IMMOBILIZATION OF ANTIMICROBIAL PEPTIDES ON SILVER NANOPARTICLES

A DISSERTATION

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By

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Candidate Declaration

I hereby certify that the work presented in dissertation entitled, "**Immobilization of antimicrobial peptides on silver nanoparticles**" has been carried out by me during the period from August 2009 to June 2010 under the supervision of Dr. Naveen Kumar Navani, Assistant Professor, Department of Biotechnology and Dr. Jeevanandam. P, Department of Chemistry, Indian Institute of Technology-Roorkee.

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And everything has initiated, almost by chance, during a visit to Nanotechnology Group at Centre of Nanotechnology, IIT Roorkee.

Life can provide us with a lot of chances (and challenges), but good luck is just a starting point. Thus, what at the end will make us to reach the goals are our strength values and certainly, all the cooperation and support from those people that once we had the chance to know.

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Table of Contents

Abbreviations

E.coli----------*Escherichia coli*

SEM----------Scanning Electron Microscopy

AFM----------Atomic Force Microscopy

AAS----------Atomic Absorption Spectroscopy

FT-IR---------- Fourier Transformation Infrared Spectroscopy

LB broth---------- Luria-Bertani Medium

MRS broth---------- deMan, Rogosa and Sharpe medium

PBS---------- Polymyxin B Sulpate

Abstract

The aim behind this project is to investigate the synergistic action of a food grade antimicrobial peptide with silver nanoparticles. The goal is to develop double edged weapons against food spoilage and pathogenic bacteria.

Silver nanoparticles were synthesised by Turkevich Method. Characterisation of the samples was performed, using SEM, AFM, Zeta seizer, AAS, FTIR and UV-Visible spectroscopy. Silver nanoparticles and nisin in combination were subjected to inhibition testing on various bacteria *viz* such as *E.coli*, *Bacillus sp*, *Salmonella* sp. It was observed that the nisin in combination with silver nanoparticles show enhanced antimicrobial activity against both Gram-positive and Gram-negative bacteria. They also exhibited lower minimum inhibitory concentration compared to either nisin or silver nanoparticles alone. Once refined, this idea can find wide variety of applications in medicine and pharmacological studies.

The other approach in this project was synthesis of silver nanoparticles using Polymyxin B sulphate. In this silver nitrate and the polymyxin B sulphate were mixed in methanol in defined concentrations and kept under the visible light for an hour. The light induced reduction of silver nitrate and thus formed silver nanoparticles were capped with polymyxin B sulphate thus confining the size of the silver nanoparticles. The silver nanoparticles thus obtained were characterized by UV-Visible spectroscopy, AFM, SEM, FTIR, Zeta-seizer and AAS. It was observed that the compounds exhibited antimicrobial activity. These silver nanoparticles showed a broad spectrum activity on both Gram-positive as well as Gram-negative bacteria.

1. Introduction

1.1 Advancements in the use of nanoparticles as antimicrobials

In recent years, there has been a growing interest and investment in conducting research on nanomaterials, tiny substances having at least one dimension within the nanometer scale, including nanoparticles, nanowires and nanofilms.[1] At the nanometer scale, these materials exhibit vast variety of different physical and chemical properties compared to their bulk materials: For *eg*: crystals in the nanometer scale have much lower melting points, ferromagnetic materials may lose their ferromagnetism when diminished to nanometer size, semiconductors act as insulators and vice versa, solids become liquids at room temperature and pressure, and inert chemicals like gold act as excellent catalysts. In addition to these, certain metal nanoparticles show anti-bacterial properties. Nanomaterials have such amazing properties because of their high proportion of surface atoms, in comparison to bulk materials. There are more atoms at the surface with unsaturated bonds, capable of taking part in interactions with biomolecules.

The antibacterial properties of nanoparticles are of interest because of their obvious potential applications. Some metal-based nanoparticles, particularly silver nanoparticles, exhibit excellent bacteriocidal and bacteriostatic properties.[2,3] It is well known that silver ions and silver-based compounds are highly toxic to as many as 12 species of bacteria, including *E. Coli.*[4] According to Kim et al.,[5] the mechanism of the inhibitory effects of silver (Ag) ions on microorganisms is only partially known. Several studies[6,7,8] have reported that the positive charge on the Ag cation plays a key role in its antimicrobial activity via electrostatic attraction between the negatively charged cell membrane of microorganism and positively charged nanoparticles. In other studies investigating the killing mechanisms of silver nanoparticles, the antimicrobial activity of silver nanoparticles on Gram-negative bacteria was correlated to the concentration of Ag nanoparticles, which governs the formation of pits in the bacterial cell wall, allowing the accumulation of silver nanoparticles to affect the membrane's permeability, resulting in cell death[9]. A report by Lok et al.,[7] states that silver nanoparticles have been found to destabilize the bacterial outer membrane and deplete the levels of intracellular ATP. Several recent publications postulate that silver nanoparticles may adhere to the surface of the cell membrane, thereby disrupting cellular functions such as

permeability and respiration. Silver Nanoparticles may cause damage, after penetration, by interacting with phosphorus- and sulphur-containing compounds, including DNA, for silver tends to have a high affinity to react with such compounds.[5]

Given the high toxicity of silver nanoparticles on bacteria, there are extensive biological, biomedical, and pharmaceutical applications[9] of nanoparticles exhibiting antibacterial properties, including widespread products where bacterial growth should be inhibited. The antibacterial activity of the nanoparticles may be used in medicine to reduce infections in burn treatment, arthroplasty, to prevent bacteria colonization on prostheses, catheters, vascular grafts, dental materials and dental resins, as well as integration into textile fabrics, or even for water treatment.[8] In addition, they can be incorporated into domestic and car air-conditioner filters, floor drain traps, shoe and insole lining, bandages or plasters, refrigerators, storage containers, and antibacterial soaps, detergents or washing liquids.

1.2 History behind antibiotics and antibacterial peptides

The search for antibiotics began in the late 1800s, with the growing acceptance of the germ theory of disease, a theory which linked bacteria and other microbes to variety of ailments. As a result, scientists began to devote time to search for drugs that would kill these infection causing bacteria. The goal of such research was to find so-called "magic bullets" that would destroy microbes without toxicity to the person taking the drug. The fact that a microorganism is capable of destroying one of another species was not established until the latter half of the 19th century when Louis Pasteur noted the antagonistic effect of other bacteria on the anthrax organism and pointed out that this action might be put to therapeutic use. It was in 1929 that Alexander Fleming discovered Penicillin, the miracle drug, derivatives of which are still in wide use. The tide began to change thereafter with the discovery of numerous antimicrobial drugs.

Antibiotics revolutionized medical care in the 20th century, but in recent years microbes have been winning the battle against the medical profession. Around the world, bacteria are mutating to defend themselves and are becoming resistant against drugs that would once have killed them. This is not unexpected as organisms constantly evolve to find ways to adapt to new circumstances. But the speed with which some strains are developing resistance is threatening the global health. The inevitable rise in the resistance has eventually eroded the utility of today's antibiotics and increased the perpetual need for new antibiotics. New

families of anti-infective agents are needed to enter the market place at regular intervals to tackle the emergence of multidrug resistance and the new diseases caused by evolving pathogens.

However, the pipeline of new antibiotics is drying up. The unfavorable economics and the long time it takes for an antimicrobial drug to reach from an initial discovery phase to the market place has made a chilling effect on the industrial discovery programs. Experts estimate that research and development in antibiotics has fallen by about 60 % in the past decade.

Historically, natural products and their derivatives have been invaluable as a source of antimicrobial drugs. Chemical substances derived from animals, plants and microbes have been used to treat human diseases since the dawn of medicine. Natural products are widely recognized in the pharmaceutical industry for their broad structural diversity as well as their wide range of pharmacological activities.[10] Owing to technical improvements in screening programs, separation and isolation techniques, approximately 20-25% of the reported natural products show biological activity, and of these, approximately 10 percent have been obtained from microbes.[11] The versatility of microbial drugs is gigantic. The microbes keep on producing novel metabolites as they move into the diverse ecological units. Soil is an intensively exploited ecological niche from which many useful natural products are derived, including clinically important antibiotics such as tetracycline, erythromycin, vancomycin etc.[12] From the microbes that have been used so far to obtain the biologically active compounds, actinomycetes and fungi in particular are the sources for the majority of commercially exploited natural products while other bacteria, on the other hand, more commonly, but not exclusively, produce peptide antibiotics like polymyxins and lantibiotics.[13] Among the antibiotic arsenal obtained from microbes so far, there 166 antibiotics and derivatives such as the lactam peptide antibiotics, the macrolides, tetracyclines, aminoglycosides, daptomycin, and others. Thus, microbes have remained as consistent sources for new antimicrobial agents overcoming new snags and challenges. As only little of the world's biodiversity has been tested for biological activity, many more useful natural lead compounds are awaiting discovery. The challenge is how to access this natural chemical diversity.

Though the number of antibiotics in nature is really huge, most of them are already known or useless (not specific for bacteria, toxic, too weak, lacking the desired pharmacokinetic

properties, etc.). This hyper abundance of antibiotics in nature implies the most crucial challenge faced by antibiotic discovery programs based on natural products i.e. rediscovery of the already known antibiotic scaffolds. Success in discovering new antibiotics from microbial natural products requires efforts to expand the range of bacteria that can be tapped for antibiotic research. This be facilitated by various strategies including exploring the little explored ecological niches or expanding conventional culturing approaches.

In these days, a lots of effort are being made to develop new compositions of nanomaterials coupled to antibacterial peptides keeping an eye on the ever increasing of resistance by bacteria to the antibacterial peptides and antibiotics. New formulation are made and used for curing the infection caused by the resistant bacteria. Taking into account the resistance of bacteria, experiments were conducted to check the activity of the Nisin and Polymyxin B sulphate in presence of Silver nanoparticles. The results showed a considerable improvement in the minimum inhibitory concentration as well as wide spread action of these newly formulated antimicrobial peptides and antibiotics. Silver nanoparticles were synthesised by using Polymyxin B sulphate as a capping agent. The prepared nanoparticles ranges from 10±5 nm and uniform in shape. These nanoparticles showed antibacterial property on both the Gram-positive as well as Gram-negative bacteria. Then work was carried out on investigation of the immobilization and synergistic effect of the antimicrobial peptide, Nisin (a well known adjuvant in food to act against bacteria) with silver nanoparticles which increased the activity as well as potency on different types of bacteria.

2. Review of Literature

Antimicrobial peptides (AMPs) are an essential part of innate immunity that evolved in most living organisms over 2.6 billion years to combat microbial challenge. These small cationic peptides are multifunctional as effectors of innate immunity on skin and mucosal surfaces and have demonstrated direct antimicrobial activity against various bacteria, viruses, fungi, and parasites.

2.1 Bacteriocins

In recent years, bacterial antibiotic resistance has been considered a problem due to the extensive use of classical antibiotics in treatment of human and animal diseases.[14] As a consequence, multiple resistant strains have appeared and spread causing difficulties and the restricted use of antibiotics as growth promoters. So, the continued development of new classes of antimicrobial agents has become of increasing importance for medicine.[15] In order to control their abusive use in food and feed products, one plausible alternative is the application of some bacterial peptides as antimicrobial substances in place of antibiotics for human applications. Among them, bacteriocins, produced by lactic acid bacteria have attracted a lot of interest.

2.1.1 Bacteriocins from lactic acid bacteria

Bacteriocins are proteins or complex proteins biologically active with antimicrobial action against other bacteria, principally, closely related species. They are produced by bacteria and are normally not termed antibiotics in order to avoid confusion and concern with therapeutic antibiotics, which can potentially illicit allergic reactions in humans and other medical problems.[16]

Bacteriocins differ from most therapeutic antibiotics in being proteinaceous agents that are rapidly digested by proteases in the human digestive tract. They are ribosomally synthesized peptides, and this fact creates the possibility of improving their characteristics to enhance their activity and spectra of action.[17]

Antibiotics are generally considered to be secondary metabolites that are inhibitory substances in small concentration, excluding the inhibition caused by metabolic by-products like ammonia, organic acids, and hydrogen peroxide. It is likely that most, if not all, bacteria

are capable of producing a heterogeneous array of molecules in the course of their growth in vitro (and presumably also in their natural habitats) that may be inhibitory either to themselves or to other bacteria. Bacteriocin production could be considered as an advantage for food and feed producers since, in sufficient amounts, these peptides can kill or inhibit pathogenic bacteria that compete for the same ecological niche or nutrient pool. This role is supported by the fact that many bacteriocins have a narrow host range, and is likely to be most effective against the related bacteria with nutritive demands for the same scarce resources[18]. Range of activity considering the antimicrobial spectrum, producing species, molecular weight, stability, physical-chemical properties and mode of action of bacteriocins, they form a heterogeneous group. There is the classic type, which has a spectrum of activity only against homologous species, and a second type, less common, which shows action against a wide range of gram-positive microorganisms. One example of this second type is nisin, which is produced by certain strains of *Lactococcus lactis subsp. lactis.*[19] Other is pediocin, produced by Pedicoccus *pentosaceus* . Nisin, produced by *L. lactis subsp. lactis*, is active against gram-negative bacteria, but only when used at high concentrations or when the target cells have been pre-treated with EDTA.

Bacteriocins are not frequently active against gram-negative bacteria. The outer membrane of this class of bacteria acts as a permeability barrier for the cell. It is responsible for preventing molecules such as antibiotics, detergents and dyes from reaching the cytoplasmic membrane. However, some studies have already reported bacteriocin activity against this group of bacteria. Examples are plantaricin 35d, produced by *Lactobacillus plantarum* and active against *Aeromonas hydrophila*, bacteriocin ST151BR, produced by Lactobacillus *pentosus,* ST151BR[7], a bacteriocin produced by Lactobacillus *paracasei subsp. paracasei* active against *Escherichia coli*; thermophylin, produced by *Streptococcus thermophilus* active against *E. coli*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* among the gram-negative species and against several Bacillus species, *Listeria monocytogenes and Salmonella typhimurium* among the Gram-positives. Bacteriocins ST28MS and ST26MS, produced by Lactobacillus *plantarum* isolated from molasses[20] inhibited the growth of Escherichia *coli* and *Acinetobacter baumanii* along with some gram-positive bacteria.

2.1.2 Classification of bacteriocins

There are a wide number of bacteriocins produced by different Lactic Acid Bacteria (LAB), and they can be classified according to their biochemical and genetic characteristics.[21]

Class I. – Lantibiotics: small (< 5 kDa) heat-stable peptides acting on membrane structures; they are extensively modified after translation, resulting in the formation of characteristic thioether amino-acids, lanthionine and methyllanthionine. These arise via a two-step process, originated from post-transductional modifications: firstly, gene-encoded Parada, J.L. et al.[22,23,24] According to Brazilian Archives of Biology and Technology 524, serine and threonine are subjected to enzymatic dehydration to give rise to dehydroalanine and dehydrobutyrine, respectively. A very well known example of this group is nisin. The lantibiotic bacteriocins were initially divided into two subclasses based on structural similarities. Subclass Ia included relatively elongated, flexible and positively charged peptides; they generally act by forming pores in the cytoplasmatic membranes of sensitive target species. The prototypic lantibiotic nisin is a member of this group. Subclass Ib peptides are characteristically globular, more rigid in structure and are either negatively charged or have no net charge. They exert their action by interfering with essential enzymatic reactions of sensitive bacteria.[5]

Class II. – Non-Lantibiotics: Bacteriocins of variable molecular weight, but usually small (<10 kDa), heat-stable, and containing regular amino-acids. This group was divided into three subgroups: Class IIa: peptides active against Listeria, the characteristic representants are pediocin PA-1 and sakacin P. Class IIb: formed by a complex of two distinct peptides. These peptides have little or no activity and it appears to be no sequence similarities between complementary peptides. In this group are lactococcin G and plantaricins. Class IIc: Small peptides, heat-stable, which are transported by leader-peptides. In this subclass are found only the bacteriocins divergicin A and acidocin B.

Class III. – Big peptides, with molecular weight over 30 kDa. In this class are helveticins J and V, acidofilicin A and lactacins A and B. Most of the low molecular weight bacteriocins are highly cationic at pH 7.0, and this seems to be a unifying feature of both the lantibiotics and non-lantibiotics.[25]

Lantibiotics are the most studied and explored industrially. Nisin, a lantibiotic usually produced by *Lactococcus lactis subsp. lactis*, is used as an additive in foods. All of the variants of nisin are active against Gram-positive bacteria, like *Listeria sp, Micrococcus sp* and also on sporulating bacteria, like *Bacillus sp* and *Clostridium sp*[22].

2.2 Nisin

Nisin is a peptide formed by 34 amino-acids (Fig-1), with a small molecular weight, below 5 kDa. Its synthesis is complex, involving processes of transcription, transduction, post-transdutional modifications, secretion, processing and signs of transduction. There are two variants of this bacteriocin: nisin A and Z, which differ from each other only by the amino-acid 27. Histidine in nisin A is replaced by asparagin in nisin Z. This bacteriocin is used predominantly in canned foods and dairy products and is especially effective when utilized in the production of processed cheese and cheese spreads acting against heat-resistant spore forming organisms such as *Bacillus* and *Clostridium.*[18] It is also effective against Gram-positive mastitis-causing pathogens.

Preference focuses on nisin, although bacteria belonging to the genus Lactococcus is also able also to produce other bacteriocins with economic potential. An example is lacticin 3147, active in a wide range of pH and with a wide spectrum of action over Gram-positive bacteria. The genetic determinants for its synthesis are located in plasmids and lacticin genes can be conveniently transmitted to different starter cultures of Lactococcus.[28] As it is active under physiological pH, it has a great potential for use with medical purposes. It has a bactericide mode of action against all Gram-positive bacteria tested so far, including food deteriorants as Clostridium sp, pathogenic microorganisms as *Listeria monocytogenes*, and those causing bovine mastitis *Staphylococcus aureus* and *Streptococcus dysgalactiae.* It is also active on human pathogens as methicilin-resistant *Staphylococcus aureus* (MRSA), vancomicin-resistant *Enterococcus faecalis*, penicillin-resistant *Pneumococcus*, *Propionibacterium acne and Streptococcus mutans.* These characteristics make lacticin 3147 very attractive for its potential application in the food industry, veterinary medicine, and eventually in the treatment of human diseases.[29]

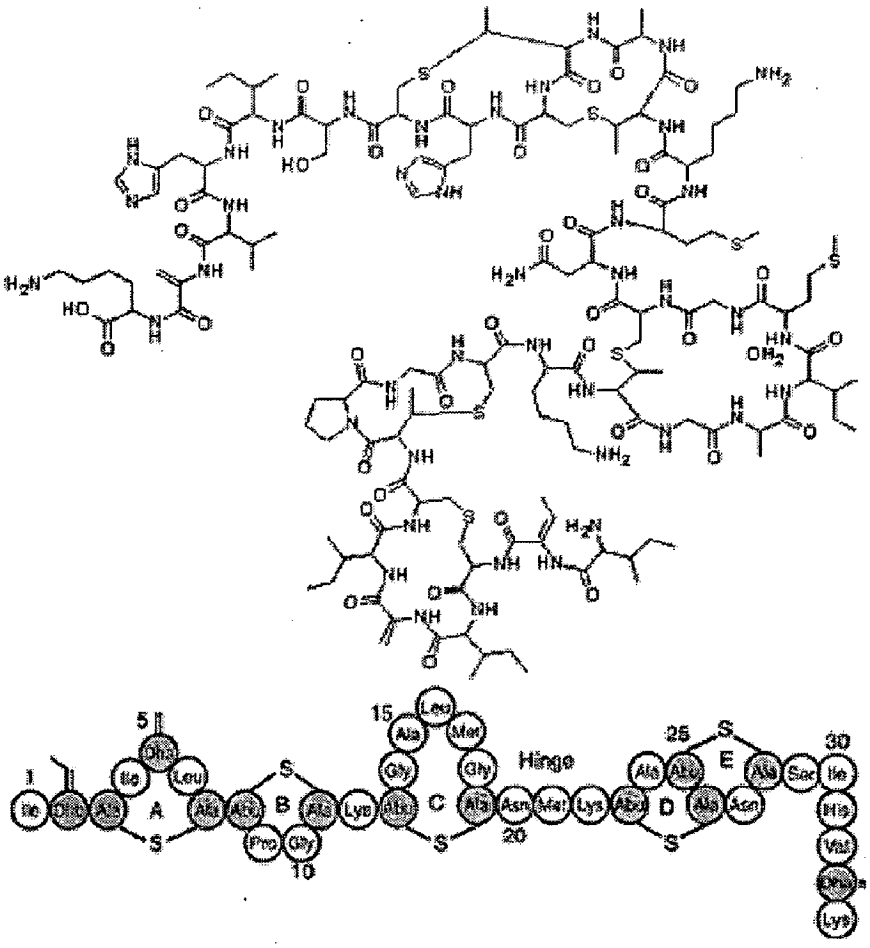


Fig-2.1 – Primary structure of nisin.

2.2.1 Mode of action

These peptides are usually effective against Gram-positive microorganisms. Bacteriocins of lactic acid bacteria may be inefficient to inhibit Gram-negative organisms because the outer membrane hinders the site for bacteriocin action, which is the cell membrane.[30] Different mechanisms of action have been proposed for bacteriocins: alteration of enzymatic activity, inhibition of spore germination and inactivation of anionic carriers through the formation of selective and non-selective pores.[31] Lactic Acid Bacteria(LAB) bacteriocins can work via different mechanisms to exert an antimicrobial effect, but the cell envelope is generally the target. The initial electrostatic attraction between the target cell membrane and the bacteriocin peptide is thought to be the driving force for subsequent events.[5] Bacteriocins may possess a bactericidal or bacteriostatic mode of action on sensitive cells, this distinction being greatly influenced by several factors such as bacteriocin dose and degree of purification, physiological state of the indicator cells and experimental conditions.[27] According to Jack et al [32], at least for the non-lanthionine-containing bacteriocins, the increased antibacterial activity observed at low pH may be the result of any one of a number of factors, including the

following: (i) aggregation of hydrophilic peptides is less likely to occur, and, thus, more molecules should be available to interact with sensitive cells; (ii) fewer molecules will remain bound to the wall, making more molecules available for bactericidal action[36a]; (iii) hydrophilic bacteriocins may have an enhanced capacity to pass through hydrophilic regions of the cell wall of the sensitive bacteria; and (iv) interaction of the non-lanthionine-containing bacteriocins with putative membrane receptors may be inhibited at higher pH values. Several features of the mode of action of the non-lanthionine-containing bacteriocins of gram-positive bacteria require further explanation: (i) the reason why, for two sensitive strains, one undergoes lysis following treatment with a Bacteriocins while the other does not (ii) for a bacteriocin to come into contact with the cytoplasmatic membrane of sensitive cells, the molecules must firstly pass through the cell wall; the mechanism of this translocation remains to be understood; and, finally, (iii) there is evidence that non-lanthionine-containing bacteriocin molecules may be adsorbed on the surface of most gram-positive bacterial cells, including sensitive, resistant, and producer strains; the influence of this is not yet fully understood.[32]

The cell wall of gram-positive bacteria allows passage of relatively large molecules, so that there is unlikely to be a requirement for bacteriocin receptors analogous to those in the outer membranes of gram-negative cells. Anionic cell surface polymers like teichoic acid and lipoteichoic acid may be important in the initial interaction of cationic bacteriocins of Gram-positive bacteria.[32] Initially, nisin was thought to act as a surfactant because of its cationic nature and because treatment of cells with nisin caused leakage of UV-absorbing material.[32] According to González-Martínez et al[22], it is possible that classes I and II use the same mechanisms of action. Peptides bond to the plasmatic membrane through electrostatic interactions with phospholipids charged negatively. So, it gets into the membrane with a reorientation that depends on the membrane potential, which is driven by the pH and phospholipidic composition. The monomers of bacteriocin form proteic aggregates that result in the pore formation with the consequent leave of ions (mainly potassium and magnesium), loss of the proton-motriz force, and exit of ATP and amino-acids. The proton motriz-force has a fundamental role in ATP synthesis, in active transport and in the bacterial movement; therefore, the synthesis of macromolecules is inhibited, as well as the production of energy, resulting in cell death. Pore formation and the consequent loss of membrane integrity induces a passive efflux of small molecules, such as potassium and phosphate ions, amino acids and ATP, through the lipid bilayer, which results in the reduction or dissipation of the proton

motive force, or at least of one of its components: Dψ (membrane potential) and DpH (pH gradient).[27]

In class I, nisin does not require a receptor on the cell membrane, since it recognizes the phospholipidic composition of the cell. Lactococin A and lactoestrepcin require binding to a specifc receptor. For class IIa bacteriocins, it is believed that the amino-terminal region has an important role in the ability to recognize cell membrane components, and they act by permeabilizing the membrane of their target cells. Studies on the mode of action of these bacteriocins indicate that antimicrobial activity does not require a specific receptor and is enhanced by a determined membrane potential.[33]

In class IIb, plantaricins EF and JK depend on the interaction between the peptides "a" and "b" for the pore formation and consequent dissipation of the membrane potential.

In class III, which includes bacteriocins with a high molecular weight, the mechanism of action is unknown, requiring more studies for its elucidation.[22]

It is likely that the secondary structures of the active peptides have an important role, since the α-helical and β-folded structures suggest an oligomerization of the monomers in the membranes, according to a mechanism of pore formation denominated "barrel-stave", with the hydrophobic sides inserted on the membrane and the hydrophilic sides forming the pore itself. Lactocin 705 is a class IIb non-lantibiotic bacteriocin, whose activity depends upon the complementation of two peptides (named Lac705α and Lac705β) of 33 amino-acid residues each[34]. A study conducted by Cuozzo et al [35] has shown that Lac705β is the active component of lactocin 705 on cell membrane, while the peptide Lac705α is involved in receptor recognition on the sensitive cells. It is suggested that lactocin 705 acts as a complex of Lac705α and Lac705β (ratio 1:4) peptides and exert its action through interaction with cell wall-associated or membrane-associated binding sites in the sensitive cells. According to this author, it is possible that the specificity of this porin complex is given by the Lac705 peptide; its net positive charge at the 14 C-terminal amino acids would neutralize the negative charge of teichoic and lipoteichoic acids in the cell wall of sensitive cells, and interact specifically and competitively with some target cell entity, allowing the Lac705β peptide to form membrane pores, which increases membrane permeabilization and causes cell death.[36a] A study shows that the broad host-range lacticin 3147 is a two-component bacteriocin, membrane-active, that is hydrophobic in nature. The author proposes that lacticin 3147 forms pores which allow K+ and phosphate to leak; the resulting change in electrical charge across

the membrane causes the immediate dissipation of the $\Delta\psi$ component (membrane potential) of the proton-motriz force. In an attempt to recover these ions, the cells use phosphate bond-dependent transport, resulting in rapid ATP hydrolysis and leading to cell death. The presence of a proton-motif force promotes the interaction of the bacteriocin with the cytoplasmatic membrane, leading to the formation of pores at low lacticin 3147 concentrations. These pores were shown to be selective for K+ ions and inorganic phosphate. The loss of these ions results in immediate dissipation of the membrane potential and hydrolysis of internal ATP, leading to an eventual collapse of the pH gradient at the membrane, and ultimately to cell death. Lacticin 3147 does not cause immediate dissipation of the ΔpH of the sensitive cells, as the membrane does not become permeable to H^+ ions.

2.3 Polymyxin B Sulphate

Polymyxin B sulfate is a mixture of polymyxins B1 and B2, obtained from *Bacillus polymyxa* strains. They are basic polypeptides of about eight amino acids and have cationic detergent action on cell membranes.

Polymyxins are a group of cyclic, polycationic peptide antibiotics with a fattyacid chain attached to the peptide through an amide linkage. These are produced by fermentation of strains of *Bacillus polymyxa*. Polymyxin B and E(colistin) are the least toxic and are the only polymyxins used clinically. These antibiotics contain a 7-amino-acid ring attached to a 3-amino-acid tail, to which is attached a fatty acyl group. Polymyxin B is a mixture containing mostly polymyxins B1 and B2. They were first isolated in 1947. Initially, only the sulphate form of polymyxin was commercially available. Colistin, which became available for clinical use in 1959, was isolated in 1949 in japan from *B.polymyxa subsp.* Colistinus. Initially it was thought to be a new antibiotic, but later it was proved to be identical to polymyxin E.

Polymyxin B1 contains several L-α, γ-diaminobuteric acid (Dab), L-threonine and D-phenylalanine residues and 3-methyl octanoic acid as a fatty acid residue.

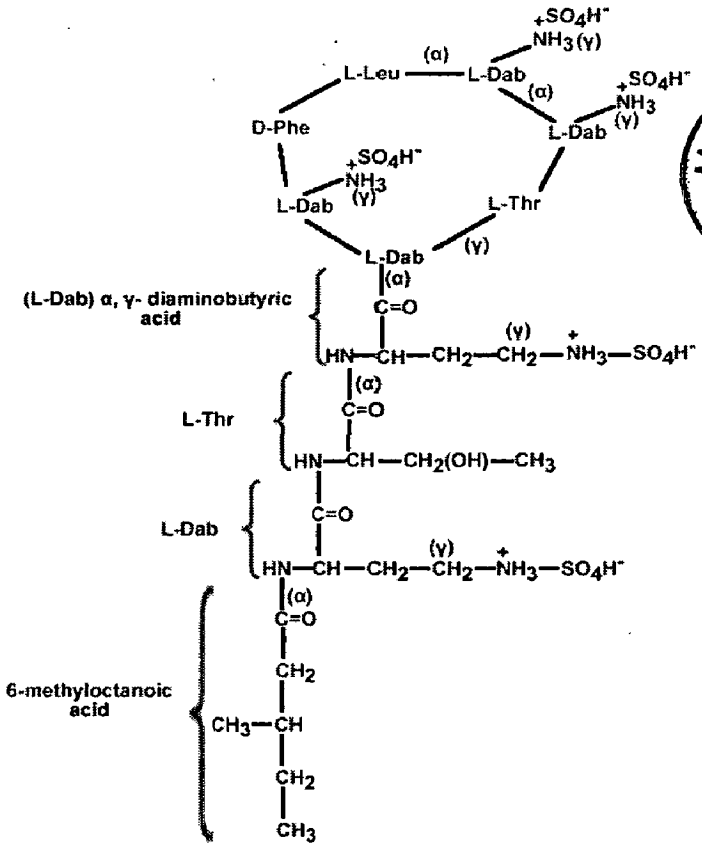


Fig-2.3- Polymyxin B Sulphate

2.3.1 Mode of action

Both polymyxin B and E (colistin) are cationic surface active compounds at physiological pH. It has been suggested that the fatty acid part of the polymyxin molecule penetrates in to the hydrophobic region of the outer membrane and the ammonium groups interact with the lypopolysaccrides and phospholipids, competitively displacing divalent cations(calcium and magnaesium) from the negatively charged phospholipid group of the membrane lipids. This displacement disrupts membrane organization and increases the permeability of the membrane.

2.3.2 Antibacterial Activity

Polymyxin B and E is active almost exclusively against aerobic gram-negative bacilli. Polymyxin B is used for infections with gram-negative organisms, but may be neurotoxic and nephrotoxic. All gram-positive bacteria, fungi, and the gram-negative cocci, *N. gonorrhea* and *N. menigitidis* are resistant. It is appropriate for treatment of infections of the urinary tract, meninges, and blood stream, caused by susceptible strains of *Pseudomonas aeruginosa.* They are inactive against gram-positive bacteria and most obligate anerobes.

2.3.3 Clinical use

Both the neurotoxicity and the nephrotoxicity of the Polymyxin B and E reflect their rather nonspecific interaction with bacterial and mammalian cell membranes. In the past, these polymyxins were used for the treatment of *P.aeruginosa* infections. Nowadays, because the availability of effective less toxic drugs such as gentamycin, tobramycin, amikacin, ticarcillin, piperacillin and cefrazidime, polymyxins are not the antibiotics of choice to treat infections caused by this bacterium. Polymyxin B and E have now been relegated to tropical preparations for dematological, otic or ophto topical preparations usually containing another antibiotic such as bacitracin, neomycin, oxytetracycline or trimethoprium.

The list of Polymyxin preparations commercially available in the United States of America are listed below.[41a]

Generic Name	**Trade name(preperation)**	**Manufacturer**
Polymyxin B sulphate plus bacitracin zinc	Polysporin (ointment)	Plizer
Polymyxin B sulphate plus bacitracin zinc and pramoxine HCL	Batadine plus (ointment)	Purdue Ferderick
Polymyxin B sulphate plus neomycin and bacitracin zinc	Neosporin (Opthalamic ointment)	Monarch
Polymyxin B sulphate plus neomycin sulphate	Neosporin (Solution for irritation)	Monarch
Polymyxin B sulphate plus neomycin	Cortisporin (optaamic suspension)	Monarch

Our new formulation of Silver nanoparticle capped with Polymyxin B sulphate is in progress.

2.4 Silver Nanoparticles and their antimicrobial properties

Since ancient times, the silver ion has been known to be effective against a broad range of microorganisms. Today, silver ions are used to control bacterial growth in a variety of

medical applications, including dental work, catheters, and the healing of burn wounds. Silver ions are also used for a number of non-medical purposes, such as in electrical appliances. The slow-release "nanosilver" linings of laundry machines, dishwashers, refrigerators, and toilet seats are also marketed and advertised. It is clear that we are exposed to a wide range of mostly unfamiliar uses of silver containing products intended to function as antimicrobial biocides. Therefore, it is necessary to elucidate the antimicrobial activity of the silver ion, which is widely used in these products.

Nanosized inorganic particles, of either simple or composite nature, display unique physical and chemical properties and represent an increasingly important material in the development of novel nanodevices which can be used in numerous physical, biological, biomedical, and pharmaceutical applications.[42,43,44] A number of recent achievements offer the possibility of generating new types of nanostructured materials with designed surface and structural properties.[45-49] The preparation of uniform nanosized drug particles with specific requirements in terms of size, shape, and physical and chemical properties is of great interest in the formulation of new pharmaceutical products.[50,51] Resistance of bacteria to bactericides and antibiotics has increased in recent years due to the development of resistant strains. Some antimicrobial agents are extremely irritant and toxic and there is much interest in finding ways to formulate new types of safe and cost-effective biocidal materials. Previous studies have shown that antimicrobial formulations in the form of nanoparticles could be used as effective bactericidal materials.[52,53] Recently, Klabunde and co-workers demonstrated that highly reactive metal oxide nanoparticles exhibit excellent biocidal action against Gram-positive and Gram-negative bacteria[54]. Thus, the preparation, characterization, surface modification, and functionalization of nanosized inorganic particles open the possibility of formulation of a new generation of bactericidal materials.

It is well known that silver ions and silver-based compounds are highly toxic to microorganisms[55,56] showing strong biocidal effects on as many as 16 species of bacteria including *E. Coli.*[57] Thus, silver ions, as an antibacterial component, have been used in the formulation of dental resin composites[58,59] and ion exchange fibers[60] and in coatings of medical devices.[61] Recently, Tiller and co-workers showed that hybrids of silver nanoparticles with amphiphilic hyperbranched macromolecules exhibit effective antimicrobial surface coatings.[62]

The aim of the project is basically to increase the potential of the antimicrobial peptides by introducing silver nanoparticles in the mixture. The second aim relies on the activity of the antimicrobial peptides to kill the micro-organisms of both Gram-positive and Gram-negative class. The scope of the project is to use these formulations in the real environment to make the food free of contamination and to make a new drug formulation which can be used for external application to kill harmful bacteria.

3. The instruments used for nanoparticle characterization

3.1 Atomic Force Microscopy

3.1.1 History

The AFM belongs to the great family of surface force apparatus (SFA). Those devices have been developed at the beginning of the 20th century to study tribology of modern machinery. They gained more and more precision as the industry and the computer sciences evolved, to reach nowadays the atomic resolution and measure forces down to the nano Newton. The AFM is in fact strongly related to the invention in 1981 of the first scanning tunnelling microscope (STM) capable of imaging a solid surface with atomic resolution in three dimensions. The scientific community acknowledged this invention by awarding the Nobel Prize to its inventors, Binnig and Rohrer, in 1986. This instrument, based on electron tunnelling between a small tip and a surface, was limited to the study of electrically conductive samples. Indeed, insulators have the particularity to rapidly charge to the same potential as the tip, and thus stop the tunnelling. Few years later, in 1985, Binnig and Rohrer developed an atomic force microscope based on the same design as their STM. It had the properties of surface imaging and surface force measuring down to the nano-scale, but this time, contrary to the STM, the instrument was not restricted to one type of surface. It could be used in different environments going from the ultra high vacuum to the liquids, to study any kinds of surfaces, including biological samples. This facilty soon propelled the AFM as popular tool for probing surfaces and commercial AFM are now available with a great variety of characteristics.

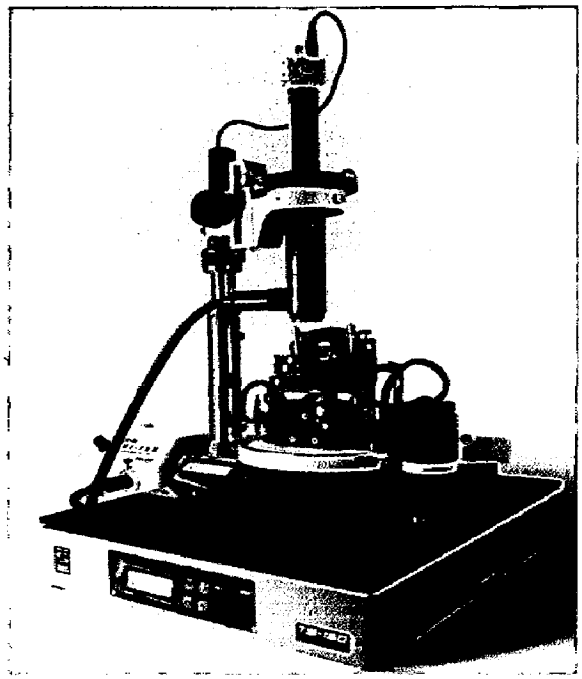


Fig-3.1 AFM used in the process of characterization

3.1.2 Working principle of the AFM

The working principle of an AFM is rather simple. It essentially consists of three systems working together: a force-sensing system, a detection system and a positioning system, the whole managed by control electronics and feedback systems, which are usually realized with the help of a computer. A sketch of the AFM setup is shown in Fig. 3. The force-sensing system is the AFM part in direct interaction with the sample surface. Usually, a flexible leaf spring, called cantilever and ended by a small sharp tip located at its free end, is used as sensor. The AFM tip is the component in contact or in near contact with the surface. The shape of the tip is generally either a pyramid or a cone, but can also be a ball. Forces acting between the AFM tip and the sample surface result in deflections of the cantilever. Depending on the forces, will have torsion and/or a bending of the cantilever while scanning a sample. Microfabricated cantilevers with integrated tip are commercially available in a wide range of dimensions and spring constants. The final radius of the AFM tip is decisive for the characteristics of the interactions with the surface.

This radius might reach few nanometers for a tip height of typically a couple of microns long. The length of a cantilever is usually hundreds of micrometers for 0.5 to 5 micrometers of thickness and tenth of micrometers width. There are two classical shapes of cantilevers, one-

beam cantilever and two-beam V-shaped (triangular) cantilever. They are mainly characterized by their normal, longitudinal, and torsional (or lateral) spring constants. Their coating, geometry and material are also important factors in AFM studies.

3.1.3 The detection system

The tip-sample interaction is detected by monitoring the deflection of the cantilever. An easy method that is actually the most used in commercial AFM is the laser-beam deflection system. A laser beam is focused on the rear end of the cantilever and reflected into a four-quadrant position sensitive photodetector (PSPD). Bendings and torsions of the cantilever result in the motion of the laser spot on the photodetector and thus, changes in the output voltage of the photo diode. The amount of bending or torsion of the cantilever is magnified since the distance between the cantilever and the photodetector measures thousands of times the length of the cantilever. Small variations in the position of the cantilever result in large displacements of the laser spot. By this way, tip displacements smaller than 1 nm are easily detectable. One major property of the detection system is its ability to record the deflection of the cantilever in the three dimensions separately and simultaneously. Thus, the AFM has the potential to measure the three components of the force vector describing the interaction of the tip with the surface. The cantilever displacements or the forces acting on the cantilever are then deduced from the measurement of the PSPD output voltage, provided that the photo diode sensitivity and the cantilever spring constants are known.

The other main detection systems involve piezoresistive cantilever (piezoresistors are integrated to the cantilever whose bending is measured through resistance change using a Wheatstone bridge) or interferometry (the laser beam reflected to the back side of the cantilever interferes with the original beam and produces an interference pattern whose intensity is related to the deflection).

3.2 Fourier Transformation Infrared Spectroscopy (FT-IR)

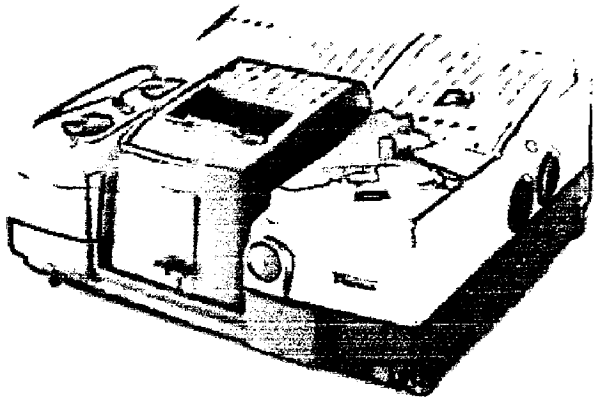


Fig-3.2 Fourier Transformation Infrared Spectroscopy

Infrared spectroscopy is one of the most powerful tools available to the chemists for identifying organic and inorganic compounds. Indeed most molecular species absorb infrared radiation. It is based on the fact that the absorbed radiation stimulates molecular vibration. These vibrations are characteristic of organic functionality, such as methyl or aldehyde groups for example. Each molecular species has a unique infrared absorption spectrum. Thus, in an ideal case, an exact match between the spectrum of a compound of known structure and that of an analyte unambiguously identifies the latter. For quantitative analyses infrared spectroscopy is less efficient than UV/Vis spectroscopy because the narrow peaks that characterize infrared spectroscopy usually lead to deviations from Beer's law. Fourier-transform infrared spectroscopy (FT-IR), offering the advantages of unusually high sensitivity, resolution and speed of data acquisition, became the standard technique for chemical characterisation. Fourier-transform instruments are detecting and measuring all the wavelengths simultaneously. In order to separate wavelengths, it is necessary to modulate the source signal in such a way that it can be decoded by a Fourier transformation, a mathematical operation.

3.3 Scanning Electronic microscopy

The scanning electron microscope consists essentially of the following: a source of electrons; lenses for focussing them to a fine beam; facilities for sweeping the beam in a raster; arrangements for detecting electrons (and possibly other signals) emitted by the specimen;

and an image-display system. Secondary-electron (SE) images, which show topographic features of the specimen, are the most commonly used type. Backscattered-electron (BSE) images are principally used to reveal compositional variations. An X-ray spectrometer is an optional extra enabling the SEM to be used for element mapping and analysis, also called energy diapersive X-ray analysis (EDXRD).

Fig-3.3 SEM

Scanning Electron Microscopy gives the morphological and topographical information of the solid surfaces that is necessary in understanding the behaviour of the surfaces. In SEM the sample is bombarde with an electron beam. The shorter wave length of the electrons creates a better resolution that optical microscopy, there by pictures of samples with higher magnifications can be recorded. For electrons to be able to pass through the sample, the sample should be conductive. In case of a non-conductive sample, a layer of gold is sputtered on the surface of the sample.

The electron beam, emitted from the filament in the electron gun, is accelerated towards the sample and focused by several collisions between the electrons from the beam and atoms n the sample will occur. As a result of these collisions some of the outer most electrons will be detached from the sample. These electrons, called secondary electrons, have relatively low kinetic energy and can easily be attracted by the detector. The detector counts the number of electrons emitted from this small area and the result is displayed as a small dot on the computer screen. A magnified image of the sample is created by scanning the electron beam over a small area, detecting and displaying the number of electrons originating from each point. Both the topography of the sample and the atom numbers affect the number of secondary electrons emitted and these factors are therefore represented in the picture. The

resulting image has shadows and perspective, much like ordinary photographs, and is consequently rather easy to interpret.

3.4 Zeta-Seizer

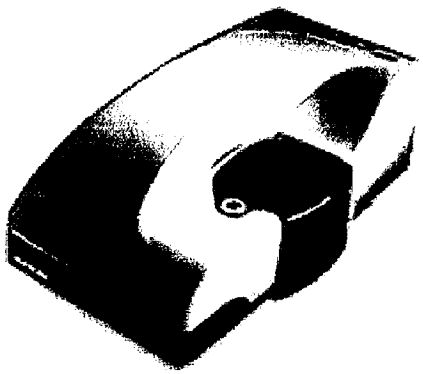


Fig-3.4 ZETA-SEIZER

Zeta potential is the electrical potential that exists at the "shear plane" of a particle, which is at some small distance from its surface. Zeta potential is derived from measuring the mobility distribution of a dispersion of charged particles as they are subjected to an electric field. Charged particles in a liquid suspension can be made to move by applying an electric field to the liquid through two electrodes. By alternating the charge between the electrodes, the particles move back and forth between the electrodes at a velocity relative to their surface charge and the electrode potential. This velocity can be determined by measuring the doppler shift of the laser light scattered off of the moving particles. Mobility is defined as the velocity of a particle per electric field unit and is measured by applying an electric field to the dispersion of particles and measuring their average velocity. Depending on the concentration of ions in the diluent, either the Smoluchowski (for high ionic strengths) or Huckel (for low ionic strengths) equations are used to obtain the Zeta potential from the measured mobilities.

3.5 Atomic Absorption Spectroscopy

Fig-3.5 Atomi Abssorption Spectroscopy

Atomic absorption spectrometry (AAS) is an analytical technique that measures the concentrations of elements. Atomic absorption is so sensitive that it can measure down to parts per billion of a gram (μg dm–3) in a sample. The technique makes use of the wavelengths of light specifically absorbed by an element. They correspond to the energies needed to promote electrons from one energy level to another, higher, energy level. Atomic absorption spectrometry has many uses in different areas of chemistry.

Clinical analysis. Analysis of metals in biological fluids such as blood and urine.

Environmental analysis. Monitoring our environment – e.g. finding out the levels of various elements in rivers, seawater, drinking water, air, petrol and drinks such as wine, beer and fruit drinks.

Pharmaceuticals. In some pharmaceutical manufacturing processes, minute quantities of a catalyst used in the process (usually a metal) are sometimes present in the final product. By using AAS, the amount of catalyst present can be determined.

Industry. Many raw materials are examined and AAS, is widely used to check that the major elements are present and that toxic impurities are lower than specified – *eg* in concrete, where calcium is a major constituent, the lead level should be low because it is toxic.

Mining. By using AAS the amount of metals such as gold in rocks can be determined to see whether it is worth mining the rocks to extract the gold.

4. Materials

4.1 Chemicals and Bacterial stocks

Nisin was a genorous gift from Dr. R. K. Malik, NDRI, Karnal., Polymyxin B Sulphate was obtained from HIMEDIA laboratories, Mumbai, India, Silver Nitrate obtained from MEREK laboratories, India and HPLC grade Methanol, Trisodium Citrate Dihydrate was obtained from Merck laboratories, Mumbai, India. *Pediococcus Sp.*, strains were generous gift from Dr. R. K. Malik Lab, NDRI Karnal and *E.Coli* Strains are generous gift from Dr. Eric Brown lab, McMaster University, Canada.

4.2 Equipments used for Characterization

The as prepared nanoparticles were analysed for UV-Visible spectrum by Molecular SpectraMax M2 / M2e Microplate Readers. The solution of as prepared silver nanoparticles was placed in a quartz curette and then analysed. Two-and three-dimensional images of the polymyxin B sulphate stabilized silver nanoparticle solutions were recorded in semi-contact mode on an NTEGRE (NTMDT) atomic force microscope (AFM). Three-dimensional surface morphology of these systems along with elemental analysis was obtained on FEI-QUANTA 200F field emission scanning electron microscope (FESEM) coupled with an energy dispersive x-ray analysis (EDAX) facility by applying an acceleration voltage of 20 kV. IR spectra were recorded on a Thermo Nicolet Nexus Fourier transform infrared (FTIR) spectrophotometer in the mid-IR range in KBr media.

4.3 Bacterial culture

The cultures *E.Coli DH-5 alpha*, *Salmonella typhi* and *Pediococcus sp.* were grown overnight on LB-Agar plates. These then are streaked for several times and a single colony was picked up to check the purity of culture. The single colony was inoculated in a 5ml LB broth, kept in 37^0C incubator overnight at 250 rpm shaking. The cultures were grown till the optical density (A_{600}) of 0.3 to 0.4 and then these were used for the microplate assays.

4.4 Microplate assays:

Broth microdilution method (96-well plate serial dilution method) for determining minimum inhibitory concentration (MIC) of the bacterial extracts

4.4.1 Principle:

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that is able to inhibit visible growth of a microorganism after overnight incubation. It can be used as a research tool to determine the *in-vitro* activity of new antimicrobials like plant extracts, bacterial extracts or synthetic molecules against desired indicator strains.

4.4.2 Materials:

- Luria Bertani Broth, Miller (HIMEDIA)
- 96-well polystyrene flat bottom plates (AXYGEN)
- 96 well plate reader(SPECTRA MAX)
- Multichannel pipette(EPPENDROF, Germany)

4.4.3 Procedure:

4.4.4 *Inoculum Preparation of the Indicator Strain (E.coli DH5α)*

- Single isolated colony from a fresh agar plate culture of *E.coli* DH5α was inoculated into a tube containing 5 ml of LB Broth medium.
- The broth culture was incubated at 37°C, 200 rpm for 12 hours.
- This culture was then subcultured (1%) into fresh 5 ml LB Broth medium tubes and incubated at 37°C, 200 rpm until the culture reached OD between 0.5 – 0.7 at 600 nm.
- The cells were diluted 10^4 times in the LB broth media for the 96 well plate assay.

4.4.5 *Serial Dilution in 96-well plate*

- The dilutions were carried out in duplicates for each sample of the bacterial extract to be tested.
- The 12^{th} well in each row contains the highest concentration (vol/vol) of the bacterial extract i.e. 500 µl/ml. 200 µl of the bacterial extract was added to the 12^{th} well.
- 100 µl of the LB Broth was added in the remaining wells.
- For serial dilution, 100 µl supernatant from 12^{th} well was transferred into 11^{th} well. The serial dilution was continued similarly up to 3^{rd} well and 100 µl of the contents from 3^{rd} well were transferred directly to the 1^{st} well (as 2^{nd} well is used as positive control i.e. without bacterial extract).

- 100 µl of the diluted *E.coli DH5α* cells were added to all the wells except 1st column wells which served as negative control.
- The final volume in all the wells was 200 µl.
- The pre-incubation reading was recorded with the help of 96 well plate reader at 600 nm.
- The plate was incubated for 12 hrs at 37°C.
- The post- incubation reading was recorded after 12 hrs of incubation.
- The change in OD of each well was calculated by subtracting the initial OD (incubation = 0 hrs) from the final OD (incubation = 12 hrs).
- OD around 0.0 indicated inhibition of growth of the indicator strain.

Table 4.4.5- A Complete profile of
Microplate assay

Column:	1	2	3	4	5	6	7	8	9	10	11	12
Concentration (v/v)	Negative Control	Positive Control	0.97 µl/ml	1.95 µl/ml	3.9 µl/ml	7.81 µl/ml	15.62 µl/ml	31.25 µl/ml	62.5 µl/ml	125 µl/ml	250 µl/ml	500 µl/ml

5. Methodology, Results and Discussion for Antibiotic Polymyxin B Sulphate capped Silver Nanoparticle Synthesis

5.1 Preparation of Silver Nanoparticles

A simple mixing of the constituents in appropriate proportions leads to the formation of silver nanoparticles. The polymyxin B sulphate is initially dissolved in water and then later a required amount of the polymyxin B sulphate solution is redispersed in methanol. The proportion is approximately less than one part of aqueous polymyxin B sulphate and rest is with methanol. 2 mM silver nitrate solution was prepared in methanol medium under vigorous stirring for about half an hour in the absence of light. Then 100 µl of as prepared silver nitrate solution is mixed with 100 µl of 60 µg/ml polymyxin B sulphate and then kept under visible light. Precaution is to be taken to prevent the evaporation of methanol. Then after an exposure time of about an hour one can observe the formation of silver nanoparticles as the colour of the solution becomes yellow. The contents were centrifuged at 15000 rpm then the precipitate was collected and re-dispersed in water. The colloid was used it for characterization purposes.

5.2 Experimental protocols and corresponding results of polymyxin B sulphate capped silver nanoparticle synthesis

5.2.1 Experimental set up of polymyxin B sulphate capped silver nanoparticle solution.

Silver Nanoparticles were prepared by using antimicrobial peptide as both reducing and capping agent. The utilization of Proteins and peptides as reducing as well as capping agents for the synthesis of silver nanoparticles has been reported[63,64] previously but the concept of reducing and capping action of antimicrobial peptides produced by bacteria is not yet reported as far as our knowledge. The experimental setup that we have used for the finding out the appropriate proportions that required for the maximum yield of silver nanoparticles is given in the fig 5.2.1. More concentration of Polymyxin B sulphate showed a red precipitate at the bottom giving a sign of aggregation of the as obtained silver nanoparticles. Therefore the adequate proportions are confined to be 35% to 70% of 60 µg/ml polymyxin B sulphate and

the rest is silver nitrate in methanol. This setup can deal with a number of reactions at a time. Antimicrobial peptides stabilized silver nanoparticles are shown an absorbance of at 410 nm which corresponds to spherical and uniform nanoparticles Fig-5.2.3. The comparison profile of the formation of polymyxin B sulphate stabilized silver nanoparticles in visible and ultraviolet radiation in given in Fig-5.2.2.

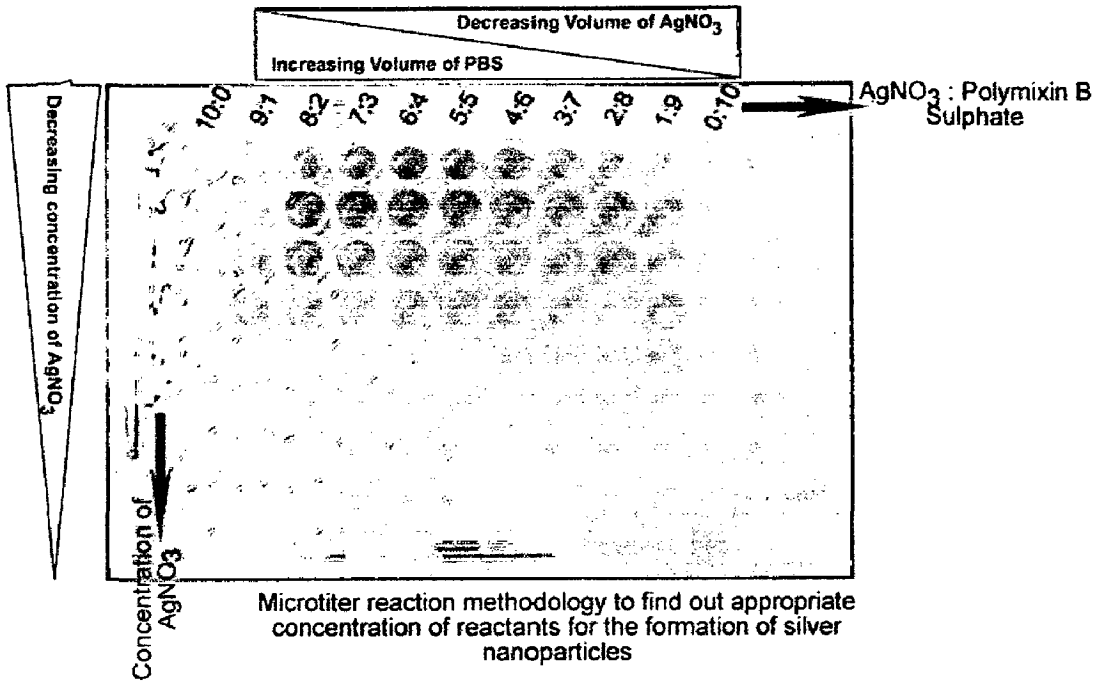


Fig.-5.2.1. Proportionate composition analysis for the formation of polymyxin capped silver nanoparticles.

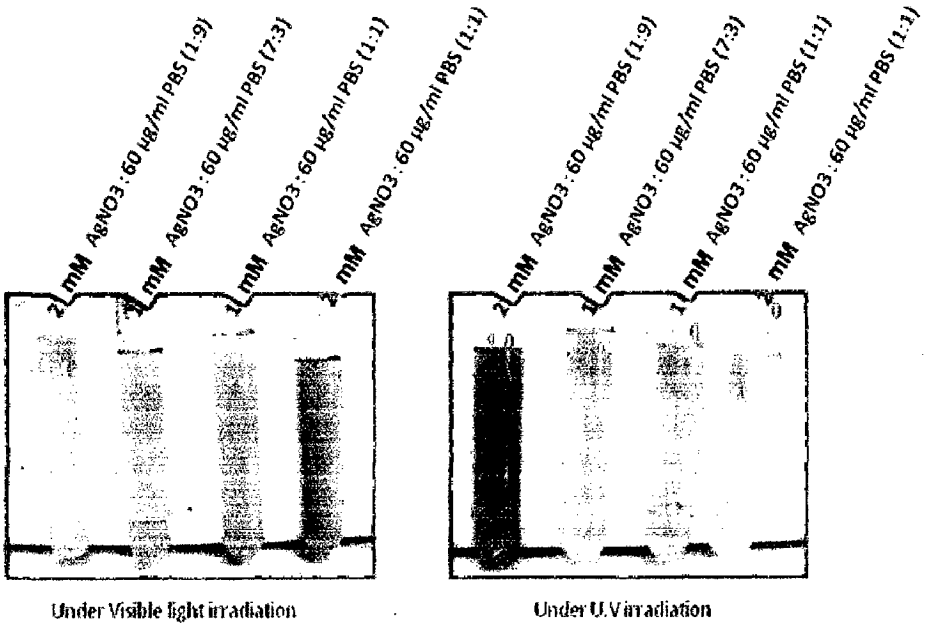


Fig 5.2.2. Comparison of visible irradiation Vs ultra violet irradiation for the formation of silver nanoparticles.

5.2.2 UV-Visible Spectrum:

The UV-Visible spectrum of the as prepared silver nanoparticles Fig-5.2.3 showed a maximum absorption at 410nm. This confirms that the obtained silver nanoparticles are sphere shaped and uniform[19]. The UV-Visible spectrum is given in the fig-10.

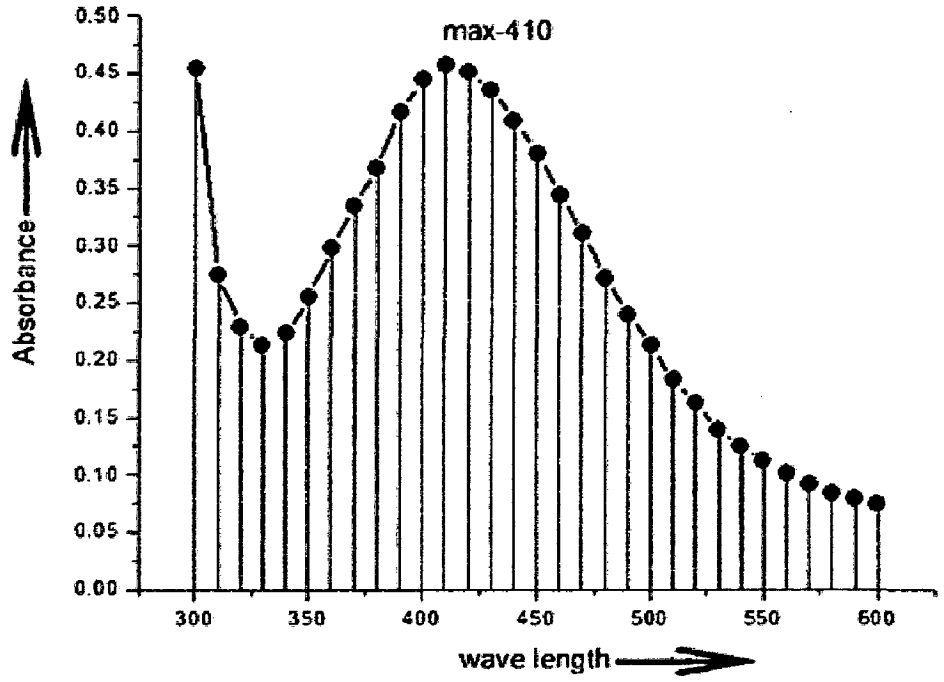


Fig 5.2.3. UV-visible spectrum of polymyxin B sulphate stabilized silver nanoparticles.

5.2.3 SEM Analysis:

The SEM analysis of the silver nanoparticles is given in the Fig-5.2.4. These are the silver nano-clusters can be visualized as round entities. Scanning Electron Microscopy Analysis of the nanoparticles is carried out by spreading out a drop of silver nanoparticle solution on a cover slip and then these are gold plated.

The stability of these nanopaticles is determined be Zeta Potential measurements. The colloidal solution is loaded in a universal dip cell and the data is collected. It was found that the value for this Colloidal polymyxin B sulphate stabilized silver nanoparticles is found to be above -60 mV which correspond to a stable solution.

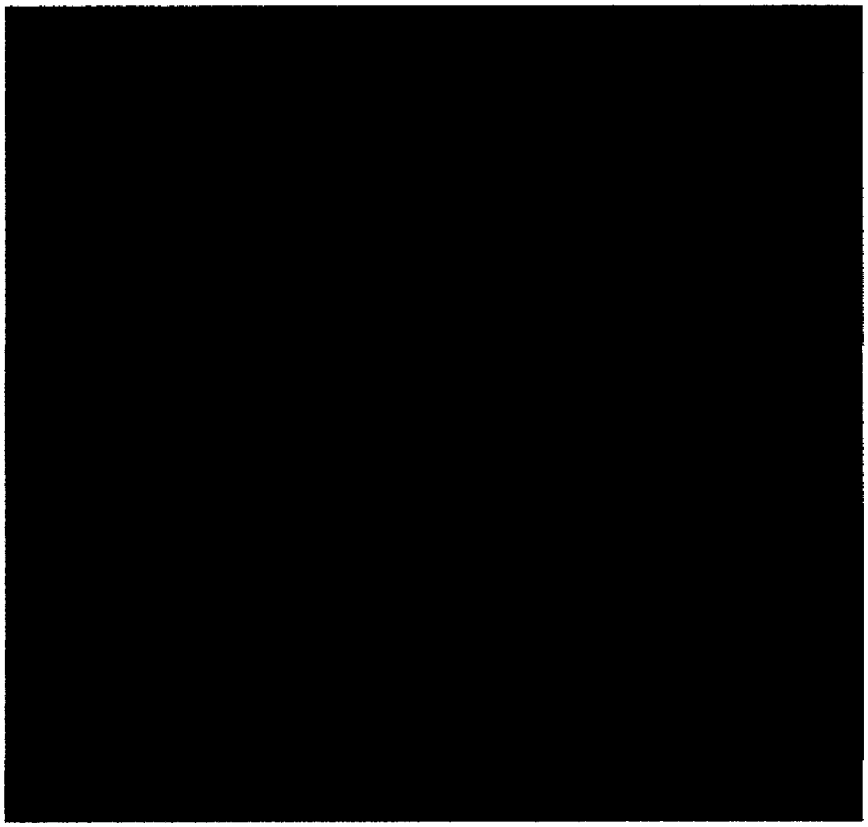


Fig-5.2.4: SEM analysis of silver nanoparticles

After two months there is a decrease in zeta potential values which states that these particles are stable for a period of around two to three months. This decrease in zeta potential is due to the process of aggregation.

5.2.4 AFM Characterization:

AFM helps us to characterize the morphology of the samples with quantitative information on several parameters such as roughness or height distributions.

The sample preparation for AFM is done by taking a drop of the polymyxin B sulphate capped silver nanoparticle solution, then disperse this on a glass slide and let it dry out completely. The as obtained nanoparticles are of size ranging between 10±5 nm as per the roughness given by AFM. The 3D picture can give us the information of the height of the nanoparticles. The Fig-5.2.5 and 5.2.6 are showing the description of the results.

The size of the polymyxin B sulphate stabilized silver nanoparticles are found by the software analysis tools of the instrument. The average sizes of the nanoparticles were found to be around 10 nm.

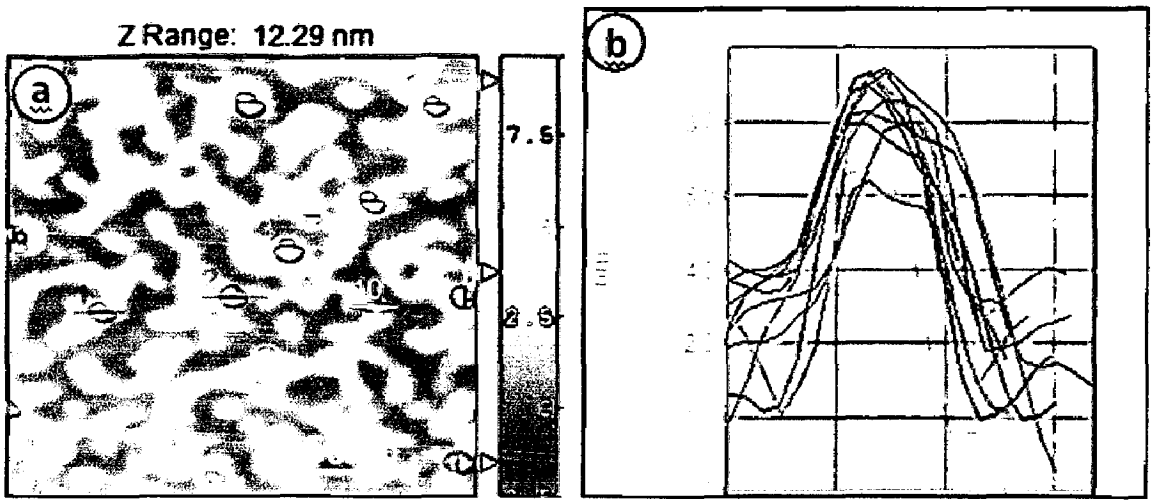


Fig -5.2.5. (a)AFM image of the polymyxin B sulphate stabilized nanoparticles. (b) Individual height of different nanoparticles

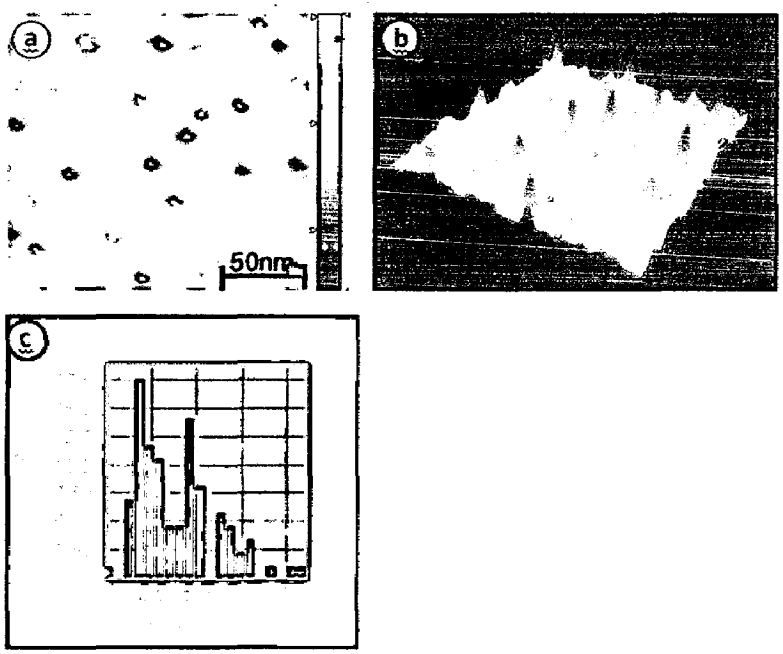


Fig-5.2.6. (a)AFM analysis. (b). 3D image. (c) Histogram showing the height of the nanoparticles

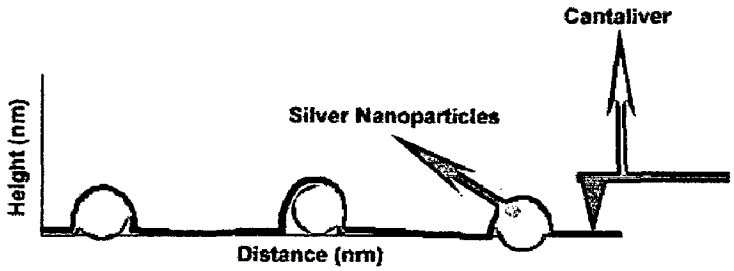


Fig- 5.2.7 Figure describing the movement of AFM tip on a glass slide with dispersed nanoparticles

5.2.5 Antimicrobial Activity:

The prepared polymyxin B sulphate capped silver nanoparticles is showing activity on both Gram-positive as well as Gram-negative bacteria. In the Fig- 5.2.8 shows the action of polymyxin B sulphate stabilized silver nanoparticles. As per the previous literature polymyxin B sulphate is itself active against Gram-negative bacteria. It has been observed that the cumulative effect of the silver nanoparticles and polymyxin B sulphate in the process of killing bacteria as the activity is observed on both Gram-positive as well as Gram-negative bacteria.

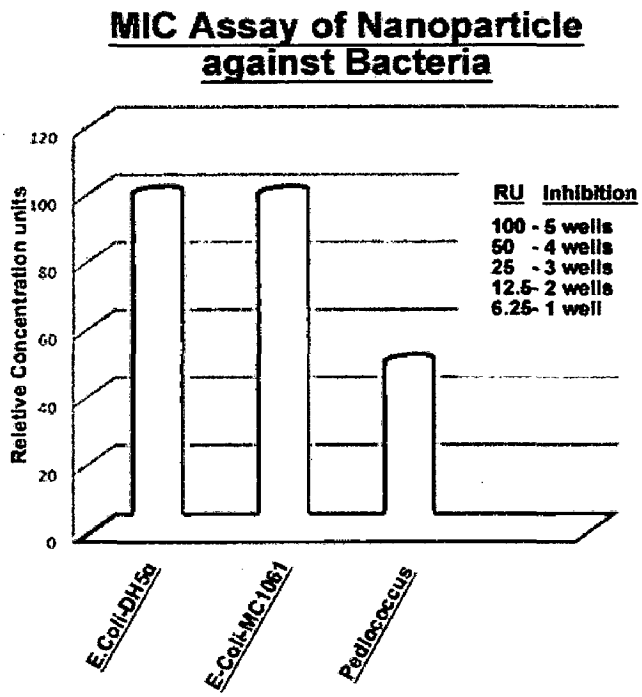


Fig-5.2.8. MIC results showing antimicrobial activity of Polymyxin B sulphate stabilized silver nanoparticles.

5.2.6 FT-IR analysis

The FT-IR studies have been made for the polymyxin B sulphate as such and polymyxin B sulphate stabilized silver nanoparticles giving a possible idea of the groups which are involved in the stabilization of silver nanoparticles before and after the action of polymyxin B sulphate. As shown in fig-5.2.9 the bonds which are disappearing are 1380cm^{-1} and 1540cm^{-1}

which corresponds to sulphate and amide vibrations respectively. Thus one can come to a possible conclusion that the amide groups and sulphate groups are involved in stabilization of the silver nanoparticles.

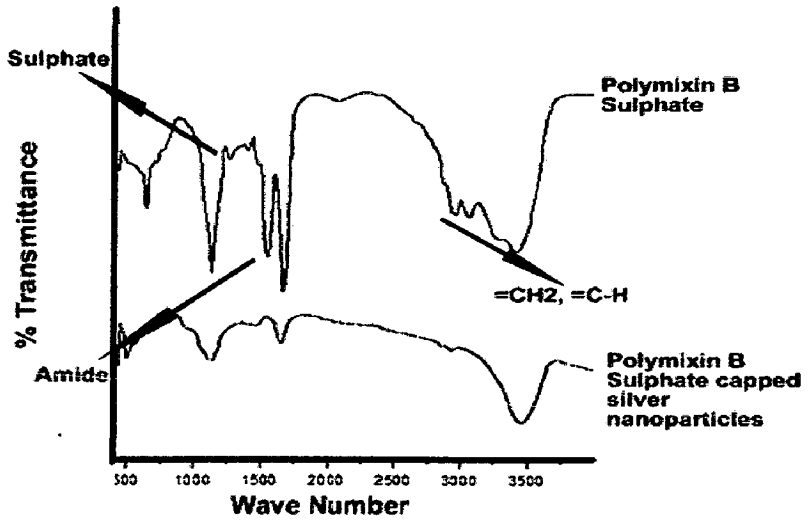


Fig-5.2.9 FT-IR results showing comparison of pure polymyxin B sulphate Vs polymyxin B sulphate stabilized silver nanoparticles.

5.3 Discussion on the polymyxin capped silver nanoparticles

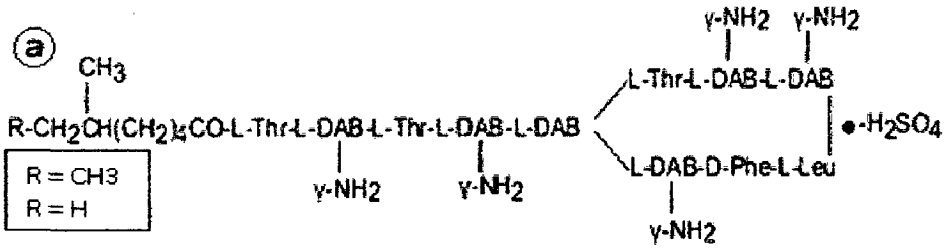
We hear by successfully synthesized silver nanoparticles by simple mixing of two different constituents in methanol medium. The hydrophilic amide groups of the polymyxin B sulphate are bound to the surface of silver nanoparticle. The hydrophobic tail of the polymyxin B sulphate surrounds the silver nanoparticle surface as a protective layer preventing the agglomeration by which the colloidal stability of the silver nanoparticles is preserved.

Several combinations produce the silver nanoparticles but by monitoring the UV-visible spectrum at 410 nm and the best proportion were found to be 1:1 mixture of 60 µg/ml polymyxin B sulphate and 2 mmol silver nitrate. There will be only single absorption band in UV-visible spectrum for spherical nanoparticles, as per the Mie's Theory. Thus the as prepared silver nanoparticles showed single absorption band which corresponds to roughly spherical nanoparticles. We had prepared silver nanoparticles by the age old process of Turkevich method and obtained fairly monodisperse silver nanoparticles of around 35 ± 5 nm and this colloid had shown only one peak at around 420 nm. The shift in the peak to 410 nm in the present one represents the effective capping action of silver nanoparticles and production of lower sized silver nanoparticles.

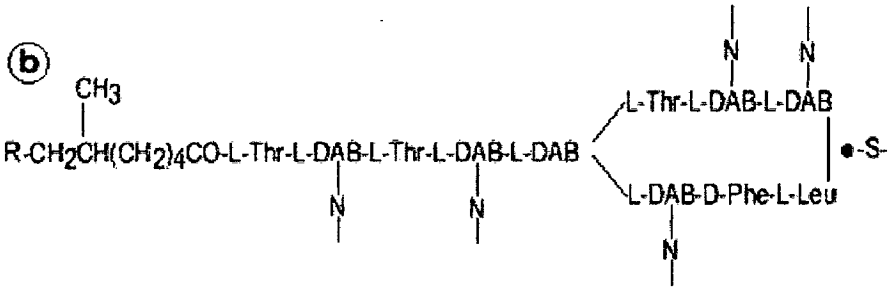
It is well known fact that the sulphide groups and amide groups usually act as active binding centres for the nanoparticle surface, controlling their shape and size. Polymyxin B sulphate being a biological antimicrobial molecule having a molecular weight of 1.3 kD proved to be a choice for the synthesis of nanoparticles as of it is having both sulphide and amide groups which act as potential candidates for the process of formation of silver nanoparticles.

As per the FT-IR studies it has been seen that there is a considerable change in the IR spectrum of pure polymyxin B sulphate to polymyxin B sulphate capped silver nanoparticles. The bands at 1380 and 1540 cm^{-1} are disappeared in the case of capped silver nanoparticles providing evidence for involvement of these groups during the formation of silver nanoparticles.

The formation mechanism in a pictorial representation has been shown in the Fig-5.3.1 which describes the actual structure of polymyxin B sulphate and its possible active form which is utilized for the capping action on silver nanoparticles. Fig 5.3.1 (a) & (b) shows the available groups present in polymyxin B sulphate that help in the surface binding on silver nanoparticles. After the described process of synthesis the predicted structure is described in Fig-5.3.1 (c). This shown the occupancy of the surface of the silver nanoparticle be polymyxin B sulphate thus confining the size by stabilizing it.



Polymyxin B Sulphate



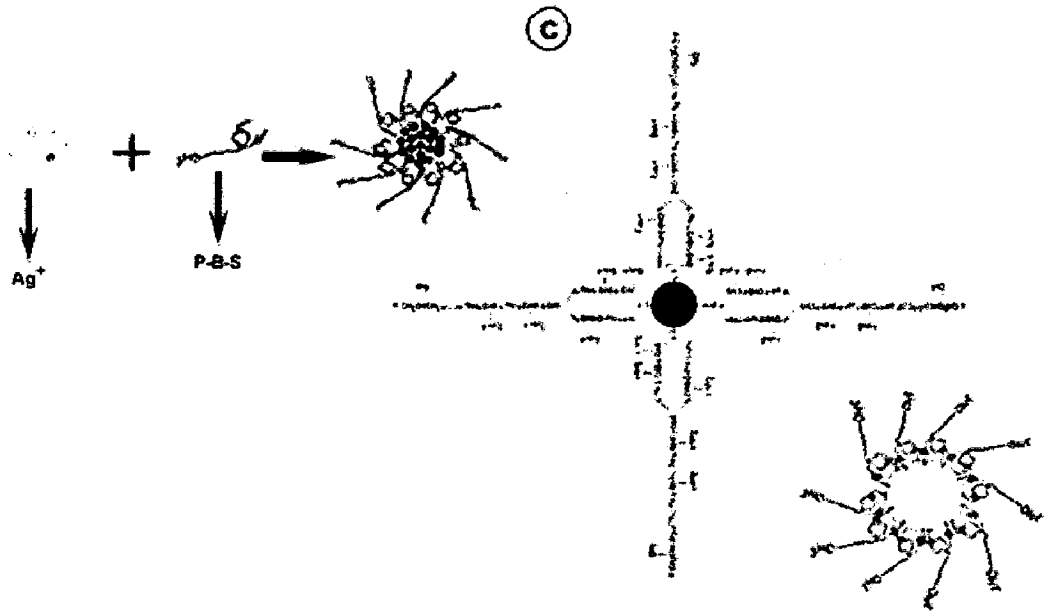


Fig-5.3.1. (a)A model of polymyxin B sulphate. (b)Reactive form of polymyxin B sulphate. (c) Polymyxin B sulphate capping action on Silver nanoparticle formation

6. Methodology, results and discussion for the synergistic effect of Antimicrobial Peptide Nisin with Silver Nanoparticles

6.1 Experimental procedures and corresponding results

6.1.1 Preparation of silver nanoparticles

50 ml Silver nitrate of 1.06 mM concentration is taken in a glass beaker. Then it is kept for boiling. As it starts boiling 1 ml of 34mM of trisodium citrate dehydrate is introduced and kept it under the same conditions for about 10 min. During this time it is observed a transition of colour from transparent to yellow- dirty yellow. The reaction is stopped and centrifuged it at a speed of 20000 rpm for about 20 min. This leads to the settlement of silver nanoparticles which as a pellet. The capping action of silver nanoparticles is shown in Fig- 6.1.4.

Now pipette out the supernatant and re-suspend the pellet in distilled water. Repeat the same for two to three times. This is done in order to remove the un-reacted components of the mixture. Preserve this by dissolving an appropriate amount of distilled water in dark conditions.

6.1.2 UV-Visible Spectral analysis

The Surface Plasmon Resonance (SPR) is observed at 412 nm (Fig-6.1.1) from the UV-visible spectrum. The sharp peak in the absorbance spectrum describes about the uniformity of the prepared nanoparticles.

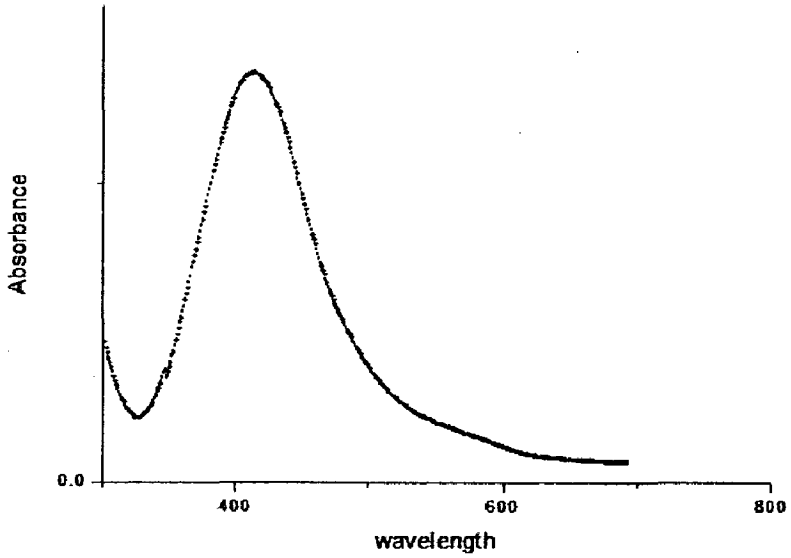


Fig-6.1.1 UV-visible spectrum of citrate capped silver nanoparticles.

6.1.3 SEM data

The Scanning electron microscopy had been carried out to check the size of the silver nanoparticles and the results show that these nanoparticles are approximately 30 ± 7 nm in diameter. The SEM and EDAX picture showing the morphology of the nanoparticles and composition is given in Fig- 6.1.2 and Fig-6.1.3.

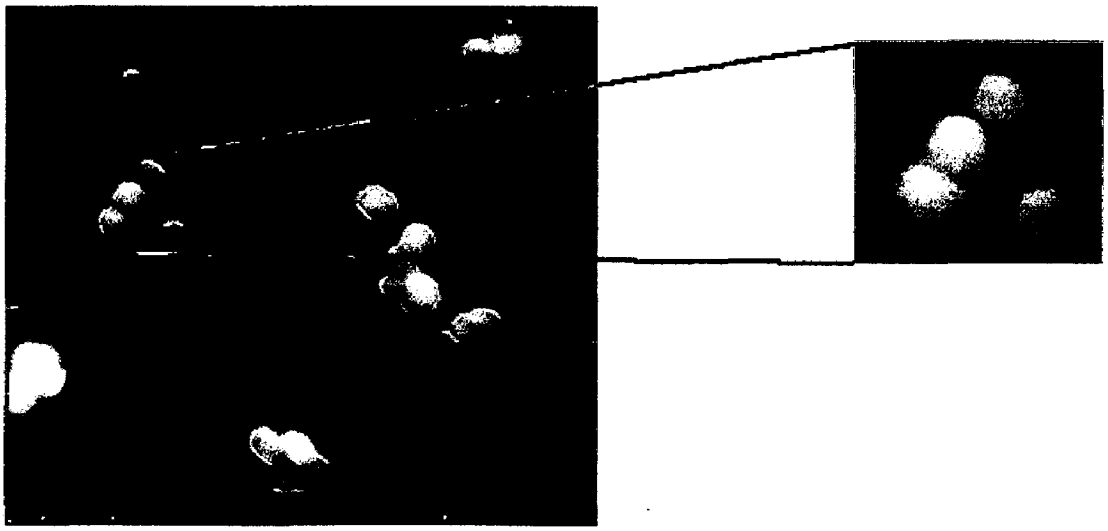


Fig-6.1.2 SEM pictures of the citrate capped silver nanoparticles.

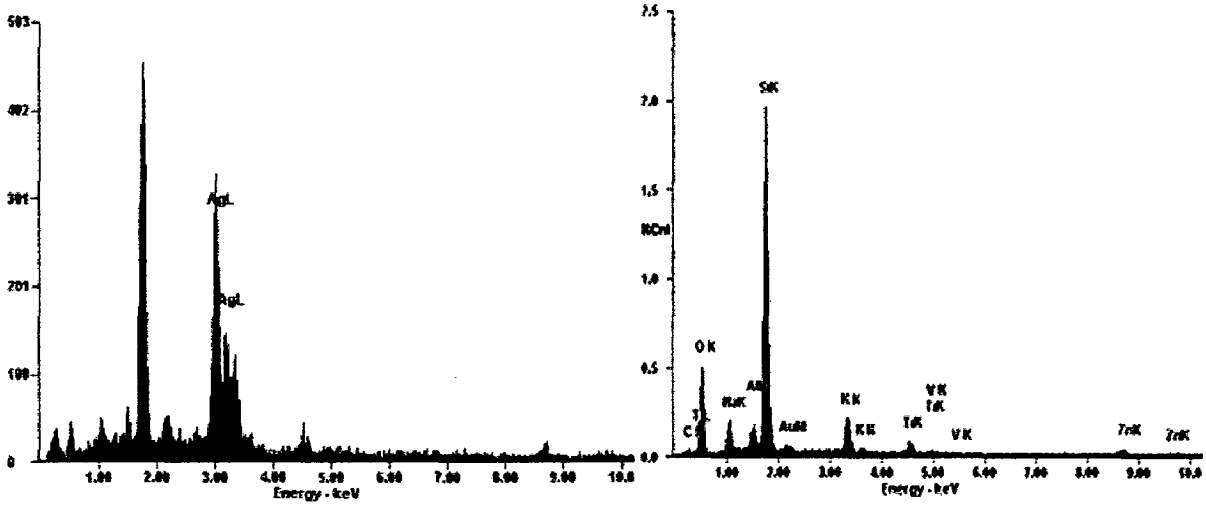


Fig-6.1.3 EDAX of silver nanoparticles and the substrate used

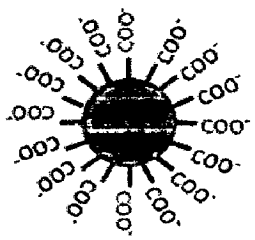


Fig-6.1.4 The pictographic representation of the silver nanoparticle

The other peaks represent the substrate (glass slide) that we have chosen to disperse the samples.

6.1.4 Zeta Potential Measurements

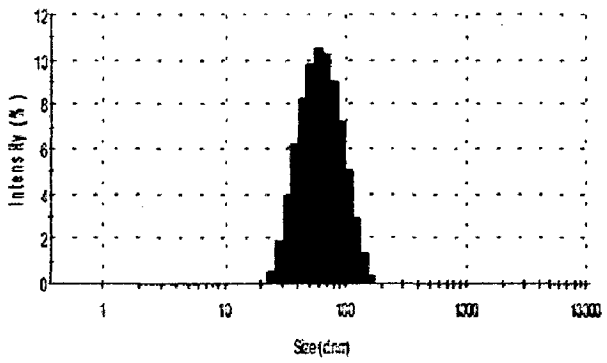


Fig-6.1.5 Size distribution of as prepared silver nanoparticles from zeta seizer measurments

The size distribution of silver nanoparticles is shown by the intensity Vs Size graph. The results shows that the size of the nanoparticles thus obtained are 40 nm as shown in Fig-6.1.5. Zeta potential values are found to be -30 mV and it shows that these nanoparticles are stable.

6.1.5 Preparation of Nisin stock obtained from Danisco

Take 70 mg of Nisin(Nisapilin) as obtained and dissolve it in 1 ml of water.

Leave this solution overnight.

The prepared solution is of concentration 70 mg/ml.

6.1.6 Optimisation of the parameters for the formation of nisin-nanoparticle mixture

The silver nanoparticle stock further used is of 26 μg/ml that is determined by Atomic Absorption Spectroscopy. 100 μl of prepared peptide solution is taken and then 100 μl of 26 μg/ml silver nanoparticle solution is taken.

Mix the both of them and then manually shake it for 5 min and then keep it for incubation at room temperature for about over night.

This leads to the binding of the peptides on the surface of silver nanoparticle.

The UV-Visible spectrum shown in the fig-6.1.1 describes that the maximum absorption of silver nanoparticles is at 412 nm.

After overnight incubation or after initial 1 hour of mixing we checked the spectrum of the solution. The peak was observed to be around 440 nm which is described in the Fig-6.1.6.

This shift in the peak is due to increase in the size of nanoparticles by the interaction of silver nanoparticles with the peptide Nisin. This is best described in the fig-6.1.7.

This is also due to the process of aggregation of nanoparticles in the presence of peptide. There are two possible hypotheses for the interaction between Nisin and a citrate-capped silver nanoparticle surface. The first is an electrostatic binding hypothesis and the second is a displacement hypothesis. The electrostatic binding hypothesis states that the attraction between the positive surface residues of nisin and the negative charge from the citrate are responsible for the strong binding of Nisin to citrate capped silver nanoparticles. In this hypothesis, the protein attaches itself to the passivating layer on the silver surface, with little direct interaction between peptide and the silver surface. The displacement hypothesis

requires citrate to be displaced by Nisin upon adsorption, with the amino acids (functional groups) lysine (amine), histidine (imidazole), and cysteine (thiol) among others that interact directly with the silver surface.

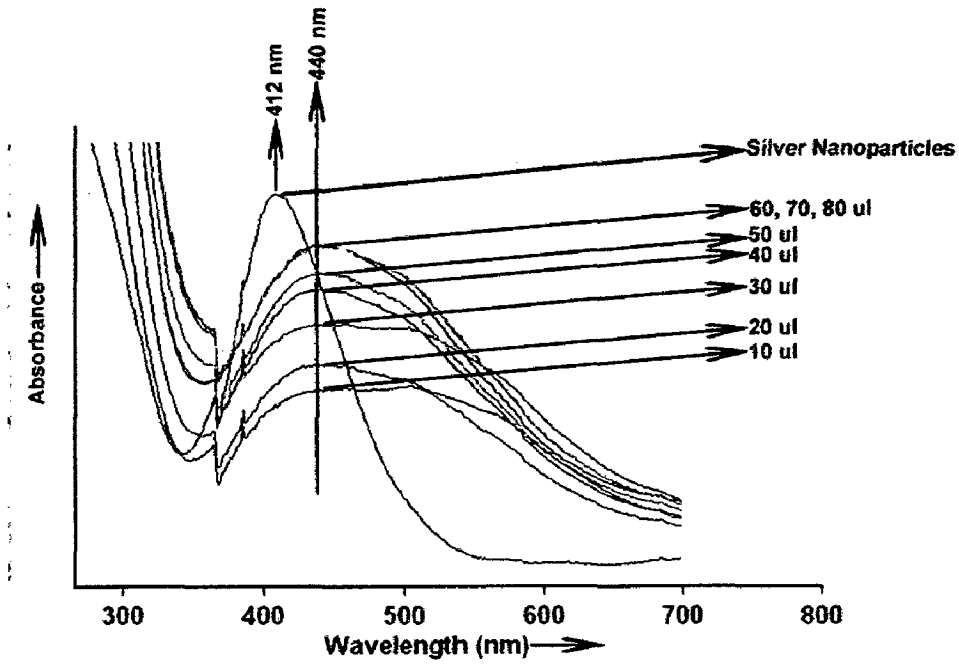


Fig-6.1.6 UV-Visible spectrum showing the shift in the SPR band of silver nanoparticles due to the presence of nisin peptide

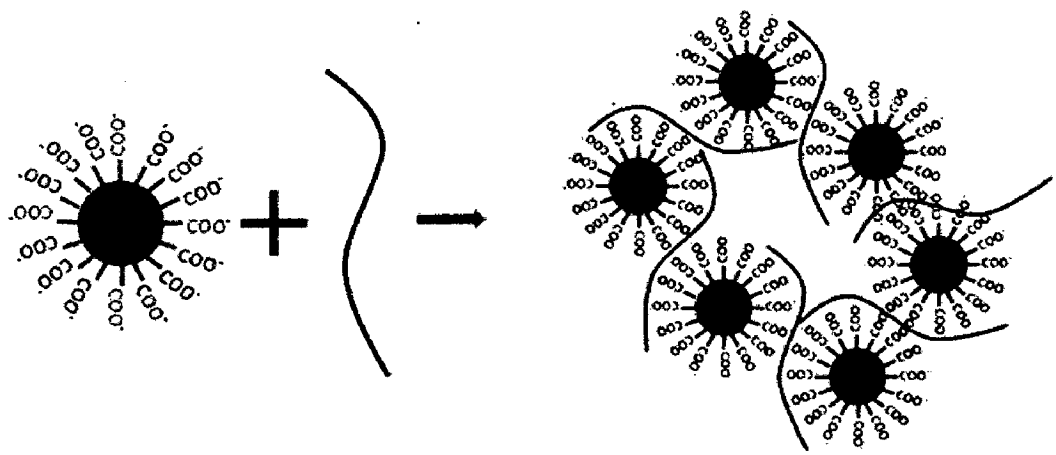


Fig-6.1.7 Pictorial representation of the arrangement of silver nanoparticle in presence of nisin peptide

6.1.7 Antimicrobial Activity

The antimicrobial assay is conducted on 96 well plates. It shows that the activity against the Gram-positive as well as Gram-negative bacteria. The activity increased after incorporation

of nisin along with silver nanoparticles. The histogram in fig-6.1.8 describes the minimum concentration of the nisin, silver nanoparticle and the mixture.

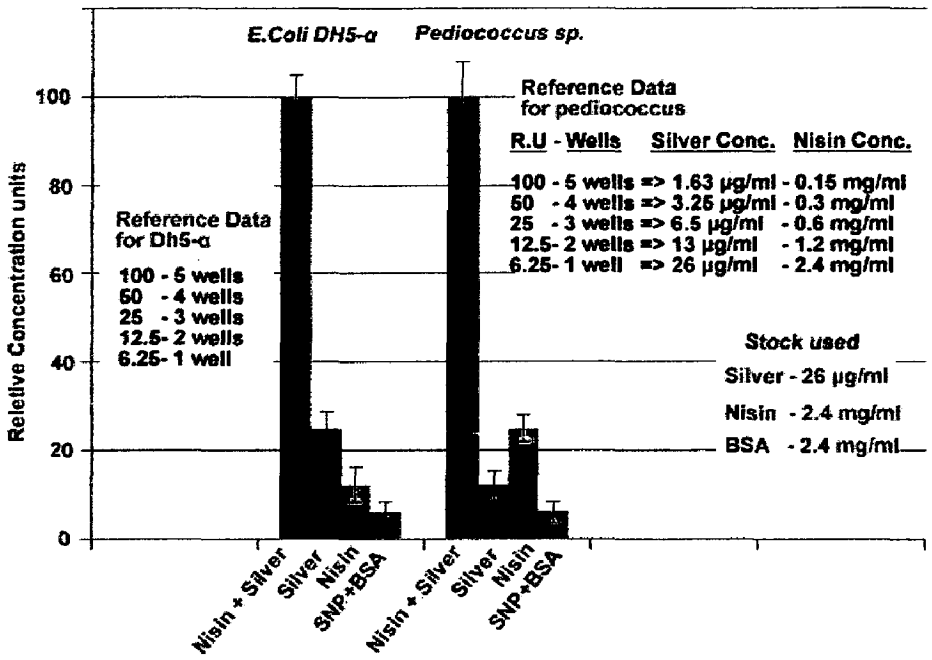


Fig-6.1.8 The antimicrobial activity of the silver nanoparticles, nisin, silver nanoparticle with nisin and silver nanoparticle with bovine serum albumin.

6.2 Discussion

Usually, when silver nanoparticles are dispersed in the LB-broth medium, most of these silver nanoparticles bound to the external surface of the bacteria either to the surface proteins or directly to the membrane as shown in the Fig 6.2.1. A few of these silver nanoparticles enter in the bacteria by utilizing the innate mechanisms of the bacteria such as endocytosis. They internalize in to the cell organelles such vacuoles, nucleolus etc.

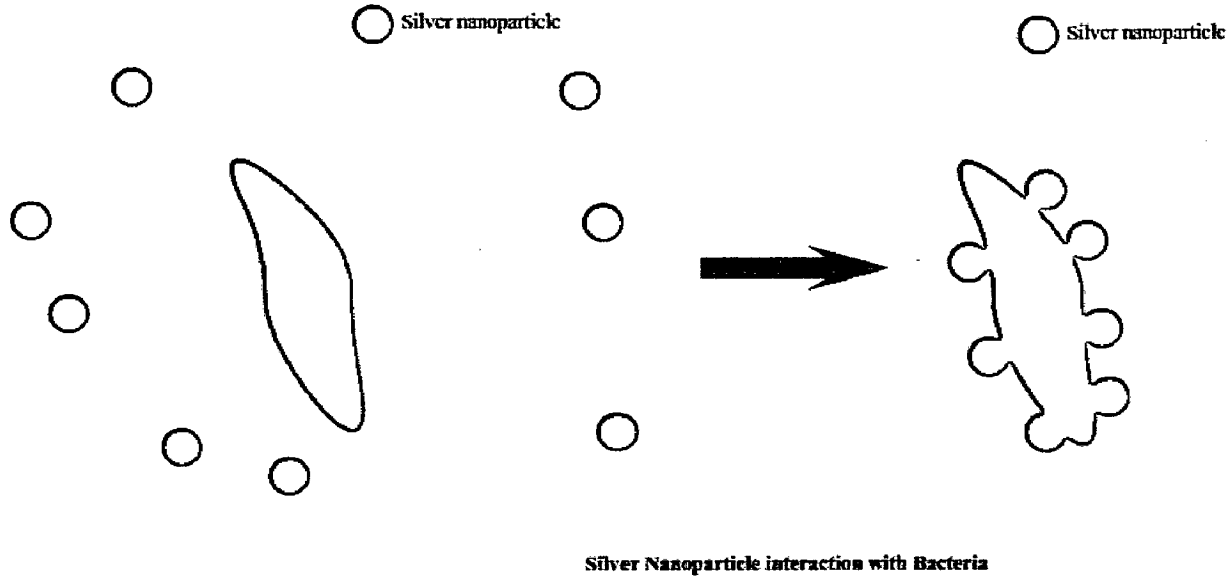


Fig-6.2.1. Interaction of nascent silver nanoparticles with bacteria

When the silver nanoparticles were act as in combination with the antimicrobial peptides the synergistic effect of both increases the bactericidal action. The antimicrobial peptide nisin shows a better affinity towards the Gram-positive bacterial cell membrane when compared to silver nanoparticles. The possible explanation of this synergistic effect of nisin with silver nanoparticle as when this aggregate encounters a Gram-positive bacteria, the nisin will leave the silver nanoparticles and start binding to the bacterial membrane and initiates the process of making pores as described in the Fig- 6.2.2.

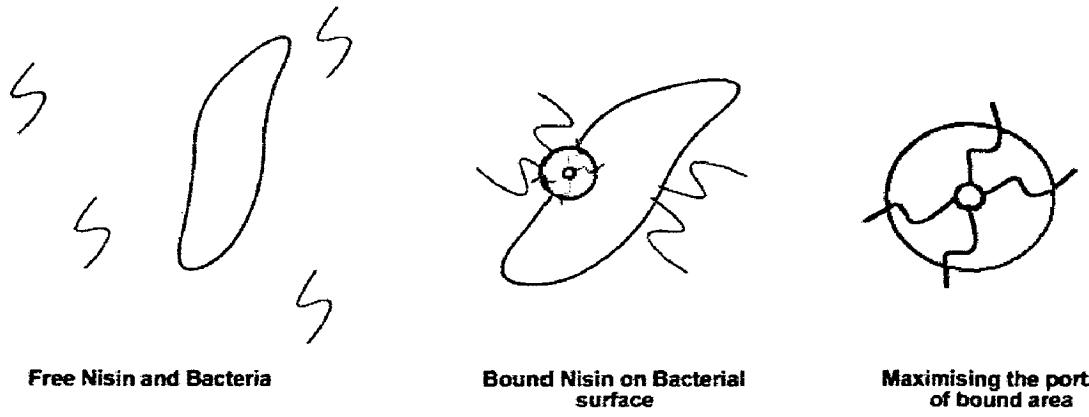


Fig-6.2.2. Different stages of Nisin binding to the bacterial surface and the maximised portion of the bound area.

The free silver nanoparticles now can invade the bacteria through the pores made by the nisin peptide (Fig-6.2.3). This lead to the death of bacteria by silver nanoparticles and antimicrobial peptide, nisin. Thus the synergistic effect of the nisin with silver nanoparticle is beneficial to decrease the Minimum Inhibitory Concentration.

In another case, these silver nanoparticles are effective against the Gram-negative bacteria too. So the nisin in combination with silver nanoparticles did show a better activity against the Gram-negative bacteria too. But the mechanism behind this mode of action is still under investigation.

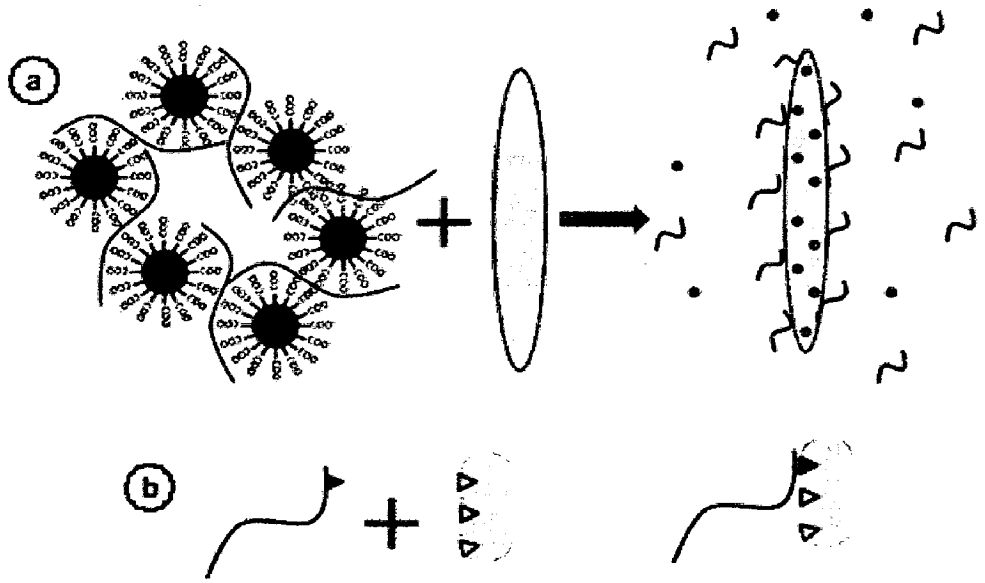


Fig-6.2.3 Pictorial description of the combined effect of the Protein nanoparticles conjugates on bacteria. (B). Specificity of the peptide towards the bacteria

7. Conclusions

In conclusion the approach towards synthesising silver nanoparticles by using antibiotic peptide polymyxin B sulphate has been successful. It had shown that the polymyxin B sulphate alone is active against Gram-negative bacteria where as silver nanoparticle capped with polymyxin B sulphate is showing inhibitory action on both Gram-positive as well as Gram-negative bacteria. Thus the procedure was successful in making polymyxin B sulphate to act on both Gram-positive and Gram-negative bacteria.

In the second approach investigation of the synergistic effect of antimicrobial peptide Nisin in combination with silver nanoparticles was successful and this showed decrease in the minimum inhibitory concentrations when compared to nisin as well as silver nanoparticles individually. And here it is showed that nisin which acts on only Gram-positive bacteria and the combination of nisin with silver nanoparticles showed activity on both Gram-positive as well as Gram-negative bacteria. Thus the results show that the food additive, nisin in combination with silver nanoparticle was having bactericidal activity on both Gram-positive as well as Gram-negative bacteria.

8. Future Work to be done

In-depth studies on the mode of action of polymyxin B sulphate capped silver nanoparticles on bacterial species.

Conformational studies of Nisin-immobilization on nanoparticles.

Investigation on mechanism of action of nisin immobilized silver nanoparticles.

Optimizing the parameters for the large scale synthesis of polymyxing B sulphate capped silver nanoparticles.

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