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Synthesis and Characterization of Cefotaxime Conjugated Gold Nanoparticles and Their Use to Target Drug-Resistant CTX-M-Producing Bacterial Pathogens

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ABSTRACT

Multidrug-resistance due to "β lactamases having the expanded spectrum" (ESBLs) in members of *Enterobacteriaceae* is a matter of continued clinical concern. CTX-M is among the most common ESBLs in *Enterobacteriaceae* family. In the present study, a nanoformulation of cefotaxime was prepared using gold nanoparticles to combat drug-resistance in ESBL producing strains. Here, two CTX-M-15 positive cefotaxime resistant bacterial strains (i.e., one *Escherichia coli* and one *Klebsiella pneumoniae* strain) were used for testing the efficacy of "cefotaxime loaded gold-nanoparticles." Bromelain was used for both reduction and capping in the process of synthesis of gold-nanoparticles. Thereafter, cefotaxime was conjugated onto it with the help of activator 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide. For characterization of both unconjugated and cefotaxime conjugated gold nanoparticles; UV-Visible spectroscopy, Scanning, and Transmission type Electron Microscopy methods accompanied with Dynamic Light Scattering were used. We used agar diffusion method plus microbroth-dilution method for the estimation of the antibacterial-activity and determination of minimum inhibitory concentration or MIC values, respectively. MIC values of cefotaxime loaded gold nanoparticles against *E. coli* and *K. pneumoniae* were obtained as 1.009 and 2.018 mg/L, respectively. These bacterial strains were completely resistant to cefotaxime alone. These results reinforce the utility of conjugating an old unresponsive antibiotic with gold nanoparticles to restore its efficacy against otherwise resistant bacterial pathogens. J. Cell. Biochem. 9999: 1–7, 2017.

KEY WORDS: CEFOTAXIME; CTX-M; ESBL; MULTI-DRUG RESISTANCE; GOLD NANOPARTICLES

The present research article is inspired by two research thrust areas. The first area could be considered as "hot" while the second area could be safely called as "continued hot." These refer to the scientific fields of "nanotechnology" and "multidrug resistance in bacteria," respectively [Grzybowski and Huck, 2016; Shaikh et al., 2016]. Dissemination of multidrug resistance owing to extended spectrum beta-lactamases (ESBLs) production by bacterial pathogens continues to be a matter of scientific concern. Unfortunately,

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due to the misuse of antibiotics on these pathogenic microorganisms, multidrug resistance has become an inevitable genetic response [Rossolini et al., 2008]. One of the most prevalent ESBLs in *Enterobacteriaceae* family spreading around the world is the CTX-M type [Rossolini et al., 2008]. In addition, plasmids, transposons, and integron gene cassettes help in rapid propagation of these CTX-M enzymes [Eckert et al., 2006]. As of now, there are approximately 172 reported variants of CTXM (http://www.lahey.org/studies/webt.asp [accessed on January 1, 2017]). Due to the worldwide prevalence of *Escherichia coli* as well as *Klebsiella pneumoniae* strains which produce ESBLs, a severe health threat has been generated especially in hospitalized patients [Hawser et al., 2009; Shaikh et al., 2015a, 2016]. In fact, *E. coli* is among the potent established causes responsible for the infections of the urinary tract (UTIs). *E. coli* is also considered responsible for soft-tissue as well as gastro-intestinal infections [Rodríguez et al., 2004]. On the other hand, *K. pneumoniae* is an important opportunistic pathogen which could cause a variety of hospital infections such as UTIs, bacteremia, and respiratory tract infections [Marra et al., 2006]. In the last few years, we have been working hard to find out the occurrence of ESBL positive strains (*E. coli* as well as *K. pneumoniae*) in different clinical specimen obtained from north Indian hospitals then assessing their resistance against third generation cephalosporins. The results showed that out of 93 ESBL producing *E. coli* strains, 71 *E. coli* strains were cefotaxime resistant, in contrast, among 74 ESBL producing *K. pneumoniae*, 58 *K. pneumoniae* strains were cefotaxime resistant [Shaikh et al., 2015a]. Recently, in 2016, we further extended our study on the said Gram-negative bacteria to identify integrons as well as CTX-M resistance marker. The outcome of the study showed increased occurrence of CTX-M-15 in the tested bacterial strains [Shaikh et al., 2016]. So far, the options to combat resistance in ESBL strains are limited. One option is to treat with combination of ESBL inhibitors and an antibiotic such as piperacillin–tazobactam or amoxicillin–clavulanate, while, the other one is to treat with carbapenems [Nicolau, 2008]. Drugs with combination of ESBL inhibitor and antibiotics are avoided in serious infections due to inoculum effect in-vitro and due to lesser efficacy than carbapenems in animal models. However, resistance to carbapenem has also increased globally [Harris et al., 2015]. Thus, new strategies are urgently required to fabricate drugs of the next generation [Azam et al., 2012].

Nanotechnology could be considered as an effective solution to these problems. In fact, nanoparticles have received attention of different interdisciplinary fields of science because of their exceptional physical, chemical, and biological properties [Jabir et al., 2012; Shaikh et al., 2015b]. There are several reports which showed that zinc oxide nanoparticles and silver nanoparticles possess potent antibacterial activity against ESBL producing strains [Bindu et al., 2015; Sultan et al., 2015]. In addition, it has been observed that the bacteria which were earlier resistant to known antibiotics such as ampicillin and cefaclor have become sensitive after loading of these antibiotics on to silver and gold nanoparticles [Rai et al., 2010; Brown et al., 2012]. Here, our team has estimated the efficacy of cefotaxime before and after loading to gold nanoparticles against two ESBL (CTX-M-15) positive bacteria (i.e., one *E. coli* and one *K. pneumoniae* strain). We took these isolates from our earlier study where they showed complete resistance to cefotaxime and the presence of CTX-M 15 marker [Shaikh et al., 2016]. To the best of our knowledge, there are no reports of using cefotaxime loaded gold nanoparticles to overcome cefotaxime resistance against ESBL producing bacterial strains. Thus, the present study is an attempt to revive an old ineffective drug into an effective drug against ESBL producing bacterial pathogens using gold nanoparticles as a tool. To achieve the same, we synthesized gold nanoparticles, conjugated them with cefotaxime, and performed characterization by UV-Visible spectroscopy, Scanning, and Transmission type Electron Microscopy methods (i.e., SEM/TEM) accompanied with Dynamic Light Scattering (or DLS) followed by pertinent antibacterial assays. The strategy outlined in the current study is expected to act as a scientific scaffold based on which a series of future nano-formulations meant for "reviving" old ineffective antibiotics into potent therapeutics could be designed.

MATERIAL AND METHODS

BACTERIA AND GROWTH CONDITIONS

E. coli and *K. pneumoniae* bacterial strains were taken from our previous study [Shaikh et al., 2016]. These strains were ESBL (CTX-M-15) positive and displayed complete resistance to cefotaxime. Before performing antibacterial test, fresh inoculum for each bacterial strain was prepared in nutrient broth medium and the culture was incubated for 18 h at 37 ± 2°C. However, turbidity was adjusted with the help of nutrient broth to the famous standard (i.e., 1.5×10^8 CFU/ml).

SYNTHESIS OF GOLD NANOPARTICLES

Method of Khan et al. [2015a] was used for gold-nanoparticle synthesis. First, 3 μl of 1.0 mM solution (Chloroauric acid) was mixed with 3 ml of Phosphate buffer (50 mM) containing 0.33 mg/ml bromelain. This reaction mixture along with a control without bromelain was incubated at 40°C temperature for 48 h. At regular time intervals, samples were removed and analyzed by using UV-Visible-spectroscopy for the confirmation of nanoparticles-formation. After complete incubation, gold nanoparticles were centrifuged at 30,000*g* for 30 min followed by washing with MilliQ water. However, ethanol (50% V/V) was used to remove extra bromelain from the sample and this sample was kept at room temperature till further characterization.

FUNCTIONALIZATION OF GOLD NANOPARTICLES WITH CEFOTAXIME

Activator 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was used to conjugate cefotaxime to in vitro synthesized gold nanoparticles [Timkovich, 1977]. A 5-ml mixture was prepared in HEPES buffer (50 mM) having 250 μg of cefotaxime and 250 μg of gold nanoparticles, followed by addition of 500 μl of 50 mM EDC in aliquots (50 μl/20 min) during incubation to assist in the formation of peptide bonds for proper binding of cefotaxime. Reaction volume was raised using double distilled water and incubated for 3–3:30 h at 40°C. The conjugates were separated from unconjugated gold nanoparticles by passing the reaction mixture through Biogel P-30 gel filtration column pre-equilibrated by NaCl (150 mM) containing

HEPES buffer (20 mM-pH 7.2). The fractions were scanned between 200 and 800 nm. The amount of fraction which exhibited the absorbance at 298/542 nm in the presence of cefotaxime conjugated with gold nano particles was pooled up.

CHARACTERIZATION OF PRE- AND POST-FUNCTIONALIZED GOLD NANOPARTICLES

For characterization, UV-Visible spectrophotometry, Scanning, and Transmission type Electron Microscopy methods accompanied with Dynamic Light Scattering techniques were used. A dual beam spectrophotometer supplied by Shimadzu (Model: UV-1601-PC; Resolution: 1 nm) was used for UV spectrophotometric analysis. For TEM analysis, gold nanoparticle drop was dried on a grid made of copper metal and coated with carbon. Subsequently, measurements were performed on JEOL, JEM 2100 TEM (Tokyo, Japan) at 200 kV of accelerating voltage. SEM was done by drying a drop of gold nanoparticles solution on glass slides and coating it with gold. The morphology of nanoparticles was examined under a scanning electron microscope (JEOL JSM 5200). Dynamic Light Scattering (DLS) X-tal- Spectrosize-300 (Marlowring, Hamburg, Germany) was used to measure the mean radius distribution of the gold nanoparticles.

DETERMINATION OF LOADING EFFICIENCY OF CEFOTAXIME ON GOLD NANOPARTICLES

Loading efficiency of gold nanoparticles was calculated as outlined previously [Gomes et al., 2014]. The nanoformulations were centrifuged at 30,000*g* for 30 min or till the total separation (i.e., gold-nanoparticles and the supernatant). Free drug in the supernatant was quantified by the same aforementioned double beam spectrophotometer (λmax 298) obtained from wave scan [Jamil et al., 2016]. The pure drug calibration curve was plotted in the linear range of 10–75 μg/ml. To calculate the percentage of drug loading efficiency, we need to find out the amount of drug attached to the gold nanoparticles. For this, we subtracted the free drug remained in the supernatant (or the drug not attached to the gold nanoparticles) from the initial amount of drug added and used the following equation:

$$\text{Loading efficiency}\ (\%) = \frac{\text{Total amount of drug} - \text{Free drug in the } \textit{sup}\text{ernatant}}{\text{Total amount of drug}} \times 100$$

DETERMINATION OF ANTIBACTERIAL ACTIVITY OF CEFOTAXIME LOADED GOLD NANOPARTICLES

Agar well-diffusion. Agar well-diffusion as described by Perez et al. [1990] was used to assess the antibacterial potential of cefotaxime and cefotaxime loaded gold nanoparticles. Mueller Hinton agar plates were swabbed with 100 μl of each test bacterial inoculums (1.5×10^8 CFU/ml). After that, two different concentrations, that is, 3.23 and 6.46 mg/L of cefotaxime and cefotaxime loaded on the gold nanoparticles were added in the well of 4 and 8 mm diameter on these Mueller Hinton agar plates. Here, sterilized double distilled water was used as a solvent to prepare stock solution, that is, 64.66 μg/ml.

Micro dilution method. Microbroth dilution method [Eloff, 1998] was employed for the determination of Minimum inhibitory-concentration or MIC-values. Sterilized double distilled water was used to prepare a stock solution (64.66 μg/ml) of cefotaxime conjugated on to gold nanoparticles. Further, stock solution was twofold serially diluted by using nutrient broth in 96-well microtitre plates for each bacterial strain. This culture having the standard turbidity of 1.5×10^8 CFU/ml was subsequently used for inoculation in each well. The plates were kept on incubation for a period of 24 h and a temperature of 37 ± 2°C. Moreover, 50 μl of INT (p-iodo-nitrotetrazoleum chloride) dye (2 mg/ml) was added in each well to assess the bacterial viability. Reddish-pink color due to bacterial viability was observed after 30 min incubation at 37°C, while, wells showing no color indicates growth inhibition. The minimum concentration of cefotaxime conjugated gold nanoparticles at which no growth or no color was observed has been considered as MIC value. However, minimum bactericidal concentration (MBC) was estimated after sub-culturing 50 μl of inoculums from the wells with no reddish pink color onto nutrient agar plate. The minimum concentration of cefotaxime conjugated gold nanoparticles where no growth was observed on agar plate was taken as MBC value.

RESULTS AND DISCUSSION

NANOPARTICLE SYNTHESIS AND FUNCTIONALIZATION

Environmental friendliness of research processes and outputs has its obvious importance. Chemicals such as citrate, borohydrate etc. when used as reducing agents to synthesize gold nanoparticles cause toxicity and also interfere with the surface modification and functionality of gold nanoparticles [Shan et al., 2008; Majzik et al., 2009]. So, instead of using chemicals, we used a natural enzyme bromelain to produce biocompatible and non-toxic gold nanoparticles. Gold nanoparticles were prepared according to the one-pot synthesis method where bromelain was used for both reduction and capping as mentioned in our previous work [Khan et al., 2015a]. Further, cefotaxime was conjugated to gold nanoparticles with the help of activator 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide.

SPECTROSCOPIC CHARACTERIZATION

Synthesis of the gold-nanoparticles was affirmed by "SPR" (also called as Surface plasmon resonance)-analysis via UV-Visible spectroscopy. Unconjugated gold nanoparticles showed absorption at 530 nm, while, cefotaxime conjugated gold nanoparticles showed reduced intensity and broadening with SPR absorption band at 542 nm (Fig. 1). This red shift of λmax value and increase in absorption suggested that changes have occurred after attachment of cefotaxime to the outer electrical layer present on the gold nanoparticles and implicated that cefotaxime has been successfully attached to the gold nanoparticles [Mangeney et al., 2002; Mukherjee et al., 2005]. In addition, the absorption spectrum of cefotaxime conjugated gold nanoparticles revealed two peaks at 298 and 542 nm which correspond to aromatic structure of cefotaxime and localized SPR band of gold nanoparticles, respectively [Jamil et al., 2016]. This confirms its binding to gold nanoparticles. Similarly, Khan et al. [2015b] also observed two peaks at 320 and

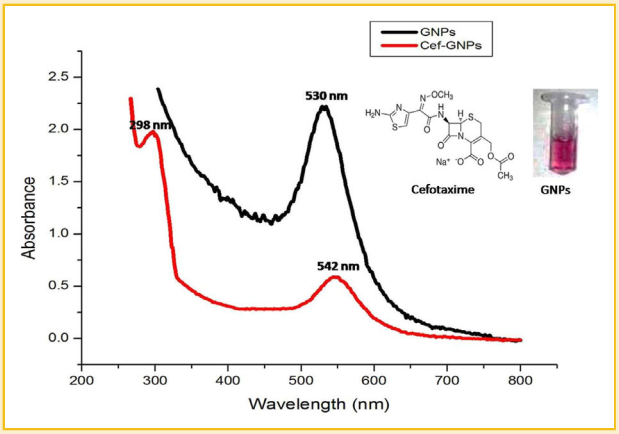

Fig. 1. UV-Vis spectra of gold nanoparticles with or without cefotaxime functionalization (here, GNPs means gold nanoparticles whileCef-GNPs means cefotaxime loaded gold nanoparticles).

525 nm for secnidazole (an antibacterial drug) and gold nanoparticles, respectively, during spectrophotometric analysis of secnidazole bioconjugated gold nanoparticles.

CHARACTERIZATION BY SCANNING AND TRANSMISSION TYPE ELECTRON MICROSCOPY METHODS ACCOMPANIED WITH DYNAMIC LIGHT SCATTERING

The unconjugated gold-nanoparticles as well as the cefotaxime conjugated gold nanoparticles were characterized using SEM (Fig. 2a and b) and TEM analysis (Fig. 3a and b). Average size of gold nanoparticles and cefotaxime conjugated gold nanoparticles were estimated to be as 6.87 ± 2.43 and 17.55 ± 2.95 nm, respectively, via TEM analysis. These results suggested that there was an increase in gold nanoparticle size owing to attachment of cefotaxime. SEM analysis showed that all these gold nanoparticles were of spherical shape and monodispersed. On the other hand, mean radius distribution of gold nanoparticles and cefotaxime conjugated gold nanoparticles were found to be 11.77 ± 3.45 and 29.72 ± 3.66 nm, respectively, by DLS (Fig. 4a and b). Actually, nanoparticles when dispersed in a liquid medium, a thin electric dipole solvent layer gets attached to it, so the size measured via DLS consists of information regarding solvent material as well as coating material along with the inorganic core. In contrast, information of hydration layer is absent when size is estimated by TEM. Thus, the size estimated by TEM and DLS may not be the same [Chu, 1991; Berne and Pecora, 2000].

CALCULATION OF LOADING EFFICIENCY

Another parameter important for the characterization of nanoparticles is estimating its loading efficiency. Here, the cefotaxime loading efficiency of gold nanoparticles was found to be 77.59%. Out of 250 μg cefotaxime added in the reaction mixture, 193.97 μg got attached to the gold nanoparticles. Loading efficiency should be high so that drug is not lost during preparation of drug conjugated nanoparticles and lesser amount is needed for its therapeutic use [Gomes et al., 2014]. Thus, we can safely state that the loading efficiency results were encouraging.

ANTIBACTERIAL EFFICACY TESTING OF ANTIBIOTIC CONJUGATED GOLD NANOPARTICLES

Now, to connect this piece of discussion with the application part, we find it important to mention that in Gram-negative pathogenic bacteria, CTXM class of beta-lactamases are the major cause of third generation cephalosporins resistance. It has been observed that in most of the cases, a bacterium which produces this class of beta-lactamases will have more resistance toward ceftriaxone and cefotaxime than ceftazidime [Bonnet, 2004]. However, out of different CTXM variants, CTXM-15 is the most common and widespread variant found in India. Our previous findings have indicated a significant increase in occurrence of $bla_{\text{CTX-M-15}}$ genes among clinical isolates of Gram-negative bacteria (i.e., *E. coli* and *K. pneumoniae*) that were isolated from hospitals located in North of India. Most of these strains were resistant to cefotaxime and other third generation cephalosporins [Shaikh et al., 2015a, 2016]. Hence,

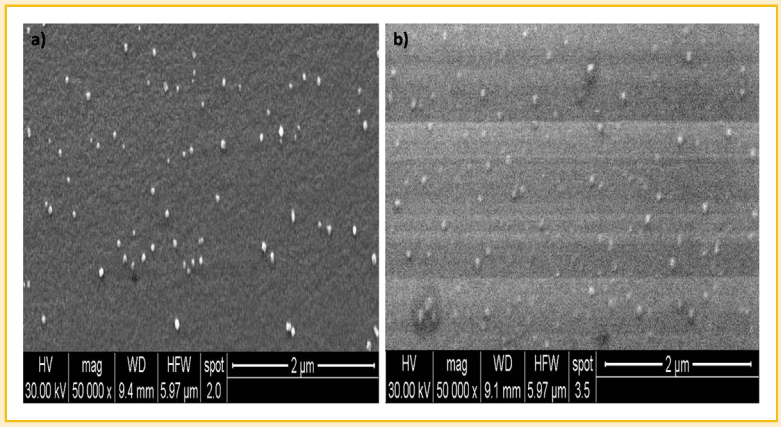

Fig. 2. SEM analysis of (a) gold nanoparticles and (b) gold nanoparticles loaded with cefotaxime.

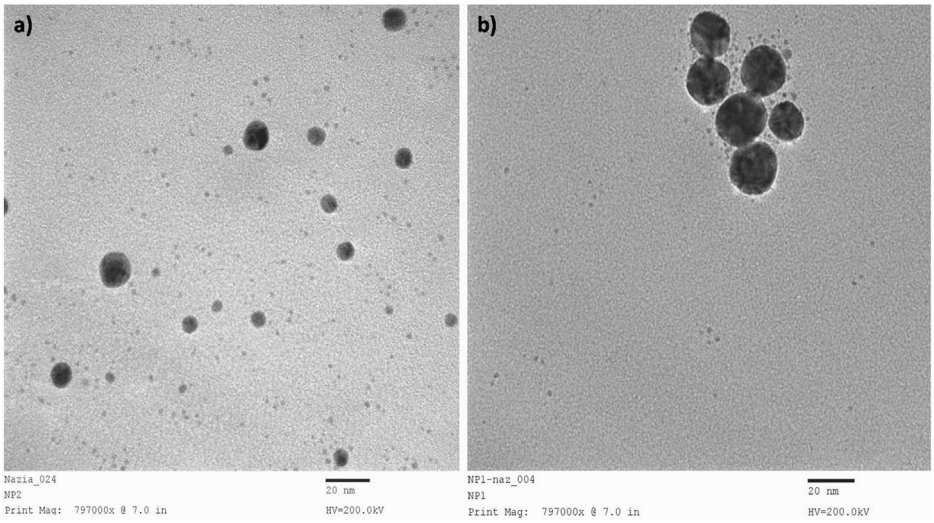

Fig. 3. TEM analysis of (a) gold nanoparticles and (b) gold nanoparticles loaded with cefotaxime.

developing new strategies to combat these drug resistant strains is the need of the hour to cope up with the dwindling stock of effective antibiotics.

AGAR WELL DIFFUSION ASSAY

Accordingly, after characterization, antibacterial efficacy of gold nanoparticles and cefotaxime conjugated gold nanoparticles against the ESBL (CTXM15)-producing strains were estimated by using agar well diffusion method (Fig. 5). Here, zone of inhibitions against *E. coli* at 3.23 and 6.46 mg/L concentrations of cefotaxime loaded on gold nanoparticles were 14 and 18 mm, respectively. However, zone of inhibitions against *K. pneumoniae* at the same concentrations were 13 and 17 mm, respectively. In addition, these strains were completely resistant to cefotaxime alone at both of the mentioned concentrations.

MINIMUM INHIBITORY CONCENTRATION AND MINIMUM BACTERICIDAL CONCENTRATION (MIC-MBC)-ASSAYS

MIC of cefotaxime conjugated gold nanoparticles was found as 1.009 and 2.018 mg/L against the study strains of *E. coli* and *K. pneumoniae*, respectively (Table I). Whereas, MBC of cefotaxime conjugated gold nanoparticles were determined to be 2.018 and

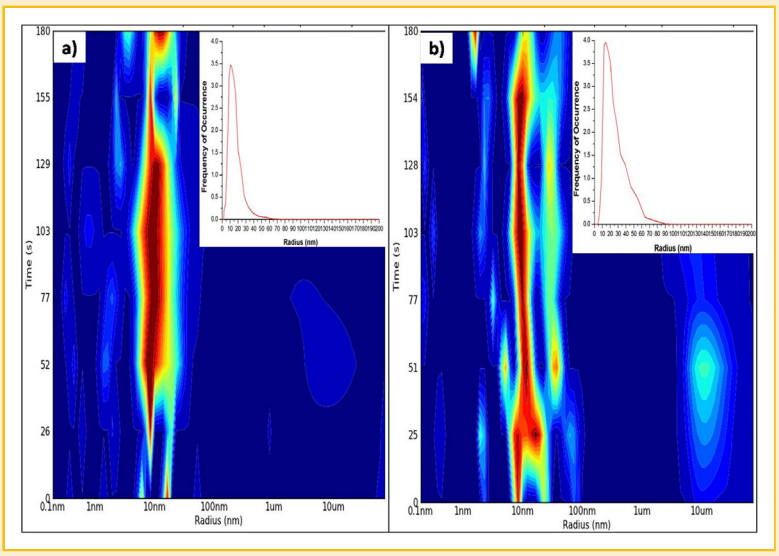

Fig. 4. DLS analysis-radius distribution (frequency of occurrence in inset) of (a) gold nanoparticles and (b) gold nanoparticles loaded with cefotaxime.

Fig. 5. Antibacterial activity of cefotaxime and cefotaxime loaded gold nanoparticles against ESBL (CTX-M-15) positive Escherichia coli and *K. pneumoniae* (here, Ce means cefotaxime while Ce + NP means cefotaxime loaded gold nanoparticles).

4.037 mg/L for the same, respectively. Various studies have been conducted to use gold nanoparticles as drug delivery tools because of their unique physiochemical properties such as non-cytotoxicity and biocompatibility [Fako and Furgeson, 2009; Cobley et al., 2011]. In 2004, Tom et al. [2004] found that reactive portion of ciprofloxacin was surface exposed when it was bound to gold nanoparticles. In another study, ampicillin retained its activity after conjugation with gold nanoparticles and showed potent effect against multi-drug resistant bacteria while gold nanoparticles alone were non-toxic [Brown et al., 2012]. In the same way, our study also implicates that cefotaxime retains its activity after conjugation to gold nanoparticles because unconjugated gold nanoparticles were inactive against the tested drug resistant strains.

MECHANISM OF OVERCOMING RESISTANCE BY CEFOTAXIME LOADED GOLD NANOPARTICLES (HYPOTHESIS)

Regarding the mechanism by which resistance was overcome in this particular piece of work, we could propose the following hypothesis. The effective concentration of cefotaxime delivered to the bacterial cell was increased due to its binding to gold nanoparticles. This in turn disrupted the cell wall before the majority of drug molecules could encounter the degrading CTX-M-15 bacterial enzyme. Hence, it was a two-way damage to bacteria. The first one could be genuinely expected to be due to the increased presence of drug molecules per unit volume of the system, that is, the cefotaxime molecules which remained untouched by the bacterial enzyme opportunistically attacked the bacteria. The second damage could be attributed to the increased porosity, that is, gold particles found easy access to the bacterial DNA which resulted in additional DNA damage. Our hypothesis is in harmony with the findings of Rai et al. [2010] who reported similar results for cefaclor (a second generation cephalosporin antibiotic) conjugated gold nanoparticles against *Staphylococcus aureus* and *E. coli*.

TABLE I. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Cefotaxime Loaded Gold Nanoparticles Against ESBL (CTX-M-15) Positive *E. coli* and *K. pneumonia*e

	Cefotaxime loaded gold nanoparticles	
Bacteria	MIC	MBC
E. coli	1.009 μg/ml	2.018 μg/ml
K. pneumonia	2.018 μg/ml	4.037 μg/ml

CURRENT RESEARCH STATUS AND FUTURE IMPLICATIONS

Currently, our research team is working on assessing the toxicity, exact mechanism of action and lethal dose of these cefotaxime conjugated gold nanoparticles in vitro as well as in vivo. In the preliminary results, these nanoparticles showed no toxicity toward human embryonic kidney cells-293 (Rizvi et al. *Personal Communication*).

Here, antibacterial assays showed that cefotaxime after attachment with gold nanoparticles got "revived" and the CTX-M-15 harboring bacterial strains which were completely resistant to cefotaxime turned susceptible to the nanoformulation. In future, we plan to work on these nanoformulations to decipher the exact molecular mechanisms involved in evading the effect of the CTX-M enzyme. Drug resistance is a major clinical concern. ESBL producing bacteria have developed resistance to even other alternatives such as carbapenems. It is natural that bacteria would eventually become resistant to other newer antibiotics as well. Hence, coming up with fresh nanoformulations of old antibiotics to overcome drug resistance, that is, "revival" of old antibiotics is of due relevance so as to keep pace with the dwindling stock of antibiotics.

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