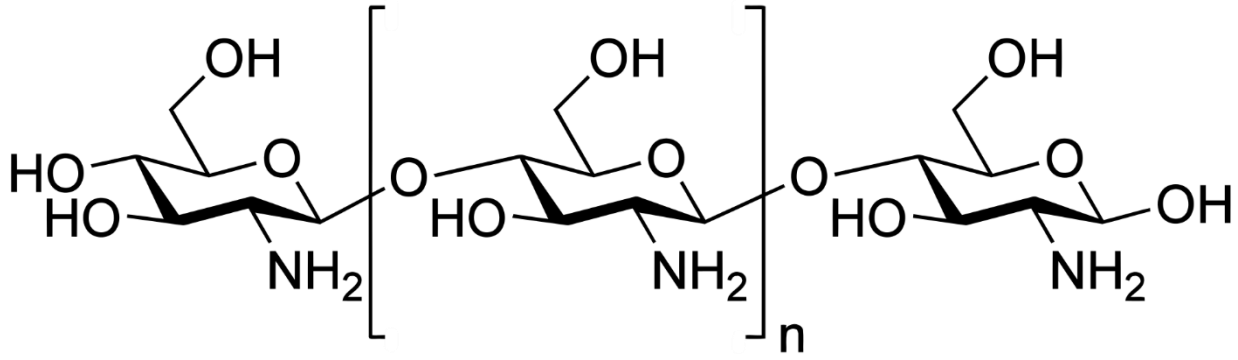
Chitosan and chitin are polysaccharide polymers containing more than 5,000 glucosamine and acetyl-glucosamine units, respectively and their molecular weights are over one million Daltons. Chitin is found in fungi, arthropods and marine invertebrates. Commercially, chitin is derived from the exoskeletons of crustaceans such as shrimp, crab and other shellfish. Chitosan is obtained from deacetylation of chitin, the cellulose like polysaccharide polymer, consisting mainly of unbranched chains of N-acetyl-D-glucosamine. Deacetylated chitin, or chitosan, is comprised of chains of D-glucosamine. When ingested, chitosan can be considered a dietary fibre. Chitosan occurs as odourless, white or creamy white powder or flakes. Fibre formation is quite common during precipitation and the chitosan may look like cotton.

Chiosan is obtained by the alkaline deacetylation of chitin. Chitosan molecule is a copolymer of N-acetyl-D-glucosamine. The sugar backbone consists of 1, 4-linked D-glucos-amine with a high degree of N-acetylation, a structure very similar to that of cellulose, except that the acetylamino group replaces the hydroxyl group on the C-2 position. Thus chitosan is poly (N-acetyl-2-amino-2-deoxy-D-glucopyranose), where the N-acetyl-2-amino-2-deoxy-D-glucopyranose (or Glu-NH2) units are linked by glycosidic bonds.

Empirical formula and molecular weight

Partial deacetylation of chitin results in the production of chitosan, which is apolysaccharide comprising copolymers of glucosamine and N-acetylglucosamine. Chitosan is the term applied to deacetylated chitins in various stages of deacetylation and depolymerization and it is therefore not easily defined in terms of its exact chemical composition. A clear nomenclature with respect to the different degrees of N-deacetylation between chitin and chitosan has not been defined and as such chitosan is not one chemical entity but varies in composition depending on the manufacturer. In essence, chitosan is chitin sufficiently deacetylated to form soluble amine salts. The degree of deacetylation necessary to obtain a soluble product must be greater than 80–85 %. Chitosan is commercially available in several types and grades that vary in molecular weight by 10000–1000000, and vary in degree of deacetylation and viscosity.

Structural Formula



Functional category

Coating agent, disintegrant, film forming agent, mucoadhesive, tablet binder, Viscosity increasing agent.

4.1.4.2 Physical Properties:

Chitosan occurs as odorless, white or creamy white powder or flakes. Fiber formation is quite common during precipitation and the chitosan may look 'cottonlike'.

4.1.4.3 Chemical properties:

Chitosan is a cationic polyamine with a high charge density at pH <6.5; and so adheres to negatively charged surfaces and chelates metal ions. It is a linear polyelectrolyte with reactive hydroxyl and amino groups (available for chemical reaction and salt formation). The properties of chitosan relate to its polyelectrolyte and polymeric carbohydrate character. The presence of a number of amino groups allows chitosan to react chemically with anionic systems, which results in alteration of physicochemical characteristics of such combinations. The nitrogen in chitosan is mostly in the form of primary aliphatic amino groups. Chitosan therefore undergoes reactions typical of amines: for example, N-acylation and Schiff reactions. Almost all functional properties of chitosan depend on the chain length, charge density, and charge distribution. Numerous studies have demonstrated that the salt form, molecular weight, and degree of deacetylation as well as pH at which the chitosan is used all influence how this polymer is utilized in pharmaceutical applications. [24]

Acidity/alkalinity: pH = 4.0–6.0 (1 % w/v aqueous solution)

Density: 1.35–1.40 g cm^{-3}

Glass transition temperature: 203° C

Moisture content: Chitosan adsorbs moisture from the atmosphere, the amount of water adsorbed depending upon the initial moisture content and the temperature and relative humidity of the surrounding air.

Particle size distribution: <30 mm

Solubility: Sparingly soluble in water; practically insoluble in ethanol (95%), other organic solvents, and neutral or alkali solutions at pH above approximately 6.5. Chitosan

dissolves readily in dilute and concentrated solutions of most organic acids and to some extent in mineral inorganic acids (except phosphoric and sulfuric acids). Upon dissolution, amine groups of the polymer become protonated, resulting in a positively charged polysaccharide (RNH3+) and chitosan salts (chloride, glutamate, etc.) that are soluble in water; the solubility is affected by the degree of deacetylation. Solubility is also greatly influenced by the addition of salt to the solution. The higher the ionic strength, the lower the solubility as a result of a salting-out effect, which leads to the precipitation of chitosan in solution. When chitosan is in solution, the repulsions between the deacetylated units and their neighboring glucosamine units cause it to exist in an extended conformation. Addition of an electrolyte reduces this effect and the molecule possesses a more random, coil-like conformation.

Viscosity (dynamic): A wide range of viscosity types is commercially available. Owing to its high molecular weight and linear, unbranched structure, chitosan is an excellent viscosity enhancing agent in an acidic environment. It acts as a pseudo plastic material, exhibiting a decrease in viscosity with increasing rates of shear. The viscosity of chitosan solutions increases with increasing chitosan concentration, decreasing temperature, and increasing degree of deacetylation.

4.1.4.4 Stability and Storage Conditions:

Chitosan powder is a stable material at room temperature, although it is hygroscopic after drying. Chitosan should be stored in a tightly closed container in a cool, dry place.

4.1.4.5 Safety:

Chitosan is being investigated widely for use as an excipient in oral and other pharmaceutical formulations. It is also used in cosmetics. Chitosan is generally regarded as a nontoxic and nonirritant material. It is biocompatible with both healthy and infected skin. Chitosan has been shown to be biodegradable.

LD50: (mouse, oral) >16 g

4.2 METHODOLOGY

4.2.1 Identification of Drug

The FTIR spectra of purchased Amikacin Sulphate was taken by IR Prestige-21, Shimadzu, Japan in the range of 4500cm^{-1} to 400cm^{-1} using potassium bromide plate technique and compared with the reference spectra (British Pharmacopoeia) to confirm the identity and integrity of the sample.

4.2.2 Analytical Method Development for Amikacin Determination:

Reference for analytical method development was taken from the official monograph of Amikacin Sulphate from British Pharmacopoeia 2010.

Mobile phase ratio was tested by varying the concentration of Acetonitrile and 0.2M potassium dihydrogen phosphate until the peak with minimum tailing and noise was obtained.

The results of the test is summarized below:

HPLC System: Shimadzu LC-20 series

Column: Agilent ODS 5 µm

- **Size:** Length = 25mm, Diameter = 4.6mm
- **Stationary phase:** octadecylsilyl silica gel for chromatography
- **Temperature:** 40 °C.

Mobile Phase:

- **Aqueous phase:** 0.2 M potassium dihydrogen phosphate previously adjusted to pH 3.0 with dilute phosphoric acid. (70%)

- **Organic Phase:** Acetonitrile (30%).

Flow rate: 1.0 mL/min.

Detection: Spectrophotometer at 200 nm.

Injection: 20 µL.

Retention time: About 2.5 min.

Run time: 4.5 times the retention time of Amikacin.

4.2.3 Preparation of 0.2M potassium dihydrogen phosphate:

13.6 gm of accurately weighed potassium dihydrogen phosphate was dissolved in the deionized Milli-Q water and the volume is made up to 1000ml. The pH of the prepared solution was adjusted to pH 3 using dilute phosphoric acid.

4.2.4 Preparation of calibration curve of Amikacin Sulphate

1mg of Amikacin Sulphate powder was dissolved in 10ml mobile phase (30% acetonitrile + 70% 0.2 M potassium dihydrogen phosphate). The resulting stock solution thus obtained contained 100 µg/ml of Amikacin sulphate. Serial dilutions were made from the stock solutions of the concentrations ranging from 25 µg/ml to 400 µg/ml.

The samples were then injected into the Shimadzu LC-20 HPLC system, running on the above mentioned parameters. The readings were taken at 200nm. The AUC values of each dilution were plotted against their respective concentrations to prepare the calibration curve of the Amikacin Sulphate.

4.2.5 Preparation of Buffer Solutions:

4.2.5.1 Phosphate Buffer pH 7.4

8.0 gm of sodium chloride, 0.2 gm of potassium chloride, 1.42 gm of di-sodium hydrogen phosphate and 0.24 gm of potassium dihydrogen phosphate were accurately weighed and diluted in 100ml of Milli-Q water. The final volume is then made up to 1000ml and the pH is adjusted to 7.4.

4.2.5.2 Phosphate Buffer pH 6.8

8.5 gm of sodium chloride, 2 gm of di-potassium hydrogen phosphate, and 1 gm of potassium di-hydrogen phosphate were accurately weighed and diluted in 100ml of Milli-Q water. The final volume is then made up to 1000ml and the pH is adjusted to 6.8.

4.2.6 Preparation of Amikacin sulphate loaded chitosan-TPP nanoparticles:

Four batches of nanoparticles were prepared by varying the amount of individual chemicals as follows:

Batch A: 10mg of chitosan, 5mg of sodium tri-polyphosphate and 5mg of Amikacin sulphate were accurately weighed. The weighed quantity of amikacin and TPP were dissolved in 1ml of water separately. The weighed amount of chitosan was then suspended in 5ml of Milli-Q water and stirred on a magnetic stirrer at 600 rpm for 15 min. Then 1-2 drops of glacial acetic acid was added to solubilize the chitosan in water with continuous stirring for another 15 minutes. When the chitosan is completely dissolved in water, the solution is then neutralized with dilute sodium hydroxide to obtain the pH 4.5. Then the drug solution is then slowly added to the chitosan soltion with continuous stirring for 15 minutes. Finally the TPP solution is added to this mixture dropwise with continuous stirring at 600 rpm. Nanoparticles forms immediately. The stirrer is continued for another 1 hour. Then the prepared nanoparticle thus obtained was ultrasonicated in bath sonicator for 15 minutes to further reduce the particle size. The nanoparticle was then centrifuged at 15000 rpm for 20 minutes. The nanoparticle pellet is collected and freeze dried for future experiments. The supernatant solution is used for the determination of drug loading by HPLC.

Batch B: 5mg of chitosan, 5mg of sodium tri-polyphosphate and 5mg of Amikacin sulphate were accurately weighed. The weighed quantity of amikacin and TPP were

dissolved in 1ml of water separately. The weighed amount of chitosan was then suspended in 5ml of Milli-Q water and stirred on a magnetic stirrer at 600 rpm for 15 min. Then 1-2 drops of glacial acetic acid was added to solubilize the chitosan in water with continuous stirring for another 15 minutes. When the chitosan is completely dissolved in water, the solution is then neutralized with dilute sodium hydroxide to obtain the pH 4.5. Then the drug solution is then slowly added to the chitosan soltion with continuous stirring for 15 minutes. Finally the TPP solution is added to this mixture drop wise with continuous stirring at 600 rpm. Nanoparticles forms immediately. The stirrer is continued for another 1 hour. Then the prepared nanoparticle thus obtained was ultrasonicated in bath sonicator for 15 minutes to further reduce the particle size. The nanoparticle was then centrifuged at 15000 rpm for 20 minutes. The nanoparticle pellet is collected and freeze dried for future experiments. The supernatant solution is used for the determination of drug loading by HPLC.

Batch C: 20mg of chitosan, 5mg of sodium tri-polyphosphate and 5mg of Amikacin sulphate were accurately weighed. The weighed quantity of amikacin and TPP were dissolved in 1ml of water separately. The weighed amount of chitosan was then suspended in 5ml of Milli-Q water and stirred on a magnetic stirrer at 600 rpm for 15 min. Then 1-2 drops of glacial acetic acid was added to solubilize the chitosan in water with continuous stirring for another 15 minutes. When the chitosan is completely dissolved in water, the solution is then neutralized with dilute sodium hydroxide to obtain the pH 4.5. Then the drug solution is then slowly added to the chitosan soltion with continuous stirring for 15 minutes. Finally the TPP solution is added to this mixture drop wise with continuous stirring at 600 rpm. Nanoparticles forms immediately. The stirrer is continued for another 1 hour. Then the prepared nanoparticle thus obtained was ultrasonicated in bath sonicator for 15 minutes to further reduce the particle size. The nanoparticle was then centrifuged at 15000 rpm for 20 minutes. The nanoparticle pellet is collected and freeze dried for future experiments. The supernatant solution is used for the determination of drug loading by HPLC.

Batch D: 10mg of chitosan, 2.5mg of sodium tri-polyphosphate and 5mg of Amikacin sulphate were accurately weighed. The weighed quantity of amikacin and TPP were dissolved in 1ml of water separately. The weighed amount of chitosan was then suspended in 5ml of Milli-Q water and stirred on a magnetic stirrer at 600 rpm for 15 min. Then 1-2 drops of glacial acetic acid was added to solubilize the chitosan in water with continuous stirring for another 15 minutes. When the chitosan is completely dissolved in water, the solution is then neutralized with dilute sodium hydroxide to obtain the pH 4.5. Then the drug solution is then slowly added to the chitosan soltion with continuous stirring for 15 minutes. Finally the TPP solution is added to this mixture drop wise with continuous stirring at 600 rpm. Nanoparticles forms immediately. The stirrer is continued for another 1 hour. Then the prepared nanoparticle thus obtained was ultrasonicated in bath sonicator for 15 minutes to further reduce the particle size. The nanoparticle was then centrifuged at 15000 rpm for 20 minutes. The nanoparticle pellet is collected and freeze dried for future experiments. The supernatant solution is used for the determination of drug loading by HPLC.

Batch E: 10mg of chitosan, 10mg of sodium tri-polyphosphate and 5mg of Amikacin sulphate were accurately weighed. The weighed quantity of amikacin and TPP were dissolved in 1ml of water separately. The weighed amount of chitosan was then suspended in 5ml of Milli-Q water and stirred on a magnetic stirrer at 600 rpm for 15 min. Then 1-2 drops of glacial acetic acid was added to solubilize the chitosan in water with continuous stirring for another 15 minutes. When the chitosan is completely dissolved in water, the solution is then neutralized with dilute sodium hydroxide to obtain the pH 4.5. Then the drug solution is then slowly added to the chitosan soltion with continuous stirring for 15 minutes. Finally the TPP solution is added to this mixture drop wise with continuous stirring at 600 rpm. Nanoparticles forms immediately. The stirrer is continued for another 1 hour. Then the prepared nanoparticle thus obtained was ultra sonicated in bath sonicator for 15 minutes to further reduce the particle size. The nanoparticle was then centrifuged at 15000 rpm for 20 minutes. The nanoparticle pellet is

collected and freeze dried for future experiments. The supernatant solution is used for the determination of drug loading by HPLC.

Batch F: 10mg of chitosan, 5mg of sodium tri-polyphosphate and 10mg of Amikacin sulphate were accurately weighed. The weighed quantity of amikacin and TPP were dissolved in 1ml of water separately. The weighed amount of chitosan was then suspended in 5ml of Milli-Q water and stirred on a magnetic stirrer at 600 rpm for 15 min. Then 1-2 drops of glacial acetic acid was added to solubilize the chitosan in water with continuous stirring for another 15 minutes. When the chitosan is completely dissolved in water, the solution is then neutralized with dilute sodium hydroxide to obtain the pH 4.5. Then the drug solution is then slowly added to the chitosan soltion with continuous stirring for 15 minutes. Finally the TPP solution is added to this mixture drop wise with continuous stirring at 600 rpm. Nanoparticles forms immediately. The stirrer is continued for another 1 hour. Then the prepared nanoparticle thus obtained was ultrasonicated in bath sonicator for 15 minutes to further reduce the particle size. The nanoparticle was then centrifuged at 15000 rpm for 20 minutes. The nanoparticle pellet is collected and freeze dried for future experiments. The supernatant solution is used for the determination of drug loading by HPLC.

4.2.6.1 Preparation of blank nanoparticle

Blank: 10mg of chitosan and 5mg of sodium tri-polyphosphate were accurately weighed. The weighed quantity of TPP was dissolved in 1ml of water separately. The weighed amount of chitosan was then suspended in 5ml of Milli-Q water and stirred on a magnetic stirrer at 600 rpm for 15 min. Then 1-2 drops of glacial acetic acid was added to solubilize the chitosan in water with continuous stirring for another 15 minutes. When the chitosan is completely dissolved in water, the solution is then neutralized with dilute sodium hydroxide to obtain the pH 4.5. Finally the TPP solution is added to this mixture drop wise with continuous stirring at 600 rpm. Nanoparticles forms immediately. The stirrer is continued for another 1 hour. Then the prepared nanoparticle thus obtained was

ultrasonicated in bath sonicator for 15 minutes to further reduce the particle size. The nanoparticle was then centrifuged at 15000 rpm for 20 minutes. The nanoparticle pellet is collected and freeze dried for future experiments.

Table: Composition of the prepared formulations

Ingredients	**NP A**	**NP B**	**NP C**	**NP D**	**NP E**	**NP F**	**Blank**
Chitosan (mg)	10	5	20	10	10	10	10
Na-TPP (mg)	5	5	5	2.5	10	5	5
Amikacin (mg)	5	5	5	5	5	10	0

4.2.7 Characterization of Amikacin loaded nanoparticles:

4.2.7.1 Drug Loading efficiency (Entrapment Efficiency)

The entrapment or loading of amikacin in nanoparticles was calculated indirectly. The prepared nanoparticles was first was first centrifuged at 15000 rpm for 20 min in Ultra centrifuge (Thermo Scientific) at 4°C. The nanoparticles settled down as pellets leaving the clear supernatant solution having untrapped drug. The supernatant thus obtained were filtered through syringe filter and injected into the HPLC system running in the parameters as mentioned previously. The drug entrapment efficiency was calculated using the following formula:

$$\textbf{Entrapment Efficiency(\%)} = \frac{\textit{Amount of drug used in formulation} - \textit{Amount of drug in supernatant}}{\textit{Amount of drug used in formulation}} \times 100$$

4.2.7.2 Particle size and zeta potential analysis by Dynamic Light Scattering Method

The particle size of the nanoparticle was measured using Zeta Sizer (Malvern ZS 90S). The prepared nanoparticles were first diluted with Milli-Q water. Based on the principle of dynamic light scattering, the zeta sizer uses an automatic variable power laser at 638 nm, 35mW power, an optical cell design, a single mode fiber, a self-protecting avalanche photodiode and a 25ns/522 channel digital auto correlator.

The parameters measured were particle size, polydispersity index, zeta potential and % intensity.

4.2.7.3 Drug and excipients compatibility study using FTIR:

Drug-polymer (chitosan) interactions were studied by FTIR spectroscopy. The IR spectra of pure drug, amikacin loaded nanoparticles and blank nanoparticles were recorded using Shimadzu IR-Prestige-21 spectrophotometer. Samples were prepared in potassium bromide (KBr) disc (2mg sample in 200mg KBr). The samples were scanned in the range of 4500cm^{-1} to 500cm^{-1}. The scanning was performed at 4cm^{-1} resolution with a speed of 2mm/second.

4.2.7.4 I*n-vitro* Drug release study of the Amikacin loaded nanoparticles:

The release study was performed by equilibrium dialysis technique at 37°C using Franz Diffusion Cell. The freeze dried amikacin loaded chitosan nanoparticle were suspended in 2ml of the buffer solution. Dialysis membrane (MWCo 12000-14000D, Himedia, India) was soaked overnight in buffer solution. The membrane is then attached to the donor portion of the Diffusion Cell and tied with thread. The receptor compartment was filled with 70ml of buufer solution and placed on the Magnetic Stirrer with heating (Spinot, Tarsons). When the temperature of the buffer solution in the receiving compartment reached 37°C, donor compartment containing nanoparticle suspension was

placed in the diffusion cell in such a way that the dialysis membrane touched the buffer solution in receiver compartment. Samples of 1ml was then collected at an interval of 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24, 36, 48, 60 and 72 hours. Sampling was done through the sampling port and after each sampling, equal amount of buffer solution was added to keep the volume in receptor compartment constant. The samples thus obtained were analysed using HPLC system using parameters described in the analytical method development. The released drug were then plotted using different pharmacokinetic models given in the result section.

4.2.7.5 Scanning Electron Microscopy :

The morphology and surface characteristics of the Amikacin loaded nanoparticles were analyzed using JEOL JSM 6360 LV Scanning Electron Microscope.

4.2.8 Preparation of Biofilm and determination of anti-bacterial activity of Amikacin nanoparticles:

Weighed quantity of nutrient agar powder was first autoclaved for 45minutes to make it completely free from microorganisms i.e. sterile. Then the agar plate was prepared by mixing the agar with distilled water. The mixture was boiled for 1 minute which melts the agar. The mixture was then cooled down to 45-50°C and poured into the petri dish. The plates were then allowed to cool and set and stored for further use.

In a 96 well microplate the suspension of nanoparticles were serially diluted with Mueller Hinton Broth (MHB). To each well 5 µl of *E.coli* cell suspension was added. Separately a blank well was also prepared by adding 100 µl of MHB and 5 µl of *E.coli* cell. A standard

well was also prepared by adding 5 µl of standard ciprofloxacin solition, 195 µl of MHB and 5 µl of *E.coli* cell.

The microplate was then placed in the incubator and incubated at 37°C for 24 hours. After 24 hours the Optical Density (OD) of each of the cell is measures at 590nm by UV spectrophotometer. The OD value of the sample is compared to the standard to determine the minimum inhibitory concentration (MIC) of the drug.

Then three agar plates which were prepared previously is taken and to each plates blank, sample and standard solutions are spread uniformly. The plates are then incubated at 37°C for 24 hours.

After 24 hours colony forming units (CFU) of each plate is calculated and the activity of sample drug is compared with the standard.

TABLES & GRAPHS

5.1 Identification of Drug Sample:

5.1.1 Fourier Transform Infrared Spectroscopy:

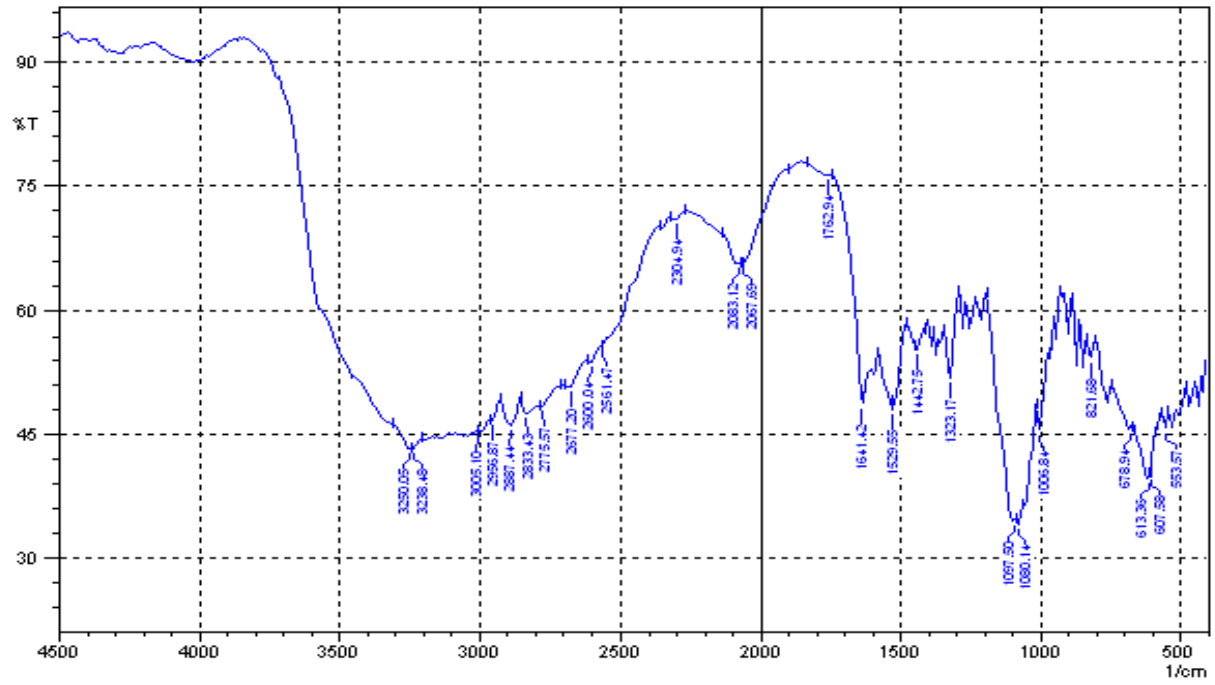


Fig 5.1.1 FTIR spectrum of Amikacin Sulphate

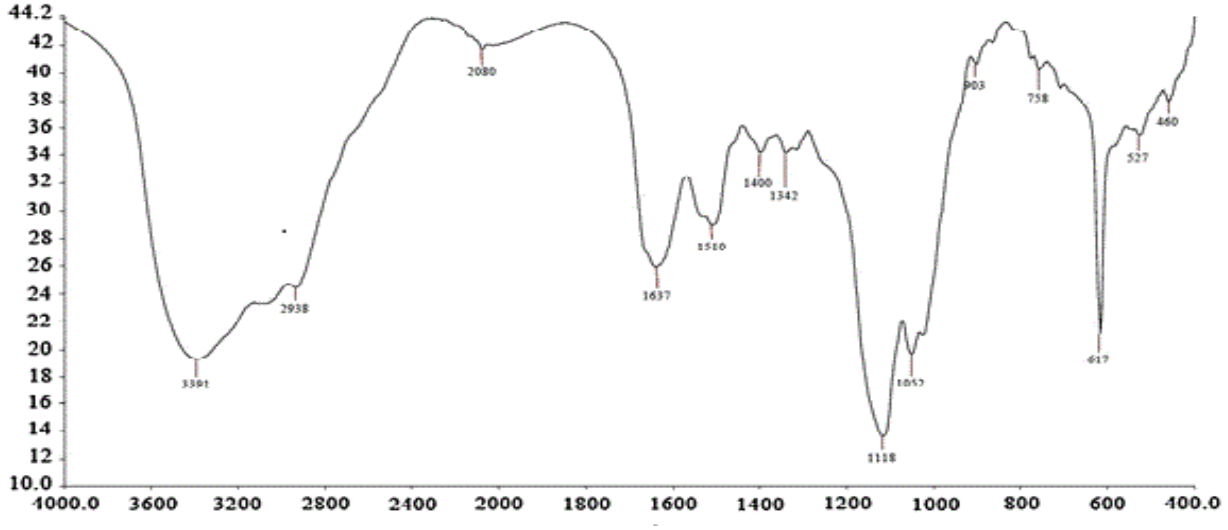


Fig 5.1.2 FTIR spectrum of Amikacin Sulphate Reference

5.2a HPLC chromatogram of Amikacin sulphate at pH 7.4:

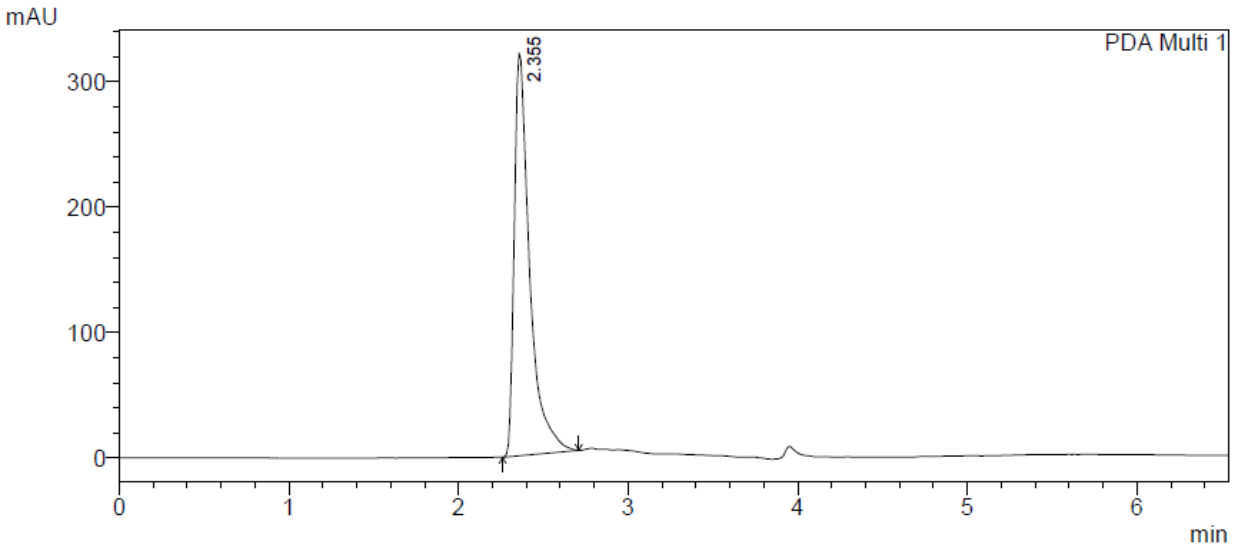


Fig 5.2a HPLC chromatogram of Amikacin sulphate at 2.35 minute.

5.2b HPLC chromatogram of Amikacin sulphate at pH 6.8:

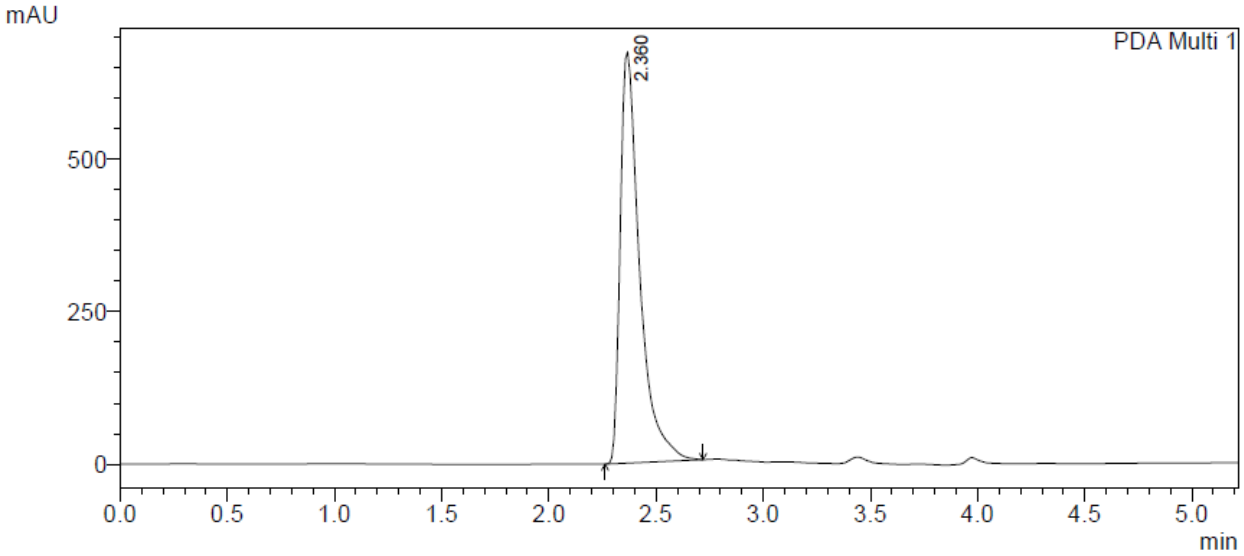


Fig 5.2b HPLC chromatogram of Amikacin sulphate at 2.36 minute.

5.3 Calibration Curve of Amikacin Sulphte:

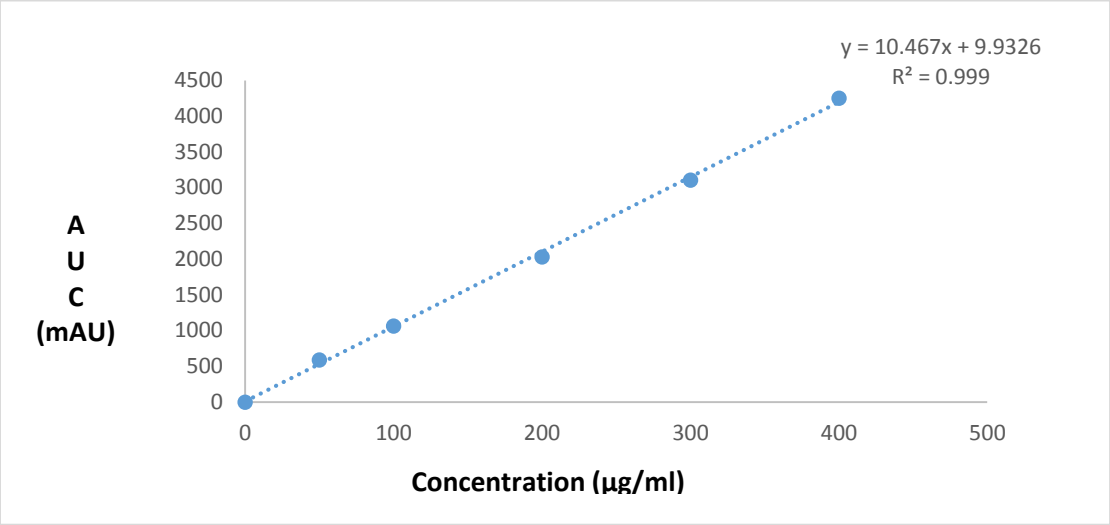


Fig 5.3 Calibration curve of Amikacin Sulphate by HPLC

5.4 Particle size, Entrapment efficiency and zeta potential of nanoparticles:

Formulation code	Particle size (nm)	Entrapment efficiency (%)	Zeta potential (mV)
NP A	270 ±0.6	70.3%	+15.3
NP B	300 ±0.9	35.2%	+18.6
NP C	342 ±0.4	63.8%	+14.2
NP D	220 ±0.9	45.7%	+19.1
NP E	295+1.3	55.4%	+14.7
NP F	310 ±0.6	44.5%	+13.8
Blank	210 ±0.8	NA	+15.2

5.4.1 DLS curve of Amikacin loaded nanoparticles size:

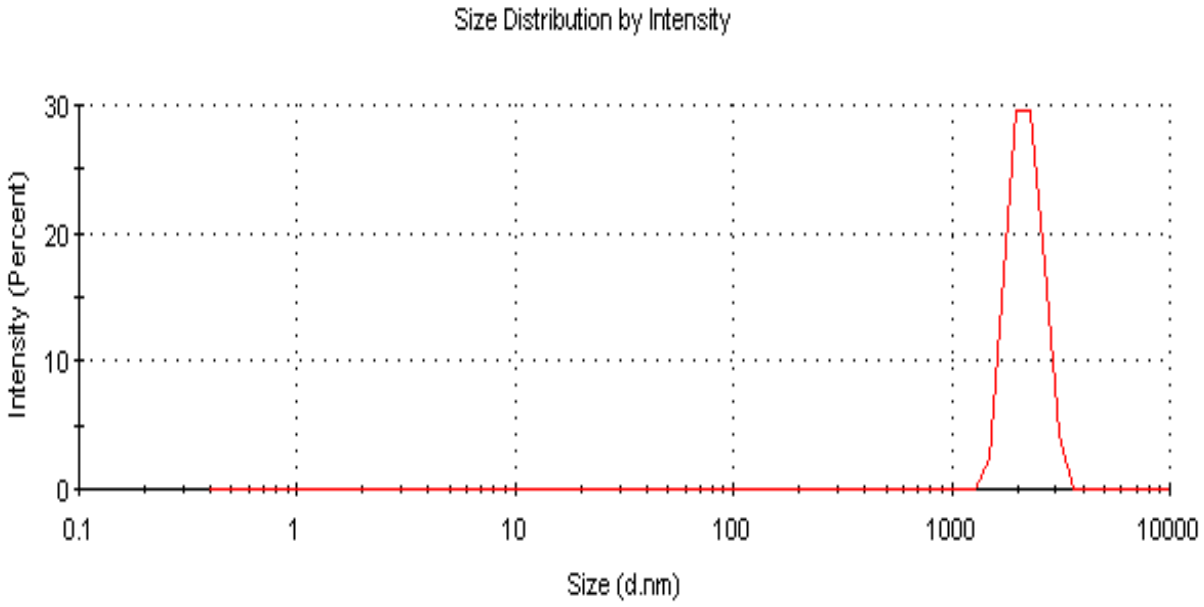


Fig. 5.4.1 DLS curve representing particle size of Amikacin loaded nanoparticles.

5.4.2 Effect of polymer (chitosan) on entrapment efficiency:

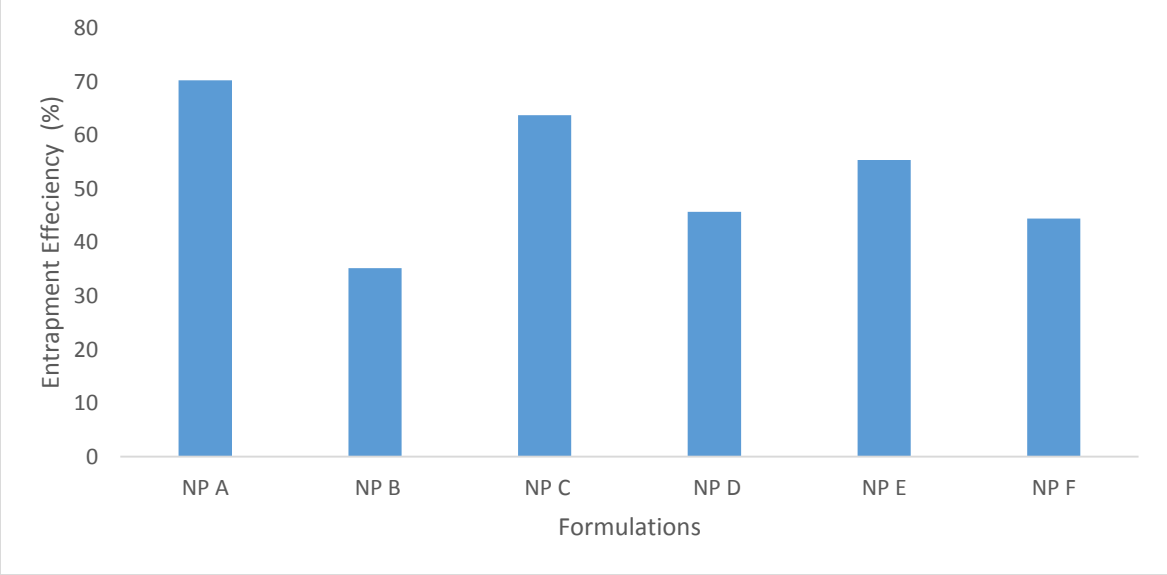


Fig. 5.4.1 Effect of polymer (chitosan) on entrapment efficiency:

5.4.3 Effect of polymer (chitosan) on Zeta potential:

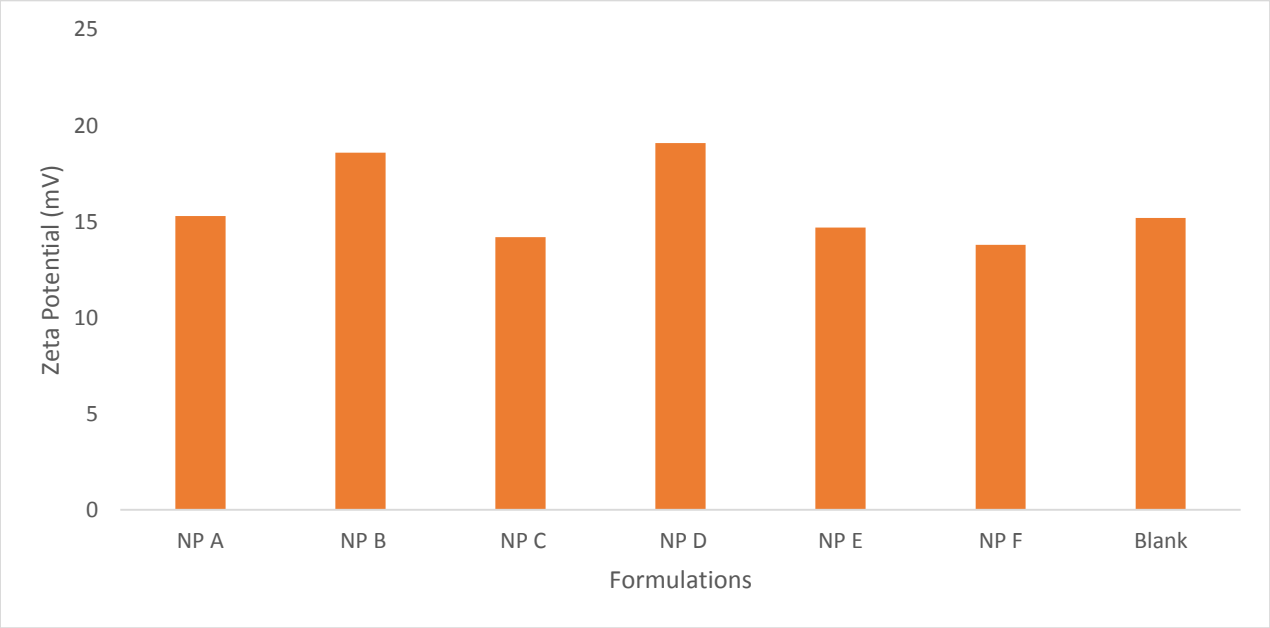


Fig. 5.4.2 Effect of polymer (chitosan) on Zeta potential:

5.4.4 Effect of polymer (chitosan) on particle size:

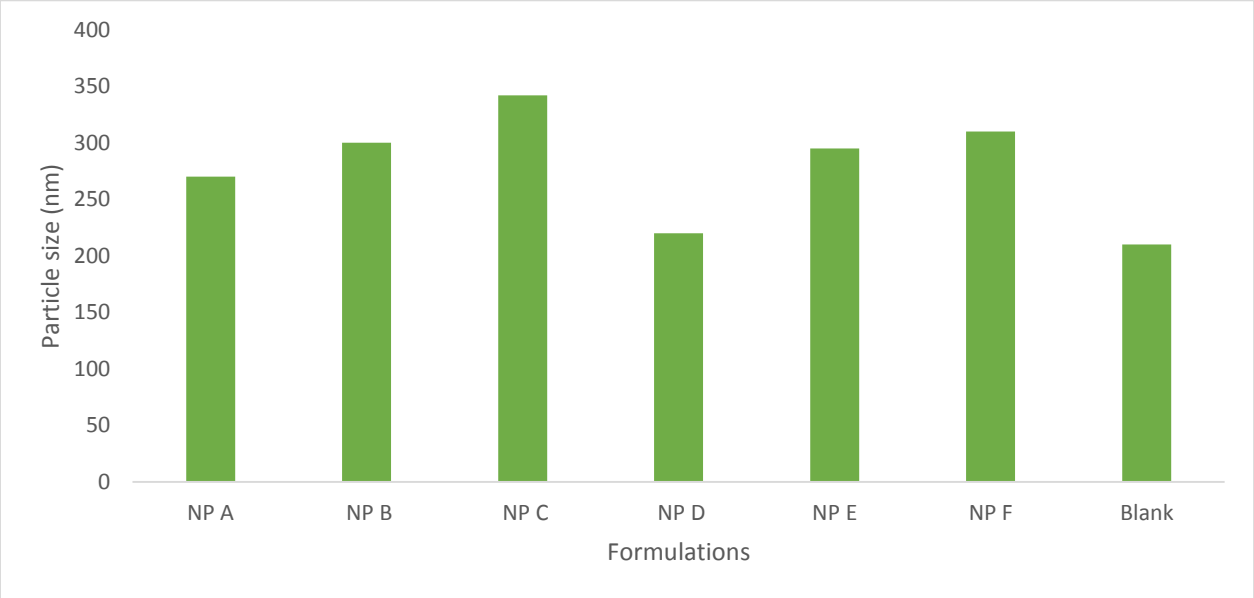


Fig. 5.4.3 Effect of polymer (chitosan) on particle size:

5.5 Fourier Transform Infrared Spectroscopy:

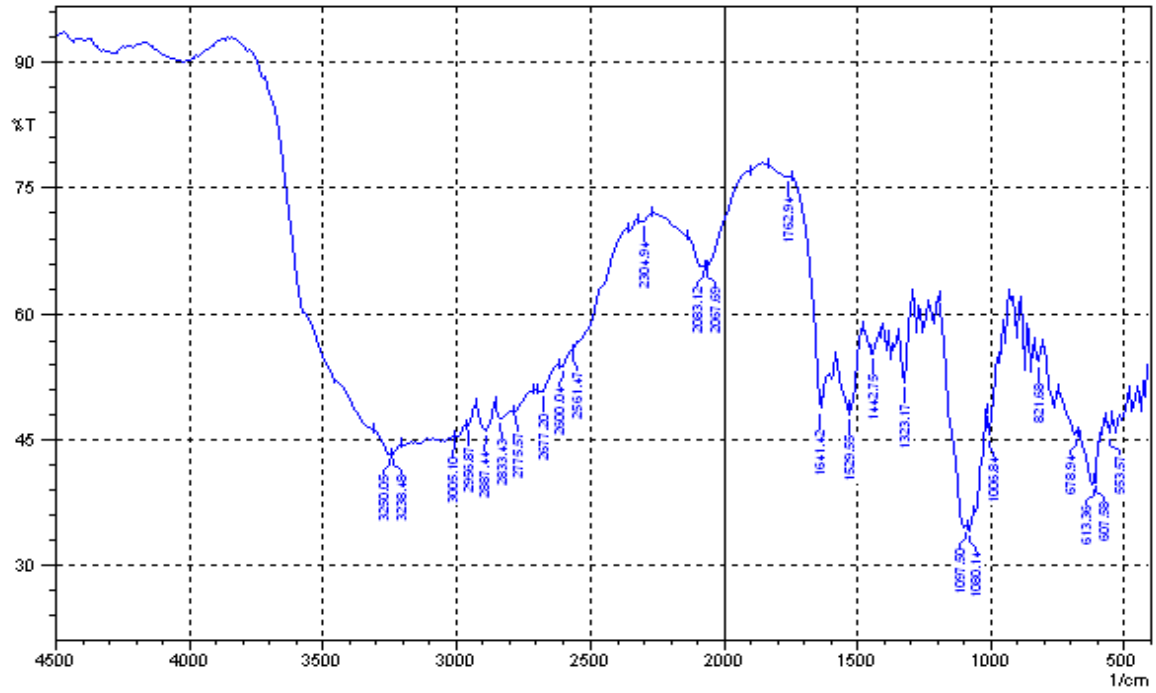


Fig. 5.5.1 FTIR spectrum of drug Amikacin Silphate

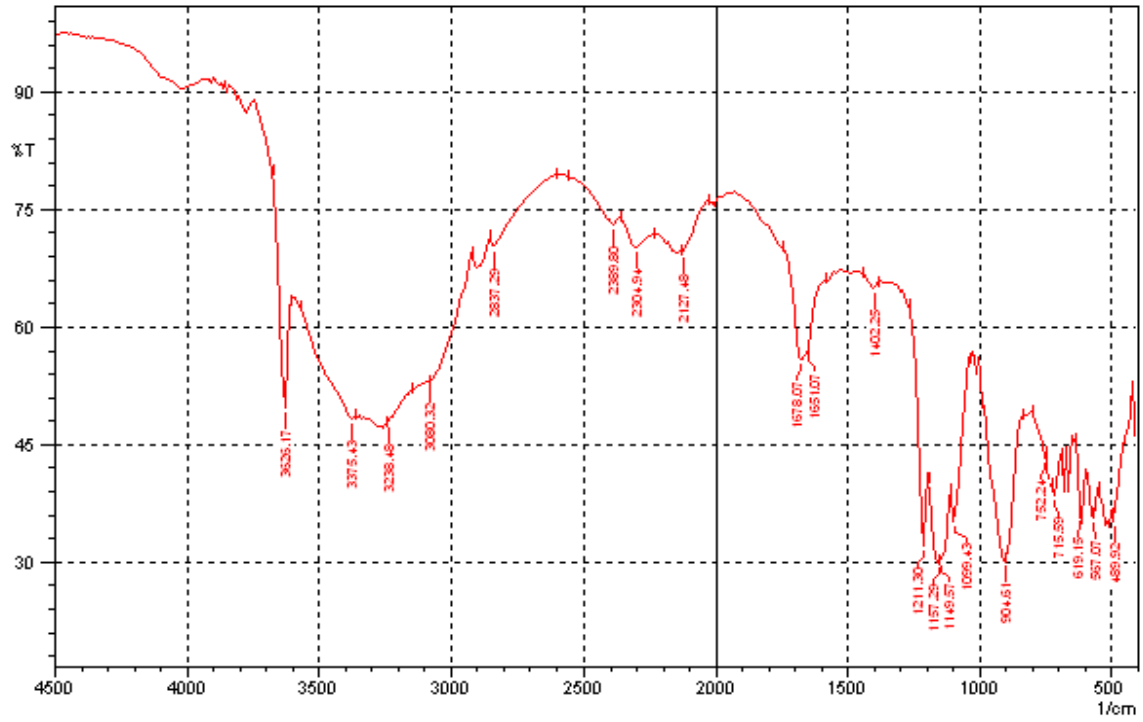


Fig. 5.5.2 FTIR spectrum of Blank Nanoparticles

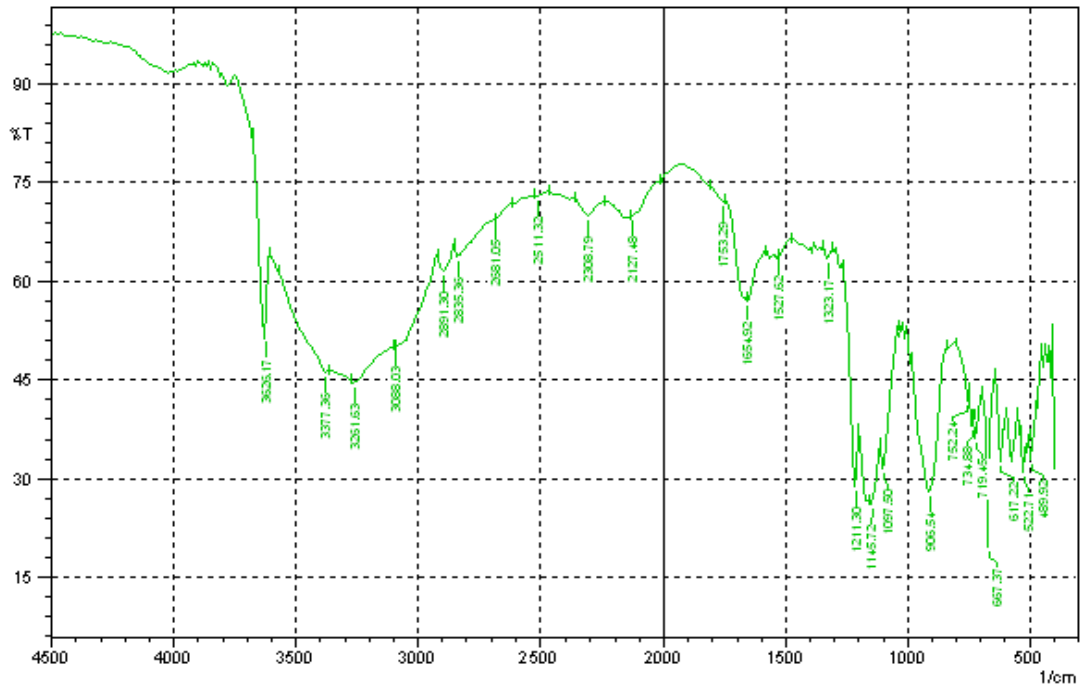


Fig. 5.5.3 FTIR spectrum of Amikacin laoded nanoparticles

5.6 Differential Scanning Calorimetry:

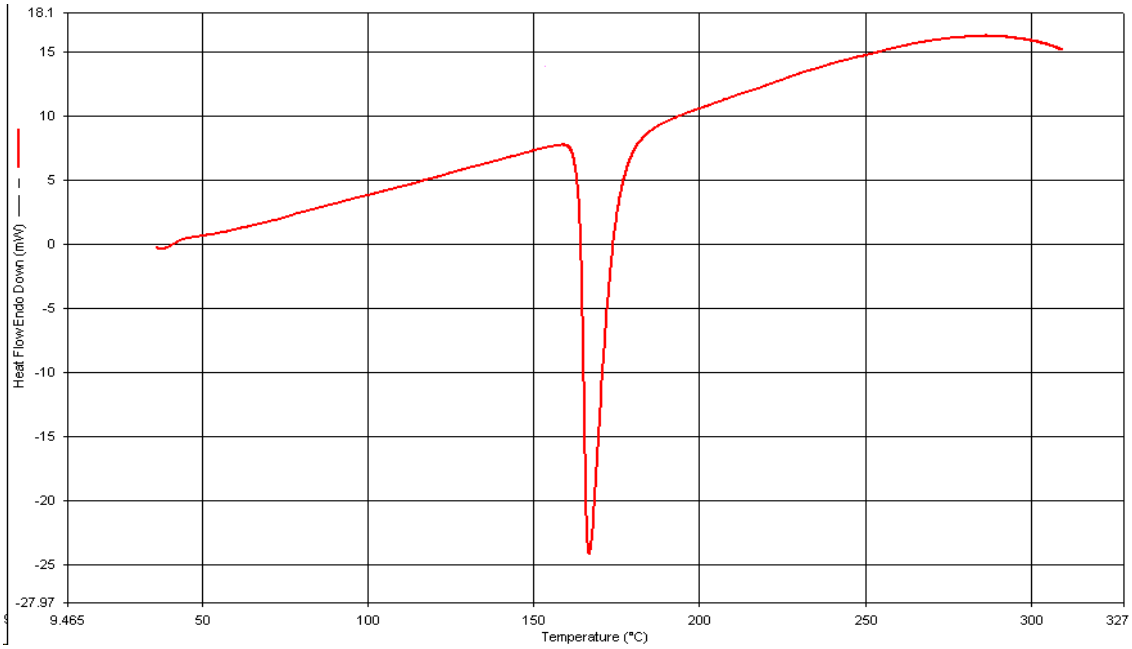


Fig 5.6.1 Differential Scanning Calorimetry (DSC) of Drug

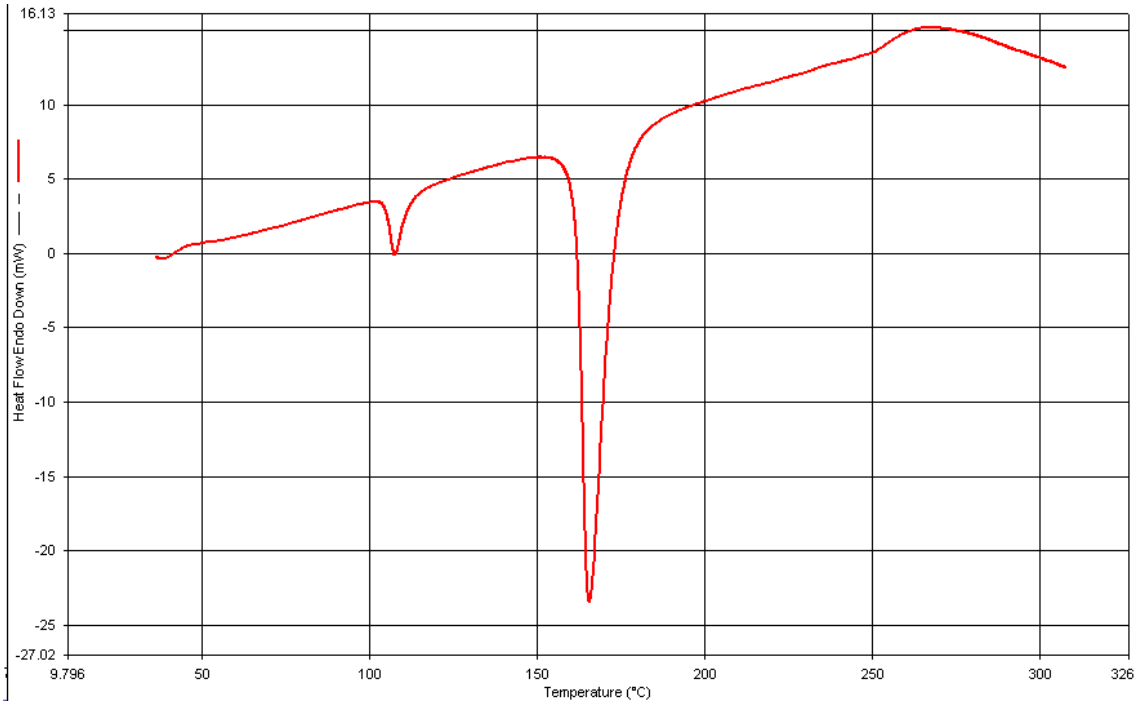


Fig 5.6.2 Differential Scanning Calorimetry (DSC) of Drug loaded nanoparticle

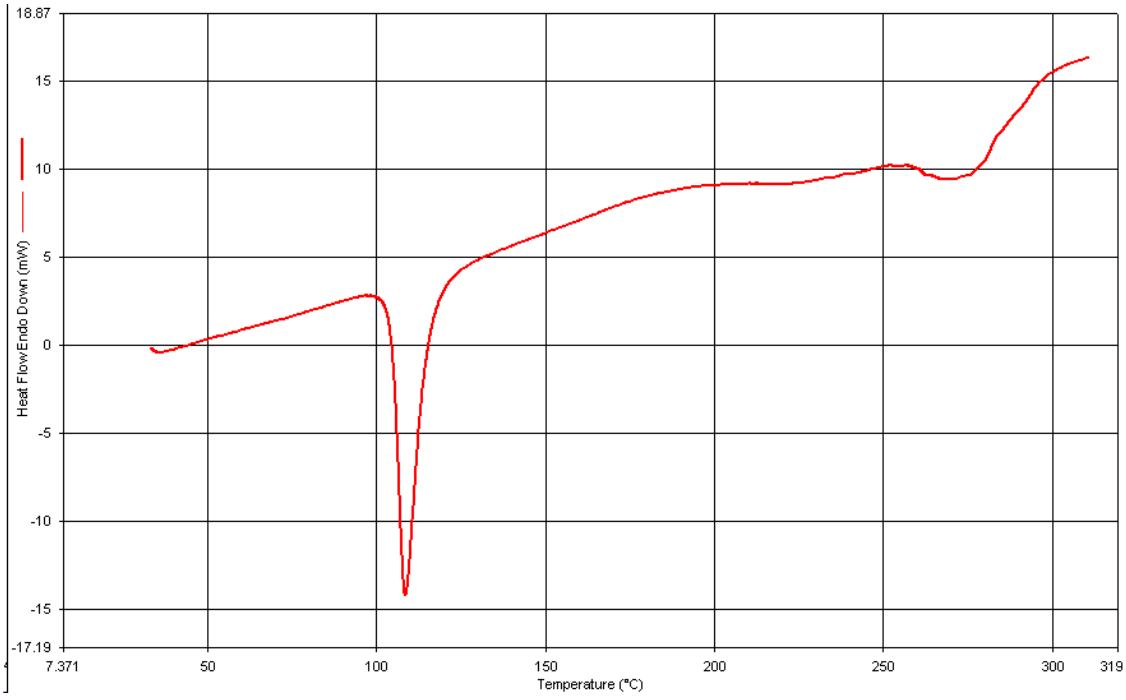


Fig 5.6.3 Differential Scanning Calorimetry (DSC) of Blank nanoparticle

5.7 Drug release model of Amikacin Sulfate Nanoparticles:

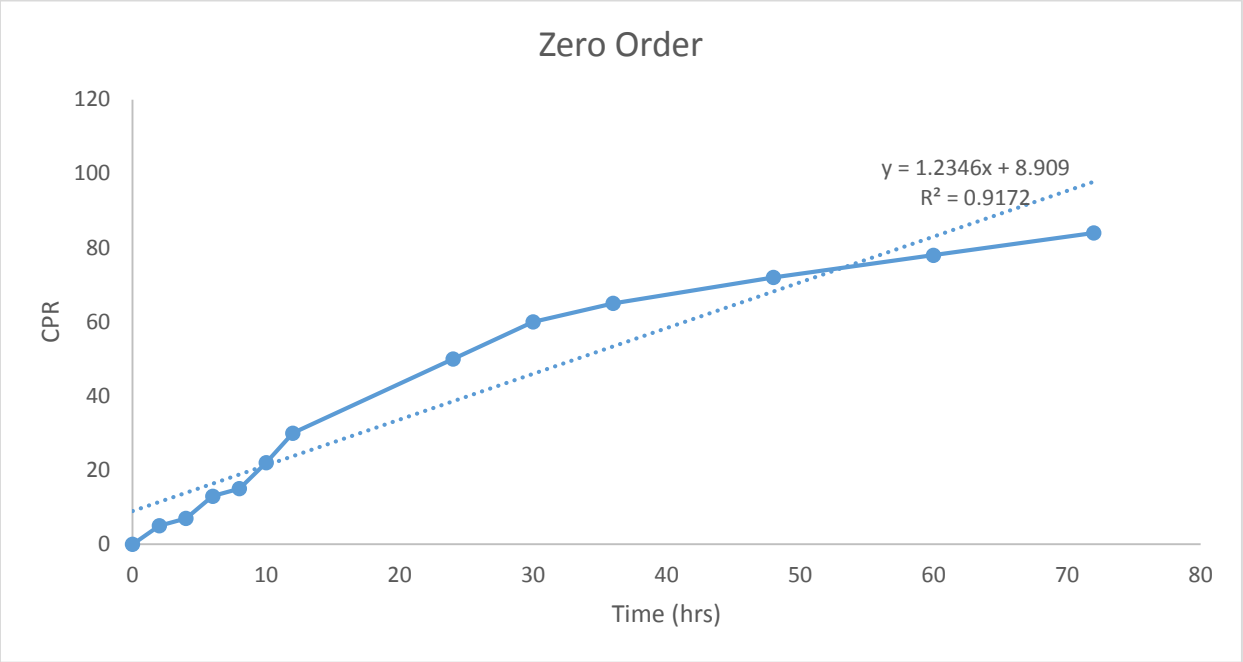


Fig 5.7.1 In vitro release of Amikacin sulfate from NP A as zero order model

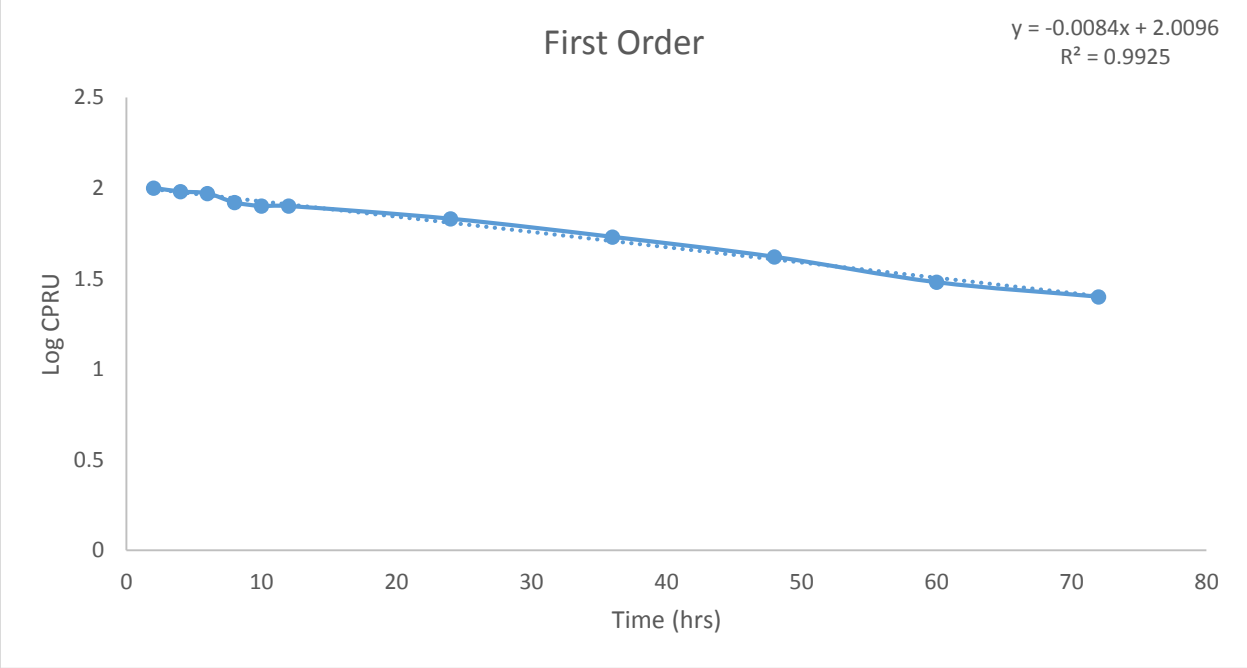


Fig 5.7.2 In vitro release of Amikacin sulfate from NP A as first order model

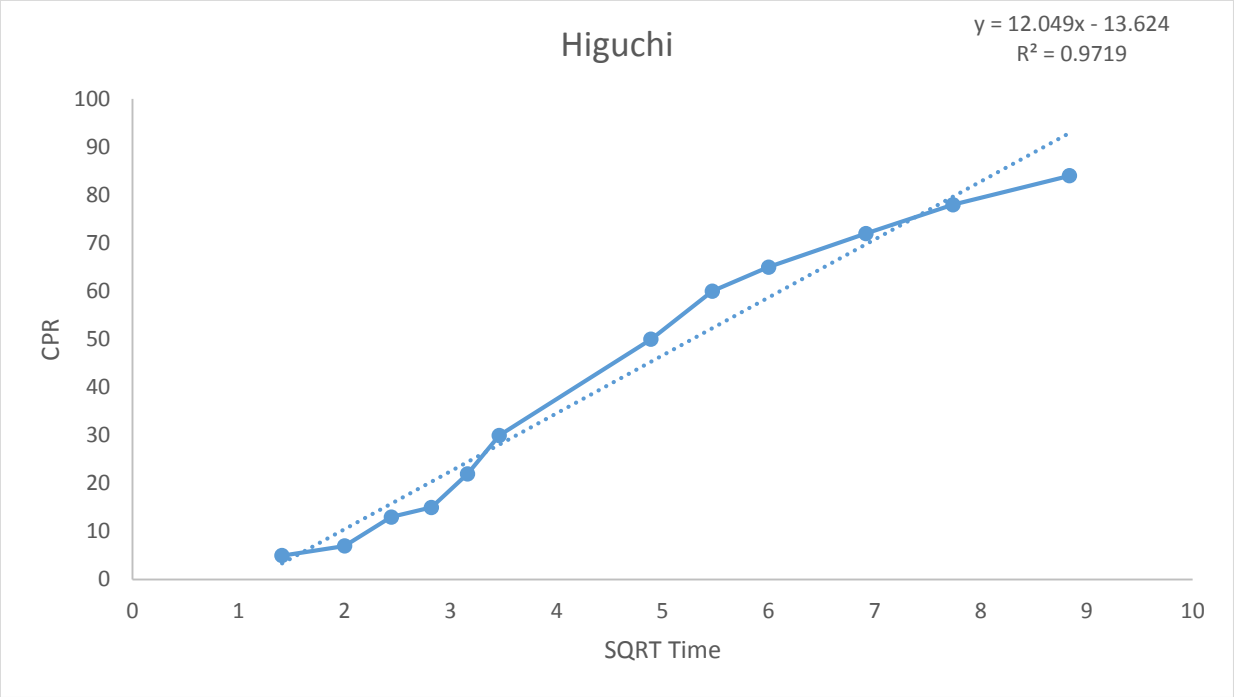


Fig 5.7.3 In vitro release of Amikacin sulfate from NP A as first Higuchi model

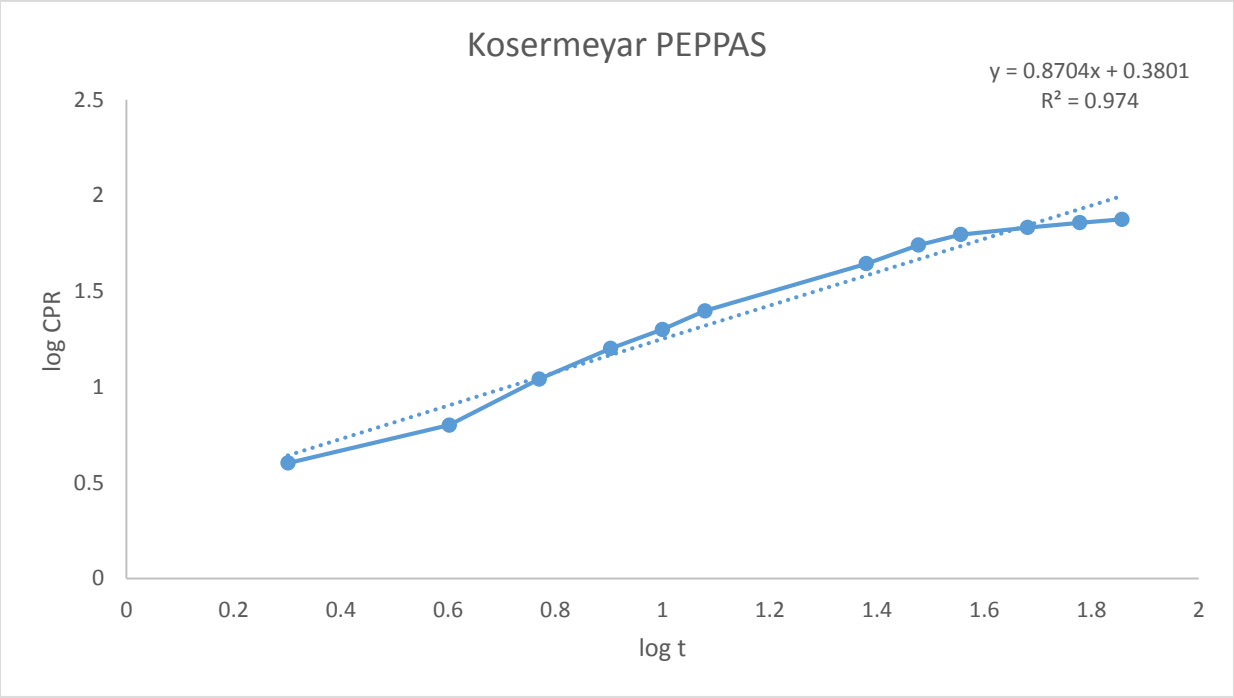


Fig 5.7.4 In vitro release of Amikacin sulfate from NP A as first Kosermeyar-Peppas model

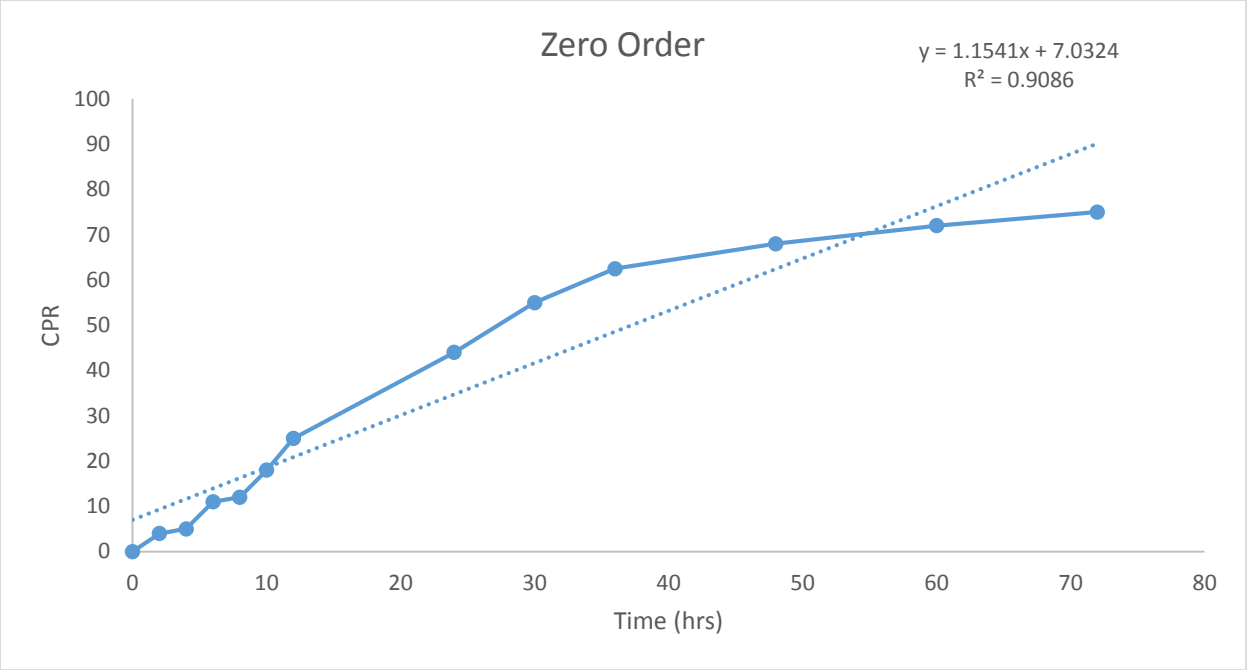


Fig 5.7.5 In vitro release of Amikacin sulfate from NP C as zero order model

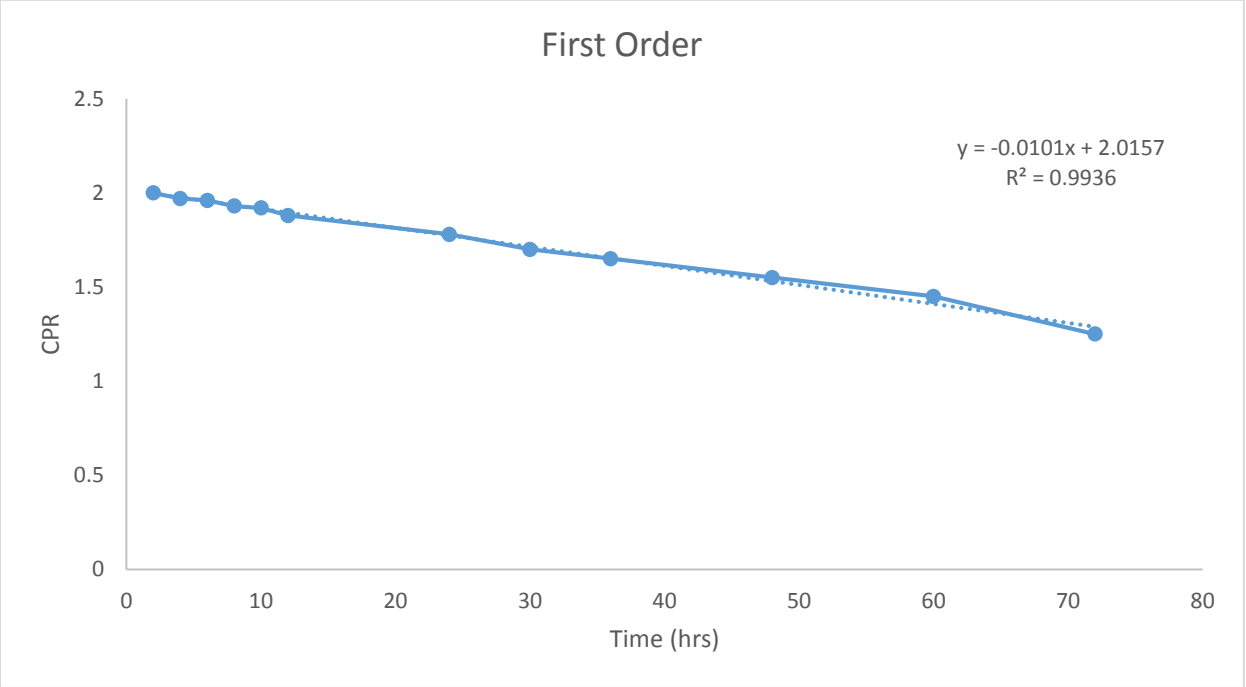


Fig 5.7.6 In vitro release of Amikacin sulfate from NP C as first order model

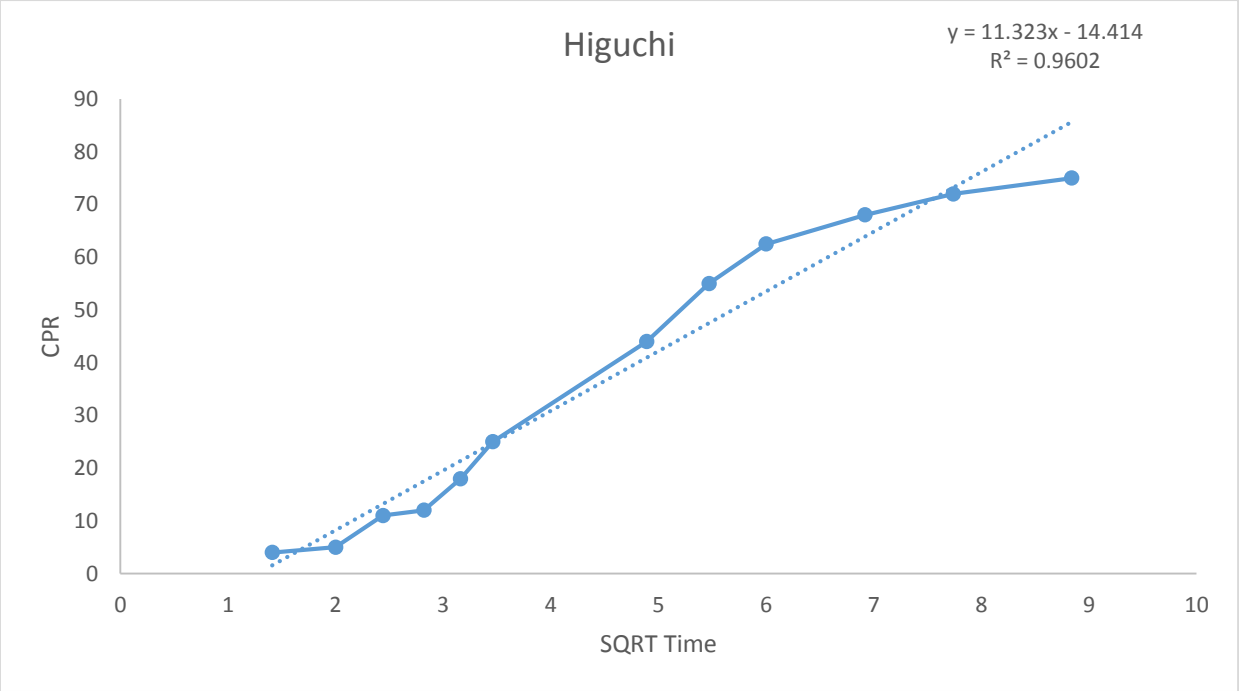


Fig 5.7.7 In vitro release of Amikacin sulfate from NP C as first Higuchi model

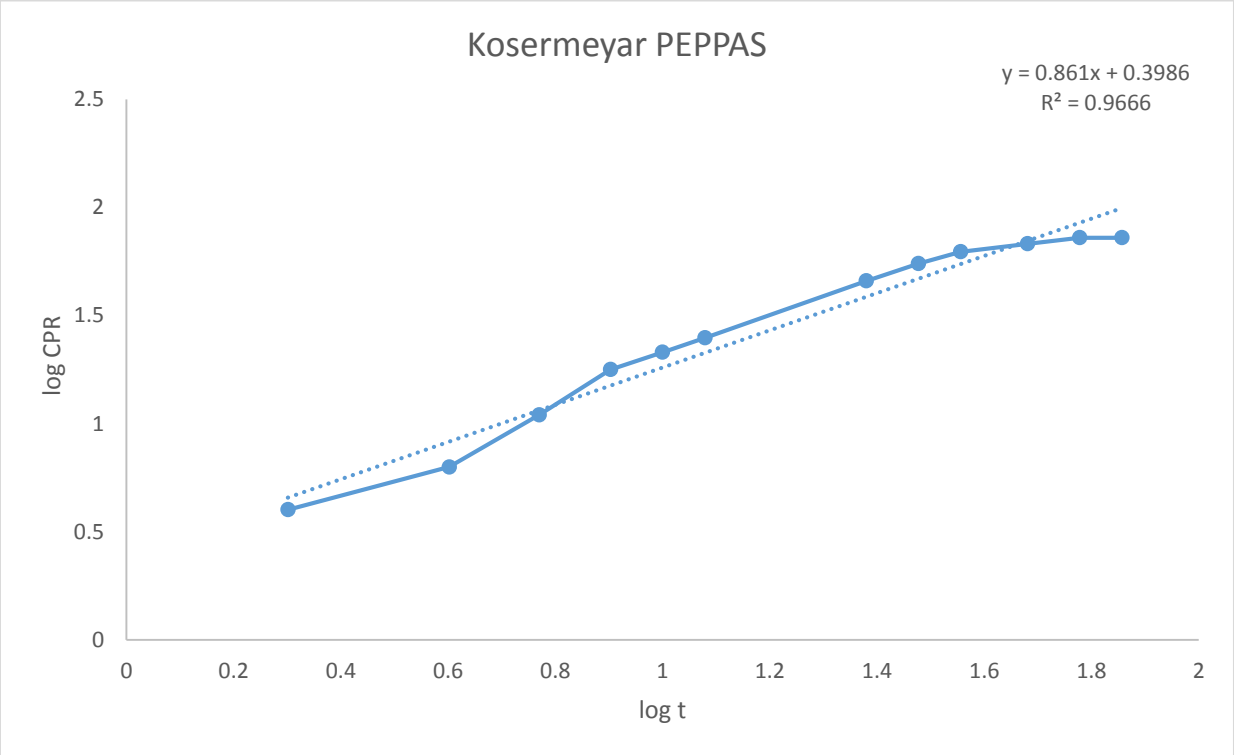


Fig 5.7.8 In vitro release of Amikacin sulfate from NP C as first Kosermeyar-Peppas model

Fig 5.7.9 In vitro release of Amikacin sulfate from NP E as zero order model

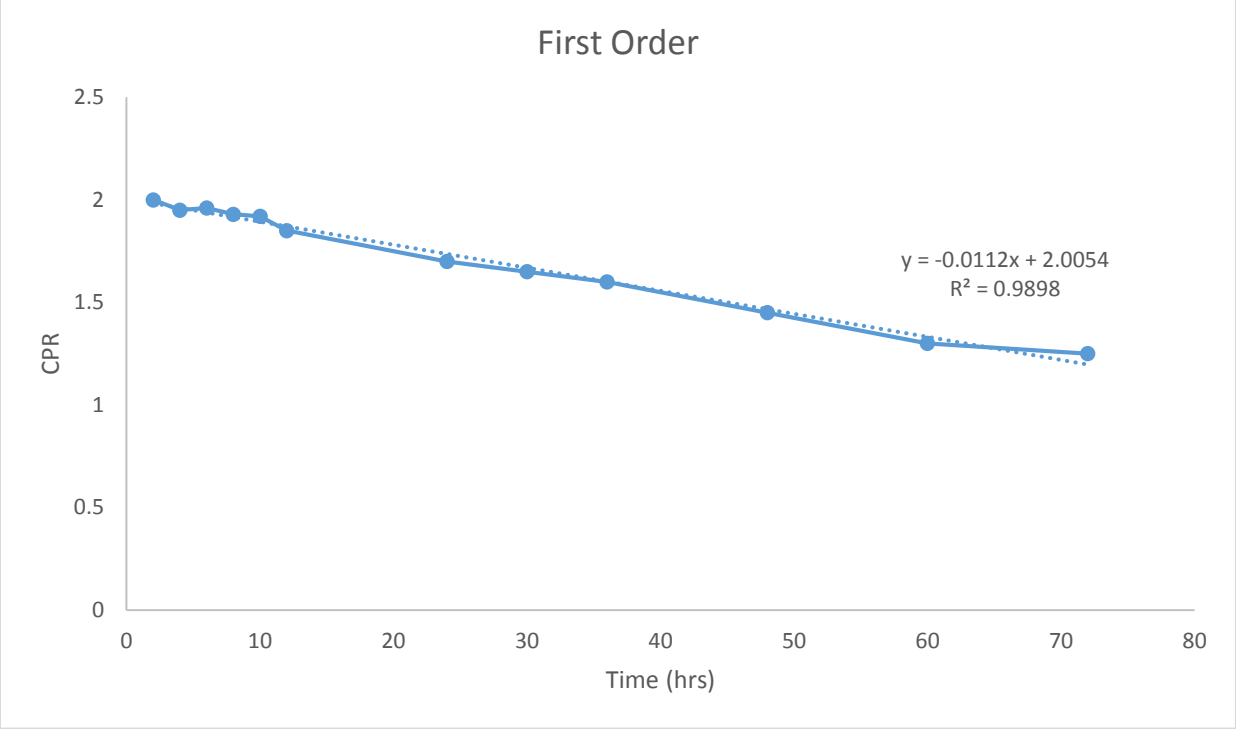


Fig 5.7.10 In vitro release of Amikacin sulfate from NP E as first order model

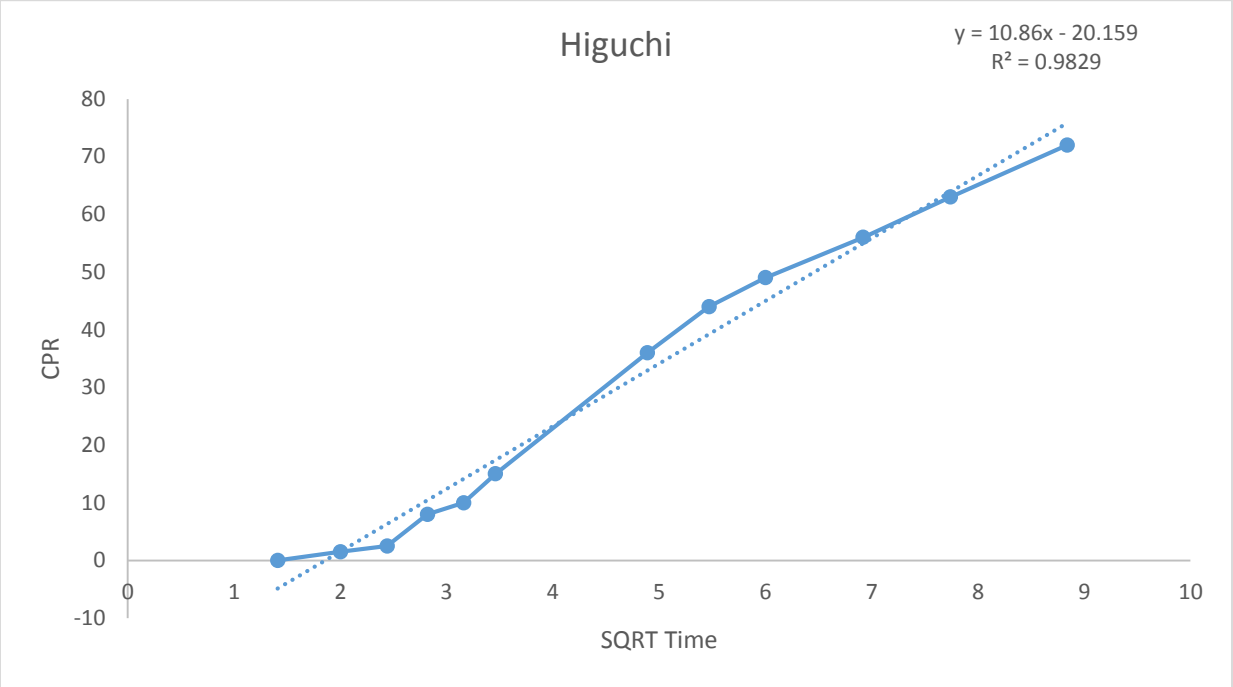


Fig 5.7.11 In vitro release of Amikacin sulfate from NP E as first Higuchi model

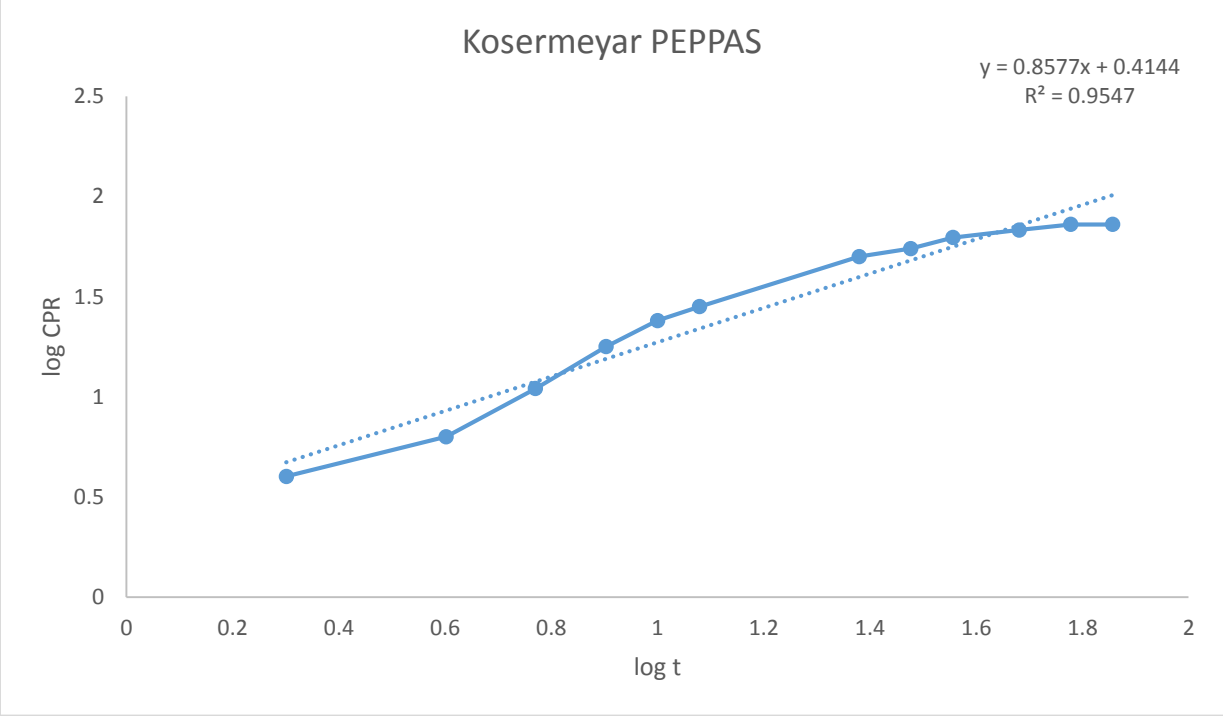


Fig 5.7.12 In vitro release of Amikacin sulfate from NP E as first Kosermeyar-Peppas model

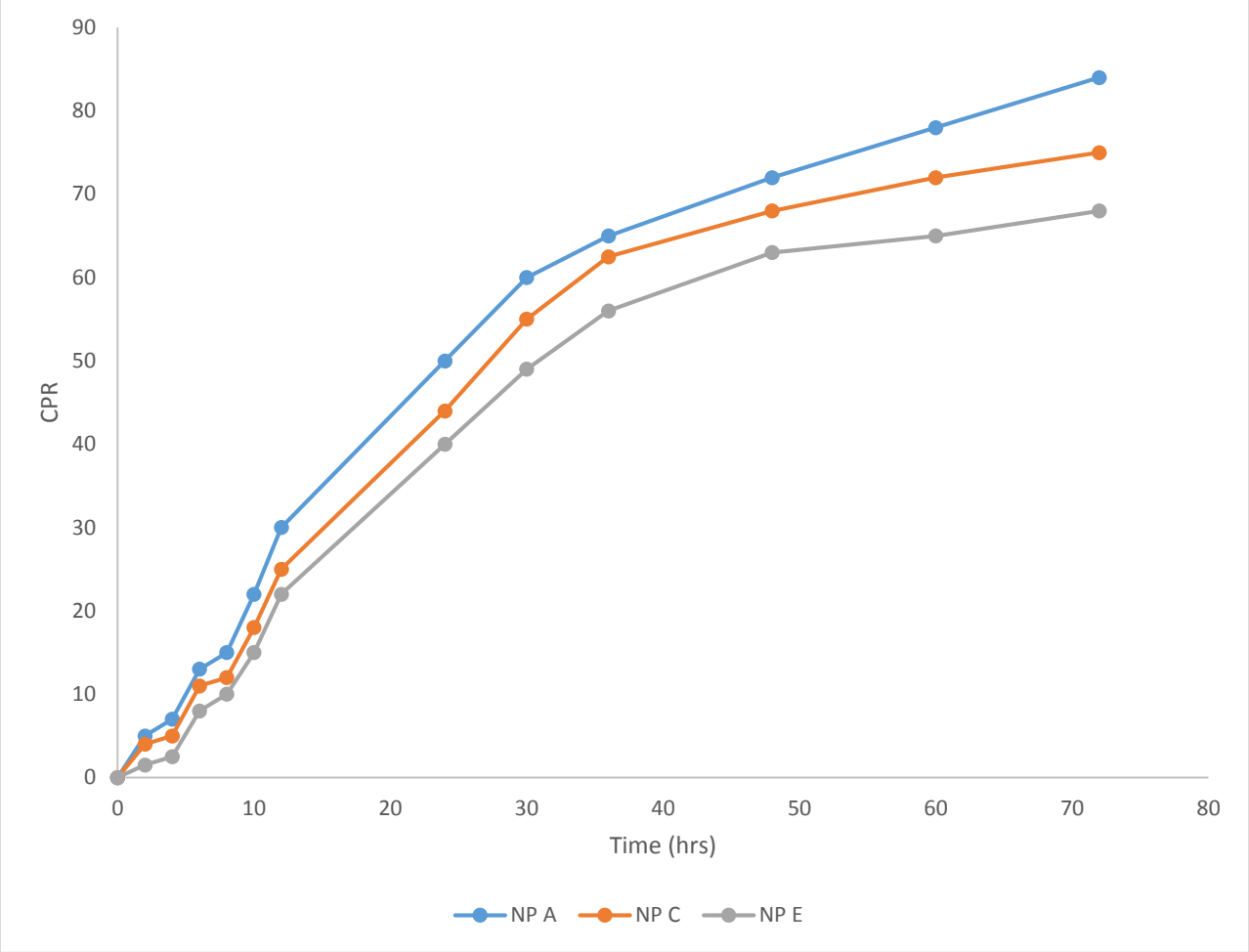


Fig. 5.7.13 Comparative release study of Amikacin nanoparticles NP A, NP C and NP E

5.8 Scanning Electron Microscopy of Amikacin Sulfate Nanoparticles:

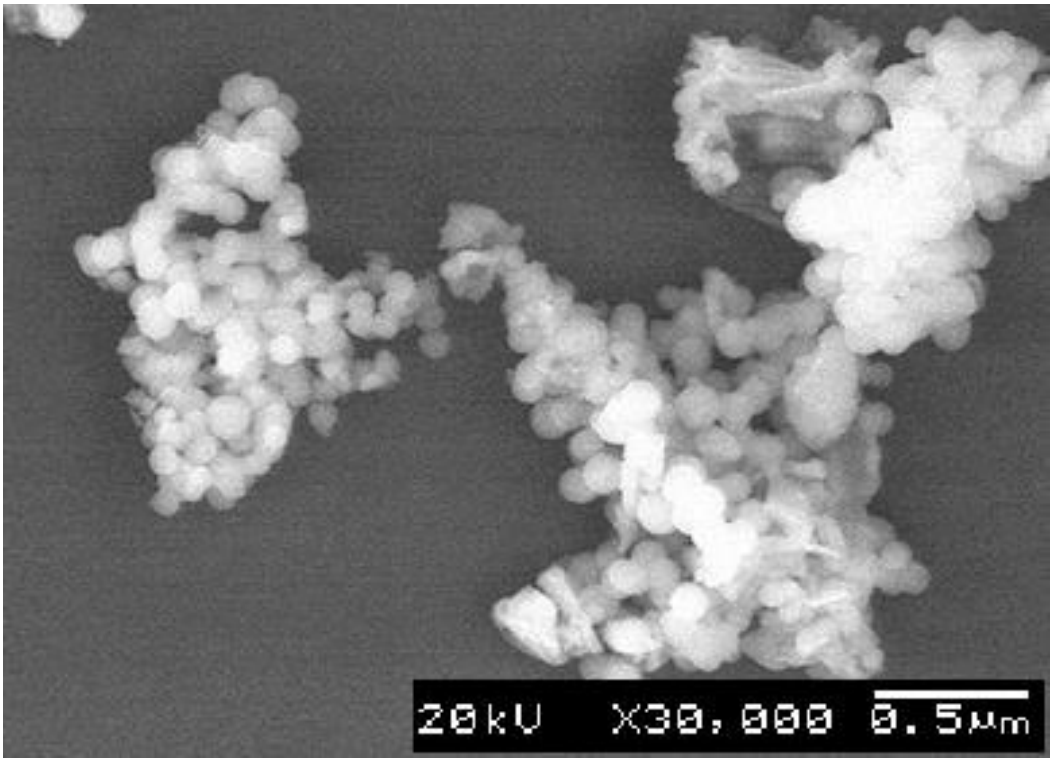


Fig. 5.8.1 Scanning Electron Micrograph of Amikacin Nanoparticles

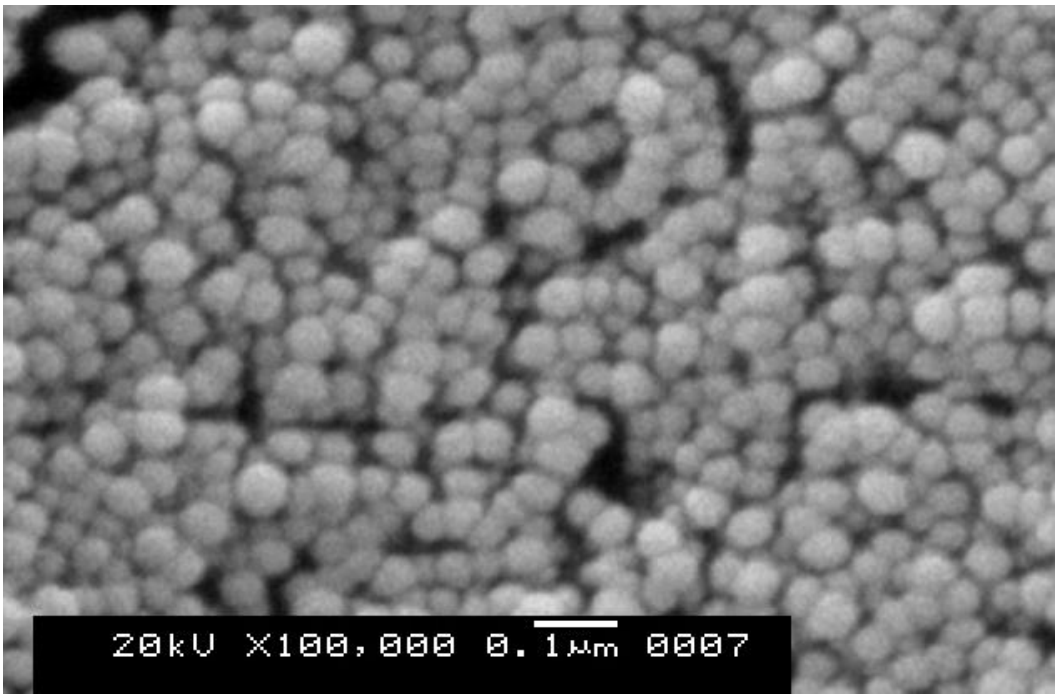


Fig. 5,8.2 Scanning Electron Micrograph of Amikacin Nanoparticles

5.9 Minimum Inhibitory Concentration (MIC) of Amikacin Sulfate Nanoparticles:

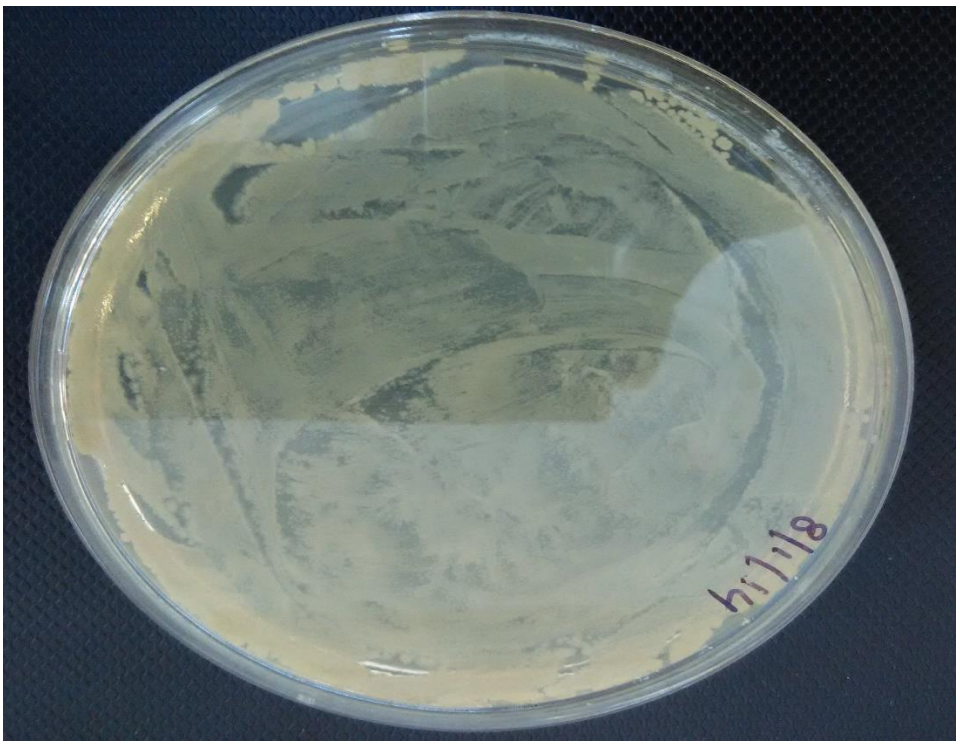


Fig. 5.9.1 Nutrient Agar plate after treating with blank nanoparticles

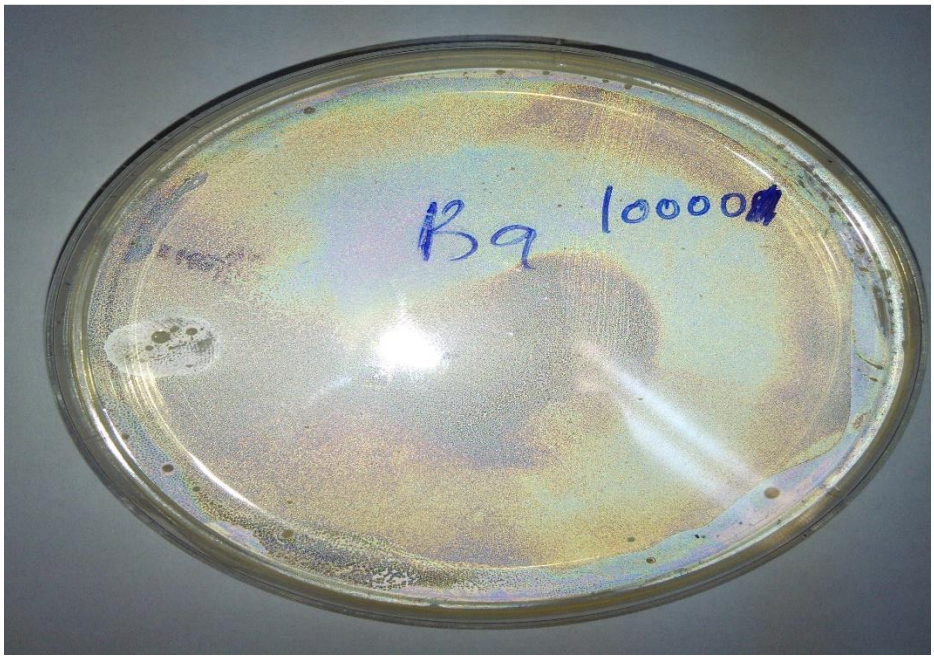


Fig. 5.9.2 Nutrient Agar plate after treating with Amikacin loaded nanoparticles

RESULTS & DISCUSSIONS

6. Results and Discussions

6.1. Identification of Drug:

6.1.1. Fourier Transform Infrared Spectroscopy:

The purchased sample of Amikacin Sulphate was identified by Fourier Transform Infrared Spectroscopy and was compared with standard FTIR of the drug given in the official monograph. The spectra was similar as shown in the **Fig 5.1** confirming the identity of the sample.

6.2. Determination of λ_{max} of Amikacin Sulphate:

The λ_{max} of the drug sample was determined indirectly by the HPLC method. Drug sample was dissolved in the mobile phase and injected in the HPLC system, running on the parameters as mentioned in the Method section. The λ_{max} was then determined by the LC Solution software of the HPLC in the post run analysis at 2.3 min as shown in **Fig.2a and Fig 5.2b.** The absorption maxima was found to be 200nm. Hence confirming the identity of Amikacin sulphate at 200nm.

6.3. Preparation of the Calibration curve of Amikcain Sulphate:

The calibration curve of Amikacin sulphate was obtained by plotting the concentrations in µg/ml of Amikacin against their respective AUC values in mAU as shown in **Fig. 5.3.** The regression equation was calculated and was used for the quantitative estimation of Amikacin for drug loading. The correlation factor (R^2 value) was found to be 0.999. This shows the linearity of the calibration curve.

6.4. Determination of particle size and zeta potential of Amikacin loaded nanoparticles:

Different batches of Amikacin loaded nanoparticles were prepared coded as NP A, NP B, NP C, NP D, NP E and NP F by varying the concentrations of chitosan, sodium

tripolyphosphate and the drug. The mean particle size of the formulations were between 210 ± 0.8nm to 342 ± 0.4nm.

The zeta potential of Amikacin loaded chitosan nanoparticles were in the range of +13.8 mV to +19.1 mV. These value of zeta potential indicates that the particles does not agglomerates.

6.5. Determination of Entrapment efficiency of Amikacin loaded chitosan nanoparticles:

Entrapment efficiency was calculated indirectly using clear supernatant obtained after centrifugation of nanoparticles and analyzing it by using HPLC. Entrapment efficiency of Amikacin in the chitosan nanoparticles were calculated using regression equation given in **Fig 5.3**. The entrapment efficiency for the prepared batches were found in the range of 35.2% to 70.3% as shown in the **Table 5.4.** This indicates the high loading capacity of amikacin in chitosan-TPP nanoparticles. Formulations NP A, NP C and NP E showed highest entrapment and those were used for release study.

6.6. Effect of polymer (chitosan) on entrapment efficiency:

Entrapment efficiency of Amikacin increases with increasing chitosan ratio to the cross linking agent i.e. sodium tripolyphosphate. Maximum entrapment was seen when the polymer to cross linking agent ratio was 2:1, further increasing the chitosan ratio did not show any significant increase in entrapment of drug. Thus the nanoparticles so prepared were optimized.

6.7 Effect of polymer (chitosan) on Zeta potential:

Zeta potential of the Amikacin loaded nanoparticles showed increased zeta potential with increasing chitosan to cross linking agent ratio. Maximum zeta potential was seen when the ratio of chitosan to sodium tripolyphosphate was 4:1 as shown in the **Fig. 5.4.3.**

6.8 Effect of polymer (chitosan) on particle size:

Particle size of the amikacin loaded nanoparticles shows increasing pattern with increase in ratio of chitosan to cross linking agent as shown in **Fig 5.4.4.** Blank formulation having no drug showed the lowest particle size.

6.9 Effect of drug release mechanism by various kinetic models:

The release pattern **Fig. 5.7.1 – Fig 5.7.13** of the prepared Amikacin loaded Chitosan nanoparticles showed a rapid initial release of the drug for the first 12 hours releasing up to 30 % of Amikacin. After 30 hours up to 60 % of the drugs were released. The formulations then showed a sustained release of drug after 30 hours.

Various kinetic model were employed to investigate drug release mechanism of the formulations using in vitro dissolution data. The in vitro release data were fitted to models representing zero-order, first-order and Higuchi's square root of time to determine the correlation coefficient, slope and intercept values. From the values of correlation coefficients, the best fitted data can be predicted. The curve fitting of the release data was carried out mainly by regression analysis. In spherical matrices, if $n \leq 0.43$, a Fickian (case-I), $0.43 \leq 0.85$, a non Fickian and if $n \geq 0.85$, a case-II (zero order) drug release mechanism model dominates. The maximum correlation coefficient has been considered as statistical parameter to designate the function with the best fit to the data.

The examination of correlation coefficient values indicated that the drug release followed the diffusion control mechanism from the Amikacin loaded chitosan nanoparticles. The data are supportive to the findings that Amikacin Sulphate (water soluble) incorporated in the Chitosan mainly released by the diffusional mechanism.

A more stringent i.e Korsmeyer-Peppas model test was applied to distinguish between the mechanisms of drug release:

$Q(t) = at^n$

Where Q (t) is a fraction of drug released after time‘t’, ‘a’ is the coefficient constant and ‘n’ is the release exponent. The values for ‘n’ was, further indicating the release following a diffusion control mechanism.

6.10 Fourier Transform Infrared Spectroscopy (FTIR):

FTIR spectral analysis was carried out in order to find out any possible chemical interactions between the drug and the polymer. The positions and the relative intensities of the absorption bands of the Amikacin loaded nanoparticles as well as that of the blank were compared with the pure Amikacin Sulfate as shown in the **Fig. 5.5.1 – Fig. 5.5.3.** The N–H bending vibration of primary aromatic amines exhibits peaks at the 1,650–1,540 cm−1 range of the spectra. The peak at 1641 cm−1 in both spectra of drug amikacin sulphate and amikacin sulphate-loaded chitosan nanoparticles formulation depicts that –NH2 group of amikacin sulphate is untreated, which represents the absence of interaction between drug and polymer. Peak at 3250 cm^{-1} represents untreated “free” hydroxyl groups of amikacin sulphate. Peaks were noticed in the mid of 3500–3000 cm^{-1} in FTIR spectra of both drug amikacin sulphate and amikacin-loaded chitosan nanoparticles. Their FTIR spectroscopy thus indicates that there are no considerable interactions among the drug amikacin sulphate and the polymer-chitosan.

6.11 Differential Scanning Calorimetry:

DSC was performed to characterize thermal changes in the melting behavior of the drug with other excipients present in the formulations. **Fig. 5.6.1, 5.6.2 and 5.6.3** depicts the thermograms of heat verses temperature for amikacin sulfate, amikacin loaded nanoparticles and blank nanoparticles respectively. The DSC thermogram showed that there were no major differences in the onset temperature and peak temperature of

amikacin nanoparticles, when compared with pure drug thermogram. Hence, it was confirmed that there were no incompatibility between drug and polymer.

6.12 Scanning Electron Microscopy (SEM):

For determining the surface morphology of the amikacin loaded nanoparticles scanning electron microscopy was performed on the formulation NP A as it showed the best entrapment efficiency and release among all formulations. JEOL JSM 6360LV Scanning Electron Microscope image of the amikacin loaded nanoparticle revealed that the particles were spherical in shape having smooth and non-porous surface, as shown in the **Fig 5.8.1 – 5.8.2.**

6.13 Antimicrobial Assay:

Three agar plates which were treated with amikacin loaded nanoparticles, standard ciprofloxacin and blank nanoparticles after incubation at 37°C were examined for the number of colonies formed in each plates. A vertical and a horizontal line were drawn in the agar plates divided them into four equal sections. The bacterial colony in each section were counted visually in each plates. The colony formation was seen maximum in blank nanoparticles averaging around 55 colony forming units (CFU). Amikacin loaded nanoparticles showed average 22 CFU. The standard ciprofloxacin showed 15 CFU. It was concluded from the results of colony forming units that Amikacin loaded nanoparticles showed promising antimicrobial action against *E.coli.*

CONCLUSION

7. CONCLUSION

In the present research work, chitosan-sodium tripolyphosphate nanoparticles incorporating drug Amikacin Sulphate was successfully prepared by Ionic Gelation method using chitosan as polymer. The method of preparation was found to be simple and economical.

The mean particle size of the formulations were between 210 ± 0.8nm to 342 ± 0.4nm with polydisperity index in the range of 0.137 to 0.315 depending on the concentration of the cross linking agent.

Among all the prepared formulations NP A, NP C and NP E showed highest entrapment efficiency. The release pattern by in-vitro studies showed an initial rapid release in 10 hours. Then the release was sustained for up to 72 hours.

Kinetic modelling studies of the release behavior showed non-Fickian diffusion of the drug release from the nanoparticles.

The results of this research work conferred potentials of Amikacin sulphate loaded chitosan-TPP nanoparticles against bacterial biofilm and could be viewed as a potential alternative to the conventional antibiotic drug delivery systems. The sustained release of the drug from the nanoparticles would be effective for reducing the dosage and the dosing frequency. As chitosan is biodegradable it is expected that it would not cause any deleterious effect or toxic response inside the body.

It appears that the amikacin loaded chitosan nanoparticles offers a promising delivery system for the enhancement of anti-microbial activity of the drug against bacterial biofilm formed inside the medical devices.

FUTURE SCOPE

8. FUTURE SCOPE

Delivering therapeutic compound to the target site is a major problem in treatment of many diseases. A conventional application of drugs is characterized by limited effectiveness, poor bio distribution, and lack of selectivity.

Nanoparticles have a great potential as drug carriers. Due to their small sizes, the nanoparticles exhibit unique physicochemical and biological properties (enhanced reactive area as well as ability to cross cell and tissue barriers) that make them a favorable material for biomedical applications.

Biofilms have been found to be involved in a wide variety of microbial infections in the body, by one estimate 80% of all infections. Infectious processes in which biofilms have been implicated include common problems such as bacterial urinary tract infections, catheter infections, infections of permanent indwelling devices such as joint prostheses, heart valves, middle-ear infections, coating contact lenses, formation of dental plaque and gingivitis.

Amikacin loaded chitosan-TPP nanoparticles designed and optimized in this research work is aimed to combat those biofilms. The optimized nanoparticles has shown promising *in vitro* anti-microbial results. *In vivo* studies of the prepared nanoparticles may show promising results in future studies and research.

REFERENCES

References:

1. Graud S, Singh D, Solid Lipid Nanoparticles: Method, Characterization and Applications, Intl. Curr Pharm J. 2012;1(11):384-93.

2. Hao J, Fang X, Development and optimization of solid lipid nanoparticles. Int J Nanomedicine. 2011;6:683-92.

3. Bjarnsholt T, Kobenhavns University, Kobenhavn N, The role of bacterial biofilms in chronic infections, Denmark. May 2013.

4. Ae Jung Huha, b, Young Jik Kwona, Nanoantibiotics: A new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era, Journal of Control Release, 2011 Dec10;156(2):128-45.

5. Vyas S.P., Khar R.K. Targeted & Controlled Drug Delivery, Novel Carrier Systems, CBS Publication, 2002, Page No.249-277,331-387.

6. Mu H, Holm R, Lipid based formulations for oral administration of poorly water soluble drugs, Int J Pharm.2013;453:215-34.

7. Loxley A, Solid lipid nanoparticles for Delivery of Pharmaceutical actives, Drug Delivery Technology; 2009.

8. VJ Mohanraj & Chen Y, Nanoparticles - A Review, Tropical Journal of Pharmaceutical Research 2006; 5(1): 561-573.

9. Jain N. K., Controlled and novel Drug Delivery, 1st edition 2001, CBS Publication; 292-301.

10. Ana Grenha, Chitosan nanoparticles: A survey of preparation methods, Journal of Drug Target, 2012 May; 20(4):291-300.

11. Desai MP, Labhasetwar V, Walter E, Levy RJ, Amidon G L, The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent. Pharm Res 1997; 14: 1568-73.

12. Swarbrick J, Boylan J. Encyclopedia of pharmaceutical technology. 2nd ed.; Marcel Dekker: New York, 2002.

13. Couvreur P, Barratt G, Fattal E, Legrand P, Vauthier C. Nanocapsule technology: A review. Drug Carrier Syst 2002; 19: 99-134.

14. Govender T, Stolnik S, Garnett MC, Illum L, Davis SS. PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug. J. Control. Rel. 1999; 57: 171-185.

15. Calvo P, Remunan-Lopez C, Vila-Jato JL, Alonso MJ. Novel hydrophilic chitosan-polyethylene oxide nanoprticles as protein carriers. J. Appl. Polymer Sci. 1997; 63: 125-132.

16. Panyam J, Williams D, Dash A, Leslie-Pelecky D, Labhasetwar V. Solid-state solubility influences encapsulation and release of hydrophobic drugsfrom PLGA/PLA nanoparticles. J Pharm Sci 2004; 93: 1804-14.

17. Magenheim B, Levy MY, Benita S. A new in vitro technique for the evaluation of drug release profile from colloidal carriers - ultrafiltration technique at low pressure. Int. J. Pharm. 1993; 94: 115-123.

18. Biofilms: Microbial Life on Surfaces, Rodney M. Donlan, 2002 Sep; 8(9): 881–890.Donlan, Rodney M. 2002. Biofilms: Microbial Life on Surfaces. Emerging Infectious Diseases. Vol. 8, No. 9: pg. 881-890.

19. Staudt C, Horn H, Hempel DC, Neu TR (2004). "Volumetric measurements of bacterial cells and extracellular polymeric substance glycoconjugates in biofilms". Biotechnol. Bioeng. 88 (5): 585–92.

20. Donlan RM, Costerton JW, "Biofilms: survival mechanisms of clinically relevant microorganisms". Clin. Microbiol. Rev. 15 (2002). (2): 167–93

21. Donlan RM. "Biofilms: microbial life on surfaces". Emerging Infect. Dis. (2002) 8 (9): 881–90.

22. Donlan RM, Costerton JW (2002). "Biofilms: survival mechanisms of clinically relevant microorganisms". Clin. Microbiol. Rev. 15 (2): 167–93

23. Suresh and Mody, "Microbial Exopolysaccharides: Variety and Potential Applications". Microbial Production of Biopolymers and Polymer Precursors. (2009)

24. Kaplan JB, Ragunath C, Ramasubbu N, Fine DH "Detachment of Actinobacillus actinomycetemcomitans biofilm cells by an endogenous beta-hexosaminidase activity (August 2003).

25. Chua SL, Liu Y, Yam JKH, Tolker-Nielsen T, Kjelleberg S, Givskov M, Yang L "Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles". Nature Communications, (2014).

26. Nadell, Carey D.; Xavier, Joao B.; Foster, Kevin R. "The sociobiology of biofilms". FEMS Microbiology Reviews, (January 2009).

27. Stoodley, Paul; Dirk deBeer andZbigniew Lewandowski, "Liquid Flow in Biofilm Systems". Appl Environ Microbiol.(August 1994).

28. Stewart PS, Costerton JW, "Antibiotic resistance of bacteria in biofilms".Lancet, (July 2001).

29. Molin S, Tolker-Nielsen T, "Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure". Current Opinion in Biotechnology, (June 2003).

30. Jakubovics NS, Shields RC, Rajarajan N, Burgess JG, "Life after death: the critical role of extracellular DNA in microbial biofilms"Spoering AL, Lewis K (December 2001). "Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials" (December 2013).

31. Nadell, Carey D.; Xavier, Joao B. "The sociobiology of biofilms". FEMS Microbiology Review, (1 January 2009).

32. M-P Mingeot-Leclercq, Y Glupczynski & PM Tulkens, Aminoglycosides: Activity and Resistance, Antimicrob. Agents Chemother.1999, 43(4) 727-737.

33. Barraud N, Storey MV, Moore ZP, Webb JS, Rice SA, Kjelleberg S, "Nitric oxide-mediated dispersal in single- and multi-species biofilms of clinically and industrially relevant microorganisms". Microbial Biotechnology, (2009).

34. Allahverdiyev AM1, Kon KV, Abamor ES, Bagirova M, Rafailovich M. Coping with antibiotic resistance: combining nanoparticles with antibiotics and other antimicrobial agents. Encyclopedia of controlled drug delivery system edited by Edith Mathiowitz, Pg: 551-564.

35. Li-Ming Zhao1 , Lu-E Shi , Zhi-Liang Zhang , Jian-Min Chen , Dong-Dong Shi , Jie Yang and Zhen-Xing Tang, PREPARATION AND APPLICATION OF CHITOSAN NANOPARTICLES AND NANOFIBERS, Brazilian Journal of Chemical Engineering, July - September, 2011, Vol. 28, No. 03, pp. 353 - 362.

36. Quan Gan, Tao Wang, Colette Cochrane, Paul McCarron, Modulation of surface charge, particle size and morphological properties of chitosan–TPP nanoparticles intended for gene delivery, Colloids and Surfaces B: Biointerfaces, Volume 44, Issues 2–3, August 2005, Pages 65–73.

37. Hui Liu, Changyou Gao, Preparation and properties of ionically cross-linked chitosan nanoparticles, Wiley InterScience, November 2008. DOI: 10.1002/pat.1306.

38. L. Zhang, D. Pornpattananangku, C.-M.J. H, C.-M. Huang, Development of Nanoparticles for Antimicrobial Drug Delivery, Current Medicinal Chemistry, 2010, pg585-594.

39. Zhilong Shi, K.G. Neoh, E.T. Kang, W. Wang, Antibacterial and mechanical properties of bone cement impregnated with chitosan nanoparticles, Biomaterials, Volume 27, Issue 11, April 2006, Pages 2440–2449.

40. Lifeng Qi, , Zirong Xu, Xia Jiang, Caihong Hu, Xiangfei Zou, Preparation and antibacterial activity of chitosan nanoparticles, Carbohydrate Research, Volume 339, Issue 16, 15 November 2004, Pages 2693–2700.

41. S. Wazed Ali, S. Rajendran, Mangala Joshi, Synthesis and characterization of chitosan and silver loaded chitosan nanoparticles for bioactive polyester, Carbohydrate Polymers, Volume 83, Issue 2, 10 January 2011, Pages 438–446.

42. Y. Xu, Y. Du Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles, Int. J. Pharm., 250 (2003), pp. 215–226.

43. J.A. Ko, H.J. Park, S.J. Hwang, J.B. Park, J.S. Lee,Preparation and characterization of chitosan microparticles intended for controlled drug delivery, Int. J. Pharm., 249 (2002), pp. 165–174.

44. A.N. Hernandez-Lauzardo, S. Bautista-Banos, M.G. Velazquez-del Valle, M.G. Mendez-Montealvo, M.M. Sanchez-Rivera, L.A. Bello-Perez, Antifungal effects of chitosan with different molecular weights on in vitro development of Rhizopus stolonifer Vuill, Carbohydrate Polymers, 73 (2008), pp. 541–547.

45. Y.L. Chen, C.C. Chou,Factors affecting the susceptibility of Staphylococcus aureus CCRC 12657 to water soluble lactose chitosan derivative, Food Microbiology, 2005 (22) (2005), pp. 29–35.

46. M. Kong, X.G. Chen, C.S. Liu, L.J. Yu, Q.X. Ji, Y.P. Xue, D.S. Cha, H.J. Park,Preparation and antibacterial activity of chitosan microspheres in a solid dispersing system, Frontiers of Materials Science in China, 2 (2008), pp. 214–220.

47. T. Takahashia, M. Imaia, I. Suzukia, J. Sawai, Growth inhibitory effect on bacteria of chitosan membranes regulated by the deacetylation degree, Biochemical Engineering Journal, 40 (2008), pp. 485–491.

48. D. Raafat, K.V. Bargen, A. Haas, H.G. Sahl, Insights into the mode of action of chitosan as an antibacterial compound, Applied and Environmental Microbiology, 74 (2008), pp. 3764–3773.

49. S. Hancock, N. Mozes, P.S. Handley, H.J. Busscher, P.G. Rouxhet (Eds.), Cell surface analysis, VCH Publishers, Weinheim (1991), p. 23.

50. Luis E. Chávez de Paz, Anton Resin, Kenneth A. Howard, Duncan S. Sutherland and Peter L. Wejse, Antimicrobial Effect of Chitosan Nanoparticles on Streptococcus mutans Biofilms, Applied and Environmental Microbiology, June 2011 vol. 77 no. 11 3892-3895.

51. Gilbert D. Aminoglycosides. In: Mandell GL, Bennett JE, Dolin R, eds. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 5th ed. Philadelphia: Churchill Livingstone, 2000:307-336.

52. Gautam Mehta and Bilal Iqbal. Clinical Medicine for the MRCP PACES. Volume 1. Core Clinical Skills. Oxford University Press. 2010.

53. Bindu LH, Reddy PP. Genetics of aminoglycoside-induced and prelingual non-syndromic mitochondrial hearing impairment: A review. Int J Audiol. 2008