

# In Vitro Structural and Functional Evaluation of Gold Nanoparticles Conjugated Antibiotics

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**Abstract** Bactericidal efficacy of gold nanoparticles conjugated with ampicillin, streptomycin and kanamycin were evaluated. Gold nanoparticles (Gnps) were conjugated with the antibiotics during the synthesis of nanoparticles utilizing the combined reducing property of antibiotics and sodium borohydride. The conjugation of nanoparticles was confirmed by dynamic light scattering (DLS) and electron microscopic (EM) studies. Such Gnps conjugated antibiotics showed greater bactericidal activity in standard agar well diffusion assay. The minimal inhibitory concentration (MIC) values of all the three antibiotics along with their Gnps conjugated forms were determined in three bacterial strains, *Escherichia coli DH5 $\alpha$* , *Micrococcus luteus* and *Staphylococcus aureus*. Among them, streptomycin and kanamycin showed significant reduction

in MIC values in their Gnps conjugated form whereas; Gnps conjugated ampicillin showed slight decrement in the MIC value compared to its free form. On the other hand, all of them showed more heat stability in their Gnps conjugated forms. Thus, our findings indicated that Gnps conjugated antibiotics are more efficient and might have significant therapeutic implications.

**Keywords** Gold nanoparticles · Antibiotics · Dynamic light scattering · Transmission electron microscope · Scanning electron microscope · Minimal inhibitory concentration · Agar well diffusion

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## Introduction

Nanotechnology is a rapidly developing field of new therapeutic and diagnostic concept in all areas of medicine [1–3]. Due to their unique characteristics, nanoparticles are considered to have wide applications in detection of biomolecules, drug delivery and release. Of them, Gnps have already been used to deliver protein-based drugs, and are of particular utility because the particles can carry multiple active groups [4–6]. The chemical, optical and electronic properties of Gnps made them well suited for applications in biosensing and therapeutic delivery. Gnps based biosensors [7, 8], drug delivery [9–11] was demonstrated to be more sensitive and effective.

Moreover, nanoparticles were shown to take up by phagocytic cells and held promises as carrier for the treatment of intracellular infections with several antibiotics [12]. It was reported that Gnps as drug carriers allow increased drug concentration at infected sites as well as reduce toxicity of the drug [13]. Thus, Gnps as carrier for

the antibacterial drug ciprofloxacin and subsequent release of the drug over an extended period of time was observed [14]. This is essential for ideal antibiotic therapy. Nano carriers were also found to be more effective for the drugs like gentamycin [15], tuberculosis drugs [16, 17], ampicillin [18–20], anticancer drugs [21, 22], anti fungal drug amphotericin B [23] etc.

For successful application of nano-antibiotic conjugation, apart from better delivery, their activities should be evaluated properly because the amount of antibiotics often given for therapy is much more higher than the dose required for killing the pathogens. This in turn could produce toxic effect, which was demonstrated in several reports too [24, 25]. For a successful antibiotic therapy, the dose should be reduced to avoid their side effects at the same time the stability should be increased to make them more economic. With the advancement of nanotechnology, functionalized nanoparticles have been used to conjugate different drugs. Among the different nanoparticles, Gnps were found to be less toxic and hence widely used for this purpose. In most of the cases, the conjugation was done by functionalized gold particles, where amino acids, glutathione, polyethylene glycol etc were used as functionalizing agents [26]. But to avoid the possible effects of these agents on biological system, we have conjugated antibiotics directly without any functionalizing agents at the time of the Gnps synthesis [27].

While there were many reports about the delivery of different drugs in nanoparticles conjugated form, little or no efforts were made, so far, to determine the efficiency, stability of antibiotics conjugated with Gnps in vitro. In this study, we compared the efficiency and stability of Gnps conjugated antibiotics with respect to their free forms in vitro. We found that the MIC of Gnps conjugated ampicillin, streptomycin and kanamycin on *Escherichia coli* DH5 $\alpha$  (Microbial type culture collection (MTCC) No.1652, India), *Micrococcus luteus* (MTCC No. 106) and *Staphylococcus aureus* (MTCC No. 96) were reduced when compared to their respective unconjugated free forms. Moreover, the activity of all Gnps conjugated antibiotics showed higher stability compared to their corresponding free forms. Thus our results suggest that antibiotics conjugated with Gnps might be used in therapy for their greater efficiency and stability.

## Experimental Procedures

### Preparation of Bare Gold Nanoparticles

Gold nanoparticles (Gnps) were prepared by the reduction of chloroauric acid (H[AuCl<sub>4</sub>]) by sodium borohydride. The normal reduction process was performed according to

the standard protocol [27]. The size of Gnps obtained by this process was 14 nm.

### Preparation of Conjugated Gold Nanoparticles using Antibiotics as Template

The combined reducing property of sodium borohydride and antibiotics were used to reduce H[AuCl<sub>4</sub>]. The seeding of Gnps was done in presence of the antibiotics (Ampicillin, Streptomycin and Kanamycin, Fig. 1) individually and thus Gnps conjugated antibiotics were formed [27].

### Dynamic Light Scattering (DLS)

The Nano-ZS (Malvern) instrument (5 mW HeNe laser  $\lambda = 632$  nm) was used for this purpose. The sample was taken in a DTS0112—low volume disposable sizing cuvette of 1.5 ml volume (path length 1 cm). The operating procedure was programmed (using the DTS software supplied with the instrument) such that there were average of 25 runs, each run being averaged for 15 s, with an equilibration time of 3 min at 25°C. A particular hydrodynamic diameter ( $d_h$ ) was evaluated several times and the result was presented in terms of distribution of  $d_h$  [28].

### Transmission Electron Microscopy

All the three Gnps conjugated antibiotics along with the free Gnps were prepared after drying on carbon coated copper grid and observed under a transmission electron microscope (FEI, Model: STWIN) with an accelerating potential of 200 KV and analyzed with TECNAI G<sup>2</sup> software.

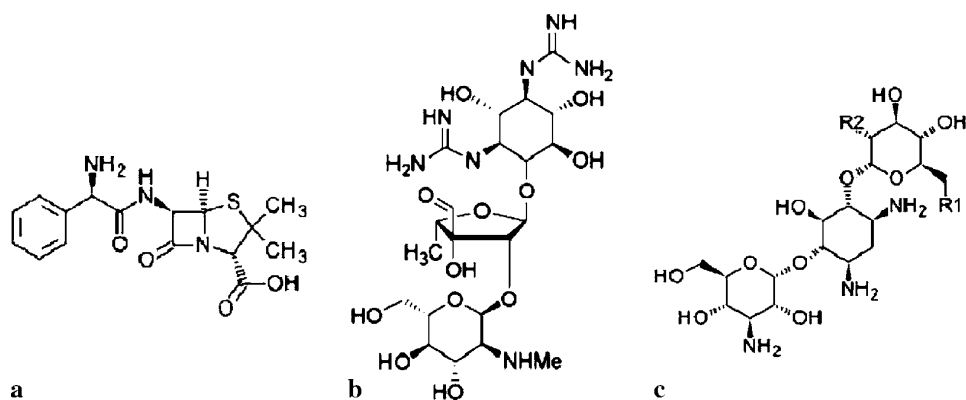
### Scanning Electron Microscopy

Gnps conjugated antibiotics along with bare Gnps were lyophilized on glass slides and then coated with gold. The samples were then observed under a scanning electron microscope (JEOL JSM 5200).

### MIC Study of Free and Gnps Conjugated Antibiotics

MIC of ampicillin, streptomycin and kanamycin along with their respective Gnps conjugated forms against *E. coli* DH5 $\alpha$ , *M. luteus* and *S. aureus* in Luria-Bertani (LB) broth were determined by standard method [29]. Each tube contained 5 ml of LB medium inoculated with 10<sup>6</sup> bacteria

**Fig. 1** Chemical structure of antibiotics. (a) Ampicillin, (b) Streptomycin, (c) Kanamycin



per ml. Decreasing concentrations of each antibiotic and their corresponding Gnps conjugated form were added to the respective tubes. After 16 h. the turbidity of each tube was measured at 600 nm using a spectrophotometer.

#### Bactericidal Activity Measurement

This assay was conducted by standard agar well diffusion method. The *E. coli DH5 $\alpha$* , *M. luteus* and *S. aureus* strains were grown on LB Broth at 37°C overnight upto a turbidity of 0.5 Mac Farland standard ( $10^8$  CFU per ml) [30]. About 100  $\mu$ l of this suspension was used to inoculate 90 mm diameter petridish filled with 35 ml of LB agar. Wells (diameter<sup>2</sup> = 0.563 cm<sup>2</sup>) were punched in the agar plates and filled with 100  $\mu$ l of either antibiotics or their respective Gnps conjugated forms. The concentrations of both the forms of antibiotics were at their respective MIC values, generally used in common laboratory purpose (50  $\mu$ g/ml for ampicillin, 10  $\mu$ g/ml for streptomycin and 50  $\mu$ g/ml for kanamycin) [31]. Plates were incubated at 37°C for overnight. Antibacterial activities were evaluated by measuring the area of zone of inhibition (diameter<sup>2</sup>). We used autoclaved water and only Gnps as negative control.

#### Results

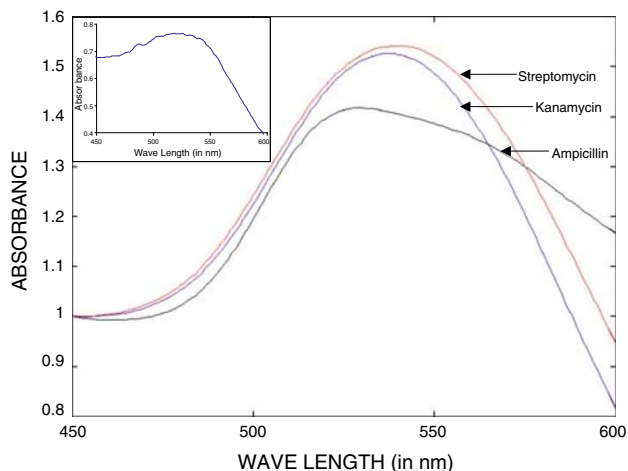
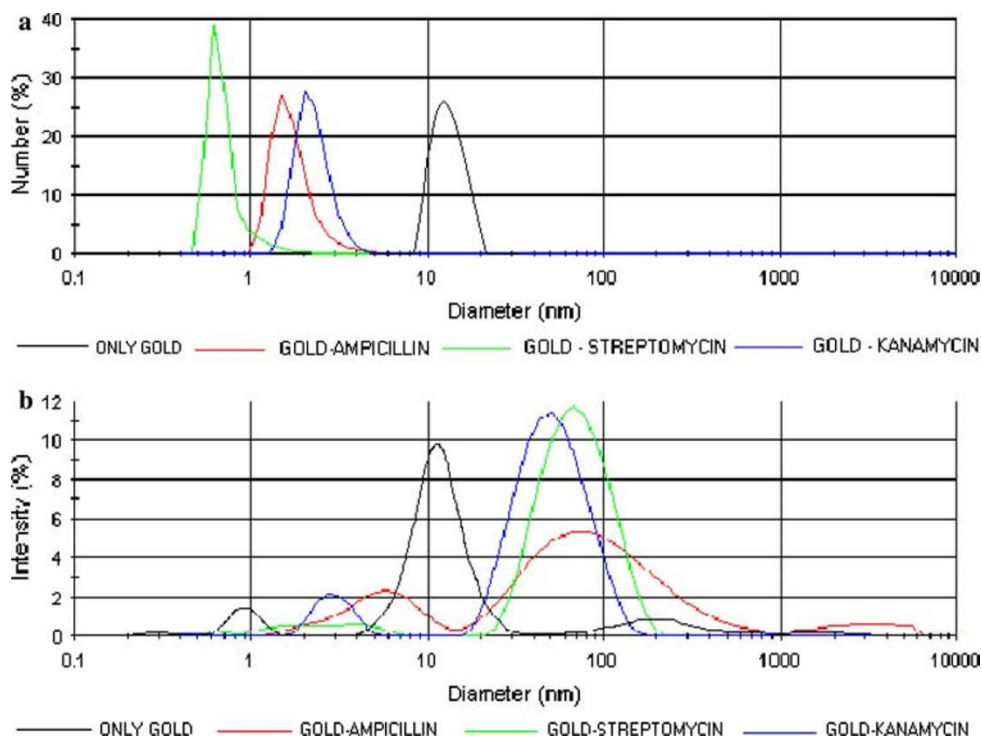
Production of Gnps on reduction with citrate or borohydride generally resulted in a size less than 20 nm but the molar ratio of reductant to H[AuCl<sub>4</sub>] was the key factor for the synthesis of Gnps below 20 nm ( $d_h$ ). We used the ratio of reductant and H[AuCl<sub>4</sub>] in such a way that the synthesized Gnps produced a size of 13.54 nm (Fig. 2a) when measured by photon correlation spectroscopy. The plasmon resonances of the Gnps varied with the diameter of the reduced particles. The plasmon resonance was obtained at 526 nm and the produced Gnps were of red wine colour (Fig. 3). Thus, larger particles appeared more bluish in colour while smaller particles showed red colour [27].

#### Conjugated with Antibiotics

The antibiotics (Fig. 1) were conjugated with Gnps by reducing H[AuCl<sub>4</sub>] with the combined reducing effect of both antibiotics and sodium borohydride. The antibiotics themselves were able to reduce H[AuCl<sub>4</sub>] to synthesize the Gnps but the reducing power was much less. It took around 4 h for ampicillin and 24 h for streptomycin and kanamycin to reduce H[AuCl<sub>4</sub>] to form Gnps conjugated nanoparticles (data not shown). Also, in case of ampicillin, the particles produced in this way formed larger aggregates and precipitated out from the solution quickly whereas streptomycin and kanamycin reduced H[AuCl<sub>4</sub>] very poorly. But the Gnps produced by using the combined reducing property of both sodium borohydride and the antibiotics showed much higher stability. The produced Gnps conjugated antibiotics appeared more bluish (Fig. 3). So, it was obvious that the size of the particles would be larger and that was reflected in the intensity distribution of the size of the Gnps (Fig. 2b). The intensity distribution was obtained due to the Rayleigh scattering (i.e., proportional to  $R^6$ , where  $R$  is the radius of particle). We found that there were distributions of large and small particles but the number distribution showed ( $\sim R$ ) that there were major numbers of particle, which have the hydrodynamic radius less than 10 nm (Fig. 2a). The colour showed bluish because of the presence of some larger particles too.

As DLS study showed size distribution of Gnps conjugated particles, we then wanted to visualize and validate the size of the particles directly. For this, we did electron microscopic study of the free Gnps and Gnps conjugated antibiotics. In the transmission electron microscopy (TEM), we observed that the Gnps conjugated with the antibiotics produce larger particles. The conjugation with antibiotics resulted an irregular but consistence change in the particles association for all the three antibiotics tested (Fig. 4b, c, d). But only Gnps showed very regular spherical shaped particles with much smaller size (Fig. 4a). We further used scanning electron microscope (SEM) to determine the conjugation of Gnps with antibiotics.

**Fig. 2** Measurement of the hydrodynamic diameter of bare and Gnps conjugated antibiotics by dynamic light scattering experiment. (a) represents the number distribution of the hydrodynamic diameter of bare Gnps and Gnps conjugated antibiotics. (b) represents the intensity distribution of the hydrodynamic diameter of the free Gnps and the antibiotics conjugated Gnps



**Fig. 3** The spectroscopic measurement of plasmon resonance of the antibiotics conjugated with Gnps. The figure in the inset represents the plasmon resonance of bare Gnps

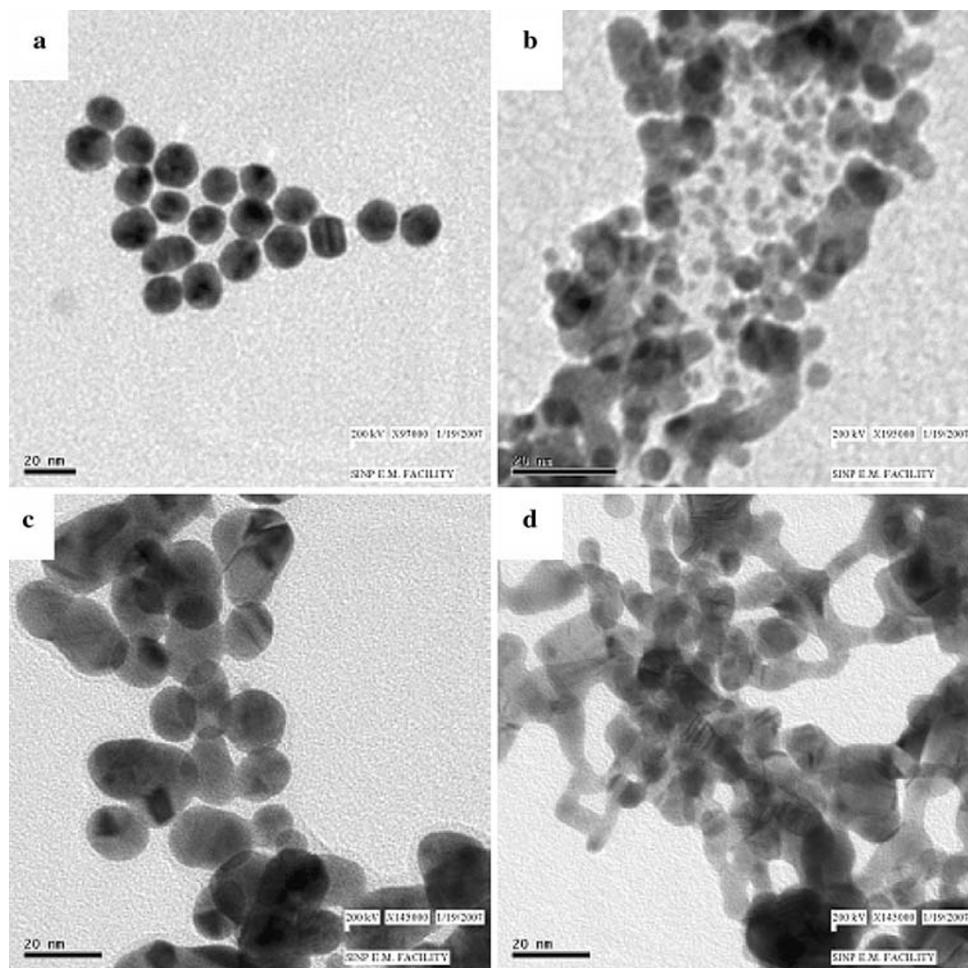
Distinct structures were found for all the three antibiotics conjugated with Gnps. Gnps conjugated ampicillin showed cubic structure (Fig. 5b), Gnps conjugated streptomycin showed rectangular rod shaped structure (Fig. 5c) and Gnps conjugated kanamycin showed extended star like structures (Fig. 5d). This observation clearly demonstrated the conjugation of antibiotics with Gnps. These structure formations were absent when pre-synthesized Gnps and antibiotics were mixed separately (data not shown).

Gnps conjugated ampicillin, streptomycin and kanamycin along with their corresponding free antibiotics were

then tested on bacterial strains *E. coli DH5 $\alpha$* , *M. luteus* and *S. aureus* by comparing corresponding zone of inhibition (diameter<sup>2</sup>). In Fig. 6, the zone of inhibition by agar well diffusion assay for *E. coli DH5 $\alpha$*  was shown to increase at a particular concentration for Gnps conjugated antibiotics compared to their respective unconjugated forms. The concentrations of all the three antibiotics taken in the above experiments were the standard concentrations used in the laboratory (50  $\mu\text{g/ml}$  for ampicillin, 10  $\mu\text{g/ml}$  for streptomycin and 50  $\mu\text{g/ml}$  for kanamycin) [31]. Similar results were obtained for *M. luteus* and *S. aureus* too (pictures not shown). We also tested a wide range of concentrations for all the antibiotics and observed that the Gnps conjugated antibiotics were more efficient than their respective free forms (data not shown). In the Fig. 7, the percentage increment in the zone of inhibition for Gnps conjugated antibiotics were compared to their respective free forms at the concentrations mentioned above. In Fig. 7a, the increment in the zone of inhibition (diameter<sup>2</sup>) of Gnps conjugated ampicillin with respect to the free ampicillin was shown for all the three bacterial strains we had tested. Similar data for streptomycin and kanamycin were plotted also in Fig. 7b and c, respectively. The percentage increments in the zone of inhibition (diameter<sup>2</sup>) for the Gnps conjugated antibiotics compared to their respective free forms were summarized in Table 1. As seen in the Table 1, kanamycin in Gnps conjugated form was more effective than its free form in the case of *E. coli DH5 $\alpha$*  and *S. aureus*, whereas streptomycin was more effective in its Gnps conjugated form in the case of *M. luteus*.



**Fig. 4** Transmission electron micrographs of free Gnps and antibiotics conjugated Gnps. (a) Bare Gnps, (b) Ampicillin conjugated Gnps, (c) Streptomycin conjugated Gnps, (d) Kanamycin conjugated Gnps



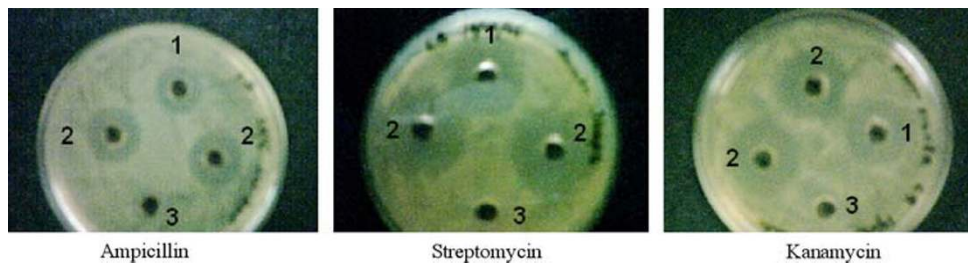
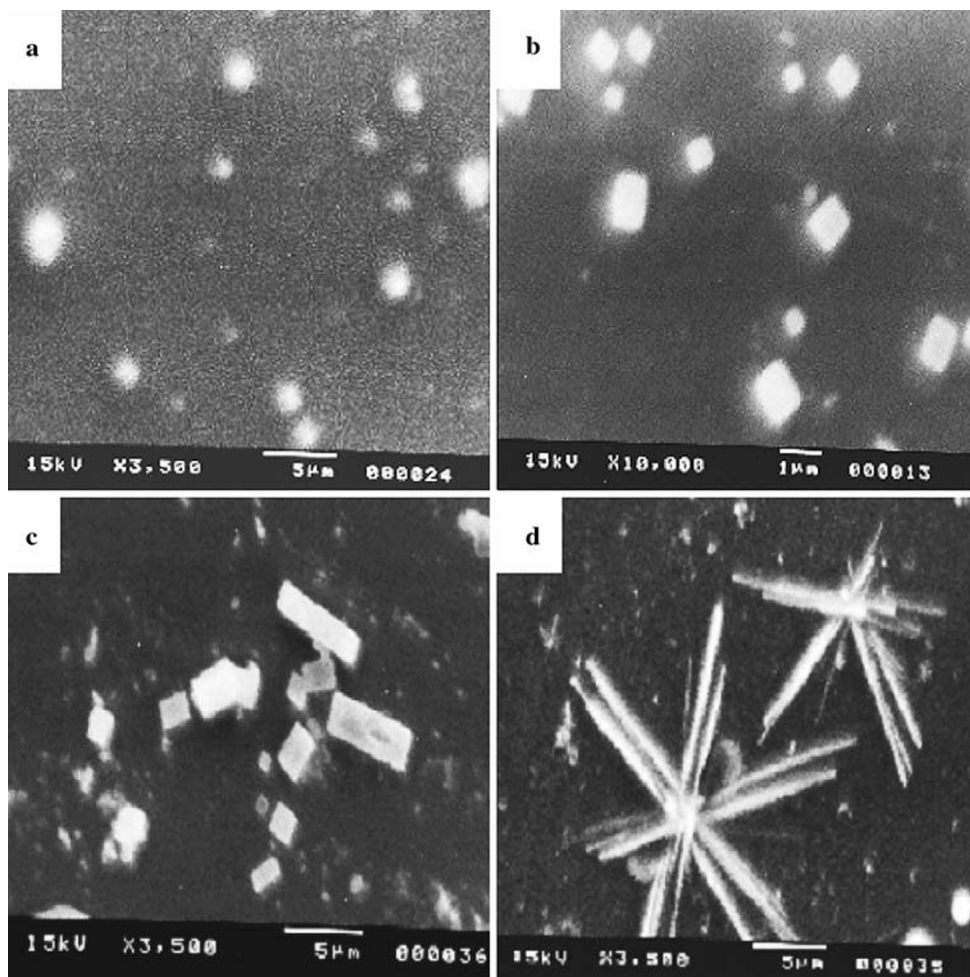
On the other hand, Gnps conjugated ampicillin showed uniform increment in the zone of inhibition compared to its free form in the case of all the three bacterial strains tested. *S. aureus* strain was resistant to streptomycin, so neither the free antibiotic nor the Gnps conjugated antibiotic produced any inhibition to their growth. Further, in one of the control experiments we determined the zone of inhibition with the mixture of previously synthesized Gnps and antibiotics. In that case, the zone of inhibition did not increase compare to the free antibiotics. Also, by adding only sodium borohydride to the antibiotics, we could not see significant increase in the activity of antibiotics (only 3–6%).

We next determined the minimal inhibitory concentration (MIC) of each antibiotic compared to their Gnps conjugated form in each bacterial strain. MIC for each of the Gnps conjugated antibiotic reduced significantly (Table 2) compared to their respective free forms. For Gnps conjugated ampicillin, the MIC value was 45  $\mu\text{g/ml}$  compared to 50  $\mu\text{g/ml}$  for free ampicillin (10% decrement), for streptomycin the corresponding values were 7 and 14  $\mu\text{g/ml}$  (50% decrement) and for kanamycin the values were 12 and 30  $\mu\text{g/ml}$  (60% decrement) in *E. coli DH5 $\alpha$* .

For other strains, the values of MIC were also reduced for all antibiotics conjugated with Gnps compared to their respective free forms (Table 2).

We then wanted to determine the stability of the Gnps conjugated antibiotics compared to the free antibiotics. Both forms of all the three antibiotics were given heat shock by incubating them at different temperature for 10 min and then their antibacterial activity was measured by agar well diffusion method. It was observed that Gnps conjugated antibiotics were more stable than corresponding free antibiotics (Table 3). The antibacterial activity of free ampicillin did not decrease much with the elevation of temperature while the Gnps conjugated ampicillin showed more activity at higher temperature. On the other hand, for free streptomycin and kanamycin, the antibacterial activities were reduced significantly but the antibacterial activity of Gnps conjugated streptomycin and kanamycin decrease slightly with the increment in temperature. One step further, we then measured the rate of functional degradation of the antibiotics (both free and Gnps conjugated forms) by storing them at room temperature. Both the forms of antibiotics were stored at room temperature (25–28°C) and used to evaluate the zone of inhibition by agar well

**Fig. 5** Scanning electron micrographs of free Gnps and antibiotics conjugated Gnps. (a) Bare Gnps, (b) Ampicillin conjugated Gnps, (c) Streptomycin conjugated Gnps, (d) Kanamycin conjugated Gnps



**Fig. 6** Comparison of antibacterial activity of antibiotics conjugated Gnps along with respective free antibiotics in *E. coli DH5α* by agar well diffusion method. (a) Ampicillin (50 µg/ml), (b) Streptomycin

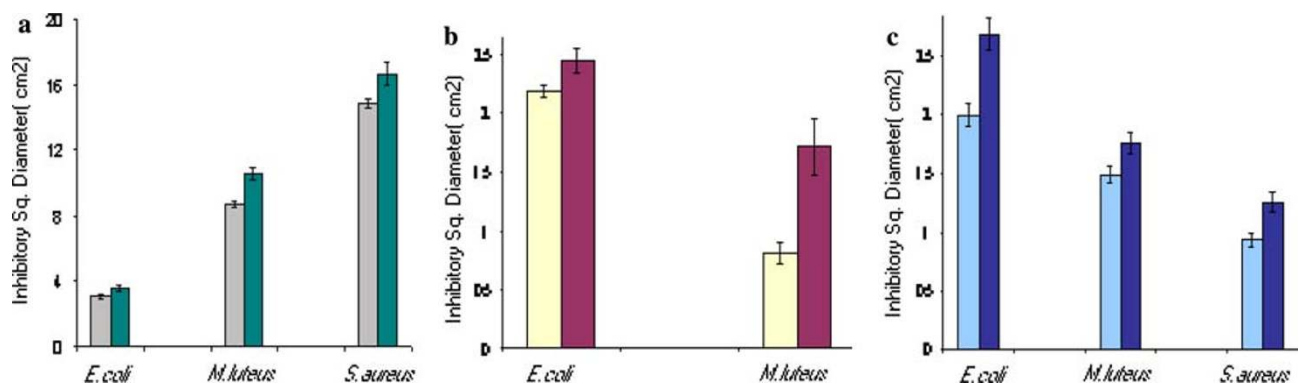
(10 µg/ml), (c) Kanamycin (50 µg/ml). The well 1, 2, and 3 represents free antibiotics; Gnps conjugated antibiotics and bare Gnps respectively in each plate

diffusion method. All the antibiotics in their respective Gnps conjugated form had more antibacterial activity compared to the corresponding free antibiotics, except Gnps conjugated ampicillin (Table 3). This is true for all the three bacterial strains tested (data not shown).

**Discussions**

Our results for the first time demonstrated that the in vitro bactericidal activity of Gnps conjugated ampicillin,

streptomycin and kanamycin were more efficient compared to their respective free forms. We had also developed a simple technique for the conjugation of antibiotics with Gnps during its synthesis step. Usually, such conjugation needs functionalization process. But we avoided the interference of such functionalizing agent in determining the bactericidal activity of the antibiotics. Using the combined reducing property of antibiotics and borohydride, antibiotics were conjugated with Gnps. The interaction between antibiotics and Gnps is likely to be mediated by



**Fig. 7** Comparative study of different antibiotics along with their respective Gnps conjugated forms in three bacterial strains. (a) Ampicillin (50 µg/ml), (b) Streptomycin (10 µg/ml), (c) Kanamycin (50 µg/ml). The data is the average of three experiments ± SD. The first column in each pair represents free form of antibiotics and the second column represents its respective Gnps conjugated form

**Table 1** Represents the zone of inhibition (in terms of diameter square) for free antibiotics and antibiotics conjugated with Gnps in three bacterial strains

Name of the bacterial strain	Name of antibiotics	Inhibitory zone in sq. diameter (cm <sup>2</sup> )		% Change in inhibitory sq. diameter
		Free antibiotics	Gnps-conjugated antibiotics	
<i>E. coli DH5α</i> (Gram –Ve)	Ampicillin	3.085 ± 0.146	3.569 ± 0.160	+15.688
	Streptomycin	2.189 ± 0.057	2.453 ± 0.102	+12.060
	Kanamycin	3.371 ± 0.164	4.545 ± 0.223	+34.826
<i>M. luteus</i> (Gram +Ve)	Ampicillin	8.740 ± 0.201	10.493 ± 0.354	+20.057
	Streptomycin	0.818 ± 0.091	1.712 ± 0.241	+109.291
	Kanamycin	2.507 ± 0.118	2.960 ± 0.149	+18.069
<i>S. aureus</i> (Gram +Ve)	Ampicillin	14.839 ± 0.321	16.659 ± 0.678	+12.265
	Kanamycin	1.588 ± 0.098	2.132 ± 0.150	+34.257

The concentrations of free as well as Gnps conjugated antibiotics are 50 µg/ml for ampicillin, 10 µg/ml for streptomycin and 50 µg/ml for kanamycin. The data is the average of three experiments ± SD. Percentage change in each case is calculated and mentioned above

**Table 2** Represents minimal inhibitory concentrations (MIC) for free antibiotics along with their respective Gnps conjugated form in three bacterial strains

Name of the bacterial strain	Name of antibiotics	Minimal inhibitory concentration (µg/ml) for 10 <sup>6</sup> bacteria/ml		% Change in MIC
		Free antibiotics	Gnps-conjugated antibiotics	
<i>E. coli DH5α</i> (Gram –Ve)	Ampicillin	50.0 ± 0.50	45.0 ± 1.50	–10.00
	Streptomycin	14.0 ± 2.00	7.0 ± 1.00	–50.00
	Kanamycin	30.0 ± 2.50	12.0 ± 1.00	–60.00
<i>M. luteus</i> (Gram +Ve)	Ampicillin	0.52 ± 0.02	0.45 ± 0.03	–13.46
	Streptomycin	22.0 ± 2.00	17.0 ± 1.00	–22.73
	Kanamycin	32.5 ± 0.50	23.0 ± 1.50	–29.23
<i>S. aureus</i> (Gram +Ve)	Ampicillin	0.45 ± 0.03	0.37 ± 0.01	–17.78
	Kanamycin	9.0 ± 0.50	5.8 ± 0.20	–35.56

The data is the average of three experiments ± SD. Percentage change in each case is calculated and mentioned above

the adsorption of the antibiotic molecules on the nanoparticle surfaces. The average particles size after conjugation were shown to decrease (Fig. 2a). This was possibly again due to the combined reducing property of both antibiotics

and borohydride in situ. However, the plasmon resonance study (Fig. 3) showed a red shift, indicating the presence of larger particles (Fig. 2b), though they were less in number (Fig. 2a). In case of Gnps conjugated ampicillin, the

**Table 3** Represents the bactericidal activity in *E. coli DH5 $\alpha$*  by agar well diffusion assay for free antibiotics and their respective Gnps conjugated form after different temperature and time stresses

Agents		Zone of inhibition for <i>E. coli DH5<math>\alpha</math></i> strain in sq. cm								
		Ampicillin			Streptomycin			Kanamycin		
		Free	Gnps conjugated	% Change	Free	Gnps conjugated	% Change	Free	Gnps conjugated	% Change
Incubated for 10 min at	26 °C	3.085	3.569	+15.688	2.189	2.453	+12.060	3.371	4.545	+34.826
	50 °C	2.806	6.141	+118.85	1.378	1.622	+17.707	2.063	3.663	+77.557
	75 °C	2.198	6.635	+201.87	1.116	1.411	+26.434	1.834	3.389	+84.787
	90 °C	2.107	6.707	+218.32	0.053	1.324	+2398.1	1.491	3.263	+118.85
Storage at room temp. (25–28°C) for	0 day	3.085	3.569	+15.688	2.189	2.453	+12.060	3.371	4.545	+34.826
	3 days	2.929	3.142	+7.272	2.126	2.361	+11.054	2.283	4.199	+83.925
	7 days	2.646	2.823	+6.689	1.486	2.049	+37.887	1.562	4.024	+157.62
	14 days	2.561	2.593	+1.250	1.055	1.483	+40.569	1.501	3.879	+158.43
	21 days	2.540	1.941	–23.583	0.913	1.345	+47.317	1.338	3.645	+172.42
	28 days	1.965	1.209	–38.473	0.547	0.945	+72.761	1.239	3.459	+179.18

The concentrations of free as well as Gnps conjugated antibiotics are 50  $\mu\text{g/ml}$  for ampicillin, 10  $\mu\text{g/ml}$  for streptomycin and 50  $\mu\text{g/ml}$  for kanamycin

plasmon resonance showed a flatten plateau in the plasmon region due to the presence of such poly dispersed particles. The dynamic light scattering study (Fig. 2b) and TEM study (Fig. 4b) also supported the above statement. In one step further, we directly showed evidences by scanning electron microscopic studies that, all the three antibiotics formed some specific three-dimensional structures when conjugated with Gnps. Also, to prove the conjugation of antibiotics with Gnps, we found that after spinning down the Gnps conjugated antibiotics, the functional activity of the precipitate (pellet-suspension) was about 60–80% and that of the supernatant was about 20–40%. Thus, majority of the antibiotic molecules were associated with Gnps.

Using standard agar well diffusion assay, we compared the bactericidal activity of Gnps conjugated antibiotics with their respective free forms. The relative bactericidal activity of Gnps conjugated ampicillin was less effective than Gnps conjugated streptomycin and kanamycin (Fig. 7 and Table 1). Consequently, for *E. coli DH5 $\alpha$*  strain, the MIC values of Gnps conjugated ampicillin decreased 10%, while the percentage decrement for Gnps conjugated streptomycin and kanamycin were 50% and 60%, respectively. Such differential activity might be due to the differences in the mode of action of the antibiotics. Ampicillin inhibits the cell wall biosynthesis by inhibiting the cross-linking reaction mediated by transpeptidase, while both streptomycin and kanamycin bind with ribosome and block translation process during protein synthesis [32]. The binding affinity of Gnps conjugated antibiotics with the said enzyme or even ribosome might be the key factor for this differential response. Although, in the control experiments, only Gnps did not show any bactericidal

activity (Fig. 6) so the antibiotics conjugated with Gnps might have a higher binding affinity to their respective targets. On the other hand, the Gnps conjugated antibiotics might have greater chance to penetrate bacterial cell membrane compared to their respective free forms. In the control experiments, we also mixed pre-synthesized Gnps and antibiotics externally to determine the bactericidal activity. None of these antibiotics mixed with Gnps showed significant increment in bactericidal activity compared to the respective free antibiotics (data not shown). Thus, only Gnps did not promote the penetration of the antibiotics into the bacterial cells. So, Gnps conjugated antibiotics might have some other mechanisms that could enhance the efficacy of the antibiotics. On the other hand, presence of sodium borohydride during the Gnps synthesis step might alter the function of antibiotics, but when we mixed only sodium borohydride with antibiotics, the functional activity of antibiotics did not increase much (only 3–6%). Thus the reduction process in our reaction condition does not change the antibiotic structure abruptly. The conjugation between the antibiotics and Gnps is probably based on the adsorption phenomenon mediated by intermolecular forces. Thus having the larger surface area of these adsorbed antibiotics in Gnps conjugated form, their bactericidal activity might increase compared to their respective free forms. However, the exact mechanisms of action of Gnps conjugated antibiotics are highly speculative and needs further study.

The Gnps conjugated antibiotics were seen to be more stable than their respective free forms. Stability of the most antibiotics is temperature and parenteral solutions dependent [33]. We introduced stresses by heat shock and by prolong storage at room temperature (25–28°C). In both the



cases Gnps conjugated antibiotics were observed to be more stable compared to their respective free forms except Gnps conjugated ampicillin during its temporal study. This was perhaps due to the close association between Gnps and antibiotics, the bond energy of antibiotic molecules were increased which in turn stabilized them. Whatever the mechanisms of such stability of Gnps conjugated antibiotics be, we showed further that at elevated temperature the Gnps conjugated forms were even more active for ampicillin. This was perhaps due to the delocalization of the electron in the carbonyl group of the  $\beta$ -lactam ring in ampicillin at elevated temperature. Elevation in temperature might induce breakage in the  $\beta$ -lactam ring of the free ampicillin, whereas Gnps conjugation might stabilize the ring and thereby allowing the delocalization of electron. Hence, in case of free ampicillin we found a decrease in the activity, whereas Gnps conjugated form showed more activity than the activity at its lower temperature. In this regard, one of the important findings was the deactivation of streptomycin at 90°C, whereas its Gnps conjugated form retained its activity at the same condition. Here also, the Gnps conjugation might stabilize the structure of the streptomycin molecules.

We found that the activity of Gnps conjugated ampicillin decreased compared to its free form after two weeks (Table 3). Actually, we observed that the Gnps conjugated ampicillin (Table 3) was precipitated out from the solution. This might be the reason for its decreased efficiency compared to its free form.

It was reported that antibiotic solutions used for longer than 7 days should be stored at 4°C, those stored at 24°C should be discarded after 7 days [34]. Our data also supported this observation. Moreover, we provided evidences that Gnps conjugated antibiotics were more stable and might withstand more harsh storage conditions, which raised a hope to use Gnps conjugated antibiotics with greater efficiency in the remote area, where proper storage condition is unavailable.

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