sciendo ASIAN BIOMEDICINE DOI 10.1515/abm-2019-0054 — Asian Biomed (Res Rev News) 2019; 13(4):149–162

Technical report

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A new methodology for simultaneous comparison and optimization between nanoparticles and their drug conjugates against various multidrug-resistant bacterial strains

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Abstract

Background: Multidrug-resistant bacteria are becoming more hazardous day by day for human health all over the world, and the scientific community is trying hard to resolve this issue by various approaches. One of the very common approaches is to bind drugs to nanoparticles and study enhanced antibacterial properties.

Objective: To compare simultaneously different types of nanoparticles, their concentration, bacterial strains and their incubation time intervals for each of the selected drug combination.

Methods: We have selected the most commonly used gold and silver nanoparticles and few examples from fluoroquinolone antibiotics to make their conjugates and study their efficacy against multidrug-resistant *E. coli* and *S. aureus* strains simultaneously, at different incubation time intervals and different concentration of nanoparticles.

Results: Gold nanoparticle hybrids do not show any significant effect. Silver nanoparticle hybrids show far better results, even at extremely low concentrations.

Conclusions: This unique and simple approach allows us to know the exact time intervals and concentration required for each nanoparticle combination to control the growth for any specific strain. This approach can be extended to any set of nanoparticles, drugs and bacterial strains for comparative purposes.

Keywords: anti-bacterial agents; colloidal silver; drug resistance; fluoroquinolones; gold colloid; metal nanoparticles

Among various approaches focused on multidrug-resistant bacteria, one approach is to bind drug molecules to nanoparticles [1, 2]. These nanoparticles act as carriers [3, 4], and in some cases, they provide synergistic effects, that is, drug bound to nanoparticles shows the enhanced efficiency of antibiotics. Many biological assays for such antibacterial or other biological studies require more than one setup to get the clear picture of the biological activity of provided samples. A new and simple methodology is required to compare the samples for their biological studies, along with variation in terms of concentration and time intervals effects at the same time. This will allow us to optimize the results quickly and get the best results from a huge number of samples.

This article focuses on metallic gold nanoparticles (AuNPs) and silver nanoparticles (AgNPs), which have various biomedical applications [5–8], and are used

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extensively because of their inert, nontoxic and bactericidal properties [9–12]. The toxicity only comes from the surfactants attached to the nanoparticles [13]. For AgNPs, toxicity can exert from the nanoparticles themselves [14]. Few reports also suggested that AuNPs have their antibacterial properties [15–17], which is contrary to a common belief. Antibacterial properties of AuNPs may be dependent on the size of the nanoparticles and bacterial strains used. However, it is confirmed that AgNPs possess a good antimicrobial activity [12]. Interestingly, few articles also suggested that molecules that have no antibacterial properties, when bound to AuNPs, possess antibacterial properties [18–20]. For the AgNPs, it was shown that capping is necessary for the antibacterial activity of AgNPs *in vivo* [21]. This suggests that the functional groups attached to nanoparticles have a great effect on their antibacterial properties [22, 23]. Few other strategies include reduction of gold salt using antibiotics, specifically Cefaclor for AuNPs [24], Ampicillin for AgNPs [25] and biosynthesis of AuNPs and AgNPs using bacterial strains and afterward functionalizing it with antibiotics, which also shows enhanced antibacterial activities as compared to pure drugs [26, 27].

We focus on fluoroquinolones that bind to nanoparticles [28–30]. Fluoroquinolones act by inhibition of type II DNA topoisomerases (gyrases) which are essential for the synthesis of DNA replication and also helpful in bacterial mRNAs (transcription) [31]. To our knowledge, there are very few reports that show binding of different fluoroquinolone drugs to AuNPs and show enhanced antibacterial properties of these conjugates resulting in an increased zone of inhibition [32]. Similar experimentation with Gentamycin has shown negative results [33]. Similarly, we only found two articles, where any of the fluoroquinolone drug bound to AgNPs can have a synergistic or an enhanced antibacterial activity against few organisms. These conjugates were studied against *Pseudomonas aeruginosa* (*P. aeruginosa*) [34], *Bacillus subtilis* (*B. subtilis*), *Klebsiella pneumoniae* (*K. pneumonia*) and *Staphylococcus aureus* (*S. aureus*) [35]. No activity against *P. aeruginosa* was observed, while a significant activity against *B. subtilis*, *K. pneumoniae* and *S. aureus* was observed. In addition, moxifloxacin fluoroquinolones bound to Au and Ag nanoparticles have been reported for *Urease* inhibitors as well [36].

Focusing on the bacterial strains, such nanoparticle-drug conjugates have been studied for *Escherichia coli (E. coli), Salmonella paratyphi* and *S. aureus* among many others [37]. Our studies reported in this article focused on clinical isolates of *E. coli* and *S. aureus*. The biological mechanism of action of fluoroquinolones toward microorganisms is well understood [38, 39]. Also, it is well known that the piperazine group of fluoroquinolones binds to the surface of AuNPs [40]. This allows us to consider that the pyridone moiety of fluoroquinolones may be responsible for the biological mechanism of action.

The effect of time intervals and the variation of concentration of the nanoparticles simultaneously could lead us to understand the role of nanoparticles in greater detail. As a result of this study, we will describe a new methodology for simultaneous comparison and optimization between nanoparticles and their drug conjugates against various multidrug-resistant bacterial strains. This approach may be extended to any type of nanoparticles and their conjugates with any class of drug molecules. Although we have focused on most commonly used combinations of nanoparticles, drugs and bacterial strains, the purpose of this study is to explore whether this methodology can be extended to any set of nanoparticles, drugs and bacterial strains. This method is easy to carry out and clearly defines a new way to compare samples for concentration and time intervals simultaneously and get the most important results in the least possible duration.

Materials and methods

Gold chloride salt (99.99%) was purchased from Guangdong Guanghua Chemical Factory Co. Ltd, China. $AgNO_3$ was purchased from Duksan Pure Chemicals Co., Ltd, Korea. Trisodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) was purchased from Fluka.

For the antimicrobial assay, Mueller Hinton Broth (MHB) and nutrient agar were purchased from Sigma-Aldrich. Bacterial strain samples of *E. coli* and *S. aureus* were obtained from Department of Microbiology, Allied Hospitals - Rawalpindi Medical University, Pakistan. The clinical isolates were collected from the samples of pus, urine and catheter tips.

Antibacterial drugs ciprofloxacin, ofloxacin and norfloxacin were obtained as gift samples from Curatech Pharma (Pvt) Ltd., Lahore Pakistan, while pefloxacin and levofloxacin were obtained as gift samples from Panacea Pharma Group, Islamabad, Pakistan.

Au and Ag nanoparticles

Au and Ag nanoparticles were prepared by using a chemical reduction method [41]. The Au and Ag nanoparticles were obtained approximately 20 and 64 nm in size, respectively. The concentration of AuNPs was calculated to be 2.87 and 0.124 nM for AgNPs.

Nanoparticles–fluoroquinolones binding studies

AuNPs (0.2 ml, 2.87 nM) or AgNPs (0.2 ml, 0.124 nM) were added to 2.6 ml of distilled water in a cuvette, which was further added 0.2 ml of 0.04 M of each of the fluoroquinolone drugs individually, to make up the final volume of 3 ml. These drugs namely ciprofloxacin, levofloxacin, norfloxacin, ofloxacin and pefloxacin were studied for their binding to AuNPs or AgNPs using UV–Vis spectroscopy [32]. The decrease in the SPR peak (hypochromic shift) or bathochromic shift was the indicator for the binding studies. In general, the binding occurs instantaneously.

UV–Vis spectroscopy

For the biological studies, Rayto RT-2100C Microplate Reader from Rayto Life and Analytical Sciences Company, Ltd., China was used in the absorbance mode. The wavelength selected was 630 nm. For binding studies, samples were prepared by combining the correct amount and appropriate ratio, as discussed earlier, from stock solutions of AuNPs or AgNPs and fluoroquinolone drugs. BMS UV-1602 double beam UV–Vis spectrophotometer equipped with a deuterium lamp and a halogen lamp from Biotechnology Medical Services K Canada Inc. was used for the spectroscopic analysis and binding studies. Quartz cuvettes were used with path lengths of 10 mm.

Biological evaluation

Antimicrobial assay of AuNP–drug conjugates

Multidrug-resistant pathogenic bacterial strains of *E. coli* and MRSA were isolated and routinely cultured overnight at 37°C with agitation in MHB under aerobic conditions. The bacterial concentration was adjusted to 1×10^6 CFU/ml by using the absorbance of the bacterial suspension. Minimum inhibitory concentration (MIC) was determined by the broth dilution method using 96-well plate reader. Inoculum was then added to MHB followed by the addition of antibiotic drug and AuNPs (or AgNPs) dispersion of varying (increasing) amounts. The fixed wavelength of 630 nm was used for absorbance.

The final volume of each well was 200 µl. Following inoculation in 96-well plate under continuous shaking, the absorbance of inoculated isolates was monitored at various time points with a final reading at 24 h.

Preparation of antibiotic stock solutions (MIC (mg/l) for reference strains)

A suitable range of antibiotic concentrations was selected for the organisms to be tested (**Table 1**).

McFarland index

With constant stirring, 0.5 ml of 0.048 M $BaCl_2$ (1.175% w/v $BaCl_2$) was added to 99.5 ml of 0.18 mol/l H_2SO_4 (1% v/v). The prepared suspension absorbance at 630 nm was in limits of 0.08–0.10 for McFarland index [44].

Dilutions according to Clinical and Laboratory Standards Institute (CLSI; M07-A10)

Each well was 5×10^5 CFU/ml after inoculation, and McFarland index was 1.5×10^8 CFU/ml. Each well contained approximately 2.5×10^4 cells. The dilutions used for antibacterial drugs were according to the suggested target MIC (mg/l) values. The bacterial growth (or inhibition) was recorded for increasing order of concentration of nanoparticles. The MHB was used for nanoparticles antimicrobial assay. The inoculum was 1×10^6 CFU/ml per well with a final volume of 200 µl which includes antibiotics, nanoparticles and media. The remaining volume was made up with water.

Frequency of readings

Following inoculation in 96-well plate under continuous shaking, the absorbance of inoculated isolates was monitored at predetermined time intervals, that is, 0, 1, 2, 4, 8, 12 and 24 h (final reading), respectively.

Table 1. Standard minimum inhibitory concentrations (MIC) for reference strains

Fluoroquinolones	*E. coli*	*S. aureus*
Ciprofloxacin [42]	0.015 mg/l	0.12 mg/l
Levofloxacin [43]	0.03 mg/l	0.12 mg/l
Norfloxacin [42]	0.06 mg/l	0.25 mg/l
Ofloxacin [42]	0.06 mg/l	0.25 mg/l
Pefloxacin [42]	0.06 mg/l	0.25 mg/l

Results

In this study, we explored the antimicrobial effects of pure nanoparticles and five different representative fluoroquinolone drugs from the second and third generation, namely ciprofloxacin, levofloxacin, norfloxacin, ofloxacin and pefloxacin, respectively, bound to AuNPs or AgNPs as hybrid nanoparticle – drug conjugates. The antimicrobial effects of drug–nanoparticles combinations were then tested on *E. coli*, a Gram-negative bacteria and *S. aureus*, a Gram-positive bacterium for comparative studies. These experiments were carried out at 37°C. The structures of fluoroquinolone drugs we used are shown in **Figure 1**.

For the antimicrobial studies using microbial plate reader, a certain absorbance observed (of any peak, at any specific time) represents the presence of bacterial strains. Since AuNPs and AgNPs also absorb strongly, their absorbance was monitored separately (without bacterial strains, media, or any added drugs) and was subtracted from the total absorbance. The resultant peaks after the subtraction of AuNPs or AgNPs absorbance are provided in all spectra below. The composition of the 96-well plate reader for pure nanoparticles and nanoparticle-drug hybrids is provided in the following section.

Composition of 96-well plates

Pure nanoparticles

No drugs were used in these experiments to differentiate between the activity of pure nanoparticles as compared to their drug conjugates. AuNPs or Ag NPs only for reference absorbance were also measured, which was subtracted from the total absorbance to represent the absorbance of bacterial strains only, representing their growth or inhibition in the wells. The total volume in each well plate was fixed to 200 µl. The amount of inoculum and media was also fixed to 10 and 80 µl, respectively. The number of nanoparticles was increased from 0 to 100 µl in 10 µl increments. The remaining volume was substantiated by an appropriate volume of water. Blank for NPs was measured separately to differentiate the absorbance of NPs from the absorbance of bacteria.

Nanoparticle-drug hybrids

The total volume in each well plate was fixed to 200 µl. The amount of inoculum, media and drug was also fixed to 10, 80 and 10 µl, respectively. The amount of nanoparticles was increased from 0 to 100 µl in 10 µl increments. The remaining volume was substantiated by the appropriate volume of water. Blank for NPs was measured separately to differentiate the absorbance of NPs from the absorbance of bacteria. Readings without drug and nanoparticles, drug only (without nanoparticles) and drug with nanoparticles were taken to differentiate the effects of drug and nanoparticles.

Activity of AuNPs and AgNPs

Effects of time intervals

Figure 2a and 2b shows the growth or inhibition of *E. coli* with pure Au and Ag nanoparticles respectively, whereas **Figure 2c and 2d** shows the growth or inhibition of *S. aureus* bacterial strains with pure Au and Ag nanoparticles,

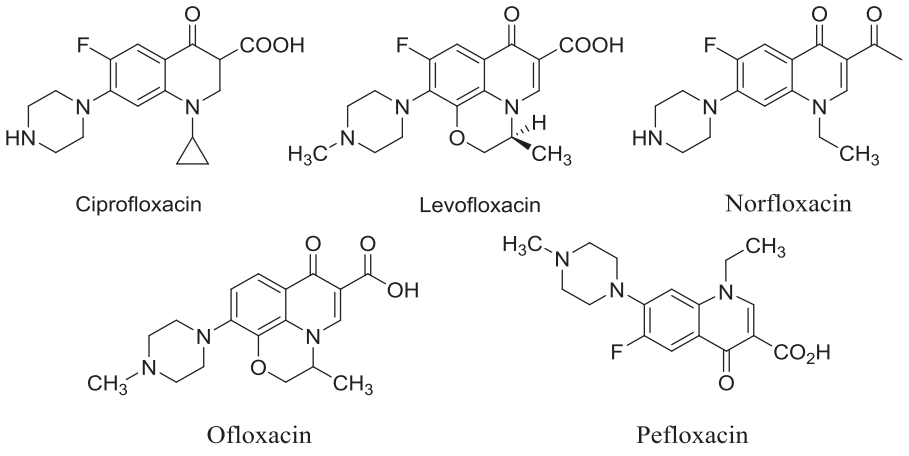

Figure 1. Few representative molecules from the second- and third-generation fluoroquinolone antibiotics are used in this study.

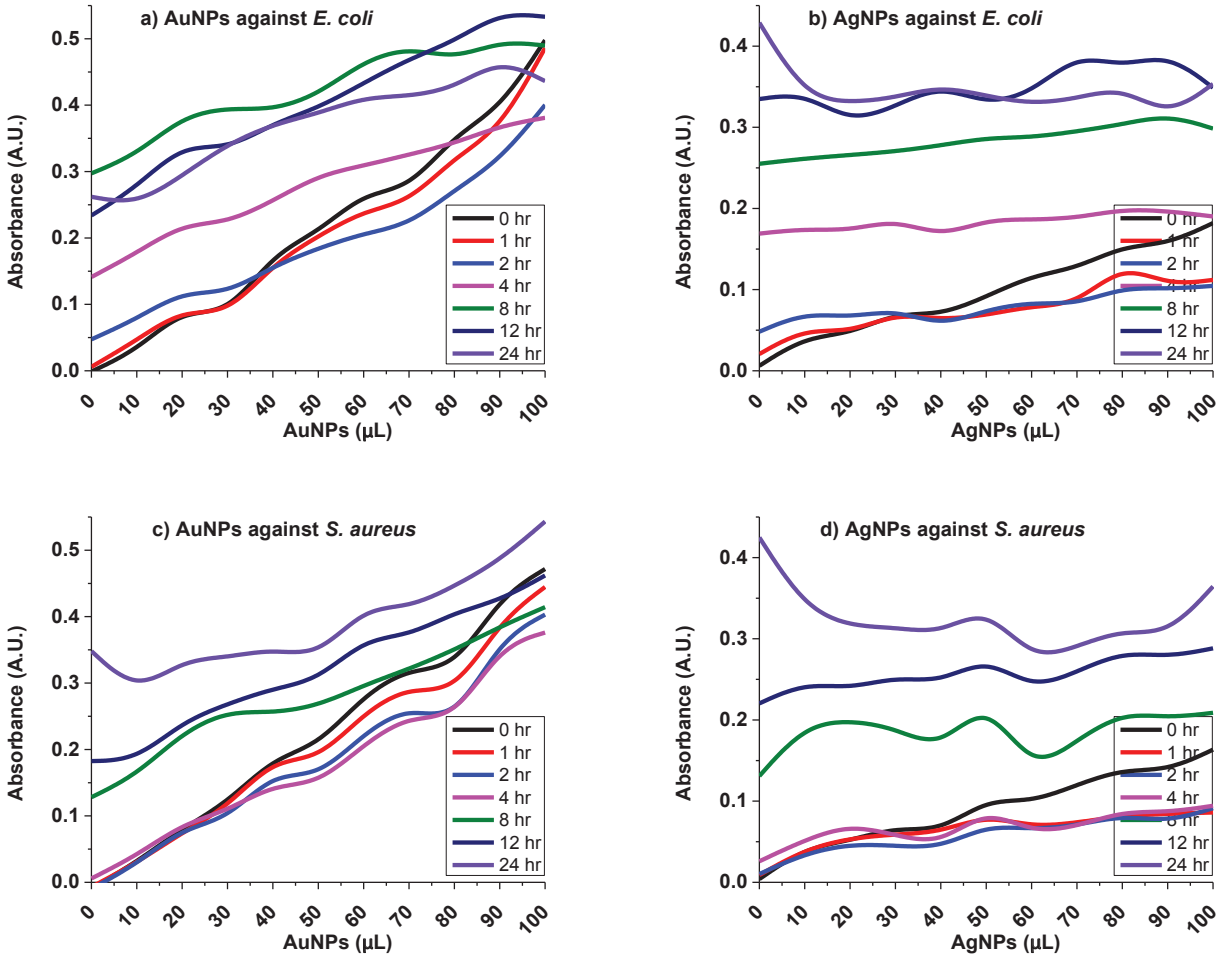

Figure 2. Absorbance of *E. coli* **(a, b)** and MRSA **(c, d)** with increasing concentration of AuNPs (2.87 nM) **(a, c)** and AgNPs (0.124 nM) **(b, d)** from 0 to 100 µl with 10 µl increments.

respectively. For AuNPs against *E. coli*, it is observed that until 2 h of incubation time intervals, the absorbance of bacterial strains is the lowest at any given concentration of Au nanoparticles, corresponding to optimal decay or control of growth of bacteria. Further going in details, for AuNP against *E. coli*, the first peak at 0 h (immediately after the addition of Au nanoparticles, black in color) shows a certain absorbance, which remains the same until 2 h of incubation time intervals, representing control on the bacterial growth. Afterward, the growth is increased until 8 h to the maximum level. The absorbance afterward slightly decreases at 12 and 24 h incubation time intervals. Hence, the growth is maximum at 8 h. For AuNPs against *S. aureus*, we observe the continuous growth until 24 h of incubation time intervals, representing no control over time. For the AgNPs against *E. coli*, we observe the similar control as that of AuNPs until 2 h of incubation time intervals. Afterward, the growth keeps on increasing with increasing time intervals. For the AgNPs against *S. aureus*, we observe control until 4 h of incubation time intervals after which the rapid growth is seen. All these results show that the maximum control is achieved in 2–4 h of incubation time intervals for these nanoparticles.

Concentration of nanoparticle effects

With increasing concentration of nanoparticles from 0 to 100 µl, the absorbance also increases, which means that these nanoparticles have no effect on the bacterial growth, probably due to multidrug- resistant nature of these organisms. The same phenomenon is observed for AuNPs against *S. aureus*. However, for the AgNPs, we see that the increasing concentration does not affect the inhibition of *E. coli* or *S. aureus* significantly. This essentially means that AuNPs have an opposite effect of increasing concentration, and AgNPs have no significant effect of the increasing concentration.

Activity of drug conjugates of AuNPs and AgNPs

The *X*-axis in the following experiments represents 12 measurements as control (no drug and no nanoparticles added), drug only (without nanoparticles) and nanoparticles added in volumes of 10–100 µl. *Y*-axis represents the absorbance in arbitrary units. All spectra contain seven different peaks (shown in different colors), representing absorbance values taken at different time intervals, that is, 0, 1, 2, 4, 8, 12, and 24 h, respectively. A trend that shows a slight increase in absorbance when going from control to drug only measurement represents that drug also absorbs slightly in the mixture at 630 nm, and that bacterial strains are resistant to that specific drug. A trend, where absorbance is decreased from control to drug, only represents that drug is effective toward that bacterial strain. In other words, bacterial strains are not resistant to that specific drug. Since the concentration of drugs was kept constant, and that the absorbance of AuNPs or AgNPs was subtracted from the total absorbance, the net increase or decrease in absorbance pattern only represents either increase or decrease in the concentration of bacterial strains. In other words, the increase (growth) or decrease (inhibition) of bacterial strains is represented by an increase or decrease of absorbance. The absorbance values at increasing concentrations of nanoparticles move upwards or downwards due to the combination of various phenomena including binding of drug to the nanoparticles, agglomeration of nanoparticles and plausible stability of overall contents present in each well. However, in this study, our focus will remain on the overall effects observed in the growth of bacterial strains or vice versa and the absorbance changed with time intervals after the addition of nanoparticles. The antibacterial studies for AuNP – fluoroquinolone conjugates and AgNP – fluoroquinolone conjugates against *E. coli* and *S. aureus* were similarly performed.

Ciprofloxacin – NPs conjugates against E. coli and S. aureus

Effects of time intervals

Figure 3a and 3b shows the growth or inhibition of *E. coli* with Au and Ag nanoparticles, respectively, whereas **Figure 3c and 3d** shows the growth or inhibition of *S. aureus* bacterial strains with Au and Ag nanoparticles, respectively, for the drug ciprofloxacin bound to a varied amount of AuNPs or AgNPs from 10 to 100 µl. For AuNP conjugates against *E. coli*, it is observed that at 12 h of incubation time intervals, the absorbance of bacterial strains is the lowest at any given concentration of Au nanoparticles, corresponding to optimal decay or control of growth of bacteria. Further going in details, for AuNP conjugates against *E. coli*, the first peak at 0 h (immediately after the addition of Au nanoparticles, black in color) shows a certain absorbance, which is slightly increased after 1 h, representing the increase in the bacterial growth. This trend keeps on slightly increasing or decreasing at various concentrations of nanoparticles, but the overall effect remains the same until 8 h, where absorbance of bacterial growth starts decreasing. Afterward, we observe a further decrease in absorbance at 12 h, where the overall absorbance is minimum, especially at the lower concentrations of nanoparticles. The last reading was observed at 24 h, which was similar to the absorbance at 12 h at the higher concentration of nanoparticles, but a higher absorbance at the lower concentration of nanoparticles was observed representing an overall increase in growth of bacteria at 24 h. As conclusion, the combination of drug ciprofloxacin with AuNPs does show a net decrease in the absorbance at 12 h, but the overall effect is not so significant. However, for AuNPs against *S. aureus*, overall results suggest that this combination of ciprofloxacin and AuNPs at any given concentration is not effective as the absorbance with time intervals keeps on increasing representing growth of bacterial strains. Further going in details for AuNP conjugates against *S. aureus*, immediately after the addition of nanoparticles, the peak at 0 h showed a certain absorbance that is slightly increased after 1 h representing the increase in the bacterial growth. The last reading was observed at 24 h, which shows the highest absorbance. For the AgNPs hybrids against *E. coli*, we observe that within 1 h, the concentration of the bacterial strains is at the lowest level, however with increasing time intervals, the concentration of bacterial strains slightly increases but does not increase from the initial level. Finally, AgNP hybrids against *S. aureus* show that bacterial concentration is decreased within 4 h of incubation time intervals to the lowest level and then increases with time significantly.

Concentration of nanoparticle effects

For AuNP conjugates against *E. coli*, the most pronounced effect is observed at 10 µl of AuNPs at 2 h of incubation time intervals, after which the absorbance is increased until the addition of 90 µl of AuNPs. It must be noted that at a concentration of 100 µl of AuNPs, the absorbance decreases representing the control in the growth of microorganisms; hence, the activity of such conjugates is increased at the higher concentration of nanoparticles. Comparing it with Ag composites of ciprofloxacin, the addition of AgNPs even at the lower concentration helps decrease the concentration of bacterial strains and consistently helps control the growth of bacterial strains.

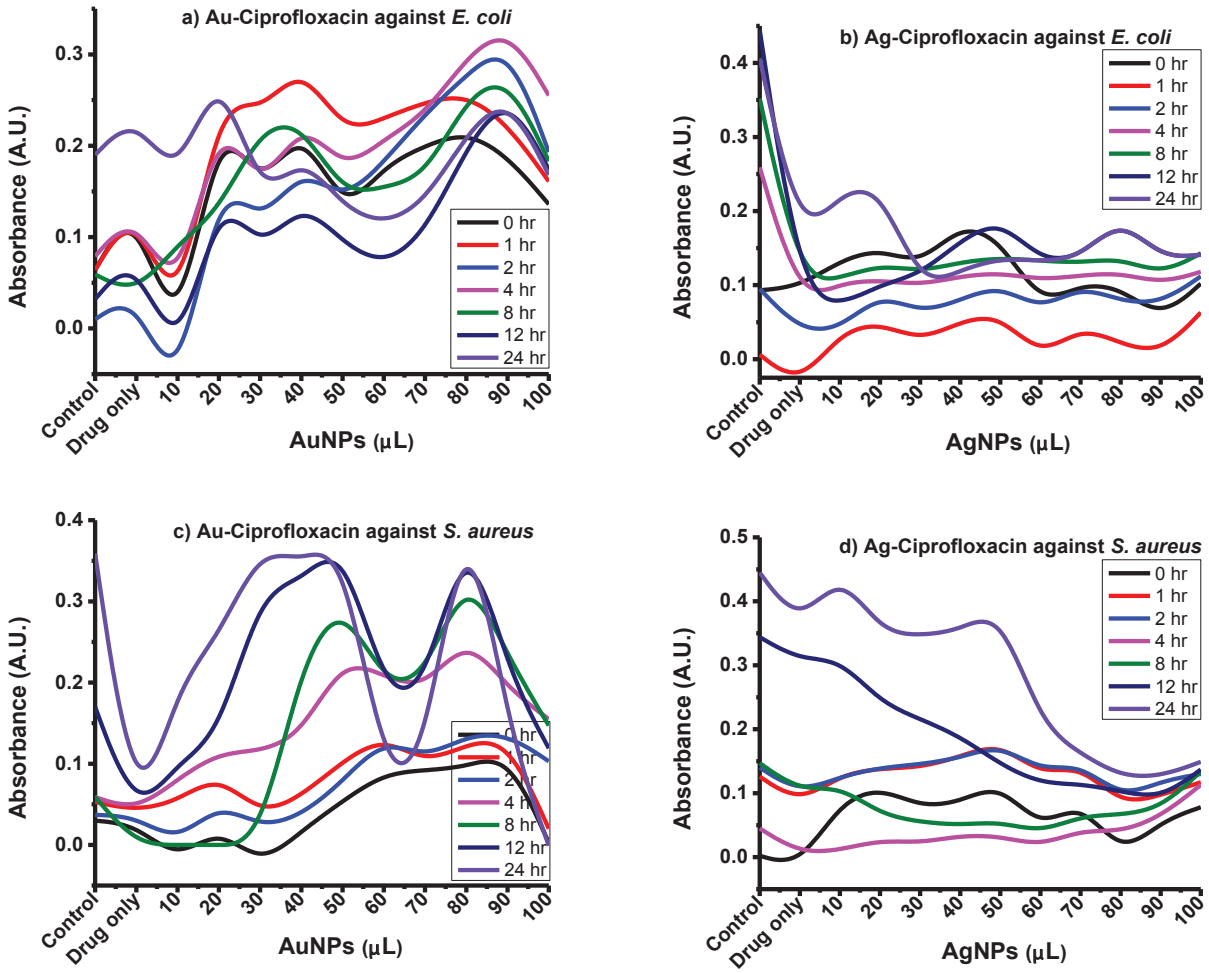

Figure 3. Absorbance of *E. coli* **(a, b)** and MRSA **(c, d)** in the absence of drug or nanoparticles (control), in the presence of drug only (ciprofloxacin) and by varying concentrations of AuNPs (2.87 nM) **(a, c)** and AgNPs (0.124 nM) **(b, d)** from 10 to 100 µl.

However, 10 µl of AgNPs at 24 h of incubation time intervals loses control, and little growth of *E. coli* is observed.

For the Au conjugates against *S. aureus*, the peak absorbance is decreased on the addition of drug ciprofloxacin, representing that *S. aureus* growth is inhibited by the use of this drug. Generally, all peaks slightly increase with the increasing concentration of nanoparticles but decreases slightly at 100 µl of AuNPs. The increasing absorbance with the increasing concentration of Au nanoparticles until 90 µl shows that nanoparticle conjugates have no effect on the inhibition of bacterial strains. However, at 100 µl of nanoparticles, the decreased absorbance represents control in the growth of *S. aureus* strains. In conclusion, the combination of drug ciprofloxacin with AuNPs does show a net decrease until 100 µl of AuNPs are added, but the absorbance was never lower than the initial levels (control experiment). Overall, a combination of AuNPs and ciprofloxacin is not effective against *S. aureus*. For AgNP conjugates against *S. aureus*, we certainly observe much better control as compared to AuNPs; however, at least 70 µl of AgNPs is required to control the growth of bacterial strains.

Levofloxacin–NPs conjugates against E. coli and S. aureus

Effects of time intervals

Figure 4a and 4b represents the growth or inhibition of *E. coli* with Au and Ag nanoparticles respectively, whereas **Figure 4c and 4d** shows the growth or inhibition of *S. aureus* bacterial strains with Au and Ag nanoparticles, respectively, for the drug levofloxacin bound to AuNPs or AgNPs. The results for AuNP conjugates against *E. coli* show that the absorbance increases after the incubation of bacterial strains and, however, slightly decreases at 12 h of incubation time intervals, which is still

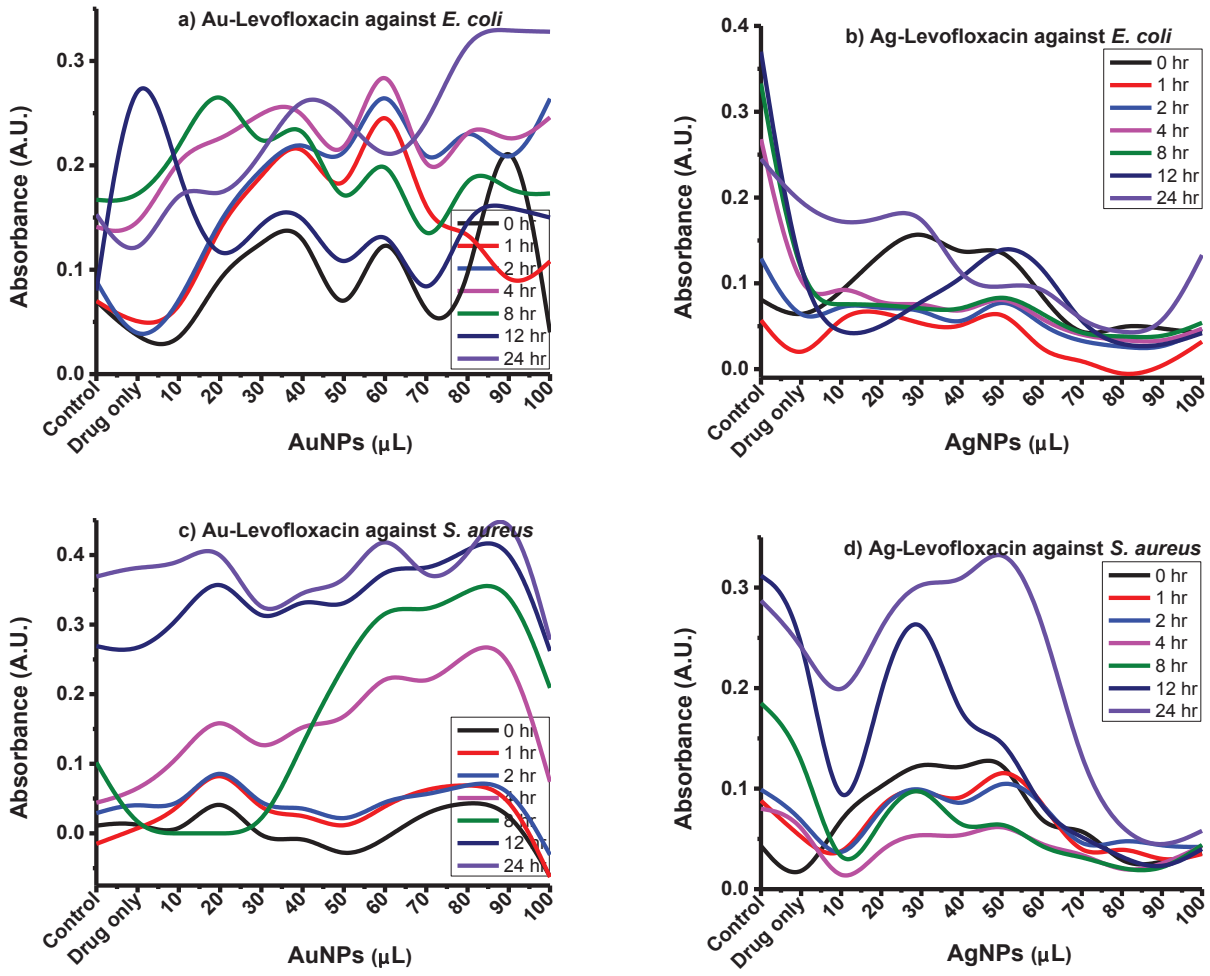

Figure 4. Absorbance of *E. coli* **(a, b)** and MRSA **(c, d)** in the absence of drug or nanoparticles (control), in the presence of drug only (levofloxacin) and by varying concentrations of AuNPs (2.87 nM) **(a, c)** and AgNPs (0.124 nM) **(b, d)** from 10 to 100 µl.

higher than the initial absorbance, measured at 0 h. AgNP conjugates against *E. coli* show similar results as of ciprofloxacin conjugates, which are far superior to AuNPs, and we observe the maximum control of growth at 1 h of incubation time intervals, that is, we observe the lowest absorption. However, after 1 h, an increase in absorbance is observed, but overall it does not increase than the initial levels, especially until 8 h, that is, the overall concentration of bacterial strains is similar to the initial levels and does not increase. For AuNP conjugates against *S. aureus*, the absorbance increases with time intervals, representing that there is no effect of drug hybrid on *S. aureus*. AgNP conjugates against *S. aureus*, however, show once again better results until 8 h of incubation time intervals, with the lowest absorbance observed at 4 h of incubation time intervals. Afterward, the increase in absorbance is observed even higher than initial levels.

Concentration of nanoparticle effects

For the AuNP conjugates against *E. coli*, we observe mixed results, essentially providing no effects at any concentration of nanoparticles. For AuNP conjugates against *S. aureus*, we observe a slight decrease in absorbance at the addition of 100 µl of AuNPs; however, the absorbance increases until the addition of 90 µl of nanoparticles. All concentrations of AgNPs shows good control against *E. coli*. AgNP conjugates against *S. aureus* show very interesting results at the addition of 70 µl or higher of AgNPs. As conclusion, we can say that the combination of levofloxacin and AuNPs is not effective against *E. coli* or *S. aureus*. However, for AgNP-levofloxacin conjugates, we observe very good effects against both organisms. A smaller concentration of AgNPs for *E. coli* and

a higher concentration of AgNPs for *S. aureus* are required to control their growth.

Norfloxacin–NPs conjugates against E. coli and S. aureus

Effects of time intervals

Figure 5a and 5b represents the growth or inhibition of *E. coli* with Au and Ag nanoparticles respectively, whereas **Figure 5c and 5d** shows the growth or inhibition of *S. aureus* bacterial strains with Au and Ag nanoparticles, respectively, for the drug norfloxacin bound to AuNPs or AgNPs. The results for AuNP conjugates against both *E. coli* and *S. aureus* are not encouraging for this combination as absorbance keep on increasing with time intervals for both cases (6a and 6c). For AgNP conjugates against *E. coli*, we observe the control until 2 h of incubation time intervals, after which the growth is observed significantly. The lowest absorption level was at 1 h of incubation time intervals as was observed with previous cases. Similarly, for AgNP conjugates against *S. aureus*, we observe control until 8 h of incubation time intervals; however, absorbance increases afterward. The maximum control observed, in this case, was 4 h of incubation time intervals.

Concentration of nanoparticle effects

For *E. coli*, the addition of AuNPs shows a negative effect, that is, the absorbance increases after the addition of nanoparticles, which is consistent for all measurements. For *S. aureus*, however, the absorbance increases slightly as compared to *E. coli*. The absorbance of *S. aureus* strains suddenly but slightly decreases at 100 µl of nanoparticles as observed

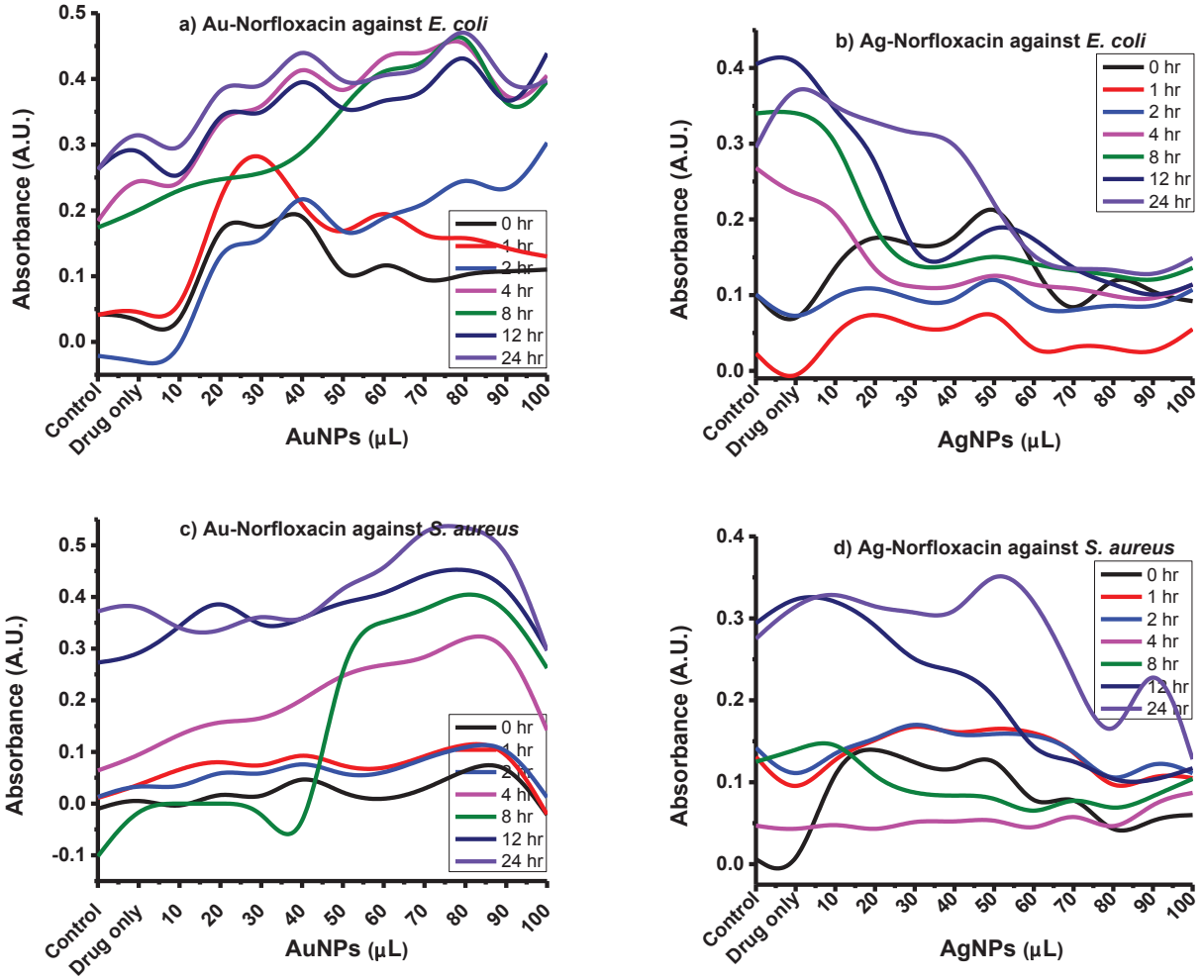

Figure 5. Absorbance of *E. coli* **(a, b)** and MRSA **(c, d)** in the absence of drug or nanoparticles (control), in the presence of drug only (norfloxacin) and by varying concentrations of AuNPs (2.87 nM) **(a, c)** and AgNPs (0.124 nM) **(b, d)** from 10 to 100 µl.

previously, representing a similar behavior as with ciprofloxacin conjugates and levofloxacin conjugates. Further investigation is required in this aspect for the effects of AuNPs at higher concentrations. For the AgNP conjugates against *E. coli*, we observed controlled effects at 50 µl and higher of AgNPs. For AgNP conjugates against *S. aureus*, 70 µl or higher of AgNPs is required for controlling the growth of bacterial strains.

Ofloxacin–NPs conjugates against E. coli and S. aureus

Effects of time intervals

Figure 6a and 6b represents the growth or inhibition of *E. coli* with Au and Ag nanoparticles respectively, whereas **Figure 6c and 6d** shows the growth or inhibition of *S. aureus* bacterial strains with Au and Ag nanoparticles, respectively, for the drug ofloxacin bound to AuNPs or AgNPs. For the results for AuNP conjugates against *E. coli*, we observed that the absorbance remains almost the same until 8 h of incubation time intervals and then decreases to the lowest level at 12 h and then again increases slightly at 24 h of incubation time intervals. As far as AgNP conjugates are concerned, 1 h of incubation time intervals provides the maximum control with the lowest absorbance required. As time proceeds, the growth of the bacterial strains is increased.

For the AuNP conjugates against *S. aureus* strains, we observe only the growth of bacteria from 0 to 24 h, which is consistent. However, the absorbance for AgNP conjugates against *S. aureus*, and we observe control until 8 h of incubation time intervals with the lowest absorbance at 4 h of incubation time intervals. In general, ofloxacin combined with AuNPs shows no effect against E. coli or S. aureus, however a reasonable activity is observed, when AgNPs are used for the conjugation purpose.

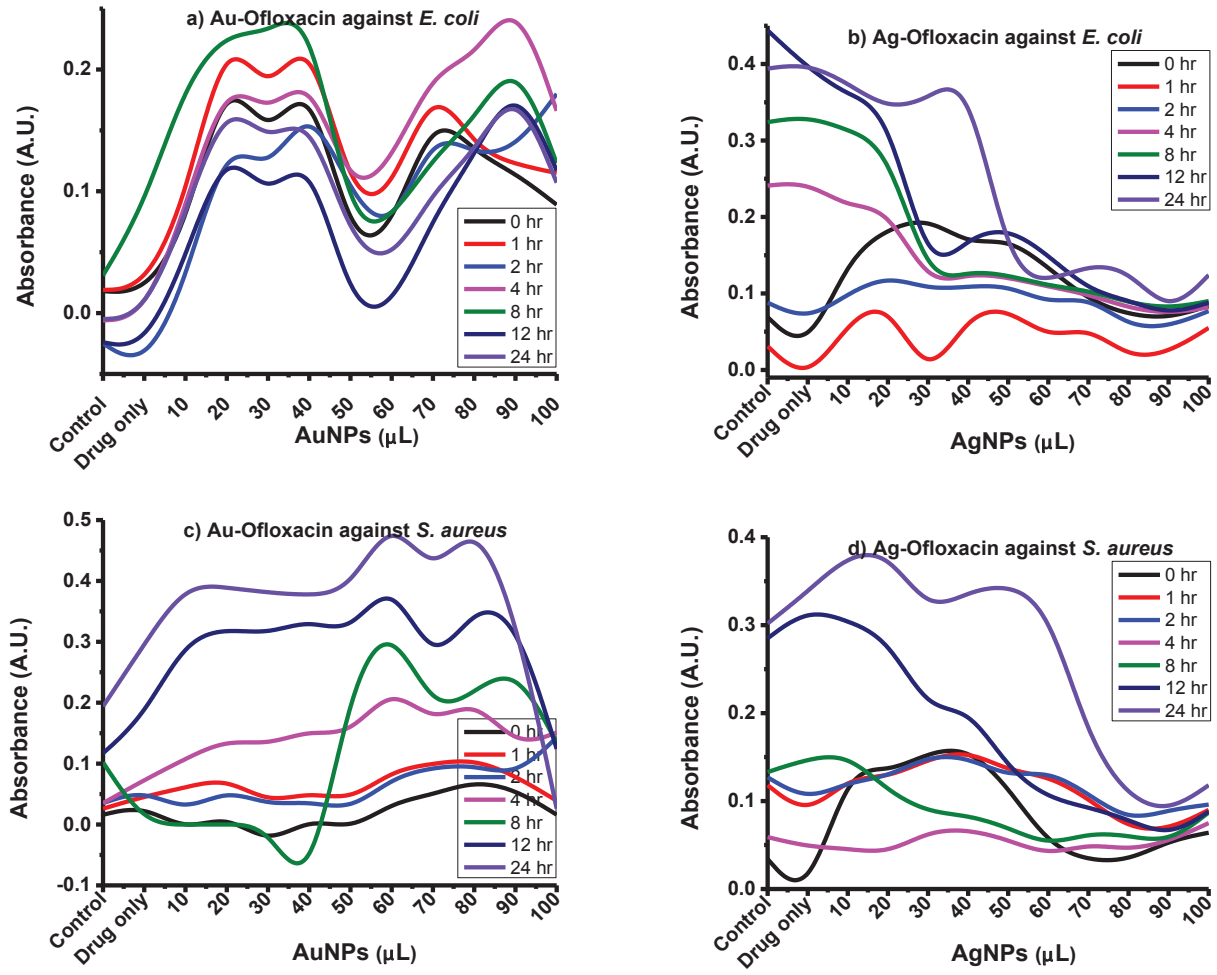

Figure 6. Absorbance of *E. coli* **(a,b)** and MRSA **(c, d)** in the absence of drug or nanoparticles (control), in the presence of drug only (ofloxacin) and by varying concentrations of AuNPs (2.87 nM) **(a, c)** and AgNPs (0.124 nM) (b,d) from 10 to 100 µl.

Concentration of nanoparticle effects

Au conjugates against *E. coli*, as usual, show a slight decrease in absorbance at the final reading of 100 µl, representing the slight control in growth at higher concentrations of nanoparticles. An unexpected decrease in the absorbance in all cases was observed at 50–60 µl of AuNPs. This is an interesting result, which we will be explored further in our future studies. Other than this unusual result, generally, nanoparticles have a negative impact, that is, the absorbance increases after the addition of nanoparticles representing the growth of *E. coli* bacterial strains. For AgNPs, 50 µl is sufficient to control the growth of *E. coli*.

For Au conjugates against *S. aureus*, similar to the previous case, the absorbance slightly increases with the increased concentration of nanoparticles. However, consistent with all previous results, the growth of *S. aureus* strains is slightly controlled at a higher concentration of nanoparticles that is at 100 µl of AuNPs. However, for the AgNP conjugates against *S. aureus*, 70 µl or higher is required for the control of the growth of bacterial strains.

Pefloxacin–NPs conjugates against *E. coli* and *S. aureus*

Effects of time intervals

Figure 7a and 7b represents the growth or inhibition of *E. coli* with Au and Ag nanoparticles respectively, whereas **Figure 7c and 7d** shows the growth or inhibition of *S. aureus* bacterial strains with Au and Ag nanoparticles, respectively, for the drug pefloxacin bound to AuNPs or AgNPs from 10 to 100 µl. For the results for AuNP conjugates against *E. coli* as well as *S. aureus* strains, we observed that absorbance increases

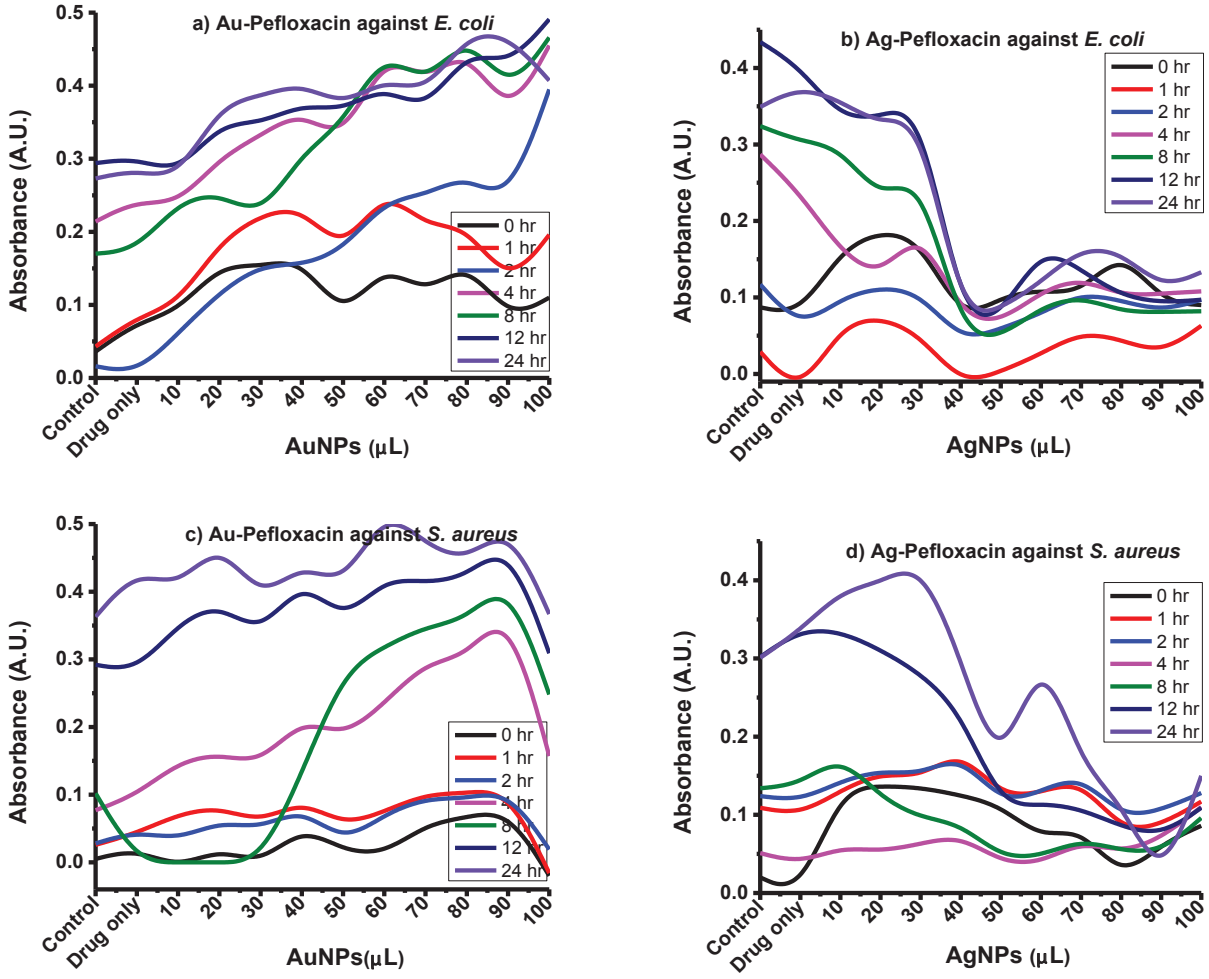

Figure 7. Absorbance of *E. coli* **(a, b)** and MRSA **(c, d)** in the absence of drug or nanoparticles (control), in the presence of drug only (pefloxacin) and by varying concentrations of AuNPs (2.87 nM) **(a, c)** and AgNPs (0.124 nM) **(b, d)** from 10 to 100 µl.

with time intervals, and there is no effect of the addition of AuNPs. However, for the AgNP conjugates against *E. coli*, we can see greater control for 1 h of incubation time intervals, controlled growth until approximately 2 h–4 h of incubation time intervals and then growth is increased afterward. The absorption remains lower than the initial levels until 8 h of incubation time intervals. However, the same conjugates are not that effective against *S. aureus*, and the growth is observed with time intervals.

Concentration of nanoparticle effects

AuNP conjugates against *E. coli* does not show any control, and the growth is observed continuously with the addition of nanoparticles. For the *S. aureus* strains, no effects of Au nanoparticle addition are observed; however, a similar decrease in absorbance is observed at the AuNPs volume of 100 µl as was observed previously with other combinations. Generally, the addition of nanoparticles enhances the absorption; hence, the

Table 2. Comparison of antibacterial effects of various antibiotic – NP conjugates against *E. coli* and *S. aureus*

Fluoroquinolone–NP hybrids	*Escherichia coli* AgNPs	*Escherichia coli* AuNPs	*Staphylococcus aureus* AgNPs	*Staphylococcus aureus* AuNPs	Conclusion
No drug (NPs only)	• Max. control until 2 h • Increasing concentration of nanoparticles has no effect on bacterial strains	• Max. control until 2 h • Growth is increased with the increasing concentration of nanoparticles	• Max control until 4 h • Increasing concentration of nanoparticles has no significant effect on bacterial strains	• No control over time • Growth is increased with the increasing concentration of nanoparticles	• Increasing concentration supports the growth of bacterial strains for AuNPs. No effect of concentration is observed for AgNPs
Ciprofloxacin	• Max. control at 1 h • Overall good control • 10 µl are sufficient to control	• Control of growth at 12 h • Minimum growth at 10 µl, increases with additional AuNPs, decreases at 100 µl	• Good control at 4 h • Growth increases with time • 70 µl are required to control the growth	• No control over time • Growth decreases at 100 µl of AuNPs	• AgNP conjugates are best effective against *E. coli* and moderately effective against *S. aureus*. AuNPs are not effective against *S. aureus* or *E. coli*
Levofloxacin	• Max. control at 1 h • Overall good control • 10 µl is sufficient to control	• Minor effects on control of growth at 12 h • No concentration effects	• Good control at 4 h • Growth increases with time • 70 µl is required to control the growth	• No control over time • Growth increases with the addition of AuNPs, decreases at 100 µl of AuNPs	• Same as above (ciprofloxacin)
Norfloxacin	• Max. control at 1 h • Growth increases with time • 50 µl is required to control the growth	• No control over time • No effect of AuNPs	• Good control at 4 h • Growth increases with time • 70 µl is required to control the growth	• No control over time • Growth slightly increases with the addition of AuNPs, decreases at 100 µl of AuNPs	• AgNP conjugates are moderately effective against *E. coli* and *S. aureus*. AuNPs are not effective against *S. aureus* or *E. coli*
Ofloxacin	• Max. control at 1 h • Growth increases with time • 50 µl is required to control the growth	• Control of growth at 12 h • Growth increases with the addition of AuNPs, minimum at 60 µl, decreases at 100 µl of AuNPs	• Good control at 4 h • Growth increases with time after 8 h • 70 µl is required to control the growth	• No control over time • Growth slightly increases with the addition of AuNPs, decreases at 100 µl of AuNPs	• Same as above (norfloxacin)
Pefloxacin	• Max. control at 1 h with controlled growth until 4 h, afterward growth increases with time • 40 µl is required to control the growth	• No control over time • Growth increases with the addition of AuNPs	• Growth is observed with time • 50 µl is required to control the growth	• No control over time • Growth slightly increases with the addition of AuNPs, decreases at 100 µl of AuNPs	• Same as above (norfloxacin and ofloxacin)

growth of bacterial strains is increased for both cases. Essentially, for the AuNPs, this combination also did not provide the required results. Coming to the case of AgNP conjugates, we see greater control at volumes of 40 µl and higher for the *E. coli* strains. For the *S. aureus* strains, we see that approximately 50 µl and higher are required for the control of growth.

Comparison of all these results for different antibiotics is shown in **Table 2**.

Conclusions

AuNPs and AgNPs were synthesized with citrate as a stabilizing agent for excluding the toxicity associated with other capping agents. Five drugs from the fluoroquinolone antibiotics, namely ciprofloxacin, levofloxacin, norfloxacin, ofloxacin and pefloxacin, were used to make drug–nanoparticles conjugates.

Antibacterial studies of pure nanoparticles and drug–NP conjugates were performed by using the above-mentioned drugs against bacterial strains of *E. coli* (multidrug-resistant) and MRSA using a new experimental setup. The absorbance of bacterial strains representing the growth or control of these strains was measured at different time intervals ranging from 0 to 24 h and at different concentrations of nanoparticles ranging from 10 to 100 µl. The results show a direct comparison of Au versus Ag nanoparticles for both bacterial strains. In nearly all cases, silver is a clear winner as compared to gold. All these studies show little to no control of AuNPs against *E. coli* or MRSA. 100 µl of AuNPs show a slight effect, but is not prominent. For AgNPs however, we observe the best effect against *E. coli*, even at the lowest concentration of AgNPs. The results differ slightly for different drugs, for example, Ag conjugates with ciprofloxacin and levofloxacin show the most promising results at the lowest concentration of AgNPs, but for norfloxacin, ofloxacin and pefloxacin, we need a relatively higher concentration of AgNPs. For the case of *S. aureus*, all drug hybrids show moderate effects, that is, relatively a higher concentration of nanoparticles is required to control their growth.

As mentioned earlier, the effects of each drug conjugate were studied concerning time intervals and concentration of nanoparticles; therefore, the graphs show the growth or decline of bacterial strains at any given time intervals for any given concentration, for which **Table 2** can be consulted.

The antibacterial results might not be significant and that most commonly used metallic nanoparticles have been used, but this easy experimental setup can be extended and clearly defines a new way to compare studies between any set of nanoparticles and allows variation in their concentration, bound drugs and bacterial strains along with their incubation time intervals. This allows for obtaining multiple data in a single experiment.

Author contributions. AS contributed substantially to the conception and design of the study. NA and MY acquired the data. AS, NA and MY analyzed and interpreted the data. AJS drafted the manuscript. NA and MY critically revised the manuscript. All the authors approved the final version submitted for publication and take responsibility for the statements made in the published article.

Acknowledgments. We are grateful to COMSATS University Islamabad, Abbottabad Campus, Pakistan and Higher Education Commission, Pakistan for research funding.

Conflict of interest statement. The authors have each completed and submitted an International Committee of Medical Journal Editors form for disclosure of Potential Conflicts of Interest. None of the authors has any conflict of interest to disclose.

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