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ORIGINAL RESEARCH

# Ciprofloxacin Functionalized Biogenic Gold Nanoflowers as Nanoantibiotics Against Pathogenic Bacterial Strains

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**Purpose:** Antibiotics are currently being rendered non-functional by the rising incidence of multi-drug resistance amongst pathogenic bacteria. Research has now been focused on developing solutions to this problem by creating new antibiotics and enhancing the functionality of the existing ones.

**Patients and methods:** In the present study, ciprofloxacin was conjugated to biogenic gold nanoflowers (GNFs) from *Bacillus subtilis* RSB64 by a robust adsorption method under optimized conditions. The resultant drug–nanoflower conjugate was characterized by UV–visible spectroscopy and Fourier transform infrared spectroscopy (FTIR). Addition of ciprofloxacin to gold nanoflowers changed the extinction spectrum towards longer wavelength. The ciprofloxacin-conjugated gold nanoflowers were tested for the drug release statistically. The prepared nanoflower–drug conjugate was subjected to an in vitro microbiological assay against different Gram-positive and Gram-negative bacterial strains to verify the effect of GNF–ciprofloxacin conjugate on the cell growth inhibitory activity of ciprofloxacin.

**Results:** The GNF–ciprofloxacin conjugates demonstrated enhanced bactericidal activity against Gram-negative bacteria as compared to Gram-positive. The enhancement of the antibacterial activity of the nanoflower–drug conjugate could be attributed to the interaction of the conjugate with phosphate/amine group of the outer membrane of Gram-negative bacterial cell wall making them susceptible to the antibacterial effect of the conjugate.

**Conclusion:** This study demonstrates the positive attributes of GNF–ciprofloxacin conjugates as a promising antibacterial therapeutic agent against pathogens.

**Keywords:** gold nanoflowers, biogenic synthesis, ciprofloxacin, drug dissolution

## Introduction

Today, the impact of nanotechnology has touched almost all spheres of life. The resilient features of nanoparticles make them appropriate for applications ranging from diagnostics to therapeutics.[1] The unique surface plasmon properties of these nanoparticles make them useful in imaging and sensing. The ability of these particles to form conjugates with many functional agents such as drugs, dendrimers, ligands, DNA, proteins and RNA enhances their utilization in drug targeting for the cure against cancers, neurological disorders, gene therapy, HIV and many other diseases.[2] Especially the biological nanoparticles have protein capping on it with a large number of functional groups which aids in the conjugation of gold nanoparticles with a number of biomolecules and chemical drug to develop a nanoparticles-based drug delivery system.[3] Over the years, gold nanoparticles

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particularly are of great interest due to their various attributes such as biocompatibility, small size, well-developed surface chemistry and stability. Due to these added advantages, gold nanoparticles have emerged as a potent candidate in drug delivery platforms.[4]

Antibiotic resistance is a widespread phenomenon in today's world.[5] The intracellular localization of the bacteria during infection makes treatment a herculean task for the antibiotics. The presence of multi-drug resistant bacteria is also contributing to the already worsened scenario. Fluoroquinolones including ciprofloxacin are in use in the medical sector since 1890. The dissemination of fluoroquinolones is often not appropriate due to their rapid renal clearance and continued efflux in prokaryotic as well as in eukaryotic cells resulting in decreased accumulation of drug, etc.[6] Therefore, nano-based drug delivery systems thus are more befitting for efficient delivery of drugs as it can assist in delivering adequate concentration and subsequently easing the burden of antibiotic resistance. Microbes are unlikely to develop resistance against drug–nanoparticle conjugate as compared to drug alone.[7]

By loading drugs into nanoparticles through polyionic complexation, physical encapsulation or chemical conjugation, the pharmacokinetics and therapeutic index of the drugs can be significantly improved in contrast to the free drug counterparts.[8]

Conjugates of gold NPs with antibiotics have been used for the selective photothermal killing of protozoa and bacteria. In the context of the antibacterial activity, Williams et al (2006) showed that gold NPs themselves do not affect bacterial growth or functional activity, whereas conjugates of vancomycin to gold NPs decrease the number of growing bacterial cells.[9] Rosemary et al (2006) reported that ciprofloxacin-encapsulated silica nanoshells synthesized from gold@silica core–shell nanoparticle displayed antibacterial activity against *Escherichia coli* DH5.[10] Gu et al have reported a similar result for the gold nanoparticles covered with vancomycin.[11] These reports and others have been focused on the chemical synthesis of nanoparticles which depend on linkers/adapters for the proper functionality of synthesized nanoparticles. These results in the toxicity of the nanoparticles.[12,13,14]

Various studies have been conducted to study the antimicrobial effect of ciprofloxacin-conjugated nanoparticles on microbial cultures. For instance, the effect of liposome-incorporated ciprofloxacin was determined on the murine model of fatal *Salmonella dublin* which resulted in enhanced activity of the drug.[15] In other study, glycosylated polyacrylate nanoparticles were conjugated with ciprofloxacin and its antimicrobial activity was assessed against *S. aureus* and *B. anthracis*.[16] Solid lipid nanoparticles (SLNs)-conjugated ciprofloxacin were also prepared in another study that resulted in prolonged drug release. The results indicated improved bioavailability and higher therapeutic efficacy.

Drug dissolution studies have a fundamental role in analyzing the release of any drug from solid and semisolid dosage forms. These aids in quantifying the amount and extent of drug release from dosage forms. Experimental release data results obtained from these studies can be examined using different mathematical models. The zeroth-order release of drug is where the rate of the drug release is independent of its concentration. On the other hand, the first-order release system is dependent on its concentration. Higuchi law indicates that the drug release from the insoluble matrix as a square root of time owing to Fickian diffusion. Korsmeyer-Peppas described the drug release that occurs in the polymeric system using a mathematical relationship.[17]

However, there are no reports of the conjugation of ciprofloxacin to biogenic gold nanoflowers and its release kinetics so far. The biogenic nanoflowers bear no additional requirement for any linker/adapter for conjugating with the drug. The protein coating on these biogenic nanoflowers itself is involved in the attachment of the drug. Therefore, they make a suitable and better alternative for the formation of ciprofloxacin–nanoflowers conjugate. For these reasons and with an applied view, we present our current study on the synthesis of nanoflowers-based drug delivery systems using biogenic gold nanoflowers and the antimicrobial activity of the conjugate was also analyzed.

# Materials and Methods

## Materials

Hydrogen tetrachloroaurates were purchased from Hi-Media Laboratories Pvt. Ltd. (Mumbai, India). NADH and ciprofloxacin were purchased from Sigma-Aldrich (United States). MiliQ water was used for solution preparation.

## Biosynthesis of Gold Nanoflowers

The biosynthesis of gold nanoflowers was carried out using bacterial strain *Bacillus subtilis* RSB64 which was isolated from the gold mines in Karnataka and has

previously been reported for the synthesis of flower-shaped gold nanoparticles.[18] The selected bacterial strain (RSB64) was grown in nutrient broth, and its supernatant containing 20 µg/mL of total protein was used to convert 1 mM aqueous solution of chloroauric acid into gold nanoparticles. The nanoparticles were maintained in powdered form using a freeze dryer (Scanvac).

## NADPH-Assisted Synthesis of Gold Nanoflowers

In order to determine the role of NADPH in the biological reduction of gold ions to nanoflowers, 100µL of supernatant solution and 100µL of 1mM hydrogen tetrachloroaurate were incubated with varying concentrations of NADPH solution (0.05mM, 0.2mM, 0.4mM, 0.6mM and 0.8mM) at 37°C. The reaction mixtures were assessed for the production of nanoflowers by observing color change of the solution and by measuring the change in absorbance at 540nm in a spectrophotometer.

## Functionalization of Gold Nanoflowers with Ciprofloxacin

Solution of ciprofloxacin (2µg) was mixed with gold nanoflower solution in 1:1 ratio at pH 6.5 and kept under stirring at 180 rpm for overnight at 37°C. The unloaded drug was removed by centrifugation and was quantified using UV spectroscopy at 320nm. The drug loading was calculated using the given formula:

$$\%\ \text{Drug loading} = \frac{\text{Amount of drug added initially} - \text{Amount of drug in supernatant}}{\text{Amount of drug added initially}} \times 100$$

## Characterization of Drug–Nanoflower Conjugate

The characterization of drug–nanoflower conjugate was performed using UV-Vis spectrophotometer and FTIR analysis. The absorption spectrum of gold nanoflowers and drug–nanoflower conjugate was obtained using Shimadzu-1800 UV–Vis Spectrophotometer. The spectra were monitored by scanning the samples in the range of 200–1000 nm at a scan speed of 2 nm/min. The FTIR measurement was done by freeze-drying the gold nanoflowers and then processed for analysis using KBr pellet method and measured in the range of 4000–400 $cm^{-1}$ using spectrophotometer (Jasco 410 Series).

## In Vitro Drug Release Studies

Ciprofloxacin–GNF conjugates were dialyzed against 30 mL of different buffers ranging from pH 6 to 9 at 37°C with continuous stirring at 100 rpm. A sample (0.5 mL) was pipetted out at specific time intervals and analyzed spectrophotometrically. Sink condition was maintained by replacing an equal volume of fresh buffer during every time of sample collection to maintain sink condition.

## Kinetic Analysis of Drug Release

The dissolution profile of the drug can be assessed using several mathematical models. The selection of an appropriate function helps in defining the drug dissolution profile. Various mathematical models (zero-order, first-order and Higuchi's model) are employed to unveil the mechanism of drug release.

Zero-order equation:

$$Q_t = Q_0 + K_0 t \quad (1)$$

where Qt is the amount of drug dissolved in time t, $Q_0$ is the initial amount of drug in the solution (most times, $Q_0 = 0$) and $K_0$ is the zero-order release constant expressed in units of concentration/time.

First-order equation:

$$\log C = \log C_0 - K_t/2.303 \quad (2)$$

where $C_0$ is the initial concentration of drug and K is first-order constant.

Higuchi's equation:

$$Q_t = K_H\ t^1/_2 \quad (3)$$

Where, $Q_t$ is the amount of drug released in time t, $K_H$ is the release rate constant for the Higuchi model.

To confirm the exact mechanism of drug release, the data were fitted according to the Korsmeyer–Peppas model. Korsmeyer et al used a simple equation to describe the general solute release behavior from controlled release polymer system equation:

$$Mt/M\infty = Kt^n \quad (4)$$

where $Mt/M\infty$ is a fraction of drug released at time t, K is the release rate constant and n is the release exponent. The n value is used to characterize different releases for cylindrical-shaped matrices. The value of n gives an indication of the release mechanism: when n = 1, the release rate is independent of time (zero-order, case II transport). n = 0.5 stands for Fickian diffusion and when $0.5 < n < 1.0$,

diffusion and non-Fickian transport are implicated. Finally, when n > 1.0, super case II transport is apparent, n is the slope value for log (Mt/M∞) versus log time curve.

## Antimicrobial Activity of Ciprofloxacin–Gold Nanoflower Conjugate

Antimicrobial activity of gold nanoflowers was evaluated using well diffusion assay against *Bacillus subtilis* (ATCC 6538), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 11103) and *Pseudomonas aeruginosa* (ATCC 25668). The test organisms were grown in nutrient broth overnight 37 °C for 24 hrs. The next day they were diluted with a saline solution (NaCl 0.9%) to achieve a turbidity of 0.5 McFarland standard ($10^8$ CFU per mL). The aliquot of each culture was homogeneously distributed onto Mueller–Hinton agar plates. Wells of 7mm each were punched into the agar plates to which 60 µL solution of ciprofloxacin (control), ciprofloxacin–GNF conjugate and gold nanoflowers (control) were added into respective wells. The concentration of the ciprofloxacin available for activity in the conjugate was 10 times less than ciprofloxacin alone. The plates were incubated overnight at 37° C. The plates were observed for the zone of inhibition around the samples and the diameter in millimeters was recorded.

## Determination of Minimum Inhibitory Concentration (MIC) of Ciprofloxacin and Ciprofloxacin–GNF Conjugate

MICs of ciprofloxacin–GNF conjugate and ciprofloxacin were determined for *Bacillus subtilis* (ATCC 6538), *Staphylococcus aureus* (ATCC 25923), *E. coli* (ATCC 11103) and *Pseudomonas aeruginosa* (ATCC 25668). The MIC value of ciprofloxacin–GNF conjugate and ciprofloxacin was evaluated according to the Clinical and Laboratory Standards Institute (CLSI) protocol. Mueller–Hinton Broth (MHB) was used for the preparation of bacterial inoculum. The turbidity of the bacterial inoculum was adjusted to 0.5 Mc Farland turbidity standard (approx. $10^8$ CFU/mL). Two-fold serial dilutions of the ciprofloxacin–GNF conjugate and ciprofloxacin in the range of 0.01–500 µg/mL were prepared and inoculated with standardized inoculum. Control tubes were maintained without the conjugate/drug. The MIC was determined after 24 hrs of incubation at 37°C by observing the visible turbidity and the final growth concentration was also calculated in terms of colony-forming units (CFU). All experiments were conducted in triplicate.

# Results and Discussion

The synthesis of gold nanoflowers by the reduction of 1mM aqueous solution of gold chloride after exposure to *Bacillus subtilis* RSB64 supernatant was confirmed via UV-vis spectroscopy and TEM image (Figure 1). Gold nanoparticles generally are known to exhibit a ruby red colour in aqueous solution due to the surface plasmon resonance (SPR) of metal nanoparticles.[18,19] During the synthesis of gold nanoflowers, the protein coating leads to nucleation of the gold ions to form the framework for the formation of the petals and assists in holding the petals of the nanoflower together by serving as a glue.[20] The GNF surface is covered with functional groups of proteins in such a way that it chemically anchors to the GNF surface and the other end remains free. The free functional groups of the molecule can thereby bind to or interact with the target molecule of interest.[21]

However, the exact reaction mechanism leading to the formation of gold nanoparticles by microbes is yet to be elucidated. But reductase activity has been previously reported to be present in microorganisms involved in nanoparticle synthesis. The reduction might be initiated by the electron transfer from NADPH by a NADPH-dependent reductase allowing the reduction of $Au^{3+}$. The color of the solution changed from yellow to pink signifying the reduction of metal ions to nanoparticles by the bacterial culture. Synthesis of gold nanoparticles was first observed in the reaction mixture containing 0.8mM of NADPH solution, i.e. after half an hour of incubation (Figure 2). The control did report color change but after a longer incubation period. The results indicate that the reductase enzyme responsible for

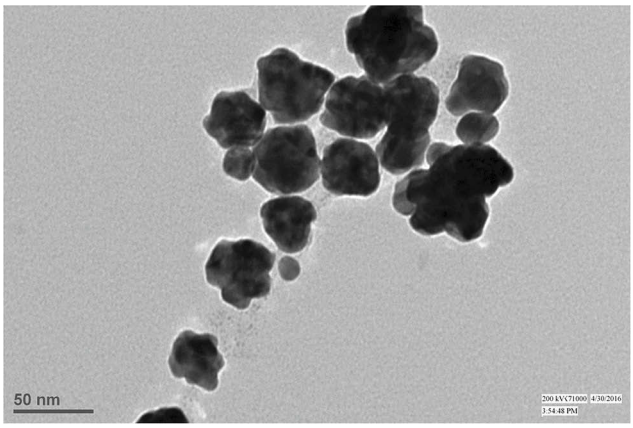


**Figure 1** HRTEM image showing flower-shaped gold nanoparticles produced by *Bacillus* RSB64.

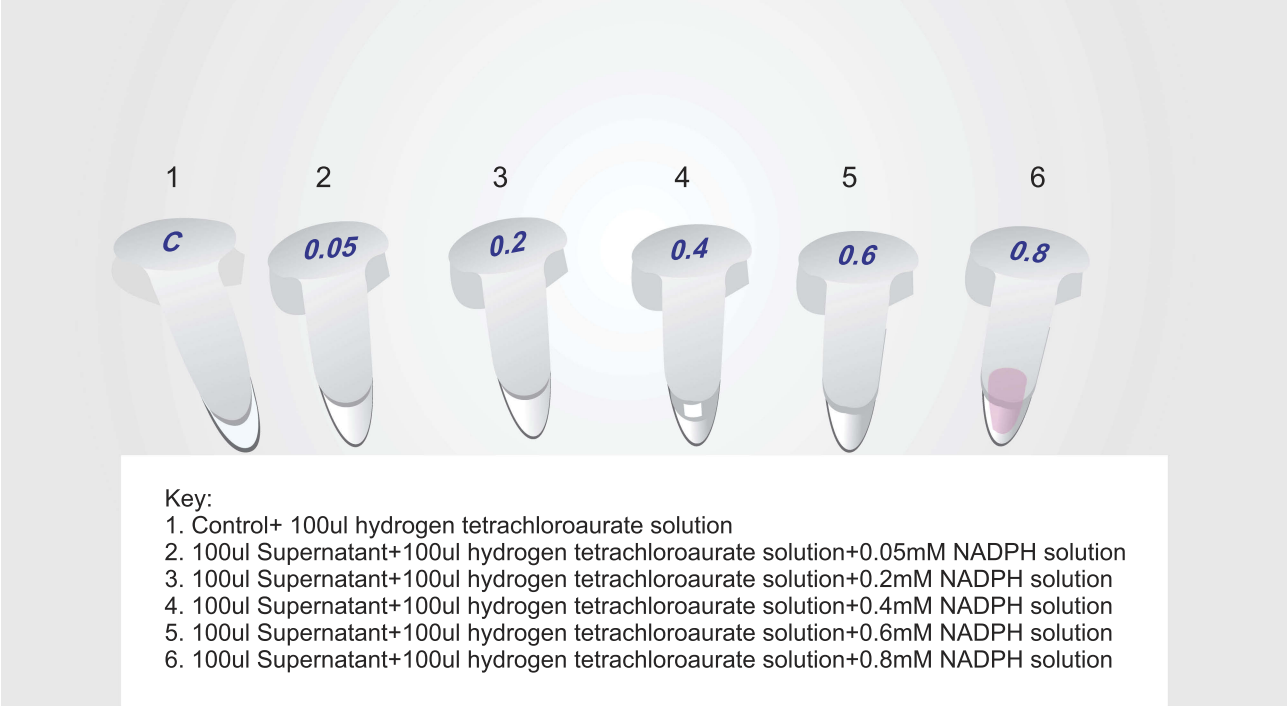


**Figure 2** Effect of NADPH on the synthesis of gold nanoflowers.

the reduction of metal ions is NADPH-dependent. NADPH is a cofactor for this biological transformation.[22] Our reports are also correlated with Kumar et al (2007) who reported the reduction of gold ions to gold nanoparticles by α-NADPH-dependent sulfite reductase and phytochelatin.[23] Nangia et al (2009) have also reported the formation of gold nanoparticles in the presence of both *Stenotrophomonas maltophilia* biomass and NADPH.[24] The reduction by NADPH facilitates a number of thiol-containing molecules such as alkanethiolates, glutathione, sulphur-containing compounds on to the surface of GNP for its functionalization.[21]

In the following step, the gold nanoflower conjugate was formed by electrostatic adsorption of ciprofloxacin onto the nanoflower surface via the amine group of ciprofloxacin and the carboxylic groups of GNF surface proteins.[25] The UV-Vis analysis of the drug–gold nanoflower conjugate indicated the shift of the absorption peak from 540nm towards longer wavelength (550 nm) after the attachment of the drug onto the gold nanoflowers (Figure 3).

FTIR spectrum analysis was used to investigate the nature of the binding of the drug to the gold nanoflowers. The FTIR spectrum of ciprofloxacin is shown in Figure 4. The vibrational frequency of stretching of the N–H bond of the imino moiety on the piperazine group of ciprofloxacin was represented by a band at 3361 $cm^{-1}$. Absorption bands at 1643 $cm^{-1}$ and 1082 $cm^{-1}$ represent a primary amine (N–H) bend of the pyridone moiety and the C–F functional group, respectively. The FTIR analysis of gold nanoflowers (Figure 4) revealed a band at 3845 $cm^{-1}$ that may be assigned to stretching vibration of the NH group present in the protein. The sample also exhibited intense bands at 3341 $cm^{-1}$ which corresponded to the strong stretching vibration of the O–H group of alcohols and phenols and band at 2062 $cm^{-1}$ referred to strong C–H stretching.[18] The band at 2800 $cm^{-1}$ might be ascribed to C–H aldehydic stretching vibrations. The characteristic band at 1636 $cm^{-1}$ indicated stretching peaks of amide I group of polypeptides/proteins.[18] Based on the spectral data, binding of ciprofloxacin to the surface of gold nanoflowers could be confirmed by the fact that the corresponding $NH_2$ peaks were broadened and shifted to higher wavelengths (from 3845 $cm^{-1}$ to 3895 $cm^{-1}$ and from 1636 $cm^{-1}$ to 1646 $cm^{-1}$). It has been reported that the nitrogen atom of the NH moiety of the piperazine group binds on the gold surface, in the case of ciprofloxacin-protected gold nanoparticles.[26] From the data, it is demonstrated that the biogenic gold nanoflowers can act as an effective system for the delivery of antibiotics.

In general, loading the drugs over GNPs using non-simple ionic interaction methods is considered a simple and effective approach as the therapeutic activities of the drug molecules would be retained without change in the drug's chemical

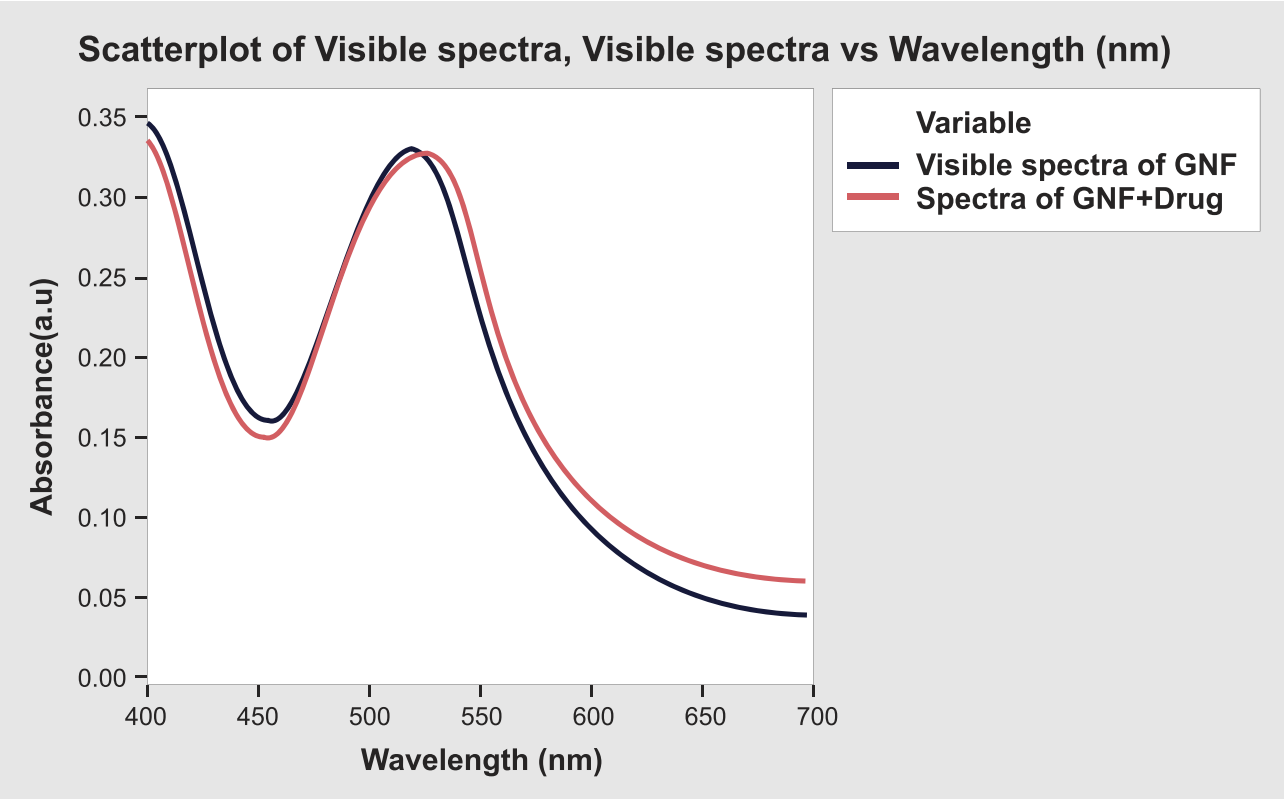


**Figure 3** Visible spectra of biogenic gold nanoflowers and ciprofloxacin-bound gold nanoflowers.

structures. The negative charge of the gold nanoflowers has been exploited for loading the positively charged ciprofloxacin on its surface. The percent loading of the drug onto the gold nanoflowers was determined based on the UV-Visible absorbance studies. The percent of ciprofloxacin loading in the GNFs was calculated and found to be 41± 2.3%.

In vitro release of ciprofloxacin from gold nanoflowers was studied in different pH conditions ranging from pH 6 to 9. The release curve followed a typical biphasic drug released behavior and results are depicted in Figure 5. Within 2hrs, approximately 15% of free drug ciprofloxacin was found to be released. The rapid release of ciprofloxacin might be caused by the release of the drug adsorbed at the surface of the nanoparticles. In addition, due to its small size, it has larger surface to volume ratio, which stimulates the burst release as suggested by Joshi et al.[27] This release can be categorized into two phases: the initial phase (2hrs) where there is a burst release of drug and the second phase (2 to 16hrs) where the drug is released in a sustained manner. $t_{60\%}$ is an important variable for assessing drug release from

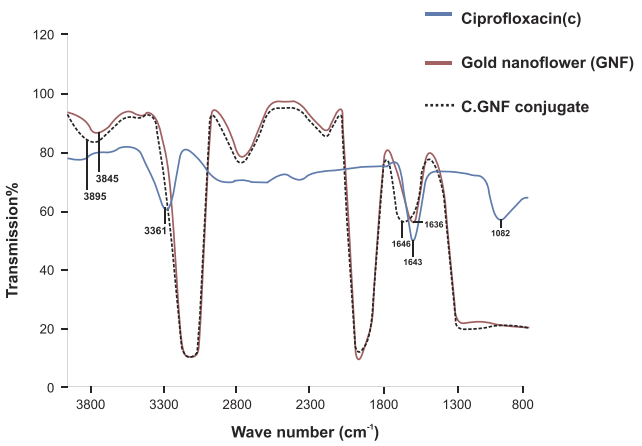


**Figure 4** FTIR spectra of ciprofloxacin, biogenic gold nanoflowers, ciprofloxacin-bound gold nanoflowers.

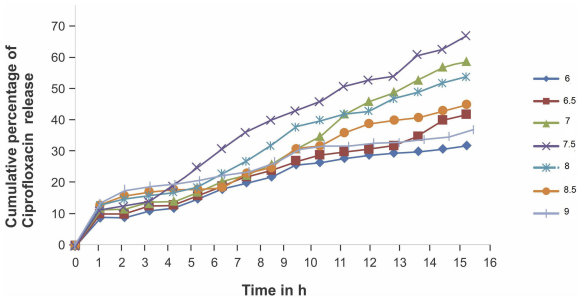


**Figure 5** In vitro ciprofloxacin release from GNFs at pH ranging from 6 to 9.

the dosage form, indicating the amount of drug available at the site of absorption. The maximum release observed at the end of 16hrs was at pH7.5 and it was approximately 67%. The release of ciprofloxacin was found to be dependent on the pH of the medium. At the end of 24hrs, 59% of the ciprofloxacin was released at pH 7, whereas it was only 32% of ciprofloxacin was released at acidic pH.

Maintaining a desired value of drug at the site of action is the main criteria of controlled release systems. Generally, the controlled release system initially releases part of the dose contained in order to attain rapidly the effective therapeutic concentration of the drug. Then, drug release kinetics follows a well-defined behavior in order to supply the maintenance dose enabling the attainment of the desired drug concentration. The in vitro drug release was fitted into different kinetic models consisting of zero-order, first-order, Higuchi model and Korsmeyer–Peppas model as presented in Figure 6. The various profiles were evaluated by the correlation coefficient ($R^2$). The highest degree of correlation coefficient determines the suitable mathematical model that follows drug release kinetics.[17]

Based on the release kinetic analysis, the release data were best fitted with first-order release with $R^2$ value being 0.9962. Hence, the drug release profile of ciprofloxacin was based on its concentration. To understand the mechanism of drug release the release data were fitted in the Kosermayer–Peppas equation which states the type of diffusion.[17] The corresponding plot for Korsmeyer–Peppas equation (Figure 6D) also shows good linearity. The release exponent "n" was found to be 1.3169, which is >0.89 which indicates super case II type of release. By considering this, it can be understood that the drug release from the conjugate was controlled by erosion mechanism.

Over all, the release studies show that the ciprofloxacin–GNF conjugates are suitable for sustained delivery of ciprofloxacin. The initial burst release will serve as a loading dose for controlling the disease spread and the sustained release phase will contribute towards a better therapeutic effect.

In the present research, we evaluated the antibacterial activity of ciprofloxacin–GNF conjugates along with ciprofloxacin and gold nanoflowers as control. The results revealed that biogenic gold nanoflowers showed no inhibition zone against the tested organisms. Gold nanoparticles have been reported to be non-toxic towards *Pseudomonas aeruginosa, E. coli, Bacillus subtilis, Vibrio chloerae, Staphylococcus epidermidis, Staphylococcus haemolyticus*, MRSA and *Shewanella oneidensis*.[28,29,30] Ciprofloxacin exhibited inhibition zone of 19 mm and 22 mm against *Pseudomonas aeruginosa* (ATCC 25668) and *E.* coli (ATCC 11103), while against *Bacillus subtilis* (ATCC 6538) and *Staphylococcus aureus* (ATCC 25923), the zone of inhibition was found to be 17 mm and 18 mm (Figure 7). The ciprofloxacin–GNF conjugate resulted in inhibition zones of 25 mm and 28 mm against *Pseudomonas* sp and

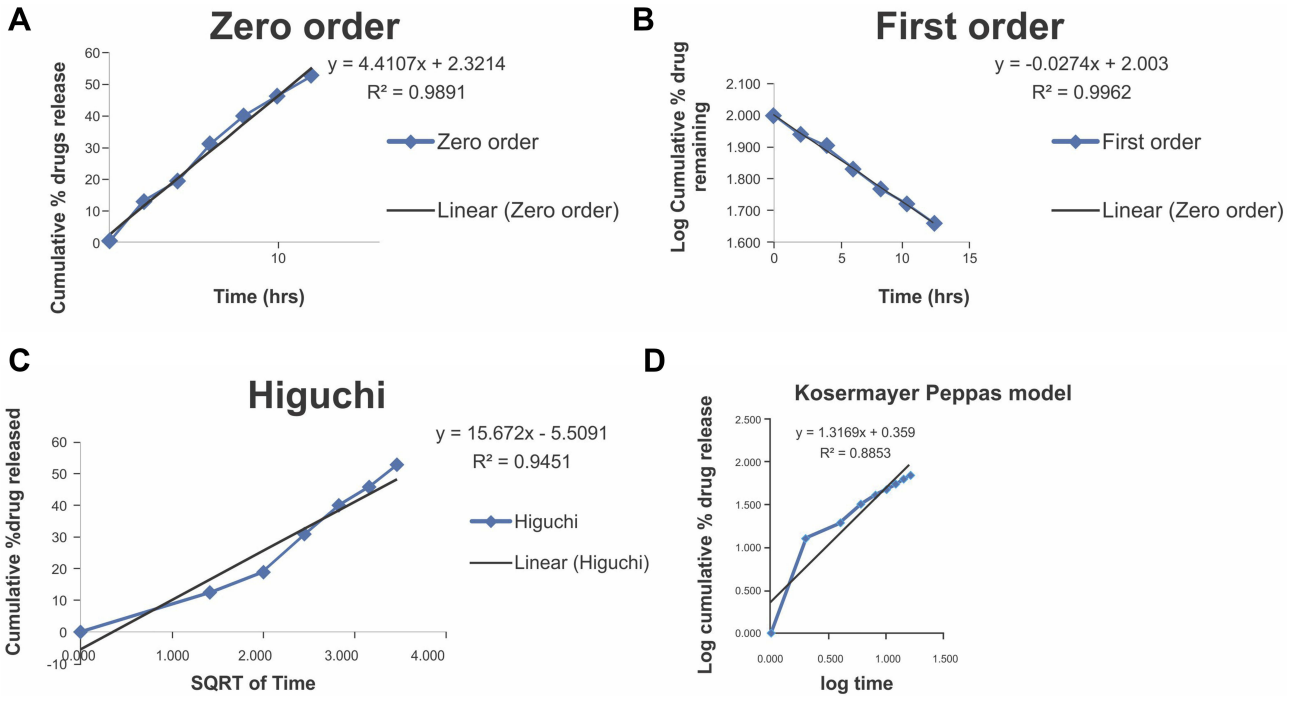


**Figure 6** Kinetic analysis of drug release: (**A**) zero-order kinetics, (**B**) first-order kinetics, (**C**) Higuchi kinetics, (**D**) Kosermayer–Peppas plot.

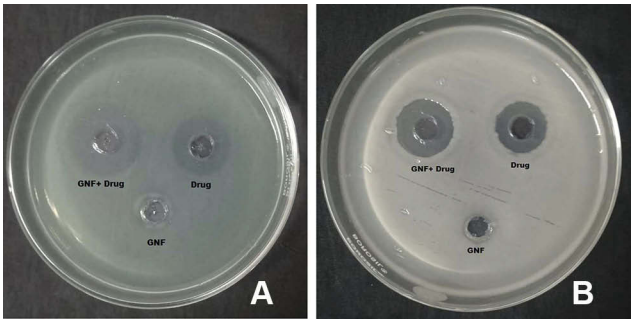


**Figure 7** Plates showing antimicrobial activity of gold nanoflowers, drug alone and drug–GNF conjugate against (**A**) *Pseudomonas* sp. and (**B**) *Bacillus subtilis*.

*E. coli* (ATCC 11103). Similarly, for *Bacillus subtilis* and *Staphylococcus aureus* (ATCC 25923), it was found to be 21 mm and 22 mm (Figure 8). These findings are consistent with Williams et al (2006) who investigated the antibacterial activity of gold nanoparticles and found them to be non-inhibitory for the bacterial growth or functional activity, while conjugates of vancomycin to gold NPs decreased the number of growing bacterial cells.[9] Similar results were also reported for gold nanoparticles by Grace and Padian (2007) who emphasized that gold nanoparticles itself did not inhibit the bacterial growth.[12] Gold nanoparticles impregnated with antibiotics such as neomycin, gentamycin and streptomycin have been tested against various strains of Gram-positive and Gram-negative organisms such as *Staphylococcus aureus, Micrococcus luteus, E. coli* and *Pseudomonas aeruginosa*. They concluded that depending on the antibiotic used, an increase in the activity of the antibiotic-colloidal-gold mixture ranged from 12% to 40%, when compared to the native antibiotic activities.

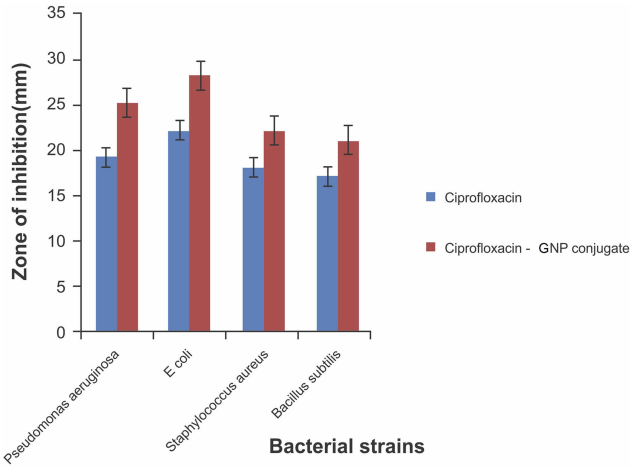


**Figure 8** Graph showing the zone of inhibition of ciprofloxacin and ciprofloxacin–GNF conjugate against the bacterial strains tested.

**Table 1** Minimum Inhibitory Concentration of Ciprofloxacin and Ciprofloxacin-Bound Gold Nanoflowers Against the Bacterial Strains*

| Bacterial Strain | | Ciprofloxacin (μg/mL) | Ciprofloxacin-Bound Gold Nanoflowers (μg/mL) |
|---|---|---|---|
| Gram-negative | *Pseudomonas aeruginosa* | 2 ± 0.1 | 0.2 ± 0.02 |
| | *E. coli* | 2 ± 0.08 | 0.25 ± 0.01 |
| Gram-positive | *Bacillus subtilis* | 3 ± 0.9 | 0.5 ± 0.02 |
| | *Staphylococcus aureus* | 3 ± 0.1 | 0.4 ± 0.01 |

**Note:** *The experiment was done in triplicate; the bacterial count was $10^8$ CFU/mL.

The MICs of the antibiotics and antibiotic-bound gold nanoflowers were tested against the various strains based on the liquid broth dilution method. It was found that MICs of all antibiotic-bound gold nanoflowers reduced significantly compared to their free forms (Table 1). The gold nanoflower-bound ciprofloxacin was found to reduce the MICs of the antibiotic from 2 to 0.21 μg/mL against *Pseudomonas aeruginosa*. Similarly, for *E. coli*, it reduced from 2 to 0.25 μg/mL. The antibacterial efficiency of ciprofloxacin increased when bound to gold nanoflowers and reduced its MIC from 3 to 0.5 μg/mL against *Bacillus subtilis,* and while for *Staphylococcus aureus,* the MIC of drug bound nanoflower was found to be 0.4 μg/mL. A similar report has also been reported for ciprofloxacin-coated gold nanoparticles against coagulase-negative staphylococci.[30]

Against *Pseudomonas aeruginosa* and *E. coli* ciprofloxacin–GNF conjugate resulted in 6 log reduction in the pathogen, while for *Bacillus subtilis* and *Staphylococcus aureus,* it achieved 5 log units reduction in the CFU/mL (Figure 9). We surmise that the higher level of antimicrobial efficacy

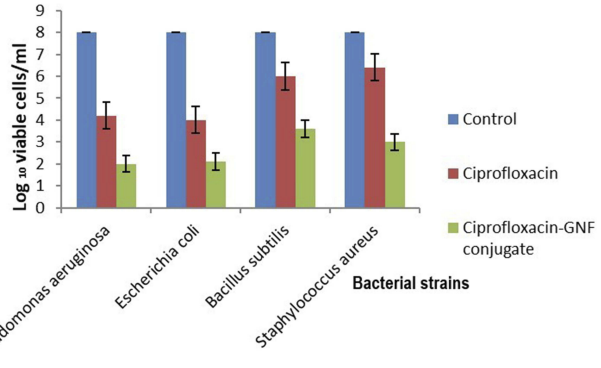


**Figure 9** Graph showing percent survival of bacterial strains against ciprofloxacin and ciprofloxacin–GNF conjugate.

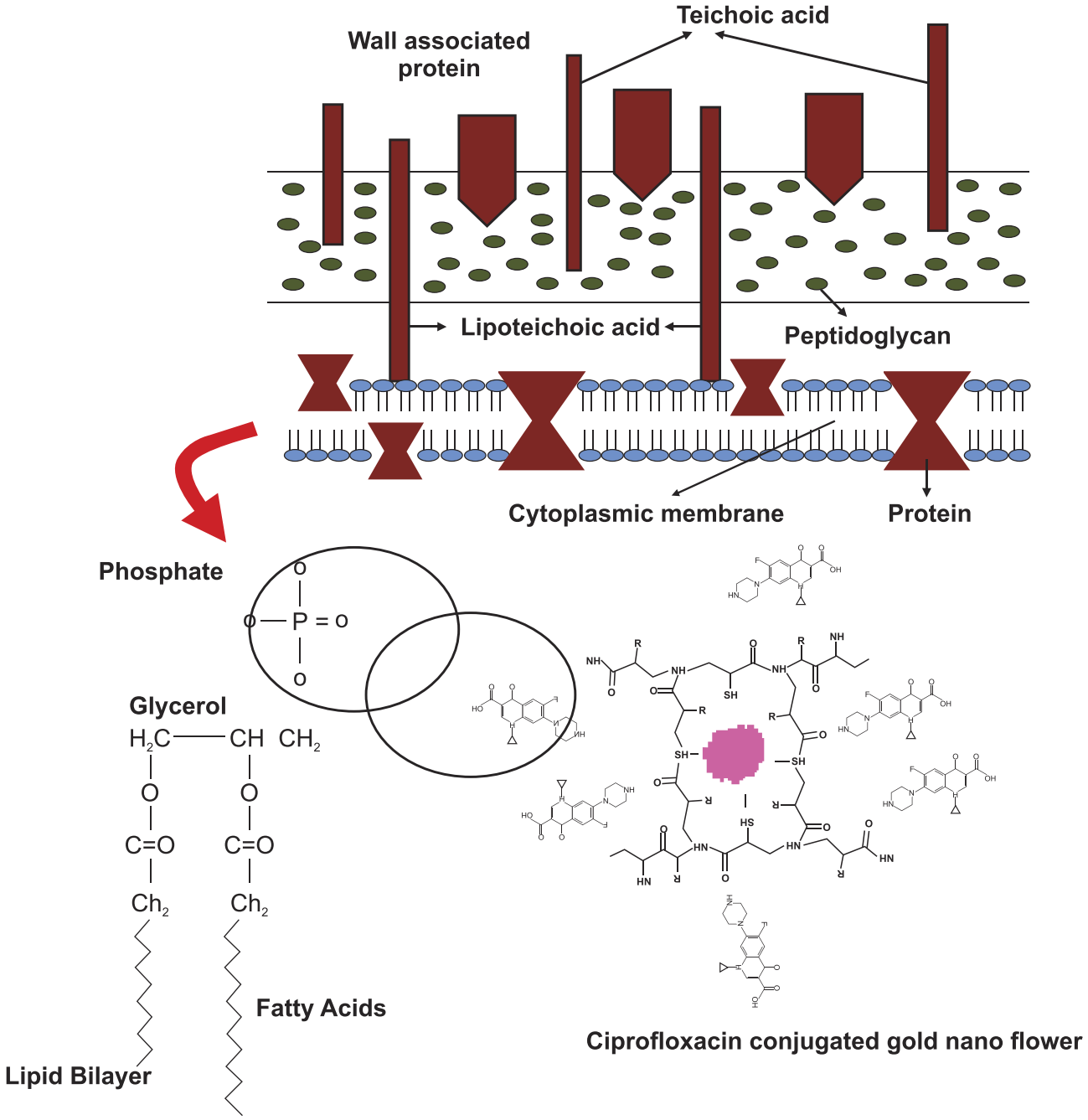


**Figure 10** Schematic representation of the interaction between ciprofloxacin–GNF conjugates against Gram-positive cell wall.

afforded by antibiotic-bound gold nanoflowers is due to the large surface to volume ratio of gold nanoflowers which facilitates adsorption of more antibiotic molecules onto gold surfaces via electrostatic attraction between the amine groups of drugs and nanoflowers. This has ensured there is an increase in the antibiotic concentration at the point of contact between bacterium and drug–nanoparticle conjugate. The gold particle functionalized with a number of antibiotic molecules now acts as a single moiety against the microorganisms.

The antibiotic nanoparticle conjugate might have entered the host cell through either of the two following mechanisms: The entire entity might have fused with microbial cell wall or membrane and release the carried drugs within the cell wall or membrane or according to the second hypothesis the entity might have bound with the bacterial cell wall and could have served as a reserve for continuous release of antibiotic which could then diffuse into the internal parts of the microorganisms. Figures 10 and 11 represent the schematic interaction of the drug–nanoparticle

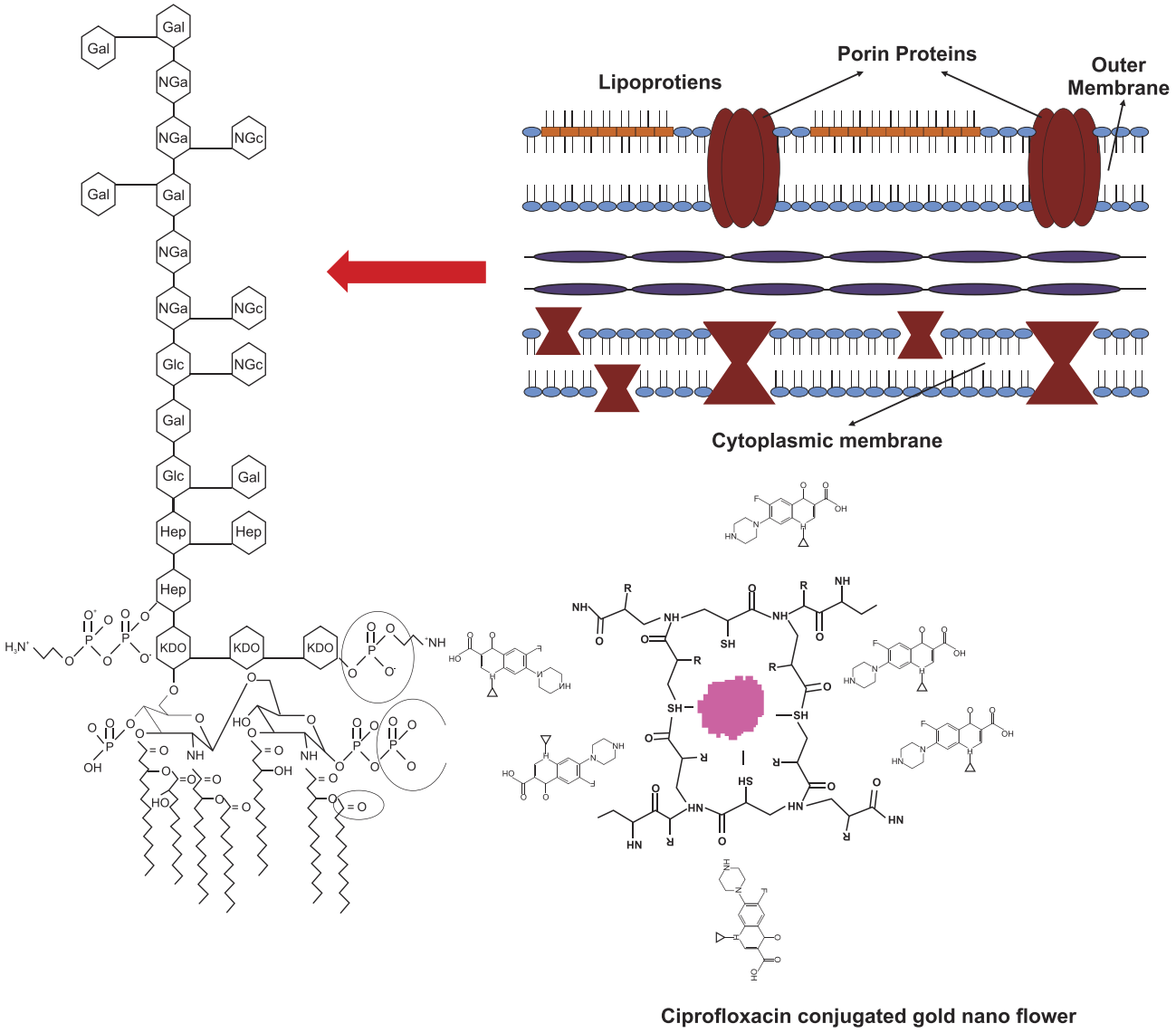


**Figure 11** Schematic representation of the interaction between ciprofloxacin–GNF conjugates against Gram-negative cell wall.

conjugate with the cell wall of Gram-positive and Gram-negative bacteria, respectively. The amine moiety of the drug–gold nanoparticle conjugate interacts with the cell wall to gain entry into the cell.

The uptake of negatively charged particles despite the unfavorable interaction between the particles and the negatively charged cell membrane has been reported in the literature.[3] Neutral and negatively charged nanoparticles have reported to show lower levels of internalization as compared to the positively charged particles due to their lesser adsorption onto the negatively charged cell-membrane surface. The negatively charged nanoparticles are believed to be taken up through their clustering and random binding at sparingly available cationic sites on the plasma membrane. For the entry, the binding of anionic nanoparticles to a lipid bilayer results in local gelation, while positively charged nanoparticles result in fluidity in gelled regions of bilayers.[31]

However, in our present study, we have once again confirmed that ciprofloxacin–gold nanoflower conjugates were more effective against various bacteria strains when compared with pure antibiotics. This finding has consolidated further the position of gold nanoflowers as biocompatible drug delivery systems without any adverse effects even on the microbial functional activities apart from enhancing the mode of action of antibiotics for a profound effect at a lower dose.

## Conclusion

In conclusion, we have demonstrated the biogenic synthesis of stable gold nanoflowers whose well-defined surface chemistry

can be exploited for the delivery of antibiotics. The exceptional feature of this biological synthesis is the non-requirement of any stabilizing or capping agent for maintaining the monodispersity of nanoparticles. The drug release curve indicated its release in a controlled manner at physiological pH. Gold nanoflowers alone are not toxic to the bacterial cells but enhanced the antibacterial potential of the drug for the Gram-negative and Gram-positive bacteria by reducing the MIC value of ciprofloxacin–GNF conjugate as compared to ciprofloxacin alone. In conjugation with antibiotic ciprofloxacin, GNF enhances the dose effect with sustained release at the site of action. Further collectively, the outcomes of this study support that ciprofloxacin functionalized gold nanoflowers with outstanding biocompatibility and stability can be used as potential nanocarrier systems for the delivery of antibiotics.

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## Author Contributions

Both authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

## Disclosure

The author reports no conflicts of interest in this work.

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