Synthesis and Characterization of the Ciprofloxacin Loaded Gold Nanoparticles

Submitted by

Afrah Nawaz

00000170772

Supervisor

Dr. Nosheen Fatima Rana

DEPARTMENT OF BIOMECIAL ENGINEERING AND SCIENCES

SCHOOL OF MECHANICAL & MANUFACTURING ENGINEERING

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY

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Author

Afrah Nawaz

Regn Number

00000170772

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Thesis Supervisor:

Dr. Nosheen Fatima Rana

Thesis Supervisor's Signature:_____

DEPARTMENT SCHOOL OF BIOMECIAL ENGINEERING AND SCIENCES

SCHOOL OF MECHANICAL & MANUFACTURING ENGINEERING

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY,

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1. Name: Dr.Umar Ansari Signature:_____

2. Name: Dr.Faheem Amin Signature:_____

3. Name: Dr.Nasir Mehmood Signature:_____ _____

Supervisor's name: Dr.Nosheen Fatima Rana Signature:_____

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ABSTRACT

Background:

Enterococcus Fecalis is a very well known pathogen that causes nosocomical infections. It is also known for its multidrug resistance. Similarly, the Escherchia coli is a known bacteria of human intestine. In normal conditions, the strains of E.coli are harmless but some harmful strains can cause severe food poisoning to the humans. The aim of this research is to evaluate the effect of an antibiotic Ciprofloxacin and its gold nanoparticles on these bacterias.
Ciprofloxacin is the antibiotic that is being used against the infections caused by various Gram positive and Gram negative bacteria. The gold nanoparticles are synthesized by the chemical reduction method and then capped by the ciprofloxacin to make it functional for use. The invitro hemolytic activity of the ciprofloxacin loaded gold nanoparticles was checked after the synthesis.

Results:

The ciprofloxacin loaded gold nanoparticles were characterized using different techniques. The size and shape was determined by the SEM and UV-Vis was used to check the synthesis. The drug loaded particles were found stable and it showed enhanced activity than the parent drug on the bacterial colonization.

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List of Acronyms

AuNPs Gold Nanoparticles

SEM Scanning Electron Microscopy

EDX Energy Dispersive X-ray Spectroscopy

Cip Ciprofloxacin

E.Coli Escherchia Coli

E.Fecalis Entercoccus Fecalis

CHAPTER 1: INTRODUCTION

The word nano has a Greek origin which means small in size. This word is being used as a prefix for the particles that ange from 9 to 1oth of a billion. The nanoparticles are defined as the particles that have more than two dimensions and range in size from 1 to 100nm(ASTM International) [1]. The nanoparticles are widely being used in different applications such as the nanomedicine, electrochemistry, inorganic chemistry, imaging, physics and biomedical sciences. But they are mostly of high benefit in the field of inorganic chemistry and nanomedicine because they can be used for the purpose of diagnosis and therapy. Currently, nanoparticles are mostly used for the drug delivery and the therapeutic purposes [2].

Nanotechnology is the branch of science that does not came into existence few decades back. It is as old as ancient civilizations of Romans and Europeans. The Romans used different concentrations of gold and silver to make the colored glasses into red, yellow and mauve (Daniel and Astruc 2004). The British museum has the famous cup of Lycurgus that was made by using the gold and silver nanoparticles (Barber and Freestone 1990). The uniqueness of that cup is the dual behavior that occurs when the cup reflects an transmits the light. The cup shows two colors of red wine an opaque pea greens and this phenomena happens because of the surface Plasmon resonance of the gold particles on the glass surface and also because of the ilver nanoparticles on the glass matrix (Barber and Freestone 1990; Louis and Pluchery 2012).

In the middle ages, the European cathedrals used windows that were colored red and purple due to the gold nanoparticles that were deposited on the glass matrix such as in the Notre Dame. The Islamic world is famous for its chemical reduction techniques that they used back in 19th century to reduce the metal oxides into the nanoparticles upon high temperature (Padeletti and Fermo 2003; Daniel and Astruc 2004).

The 15th and 17th century is known for the refined techniques of glass forming in which precipitation was used to deposit the colloidal gold to the glass(Daniel and Astruc 2004). Michael faraday is known as the finest chemist who was the first to introduce the chemical synthesis of metal nanopartilces in 1857. He used chloroauric acid solution and reduced it with carbon disulfide, red colored solution was obtained which was named as ruby fluids.

Another method was introduced by the Zsigmondy in 1906 and used the method to obtain the mono dispersed gold nanoparticles (Overbeek 1984). This method was further improved and

improvised by another scientist Turkevich (Turkevich, Stevenson et al. 1951). In this method, citrate was used to reduce the chloroauric acid and gold nanoarticles were synthesized. Same method was used to synthesize the silver nanoparticles

In 1618, first book on the colloidal gold was published by the famous doctor and philosopher Francisco Antonii. This book contain the knowledge about how to synthesize the gold nanoparticles and its various applications in different fields of medicine and therapeutics.Thou the history of nanotechnology and nanopaticles is not very new but the main discoveries and developments occurred in past few decades. The new methods were made and optimized in past few years that are playing vital role n revolutionizing this domain.

A method was developed by the British researchers Faulk and Taylor to conjugate the antibody with the gold nanoparticles for the purpose of antigen visualization.

A Noble laureate, Richard Feynman, in December,1959 gave the concept of nanotechnology ia lecture at California Institute of Technology for the very first time. In 1970, Japanese researcher Norio Taniguchi, (Corbett, McKeown et al. 2000), gave definition of nanotechnology as "Nanotechnology majorly consist of processing of, seperation of, deformation and consolidating materials by atom or by molecules". A successful implementation of the application of gold nanoparticles was seen in 1997, when a patient of rheumatoid Arthritis was treated. The reports containing the data on the clinical trials were published in 2008.

Out of all the different kinds of nanoparticles, gold nanoparticles are highly in demand because of their unique and beneficial properties. The gold nanoparticles show high surface plasmon property because of the large surface area and is more conducting than silver [3]. Their nanometer size makes it possible to interact easily and efficiently with the living cells [4]. The unique properties of the gold nanoparticles increase their rententiont time in the tissue and cells thus making them beneficial for the therapeutic purposes. Every drug delivery system is dependent on the carriers that will the target the specific sites where the traditional methods of treatment cannot reach as efficiently as the nanopaticles [5].

The use of gold in the synthesis of nanoparticles is In practice due to their inert nature and least toxicity as the dug delivering agents [6]. Different types of nanocarriers are there such as the nanorods, nanostars, dendrimers, quantum dots, polymer gels etc. all of them have wide applications in many domains like imaging, medicine, mechanics, ultrasound, PET, CT scan, MRI etc [7].Different techniques are used to synthesize Gold nanoparticles [8]. The gold

nanoparticles can be prepared by the green synthesis or the chemical reduction method. The chemical reduction methods include many procedures but the most appropriate and optimized is that of Michael Faraday's reduction method. In this method he utilized the citrate to reduce the chloroauric acid into gold nanoparticles, all this procedure takes place in the water. The gold nanopaticles have numerous applications including the microbial and cancer therapeutics, molecular imaging etc [9].

The gold nanoparticles can be fabricated into different sizes ranging from 1nm to150nm depending upon the size that is required for the activity [10]. Their tremendous structural designs make their surface properties unique thus enable them to allow the interaction of various targeting agents either though capping, conjugation, adsorption or loading on the surface. Apart from that, these particles are non toxic to human cells and tissues and show great biocompatibility [11].The transport of therapeutic compounds by the gold nanoparticles as the drug delivery system can be controlled by operating the physical, optical and chemical parameters of the gold nanoparticles [12]. The gold nanoparticles have fascinating properties tht play important role in thei interactions [13-14]. The size of the gold nanoparticles ranges from 1nm to 8um. They come in various shapes such as nanostars, nanorods, octahedral, spherical rings, tetrahedral and decahedrals etc.

The reason for gold nanopartcles to be preferred by the chemist is because of their optical properties and compatibility. Being a good therapeutic and diagnostic agent, they are transported easily to the diseased cell [15]. Due to small size, they are preferably introduced in the cells and tissues of the host. They can be used as the electrode sensors because of the electronic properties [16]. The nanopaticles can be characterized using different techniques. This is done in order to find out the nature of the nano material. The characterization can be done using Scanning electron microscopy (SEM), Transmission electron microscopy (TEM), Uv-Vis spectroscopy, X-rays, Fourier transform infrared spectroscopy (FTIR) and X rays diffractometry (XRD) [17].

By changing the bio distribution, the nanoparticles enhance the drug efficiency an also reduce the nonspecific toxicity of the antimicrobial drug because it is strongly compatible and it provides the protection to the nucleic acid from degradation. They can effectively deliver the drug in vivo also making them the most efficient drug delivery system. Nanoparticles: (As efficient drug delivery vehicles)

The innovation in nantechnology has made a great impact on the clinical therapeutics in the past

few decades (Hu, Aryal et al. 2010). Alotof work has been done to make the significant improvement and modifications in the field of nanomedicine in microbial studies for the purpose of diagnosis, detection and the treatment of microbial infectons. The field of nanomedicine has gained much success because of its capability to increase the drug compatibility and bio availability. The ability to cross the blood brain barrier, efficiently transport the hydrophilic and hydrophobic drugs and specifically targeting the infectious sites (Babu, Templeton et al. 2013).

The use of nanocarrier is done enormously because of their small size, unique shapes and inert nature. They are non cytotoxic to the human cells and have high biocompatibility as compared to the parent drug.Nanoparticles have very specific size related properties which provide endless chances and options for various shocking discoveries. The nanopaticles most of the time produces unexpected results and are source of great unconventional and unusual applications. The nanopaticles also occur in nature and are foun in many natural products such as the organic compounds and inorganic compounds. The organic compounds include polysaccharides, proteins and viruses while the inorganic compounds include alumina,silicates, gold, magnesium, copper etc.

Due to diversity in the nanoparticles and unique properties, they are of great interest to be used in the biological systems. The nanoparticles because of their small size have always been closely related to the bio molecules due to similarity in size. This fact makes them good option to be utilized in the hybrid systems. Now a day, scientists are using various methods to combine the functionalities of the living molecules and other organic molecules that are than incorporated in the nanostructures. The gold nanoparticles can be prepared by the green synthesis or the chemical reduction method. The chemical reduction methods include many procedures but the most appropriate and optimized is that of Michael Faraday's reduction method. In this method he utilized the citrate to reduce the chloroauric acid into gold nanoparticles, all this procedure takes place in the water. The nanopartices are widely being used in the biomedical applications where biologically active molecules are interacted with the nanoparticles. The nanoparticles can be capped with the ciprofloxacin Hcl to make it functionalized. This complex was seen to have a good anti bacterial activity as compared to the parent drug. The gold solution is reduced by chemical method to synthesize the drug loaded nanoparticles.

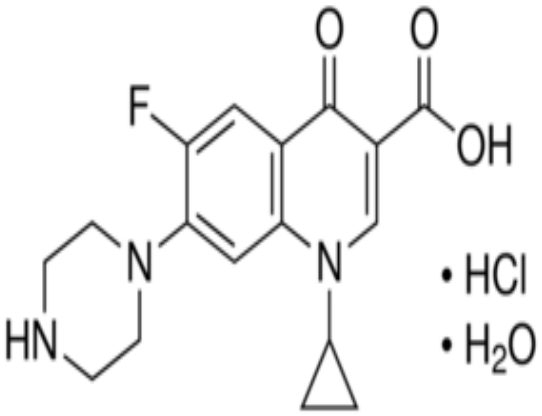

Figure 1.1 Structure of Ciprofloxacin Hcl

Ciprofloxacin is a well known antibiotic that is used to treat a huge number of bacterial infections. The infections could be off joints or bones, abdomen or respiratory tract, skin infection or urinary tract infection. This antibiotic is approved by the World Health Organization to be the safest and most effective antibiotic for the mankind against microbial diseases. The reason we chose ciprofloxacin is because it is from the class of fluoroquinolones. These drugs are fluorescent and can be probed using different techniques even with the low concentration of the drug. Fluoroquinolones such as ciprofloxacin [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-piperazinylquinolone-3-carboxylic acid], clinofloxacin, sparofloxacin, levofloxacin, and norfloxacin are antibacterial agents that are being used against several microbial infections.

Only limited information is available on the nature of binding of the ciprofloxacin with gold nanoparticles. As far as the mode of action is concerned, the fluoroquinolones are well known anti bacterial agents that target two important enzymes of bacteria. One is the DNA topoisomerase IV and the other is the DNA Gyrase. The DNA gyrase is responsible for introducing the supercoiing in the DNA of bacteria. On the other hand, DNA topoisomerase IV causes the recognition of the crossovers in DNA and therefore is a decatenating enzyme. Both the enzymes are essential for the division and survival of the bacteria.

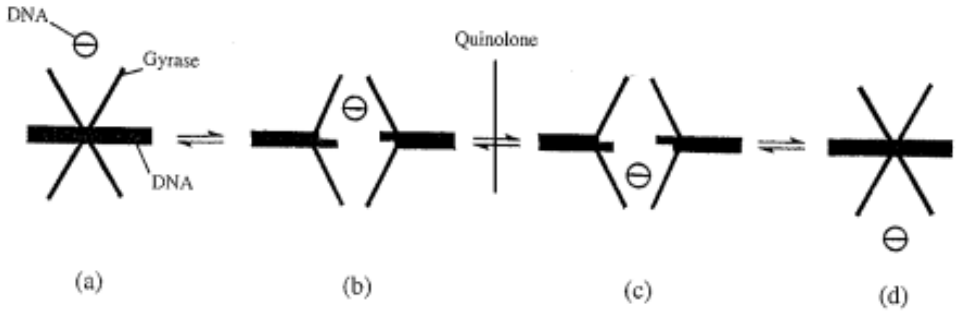

Figure 1.2 Pathway of DNA Gyrase in DNA molecule

Enterococcus fecalis is a gram negative bacterium that resides in the gastrointestinal tract of the humans and mammals. This bacterium is usually harmful but can also be a great threat as it is resistant to a braod range of antibiotics such as macrolides, clindamycin, chloramphenicol, tetracyclin, trimethoprim sulfamethoxazole, and penicillin, , rifampin, cephalosporins, nalidixic acidaminoglycosides, vancomycin (a drug of last hope for multi resistant enterococci) respectively. Due to the drug resistance, its pathogenicity is increased n it can cause many nosocomical infections such as urinary tract infection, bacteremia, surgical wound infection and endocarditis. It can also cause infection in the root canals of teeth.The mutations or the genetic material exchange in this bacterium is responsible for its multidrug resistance. All this situation make it a challenge in this domain to overcome all these issues which can be one by (1) using the approach to develop a drug conjugate with nanoparticles (2) synthesizing a novel formulation that can not only help in the inhibition of the bacterium growth but also be beneficial and safe to the cells and tissues rather than causing damage to living cells and tissues.

For this purpose, drug conjugates with gold nanoparticles play an important role in the inhibition of the bacterial growth. This method enhances the effectiveness of the parent drug against certain bacterium. It also helps in reducing the dosage of the drug because the retention time of the drug is increased. In this way , it can target the inhibition of energy metabolism in bacterium, breakdown of cytoplasmic membrane and inhibition of nucleic acids in the cells of bacterium.

1.1 Phase 1:

Synthesis, optimization and characterization of the ciprofloxacin loaded gold nanoparticles.

The research study is divided into two phases, in the first phase our target is to inhibit the

bacterial colonization and for that purpose we will do the seed formation of gold nanoparticles . the ciprofloxacin which is our required drug will be loaded to the nanoparticles by a one step method.

1.2 Phase 2:

Effect of the ciprofloxacin loaded gold loaded gold nanoparticles on E.Fecalis colonization:

In the second phase, the effect of the formulation will be checked against the gram negative and gram positive bacteria that cause many infections in the humans and mammals. The gram positive bacterium includes the Enterococcus Fecalis whereas the gram negative bacterium includes the Escherchia Coli. To check the potential of the ciprofloxacin coated gold nanoparticles , the nanoparticles are investigated through different parameters such as the salt effect, temperature, pH and stability check.

The invitro antibacterial activity is done to check the potential of nanoparticles as compared to the parent drug. Less hemolysis was observed in the drug loaded gold nanopaticles and was found more biocompatible. The amount of effective dose required for the inhibition of the bacterium was also reduced. The inhibition zones were analyzed for both the bacteria.

Aim:

The aim of this study is to synthesize the gold nanoparticles loaded with drug and check its effect on the bacterial colonization of E.Fecalis and E.Coli.

Objectives:

- Synthesis and optimization of the gold nanoparticles.
- Drug loading.
- Characterization of the drug loaded gold nanoparticles.
- Effect of drug loaded gold nanoparticles on the E.Fecalis and E.Coli.

CHAPTER 2: LITERATURE REVIEW

In metal nanoparticle synthesis, the most essential and challenging task has been control of shape and size as these are major considerations in deciding the surface and catalytic activity of the nanoparticles. (Nikoobakht, B., & El-Sayed, M. A. (2003)). A few properties of gold nanoparticles make them a great vehicle for drug delivery. They can be fabricated to have a range of sizes: from 1 nm to 150 nm. Various targeting agents can be coated on their surfaces. Moreover, these essential properties are biocompatible and non-toxic.They also have great chemical, physical and optical properties. These properties can be used through innovative approaches to control pharmaceutical compound transport. Citrate reduction technique is used for colloidal gold preparation. Gold nano-rods, silica - gold nano-shells and empty gold NPs are a few examples of the various structures gold nanoparticles can be fabricated into. Noble metal nanoparticles are distinct from different nano-platforms like semiconductor quantum dots, magnetic nanoparticles and polymeric nanoparticles by their single surface plasmon resonance (SPR), which has a small particle size, enhances all the radiative and irradiative properties of the nanoparticles (Alaqad, K., & Saleh, T. A. (2016).

The high surface-to-volume ratio and the increased number of atoms at the grain boundaries give nanoparticles their uniqueness. They turned out to be noteworthy materials in the progression of different novel devices that are utilized in various biological, physical, pharmaceutical and biomedical applications. The phenomenal usefulness of NPs is for the most part in view of their size. Among NPs, gold nanoparticles are broadly utilized as a catalyst for medicinal treatment, gene therapy, and diagnostic purposes.

The principle preferred standpoint of gold NPs is that they are anything but difficult to synthesize by chemical reduction method and they have low toxicity in contrast to different nanomaterials. To improve applications of nanoparticles, different techniques have been used for synthesis for various dimensions and to functionalize their surface. The principle challenges in creating distinctive techniques are their high purity and low polydispersity. With a specific end goal to control the size and shape of NPs, different reducing agents, stabilizers, and solvents have been used in the synthesis of NPs. The effect of different stabilizers on particle size and morphology has also been considered (Shamaila, S., Zafar, N., Riaz, S., Sharif, R., Nazir, J., & Naseem, S. (2016).

The gold is being investigated as for its antibacterial and antifungal properties. It has been used both as nanoparticles as well as ionic states for the checking of its activities against bacterial strains and fungal strains. The gold complexes such as Au (I) and Au (III) both have been found to exhibit some antibacterial activity. On the other hand, gold has shown its antifungal properties as well. But over all research suggest gold to be not really bactericidal. Or if shows some activity than it is very weak and must be at higher concentrations (Zhang, Y., Shareena Dasari, T. P., Deng, H., & Yu, H. (2015). There could be a reason for gold nanoparticles that they appear to have antibacterial activity. This might be because of any chemicals that are left in the nanoparticles after the synthesis and are not properly removed after washing of the gold nanoparticles. This might be the chemicals present in the nanoparticles coating or in the gold ions. But similarly, gold nanoparticles can be very efficient carriers of antibiotics and other drugs. In this way, they play an important role in enhancing the antibacterial activity of many drugs and antibiotics meanwhile reducing the effective dose and also the side effects that are usually caused by the high dose of drug (Zhang, Y., Shareena Dasari, T. P., Deng, H., & Yu, H. (2015).

Although gold nanoparticles have many unique properties in common, but one of it is surface Plasmon peak which is very important parameter. This property is characteristics of those particles that have very small size almost close to the wavelength of light. It is a physical concept that tells about the oscillations occurring because of the electrons Biocompatibility can be increased and cytotoxicty can be reduced by the coating and capping of the gold nanoparticles. The capping reduces the toxic effect of the bare nanoparticles that can be harmful if administered without any coating (Matulionyte, M., Dapkute, D., Budenaite, L., Jarockyte, G., & Rotomskis, R. (2017).

The capping of the nanoparticles or coating can be of great benefit because it can increase the retention time of the desired nanoformulation in the cells and tissues thus enhancing the effect of that particular drug or formulation. The synthesis can be done along with many vectors such as viral and non viral vectors. Many cationic vectors are also been used such as polyethyleneimine (PEI), polypropyeneimine etc (Teimouri, M., Nia, A. H., Abnous, K., Eshghi, H., & Ramezani, M. (2016).

CHAPTER 3:

3.1 Materials and Methods

Experimental

3.2 Material and Instruments

Chloroauric acid ($HAuCl_4$), sodium borohydride ($NaBH_4$), sodium citrate tribasic dehydrate ($C_6H_5O_7{\bullet}_2H_2O{\bullet}_3Na$) ,sodium chloride, 2-propanol, ethanol, nutrient agar, Luria bertani, phosphate buffer saline (PBS), Triton X-100, sodium hydroxide, sulphuric acid were purchased from Sigma Aldrich. Ciprofloxacin HCl ($C_{17}H_{19}ClFN_3O_3$) was used as a drug. A digital pH meter model 510 (Oakton, Eutech) equipped with a glass working electrode and a reference Ag/AgCl electrode was used. Ultrapure distilled water was used throughout the experiments. Shimatzu UV-Vis 1800 spectrophotometer was used to record the spectra.

3.3 Synthesis of Gold nanoparticles:

The procedure used for the synthesis was followed by the Turkevich method. 25ml of 375ul gold salt solution was prepared in the deionized water. The gold solution was stirred on water bath for 30 minutes at 4^0C. Crushed ice was used to reduce the temperature to 4^0C. When the temperature was maintained to 4^0C, the 0.5ml solution of 25mM sodium citrate tribasic dehydrates was added to it. The solution was than stirred for two hours and temperature was maintained at the 4^0C. After two hours of stirring the 1.5ml of 2.64mM sodium borohydride was added to the solution. The stirring was further continued for four hours at 4^0C. The change in the color of the solution was seen. The color changed to red wine which indicates the synthesis of the gold nanoparticles.

The solution was characterized by UV-vis spectroscopy. The UV spectra of the nanoparticles can be seen in the figure. The UV Vis spectrum of the gold nanoparticles was recorded at 520nm. The solution was diluted while taking the UV vis spectrum.

3.3 Drug loading:

The same stock solution of the gold nanoparticles was taken for the drug loading. The stock solution will act as gold seeds. The ciprofloxacin hcl was used as drug. 5ml of 1mM solution of ciprofloxacin was prepared in distil water. The ciprofloxacin solution can also be prepared in the organic solvents such as methanol and 2 –propanol. The solution was vortexed to dissolve the drug completely. The pH of the solution was maintained at 9.2 as the ciprofloxacin hcl only work in the basic conditions.

50ml of the stock solution (gold seeds) were taken and placed on the dry shaker. 1000µl of the ciprofloxacin hcl solution was added to the nanoparticle solution with constant stirring. The solution will start changing the color from wine red to purple. Soon the color is further change to bluish purple and then to blue leading to the aggregation. The solution of drug loaded gold nanoparticles was than characterized by the UV vis spectroscopy. A peak shift was observed in the spectrum of the drug loaded gold nanoparticles as compared to the gold nanoparticles.

Invitro stability studies for the Ciprofloxacin loaded gold nanoparticles

To check the stability of the ciprofloxacin loaded gold nanoparticles, different tests were performed to check certain factors (1) Salt effect (2) ph test (3) heat test (4) solution stability test.

3.4 Salt Effect

In this test, the effect of different concentrations of sodium chloride (NaCl) was studied. Three concentrations of NaCl were prepared i-e 2M, 3M, and 4M. The 2ml of ciprofloxacin gold nanoparticle solution was taken in the beaker and 2ml of NaCl solution was added to the beaker. Same protocol was performed for the other concentrations as well. The solution was kept for 24 hours at 37^0C. After 24 hours, the UV vis spectra of the solutions was recorded.

3.5 Heat effect

Effect of heat on the drug loaded gold nanoparticles was also observed. For this purpose, 10 ml of the ciprofloxacin loaded gold nanoparticles were taken in a flask and heated on 30^0C for 3o

mints. Same protocol was repeated at the temperature of 40^0C, 60^0C and 100^0C. Than the UV vis spectra of the different solutions was recorded.

3.6 PH test

This test was performed to observe the effect of pH on the ciprofloxacin loaded gold nanoparticles. The test was performed on three different PHs i-e 3.3, 8.5 and 9.2. Than the UV vis spectra was recorded.

3.7 Characterization of Ciprofloxacin Loaded Gold nanoparticles (AuNPs-Cip)

Ultraviolet Visible Spectroscopy (UV-Vis)

Gold nanoparticles absorbance was recorded using the UV vis spectroscopy. The device use for this purpose was UV-2800 BMS Scientific Technical Corporation (PVT) Ltd. The UV vis spectroscopy is a technique that is used widely in clinical, biochemical and chemical studies. It works on the principle of reflected beam of light. The extent of absorption is measured when a beam of light falls on the sample and then the reflected light will give the absorbance which is measured by the light sensor. A glass cuvette containing the sample is placed in front of the lens and the beam of light will pass through it. The beam is split into two parts, one half passes through the cuvette containing the sample and the other half passes through the cuvette that contains the solvent only which acts as a reference.

In this device, the absorption can be measured at the desired wavelength and the range can also be changed accordingly and can be adjusted as required. The plotted graph gives the wavelength on x-axis and absorbance on the y-axis. The maximum absorption which is observed at a certain specific wavelength is called as the lambda max. Lambda max obeys the Beer Lambert Law. According to this law it measures the electronic transition of molecules. The molar concentration of the sample is proportional to the absorbance value of the sample. We call the absorption value as the molar absorptivity. Beer Lambert Law states that

$$A=EcL$$

In this equation, E is the molar absoptivity, A is the absorbance, c is the sample concentration in moles/litre, L is the length of the light path through the cuvette in cm. so

$$E=A/cL$$

Due to this law, the UV vis is very helpful for the quantitative analysis

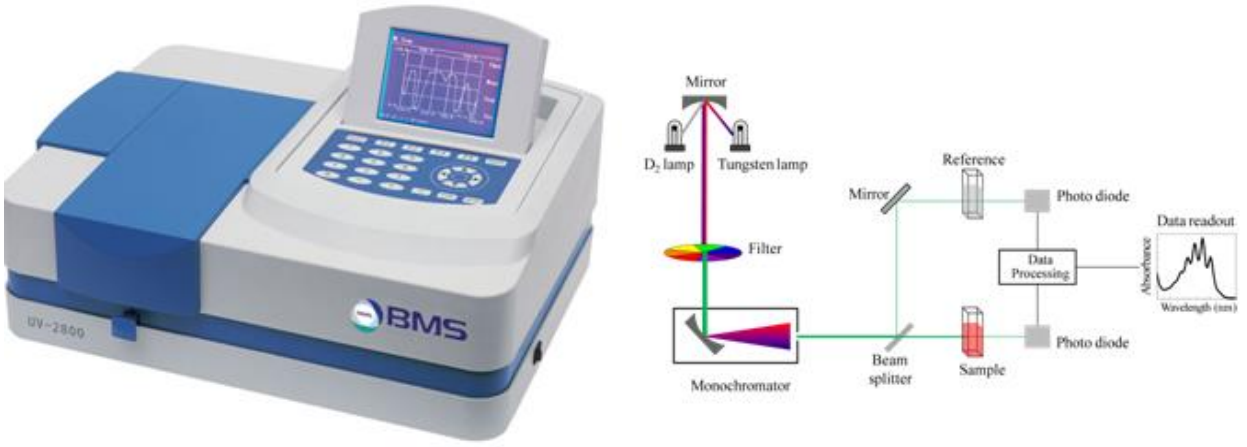

Figure 3.1 UV vis Spectrophotometer and Schematic Diagram of UV vis spectrophotometer

Scanning electron microscope (SEM)

A scanning electon microscope is a device widely being used for the imaging of the samples to the nano scale. The scanning electron microscope makes use of the beam of electron. The beam of electrons scans the surface of the sample and scans the image. The beam of electrons basically interact with the molecules and atoms in the sample which give rise to varied signals thus providing the information about the composition, morphology and topography of the sample. The raster scan pattern is used by the beam of electrons to scan. Detected signal along with the position of beam of electrons give the combine effect and an highly refined image is produced. More than 1 nanometer resolution can be checked with the Scanning Electron Microscope. The conventional SEM uses high vacuum for imaging where as if we have environmental SEM we can use low vacuum and wet conditions.

Imaging can also be done at high temperatures but that is done with the specified instruments. When the beam of electrons interacts with the sample atoms it causes the atoms to emit

secondary electrons. The device detects these secondary electrons depending upon the topography of the sample to be checked. The image can be created when the secondary electrons are detected by the detector, the sample is scanned and the topographic image of the specimen is created.

In this imaging technique, it is important to note whether the sample can withstand the high energy electron beam and the vacuum conditions or not. So the sample is prepared accordingly. The sample needs to be small enough so it can be adjusted and fixed on the specimen stage. Stub is used to mount the sample and a conductive adhesive is used. Some of the SEM machines have facility that they can provide the rotation so that the sample can be viewed at 360° rotation.

Figure 3.2 Scanning Electron Microscopy

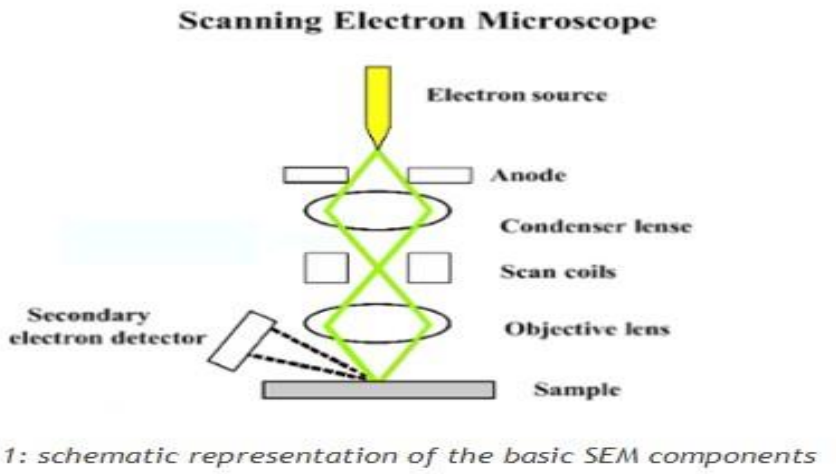

Figure 1: schematic representation of the basic SEM components

Figure 3.3 Schematic Diagram of Scanning Electron Microscopy (SEM)

Energy-dispersive X-ray spectroscopy (EDX)

Energy dispersion X-Ray is a characterization technique that is used for the elemental analysis and also for the chemical characterization. The technique uses some kind of source of X-ray that excites the sample. Each element that is being characterized by this technique has its own characteristic properties thus having a specific structure which give it characteristic electromagnetic peaks. The emission spectrum shows those peaks and that is the principle of the spectroscopy. High energy beams such as electron or protons are used for the emission or in any other case X-ray beam can be used to study the desired sample. The X-ray beam is of high intensity so it is more often used.

The electrons in a sample or the specimen are in ground state and are not excited. So to excite those electrons, the beam is subjected on the atoms of the sample. This causes the excitation of electrons in the inner shells of the specimen atoms. Due to this excitation, the electrons leave its place in the inner shell and jumps to the outer shell which is of higher energy. This causes the hole in the inner shell. When the hole is created in the inner shell, it creates an energy difference in the between the shells. The energy difference emits the X-rays beam.

This emitted beam of X-rays has a certain number and energy that can be measured using the energy dispersive spectrometer. Each X-rays have energies that are specific for certain

composition of the sample and its elements. These energies are characteristics of that energy difference that is present between lower and higher energy shells and also the atomic structure and elements of the specimen. The EDS allows all the elemental analysis of the sample to be characterized.

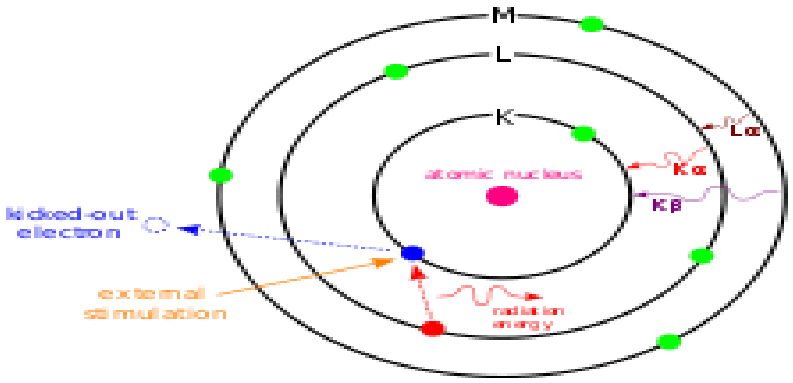

Figure 3.4 mechanism of EDS

Materials and Methods: (Phase II)

Bacterial Strains and Growth media:

Two of the bacterial stains were used for the research study. One stain was of the Enterococcus Fecalis which was gram positive and the other was of the Escherchia Coli which is gram negative. The one strain of bacteria Escherchia Coli was grown in the Luria Bertani because it shows best growth in it. The other strain of Enterococcus Fecalis was grown in the M17 media with 3-(N morpholino) propanesulfonic acid (MOPS). The E.Coli and E.fecalis were grown in the culture tubes and the culture was incubated overnight at 37°C for 24 hours. The growth of the strains was done in the culture tubes and the 10ml media was used for pre culture. The LB agar was prepared in distilled water and was autoclaved at 121°C. After it was cooled to room temperature, plates were prepared.

Invitro Antibacteral Activity

Bacterial culture

The bacterial strains were cultured in the culture tubes and we repeated the culture twice. 50µl of the pre culture was taken in 10ml of the M17 media for Enterococcus Fecalis and in Luria Bertni media for Escherchia Coli. The tubes were incubated at 37°C for 24 hours. After 24 hours the optical density of the pre culture at 600nm. The O.D comes out to be 0.8-1.0 at 600nm, so we stopped the culture at this O.D as it is the optimized one. Now these cultures were used for the antibacterial activity.

Antibacterial Evaluation of AuNPs-Cip Using the Quantitative Method

The LB agar was used as a media on plates for the growth of colonies. After preparing, the agar was brought to room temperature and then agar was poured on the Petri plates. 20ml of agar media was poured on each Petri plate. This method is known as the disc diffusion method described by Yin et al. (2010). According to this method, the antibacterial activity of antibacterial material is checked. For this purpose, we selected the Gram positive bacteria Enterococcus Fecalis and Gram negative bacteria Escherchia Coli. The bacteria inoculation was done by streaking the bacteria on the plate with the help of the spatulas made by the Pasteur pipettes. When the streaking was done the paper disc of 5mm was placed in the Petri plates. We made different concentration samples and poured 10µl of each on the disc. The Chloramphenol was used as positive control and 10µl of it was poured on the disc. Distilled water was used as the negative control and 10µl of it was poured. After pouring the sample on the disc, the plates were left undisturbed for few hours so that the sample can properly diffuse in the plates. After few hours, the plates were placed in the incubator at 37°C for 24 hours. The zone of inhibition was checked after 24 hours.

Hemolysis

Hemolysis is a mechanism in which the destruction of the red blood cells occurs. The red blood cells burst and the hemoglobin is released in the blood streams. The reason for hemolysis can be many such as drug cytotoxicity, blood diseases or any reaction in blood. In our research study, we performed this test to check the cytotoxicity of the ciprofloxacin loaded gold nanoparticles for the blood cells.

For this purpose, the hemolytic activity was carried out on the red blood cells of healthy person. 15ml of blood was taken from the healthy person. 4ml from that blood sample was taken and 8ml of Phosphate buffer saline was added to it. The blood was than centrifuged at 10,000 rpm for 10mins. After the centrifugation, the serum and blood cells were separated. The serum was discarded and the pellet containing the red blood cells was taken. The pellet was further washed for three times at 10,000 rpm for 3minutes with PBS to remove all the supernatant properly. When washed thrice with PBS, the pellet was diluted with the PBS.

The sample was prepared from the stock solution of ciprofloxacin loaded gold nanoparticles and the PBS was used as the solvent. We prepared different concentrations such as 1, 5, 10, 25, 50, 100 and 150 µg/ml. The positive and negative controls were also used. The PBS was used as the negative control and 0.5 %Triton X-100 was used as positive control. We took 1ml from each concentration and added each to 1 ml of the blood cells suspension. The 1ml of PBS as negative control and Triton X-100 as positive was also added to the blood cells suspension. We kept these samples for 1 hour and 4 hours for the incubation at 37°C in the incubator. After the incubation period, the samples were centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected and used for further analysis while the pellet was discarded. The samples were analyzed at 550nm using the UV-2800 BMS Scientific Technical Corporation (PVT) Ltd spectrophotometer. The sample was poured in the cuvette and the absorbance was checked. The positive control will give the 100% absorbance and the negative control will give the 0% absorbance. The hemolysis percentage was obtained using this equation

$$\textbf{Hemolysis (\%)} = \frac{\textbf{Absorbance of sample – Absorbance of negative control}}{\textbf{Absorbance of positive control-Absorbance of –ive control}} \times 100$$

Statistical analysis

Statistical analysis was done using different software. The graphs were done using the origin version 8.5. One of the analysis was done using the Microsoft excel and Graph pad prism version 6.0. The SEM image was done by image j software.

Results and discussion

When the red wine color solution of nanoparticles was taken and the ciprofloxacin solution was added to it. The color changed from wine red color to purple and then to bluish purple and finally to blue. This shows the reaction taking place with the passage of time and loading of the drug on the nanoparticles. The bluish purple color change shows the aggregation of the drug loaded gold nanoparticles. The reason for the aggregation was found to be the higher concentration of drug which was not optimized properly, that caused the particles to aggregate.

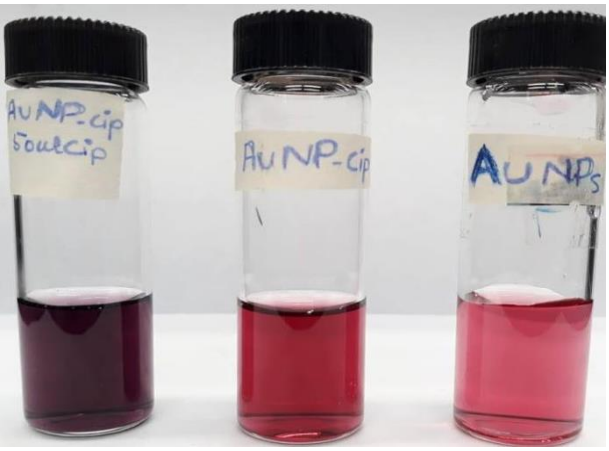

Figure 4.1 Optical visualization of (A) AuNPs (B) AuNPs-Cip

U.V spectroscopic Analysis

The gold nanoparticles and the ciprofloxacin loaded gold nanoparticles have their characteristics optical properties that depend on their shape, size, conductivity and distribution of size. Due to this they show characteristic surface Plasmon peaks at specific wavelengths. For this reason, we find the U.V visible spectroscopy to be the best way to do the analysis of the surface Plasmon peaks of the samples. The gold nanoparticles give their characteristic peaks at 520-550, the ciprofloxacin is a drug that gives its surface Plasmon peak at 277nm and ciprofloxacin loaded gold nanoparticles give their characteristic surface Plasmon peak at 580-650nm. When taking the U.V spectra of drug loaded nanoparticles, we found that it shows a peak shift from 520-550nm to580-600nm which confirms the formation of the dug loaded nanoparticles. Also when we recorded the U.V spectra of the drug loaded sample in the wavelength range of drug that is 250-

300nm, it gave a surface Plasmon peak at the 277nm which shows the presence of ciprofloxacin in the nanoparticles an confirms the drug loading.

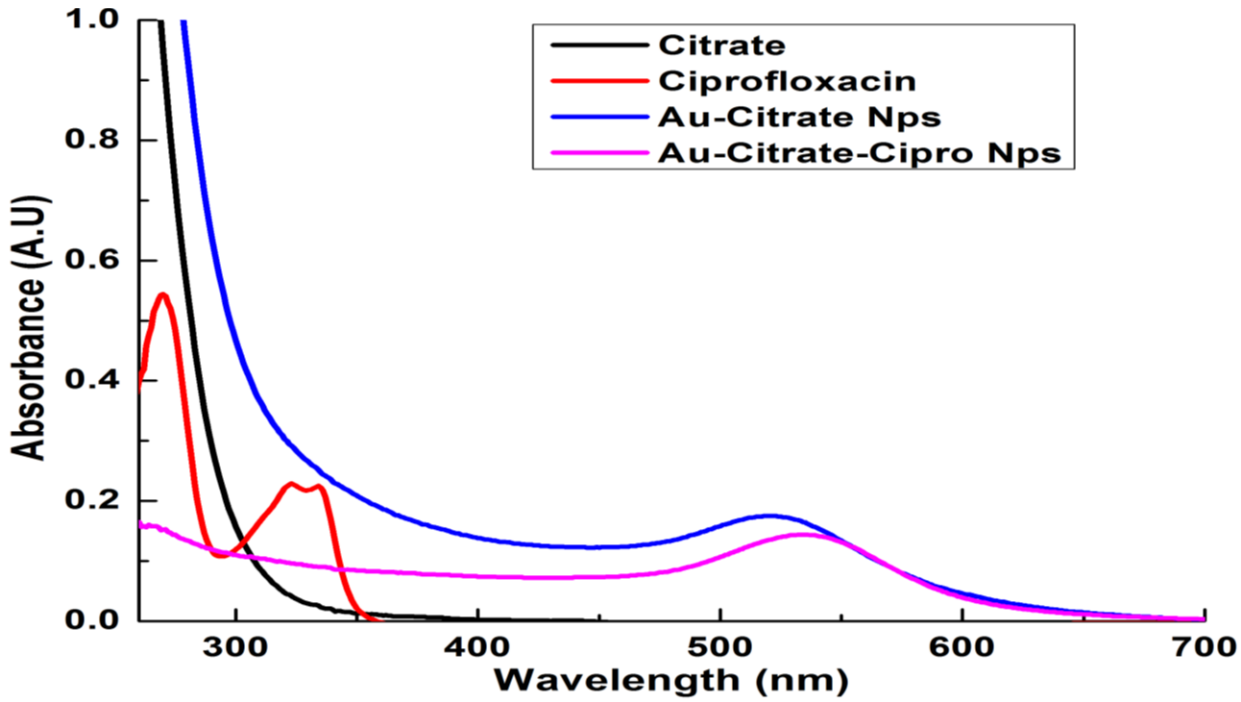

Figure 4.2 Comparative UV-Vis spectra of AuNPs, AuNP-Cip,, citrate and ciprofloxacin.

in this figure, the shift can be seen in the surface Plasmon peaks of the bare gold nanoparticles and the ciprofloxacin loaded drug nanoparticles. This wavelength shift shows the loading of drug on the gold nanoparticles.

Drug loading efficiency of AuNPs-Cip

The drug loading efficiency of the cip loaded gold nanoparticles was checked by fining the unbounded drug in the supernatant. It is understood that not all the drug will be loaded to the nanoparticles and some will be present in the free form. For that we need to take the U.V spectra of both the pellet and the supernatant.

The U.V spectra of pellet gave the peak at 580nm and supernatant gave at 277nm. To find out if there is any unbounded drug in the sample, we plotted graph with different molar concentrations of the ciprofloxacin such as 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6mM. The molar concentrations of the

drug were plotted on the x-axis. The graph shows increase in the absorbance of the ciprofloxacin with the increase in concentration. The U.V spectra's of each of the molar concentrations was recorded and was plotted on the y-axis. The linear graph of calibration was formed and the slope was calculated. To find the unbounded drug in the solution, the Beer Lambert's Law was used which says that

A=EcL

Where A is the absorbance of the required solution, E is the extinction co efficient, L is the path length of the cuvette and c is the concentration of that compound in the solution.

According to this equation, the unbound drug found to be 0.0016 mg from 0.05 mg of the AuNPs-Cip. The drug loading can be found by this equation

$$\text{Drug Loading (\%)} = \frac{\text{Total amount of Drug- Amount of Drug in supernatant}}{\text{Total amount of drug}} * 100$$

According to this equation, the drug loading efficiency or the amount of Ciprofloxacin bounded to AuNP-Cip was calculated to be 46.6%.

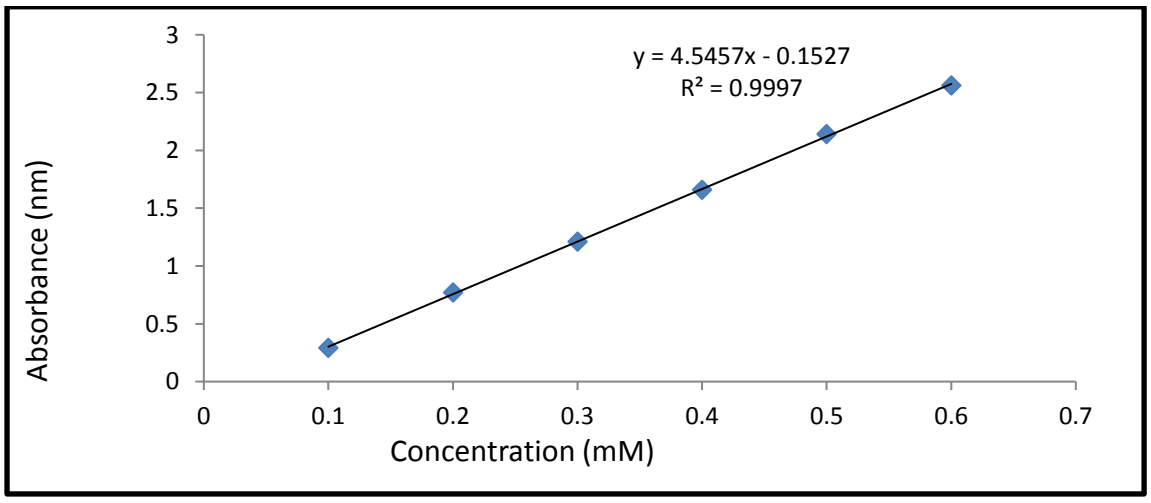

Figure 4.3 (A Increase in absorbance by increase in molar concentration

(B) Determination of slope

The graph shows the slope of to be 4.545 (figure). The unbound drug is calculated by this calibration curve of the ciprofloxacin with different molar concentration

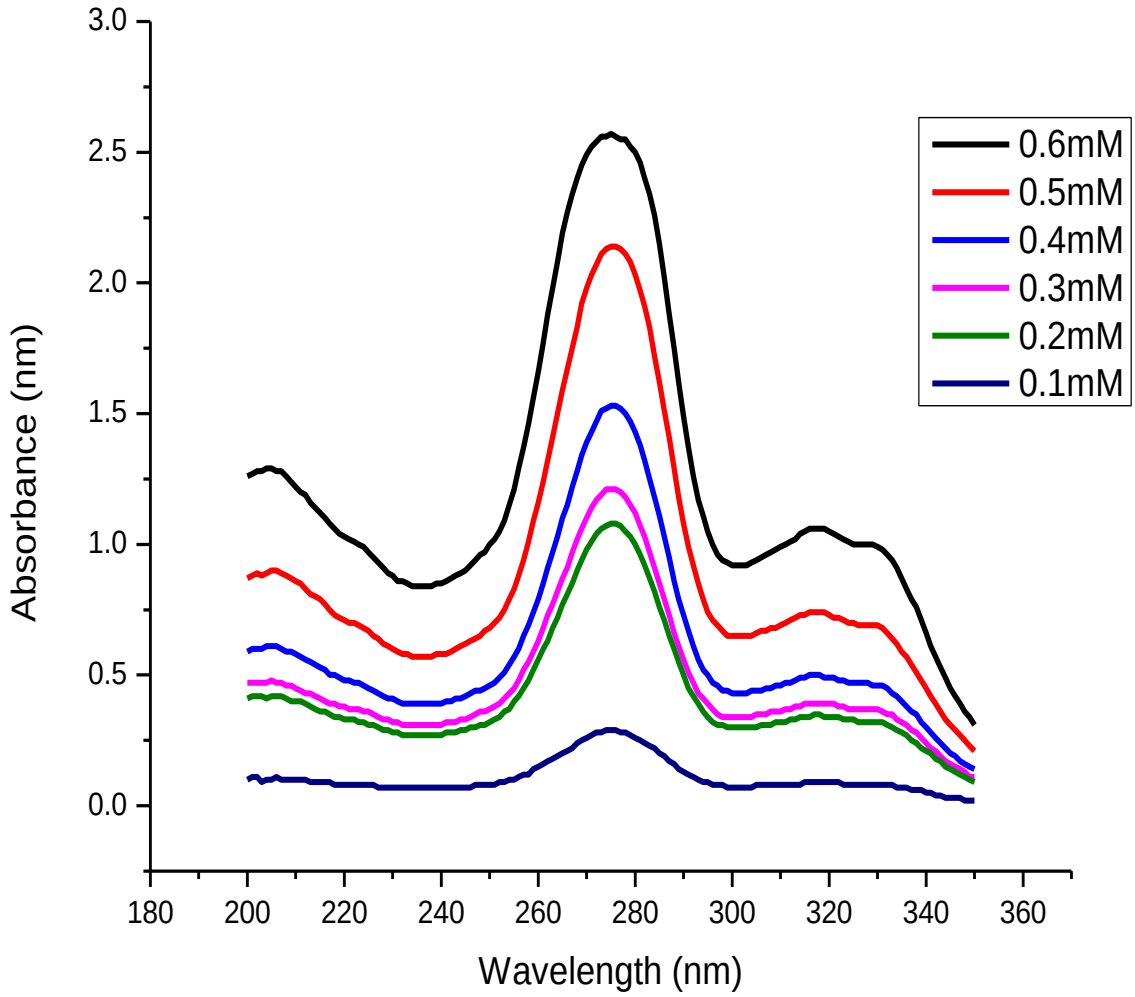

Figure 4.4 Different molar concentrations of Ciprofloxacin Hcl (mM)

Stability Test

The stability test was performed to check the optimized concentration of ciprofloxacin in the solution.

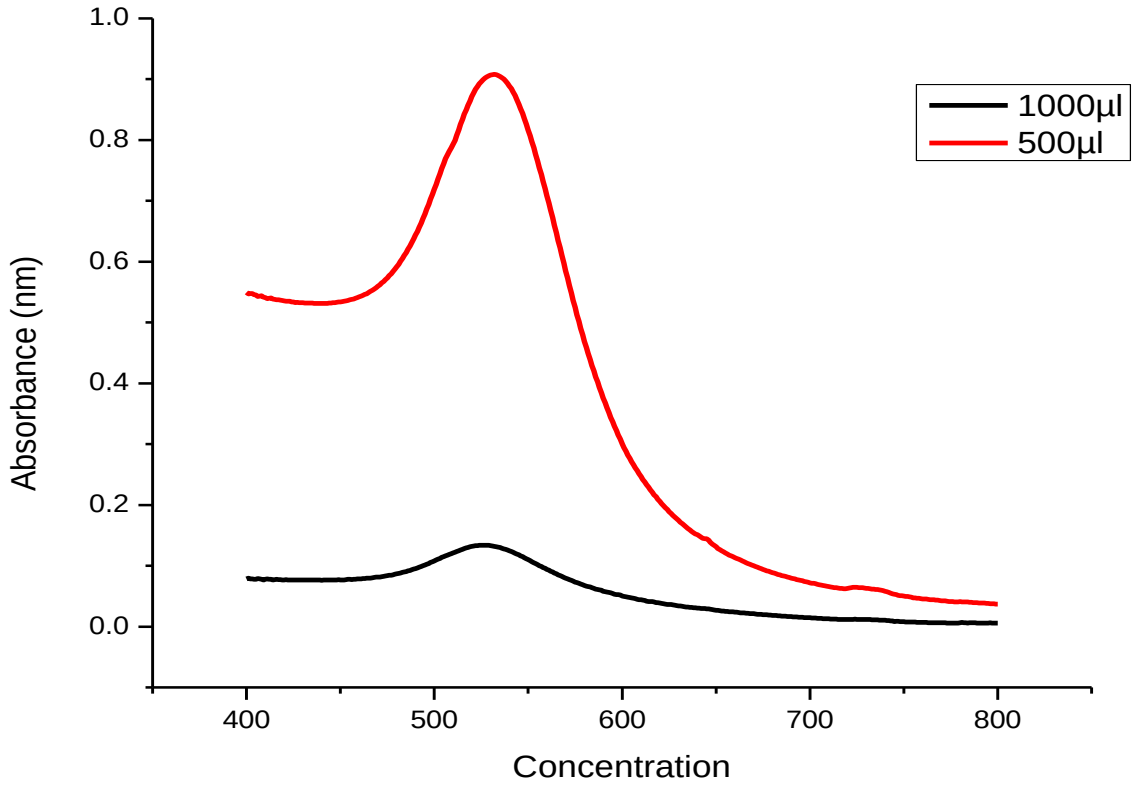

Figure 4.5 Concentration graph of AuNPs (A) at 1000µl (B) at 500µl

The graph shows the decrease in the absorbance of the AuNPs-Cip with the higher concentration of the drug. The solution turned to blue color which shows the aggregation of the nanoparticles.

Higher the concentration of the ciprofloxacin in the sample, lower the stability of the ciprofloxacin loaded gold nanoparticles

PH Test

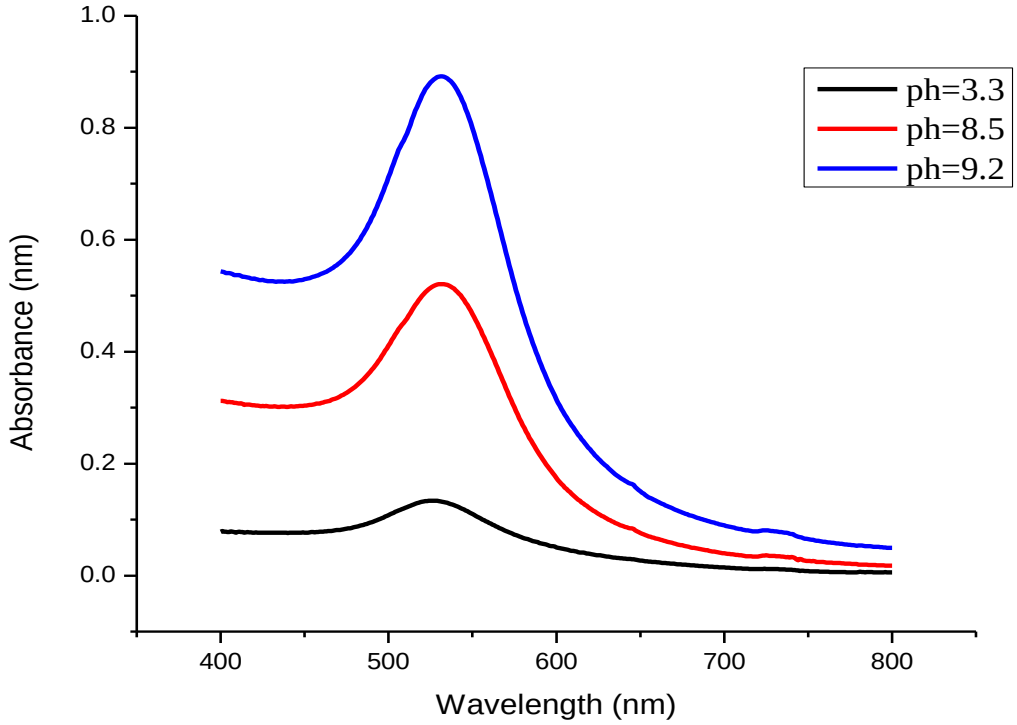

Figure 4.6 Effect of changing pH on the AuNPs-Cip

The pH test was performed to find out the best optimized pH

To optimize the pH, the solution was checked at different pHs and the best optimized pH was found to be 9.2. The reaction occurs efficiently at the basic pH rather than the acidic pH.

Temperature Test

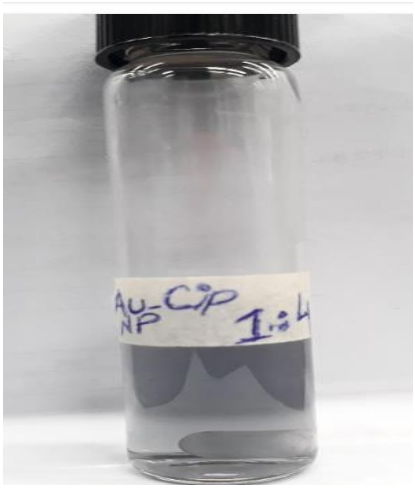

Figure 4.7 shows aggregation of AuNPs-Cip at 100°C

The effect of temperature was observed in the AuNPs-Cip. The test was performed at different temperatures and best optimized temperature for synthesis of nanoparticles was found to be 4°C. The aggregation of the nanoparticles was observed at the high temperature such as 100°C.

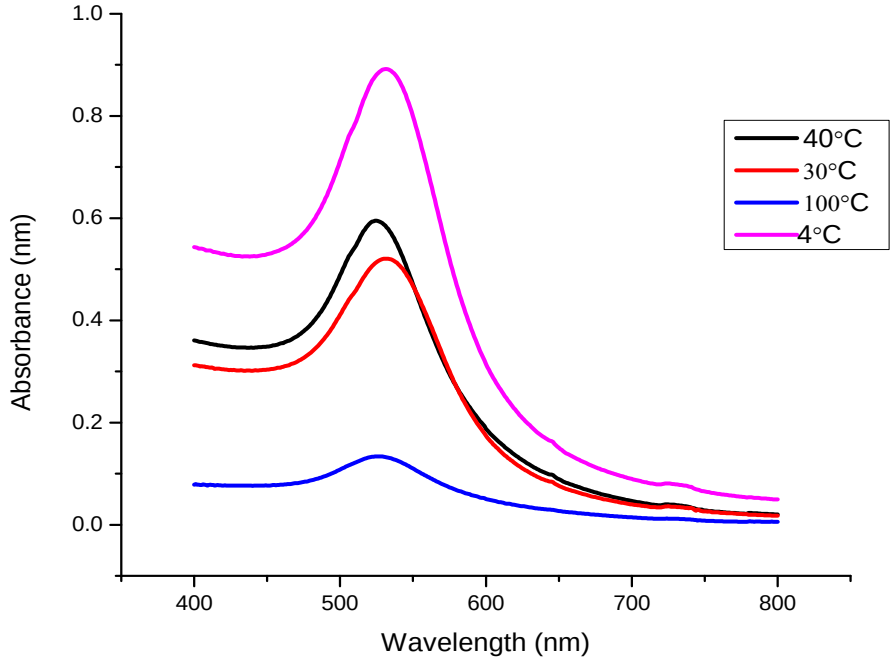

Figure 4.8 Optimizing the reaction by changing temperature

Salt Effect

Effect of NaCl was found to be negligible on the ciprofloxacin loaded gold nanoparticles**.**

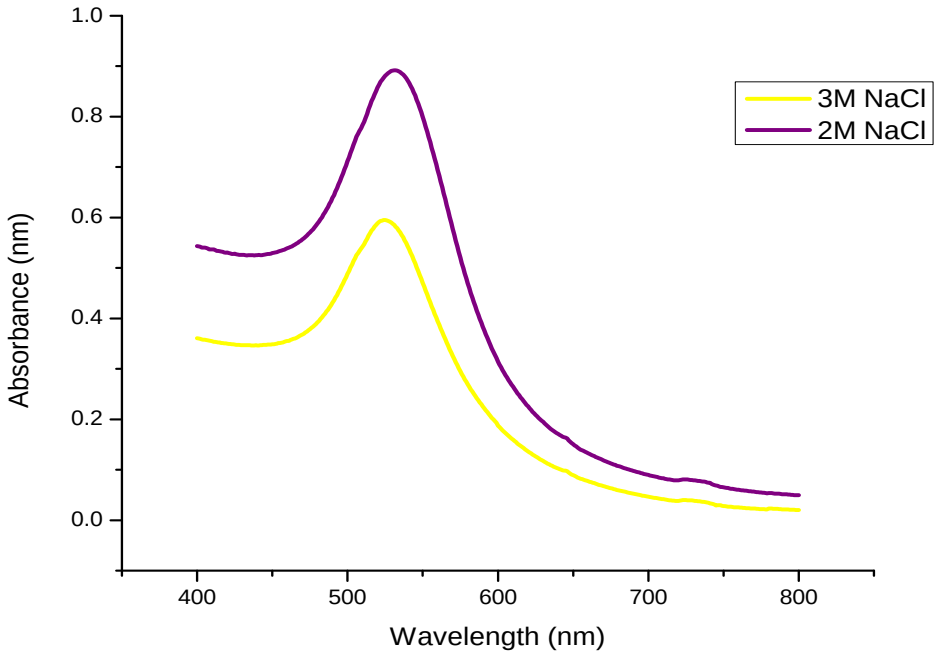

Figure 4.9 Effect of NaCl on the stability of AuNPs-Cip, after 24h

Energy Dispersive X-Ray (EDX)

For the elemental analysis of the sample, EDS was performed.

The composition clearly shows the presence of Au which is 83.2% in the EDS analysis report. Apart from that the analysis also shows the presence of Na, Cl, and O which confirms the synthesis of the nanoparticles**.**

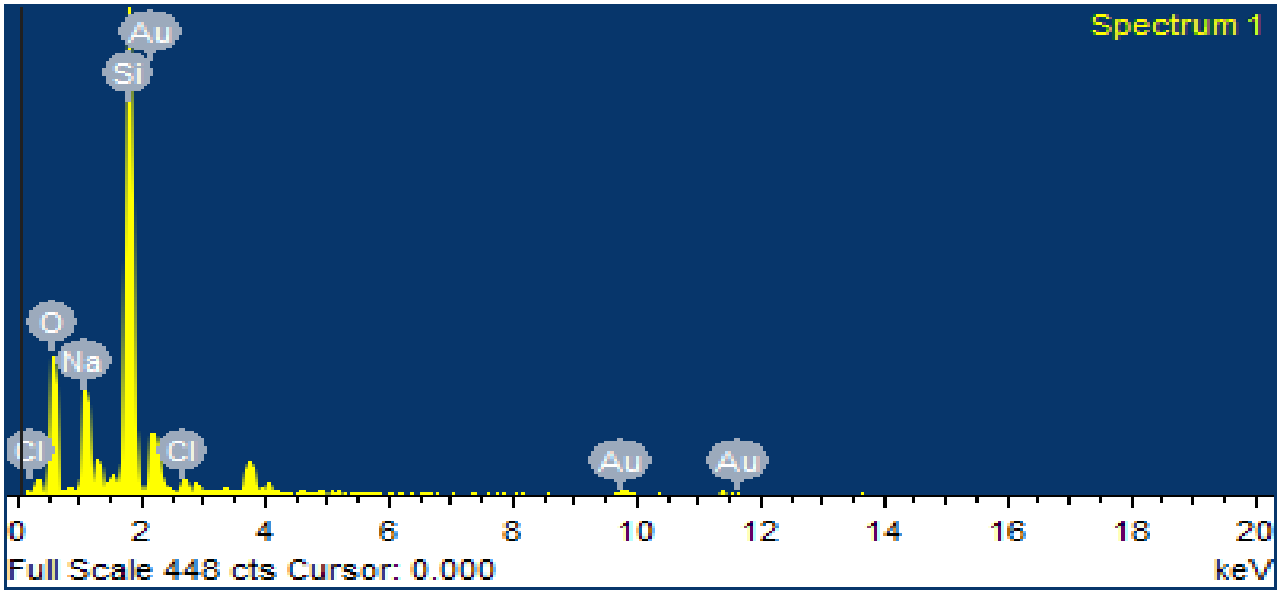

Figure 4.10 Energy dispersion spectra of AuNPs-Cip

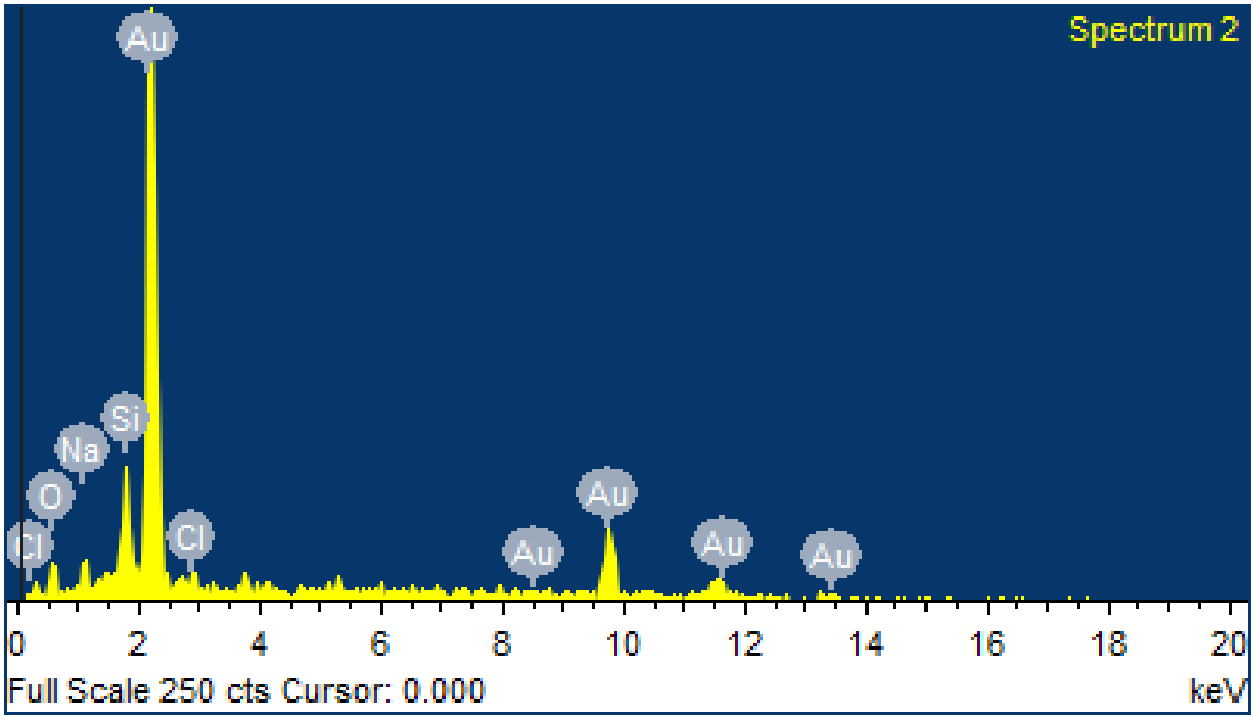

Figure 4.11 Energy dispersion spectra of AuNPS-Cip

Table 1 Elemental composition of AuNPs-Cip

Element	Weight%	Atomic%
O K	9.68	46.95
Na K	2.32	7.82
Si K	3.56	9.85
Cl K	1.16	2.55
Au M	83.28	32.82
Totals	100.00	

Scanning Electron Micoscopy

The scanning electron microscopy was performed to find out the size, shape and morphology of the nanoparticles.

The size and shape of the ciprofloxacin loaded gold nanoparticles was determined.

Hemolysis

The hemolytic activity of the sample clearly indicated the hemocompatibility of the ciprofloxacin loaded gold nanoparticles. The different concentration of AuNPS, AuNPs-Cip and Ciprofloxacin at 1, 10, 25, 50, 100, 150 µg/ml showed the hemolysis .It was indicated that the AuNPs-Cip are more compatible to the blood cells as compared to the parent drug. Least hemolysis was shown by the drug loaded nanoparticles and maximum was shown by the pure drug.

The increase in concentration of all the sample showed a relative increase in the hemolytic activity and the maximum of the hemolysis shown by the AuNPs-Cip was around 4%.The hemolytic behavior increases with the increase in the concentration of the drug and the nanoparticles. But overall the percentage of hemolysis was found to be safe according to the IS O/TR 7406.

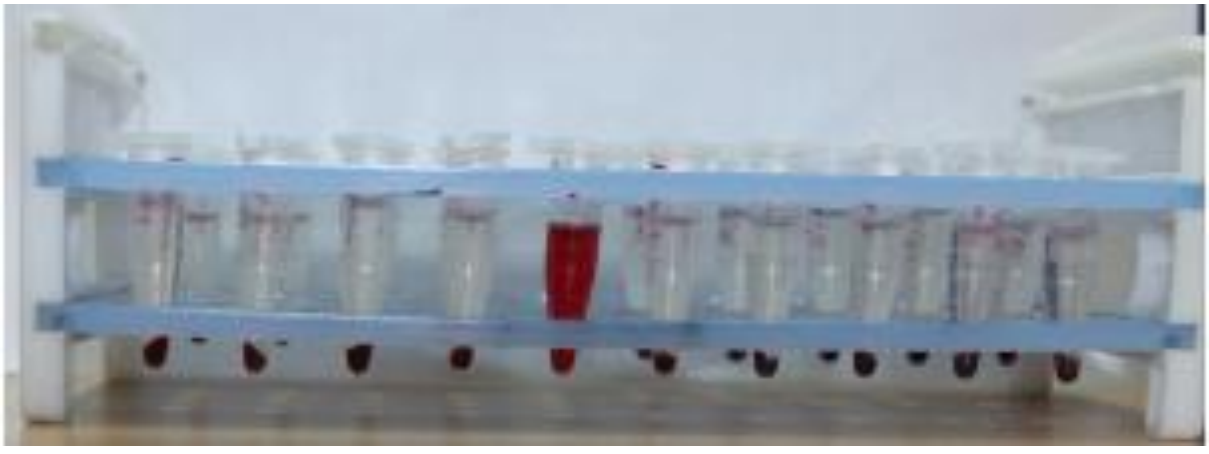

Figure 4.13 Effect of AuNPs-Cip, AuNPs and Ciprofloxacin on the red blood cells

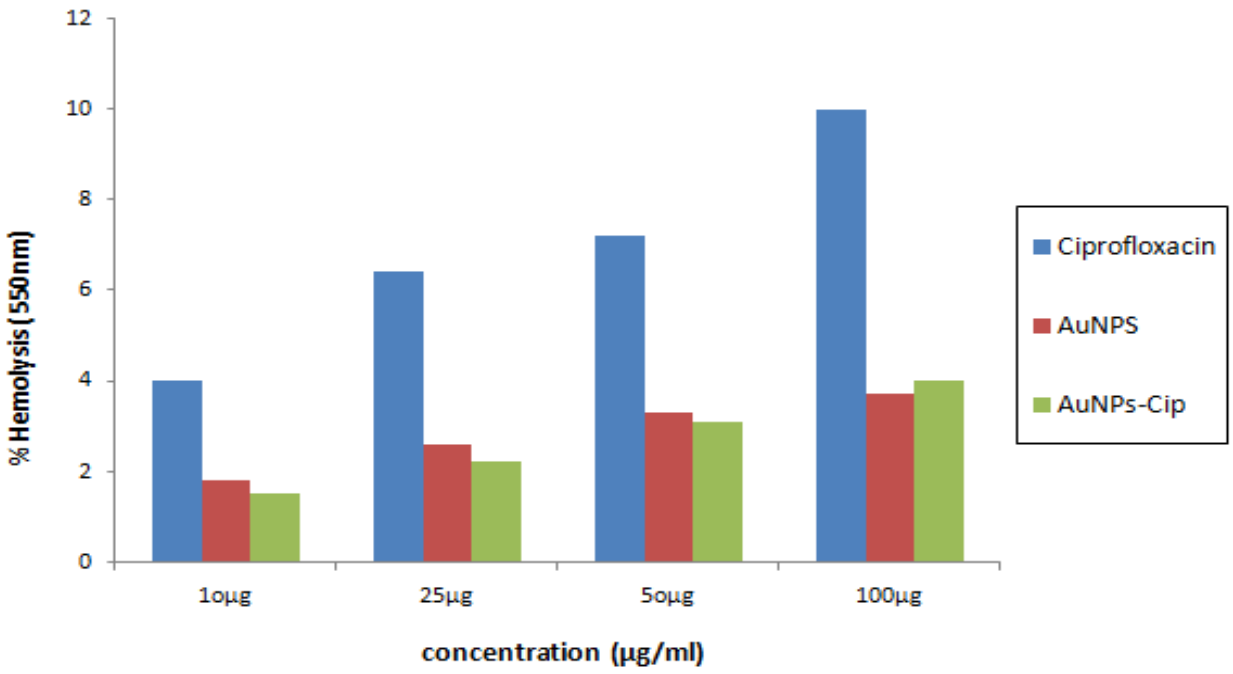

Figure 4.14 % hemolysis of the red blood cells by the AuNPs-Cip, AuNPS and ciprofloxacin.

The bars represent the percentage hemolysis of the red blood cells.

Results (Phase II)

Invitro Antibacterial Activity of AuNPs-Cip

The antibacterial activity of the pure drug, bare gold nanoparticles and the ciprofloxacin loaded gold nanoparticles was investigated against the gram positive bacteria which was the Enterococcus Fecalis and gram negative bacteria which was the Escherchia Coli. The results were seen which showed significant effect of the samples on the growth of bacterial strains. The results showed that the bacterial strain Enterococcus Fecalis showed the inhibition zone of 24mm with the drug loaded sample and 22.3 mm zone of inhibition value is seen in the E.Coli with the drug loaded sample.

The pure drug ciprofloxacin showed the zone of inhibition value of 21mm for Enterococcus Fecalis and 20mm for the E.Coli. These zones of inhibitions were obtained by the 20µg/disc of

the sample solution. The positive control was chloramphenicol which also showed zone of inhibition in 11 to 24mm range for E.Coli and E.Fecalis.

Table 2 MIC of AuNPs-Cip and Ciprofloxacin

ENTEROCOCCUS FECALIS	MIC (µg/ml)
AuNPs-Cip	20
Ciprofloxacin	20

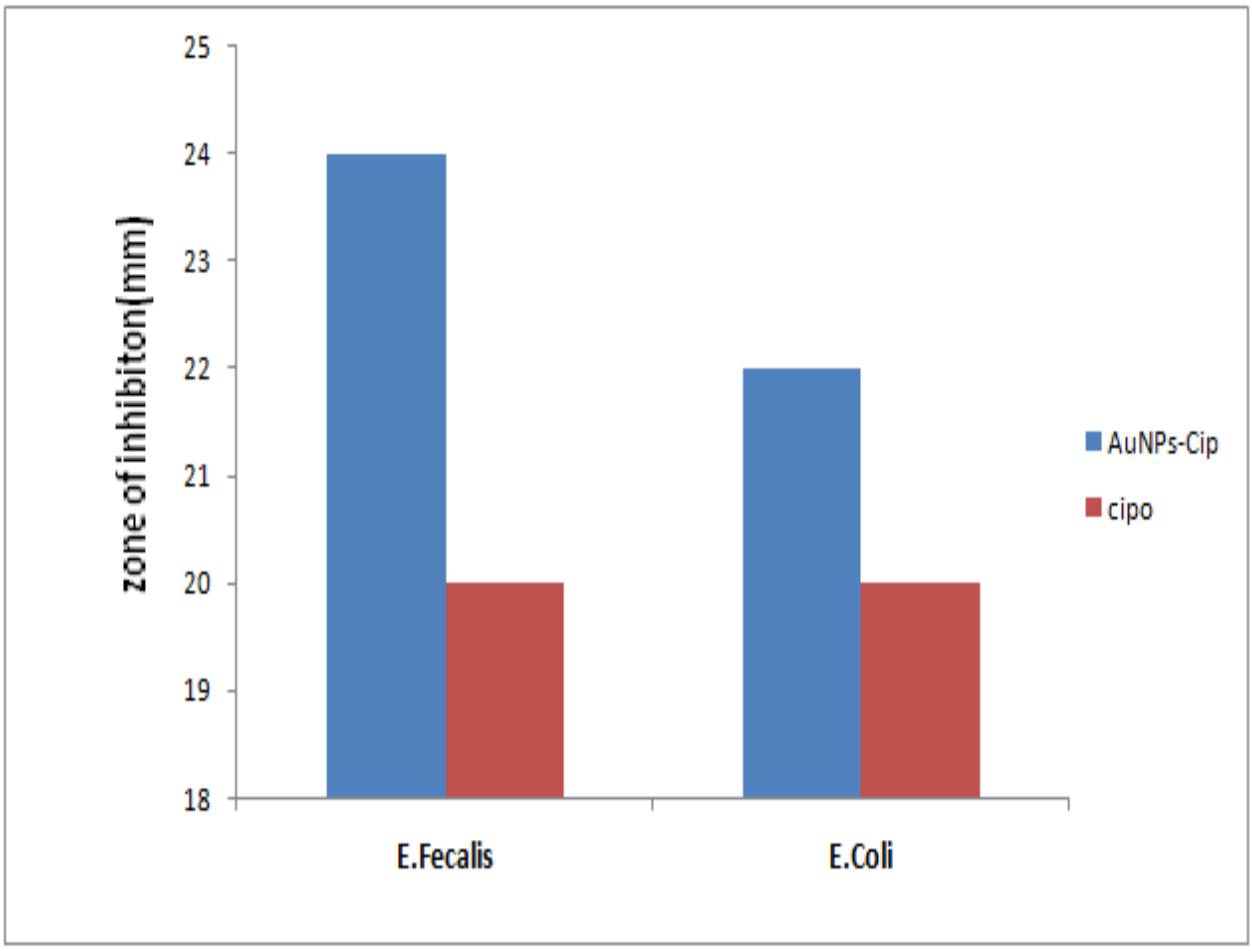

Figure 4.15 Antibacterial activity of AuNPs-Cip against gram positive and gram negative bacteria

The graph shows the antibacterial activity of the ciprofloxacin loaded gold nanopaticles and pure drug ciprofloxacin against the E.Fecalis that is gram positive and E.Coli that is gram negative bacteria. The difference in the inhibition zones can be seen in the table.

Table 3 Inhibition zone values of bacterial strains

Bacterial strain	Inhibition zone value
Enterococcus Fecalis	24mm
Eschechia Coli	21.3mm

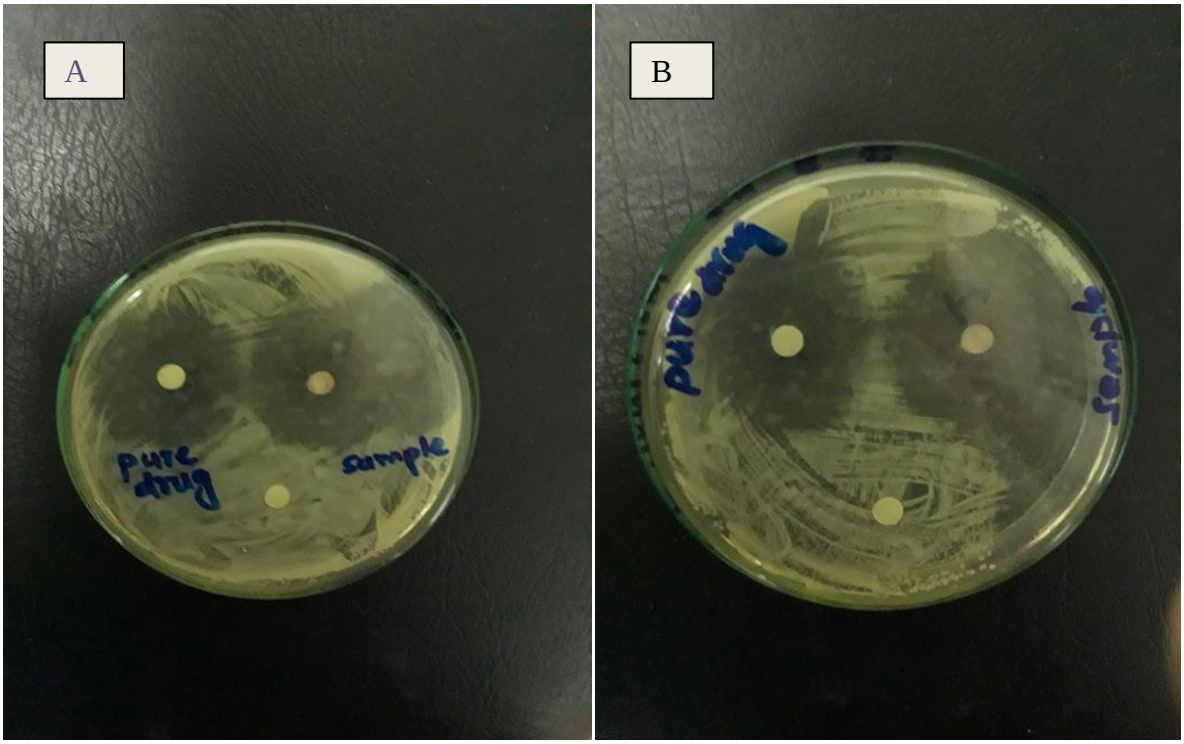

Figure 4.16 Zone of inhibition of ciprofloxacin and AuNPs-Cip in E.Fecalis
(A) Inhibition zone of AuNPs-Cip at 20µg/ml
(B) Inhibition zone of AuNPs-Cip at 30µg/ml

The figure shows the zone of inhibition of the drug loaded gold nanoparticles which is 24mm and that of the pure drug to be 20.1mm. . The negative control shows no inhibition.

Table 4 Inhibition zone values of E.Fecalis at different concentations

Inhibition zone value	20µg/ml	30µg/ml
Enterococcus Fecalis	22.7mm	24mm

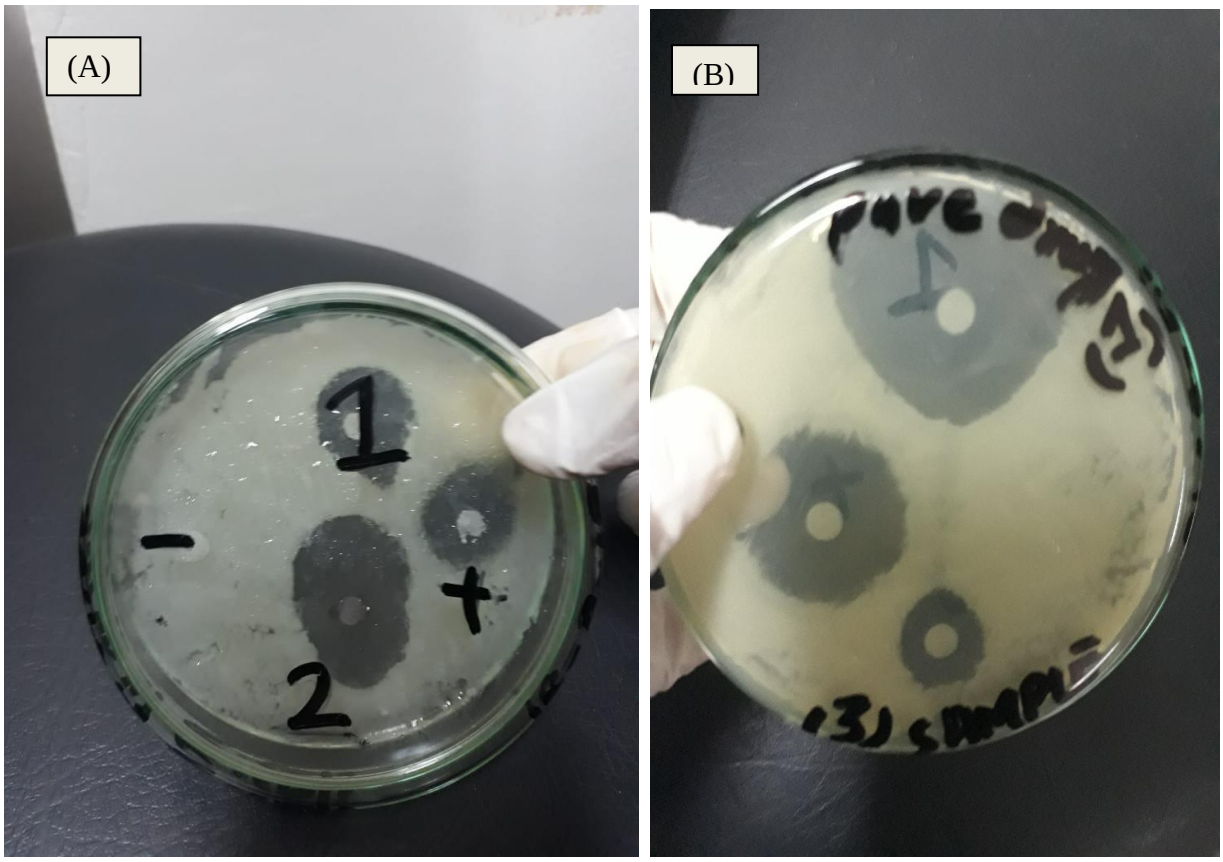

Figure 4.17 Inhibition zones of AuNPs and ciprofloxacin against E.Coli
(A) Inhibition zone with 20µg/ml of AuNPs-Cip
(B) Inhibition zone with 30µg/ml of AuNPSs-Cip

Table 5 Inhibition zone values of E.coli at different concentrations

Inhibition zone value	20µg/ml	30µg/ml
ESCHERCHIA COLI	20.3mm	22..3mm

Conclusion

The basic idea of this research was to design or synthesize the nanoformulation that can work as a drug delivery system for the antibiotic Ciprofloxacin and can be more effective than the parent drug alone. In this research, we have come to this conclusion that the Ciprofloxacin loaded gold nanoparticles have shown better results as compared to the pure drug ciprofloxacin. Not only this, it has been more compatible and less toxic to the cells and tissue. The hemolytic activity of this AuNPs-Cip was less than 5% than the parent drug. Another advantage so far is that the amount of the effective dose has been reduced than the pure drug which can reduce the side effects of the drug to a sufficient level. The biocompatibility of this formulation makes it preferable for the targeted drug delivery to the targeted tissues and cells.

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