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3,6-Di(pyridin-2-yl)-1,2,4,5-s-tetrazine capped-gold nanoparticles as an efficient antibacterialagent against gram-positive *Bacillus subtilis*

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In current work, we present 3,6-di(pyridin-2-yl)-1,2,4,5-s-tetrazine (pytz) capped gold nanoparticles (TzAuNPs) as an potent antibacterial agent against Gram-positive bacteria. The antibacterial properties of TzAuNPs have been studied using Gram-positive *Bacillus subtilis* (ATCC 11774) by the plate count method and TzAuNPs is found to be highly effective against the strain. Furthermore, TzAuNPs also show significantly better antimicrobial activity against the bacterial strain when compared to pytz. The effect of TzAuNPs on the bacterial cell wall is also investigated by TEM analysis. This new antimicrobial material could be promising in future for the treatment infectious diseases caused by Gram-positive bacteria.

Keywords: Antibacterial activity, Antibacterial mechanism, Gold nanoparticles, TEM analysis

As a result of increasing microbial resistance to multiple antimicrobial agents and development of resistant strains, there is an increasing demand for novel antimicrobial materials with superior performance for disinfection applications[1–8]. Consequently, new agents and methods for bactericidal treatment of infections caused by bacteria are urgently needed. In this regard, nanomaterials are particularly effective and have shown great potential as a new drug to kill or inhibit numerous microorganisms.[9–13] The antibacterial activity of nanoparticles is dependent on their direct interaction with the bacterial cell wall and they can, therefore, act as antibiotics without entry into the cell.[14,15] Other possible modes of antibacterial action for nanoparticles include the generation of reactive oxygen species (ROS), penetration through the cell membrane and interactions with cellular DNA and proteins. As a result of these multiple modes of action, the use of nanoparticles as antibacterial agents would provide a greater barrier to bacteria developing resistance mechanisms that is the case for conventional therapeutics.[7] Several metal nanoparticles (NPs) (e.g., silver, copper, gold) have been synthesized and tested for antimicrobial activity against several pathogenic bacterial strains. Gold nanoparticles (AuNPs) have recently attracted a lot of attention compared to other metal NPs due to low toxicity, chemical stability and easy surface functionalization. AuNPs have been extensively used in drug delivery applications, intracellular gene regulation, bioimaging (as contrast agents), anti-inflammatory therapy and anticancer therapy (photodiagnostic and photothermal therapy).[16–22] Furthermore, the antimicrobial activity of gold nanoparticles has been recently demonstrated.[15,21–25]

In this work, a simple, eco-friendly, and cost-effective synthesis method of AuNPs using 3,6-di(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazine(H_2pytz) as reducing agents has been reported. This 3, 6-di-(pyridin-2-yl)-1,2,4,5-tetrazine (pytz) capped gold (TzAuNPs) nanoparticles have been synthesized to investigate their potential against bacterial growth. The antibacterial activity of the TzAuNPs against Gram-positive *Bacillus subtilis* and Gram-negative *E. coli K12* has been studied.

Materials and Methods

The syntheses of 3, 6-di(pyridin-2-yl)-1,2,4, 5-tetrazine (pytz) capped Au(0) nanoparticles were carried out according to the method reported earlier.[26] Standard strain of *Bacillus subtilis* (ATCC 11774) was utilized to determine antibacterial activities.

3,6-Di(pyridin-2-yl)-1,2,4,5-*s*-tetrazine (pytz) capped-gold nanoparticles (TzAuNPs)

The synthesis of 3,6-di(pyridin-2-yl)-1,2,4,5-*s*-tetrazine (pytz) capped-gold nanoparticles (TzAuNPs) was achieved by the reduction of $HAuCl_4 \cdot 3H_2O$ with 3,6-di(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazine (H_2pytz). A 100 mL round bottom flask containing solution of 3,6-di(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazine (0.12 g, 0.5 mmol) in ethanol (50 mL) was placed in an ultrasonicator bath and hydrogen tetrachloroaurate(III) (0.3 g, 0.75 mmol) in 10 mL water was added to it drop wise. After few minutes, a reddish black precipitate started to appear slowly indicating the formation of gold nanoparticles. The resulting particles were isolated by centrifugation (10 min, 12000 rpm) and washed subsequently with water, ethanol, and dichloromethane. The product was then dried in vacuum oven and kept in vacuum desiccator for further characterization.

Characterization of nanoparticles

Powder X-ray diffraction (XRD) patterns were obtained on a Philips PW 1140 parallel beam X-ray diffractometer with Bragg-Bretano focusing geometry and monochromatic CuKα radiation (λ = 1.540598 Å). Transmission electron microscopy (TEM) images were collected by using JEOL JEM-2100 microscope working at 200 kV. TEM samples were prepared by sonicating an aliquot of the sample in ethanol for 15 min and 1 μL of this suspension was drop-casted onto carbon-coated, 300 mesh copper grids. Grids were allowed to dry in descicator prior to imaging.

Antimicrobial test for TzAuNPs

The antibacterial activities of TzAuNPs were measured by serial dilution method. The 50 mg of nanoparticles were as suspended in 6 mL of Muller-Hinton Broth and inoculated with 1 mL of 18 h stock culture of *Escherichia coli K12* and *Bacillus subtilis*. Aliquots of each sample were diluted and 200 μL plated on sterile Petridishes, covered with Muller-Hinton Agar. The plates were incubated for 20 h at 37 °C. After the incubation period, the colony forming units (CFU) of each plate were determined. Antimicrobial activity of pytz was also tested by the same method to compare its activity with TzAuNPs.

Minimum inhibitory concentration (MIC) of TzAuNPs

Bacillus subtilis was respectively cultured in Muller-Hinton broth at 37 °C on a shaker incubator at 200 rpm for 6 h. Then the concentration of microorganism of 1×10^8 CFU mL^{-1} dilution was fixed at 0.1 optical density at 600 nm with adding medium and then again diluted to 1×10^6 CFU mL^{-1} with medium. We mixed bacterial suspension (1×10^6 CFU mL^{-1}, 100 μL mL^{-1}) with the solutions of pytz capped-gold nanoparticles with different concentrations in medium and the final volume of the solutions were adjusted to 10 ml with medium. The solutions were shaken at 37 °C on a shaker incubator at 200 rpm for 24 h. The bacterial viability was determined by measuring optical density at 600 nm ($OD_{600\ nm}$). Simultaneously, aliquots of each solution were diluted and plated on Muller-Hinton Agar. The plates were incubated at 37 °C for 20 h and colonies were counted and compared to those on control plates to calculate changes in the cell growth inhibition. The percentage of cell growth reduction (*R*, %) was calculated using the following equation:

$$R = \left\{\frac{C_0 - C}{C_0}\right\} \times 100$$

Where C_0 is the number of CFU from the control sample and C is the number of CFU from treated samples. Each concentration was prepared and measured in triplicate.

Antibacterial growth kinetics of Bacillus subtilis

Approximately 1×10^7 CFU mL^{-1} bacterial suspension was inoculated with different concentrations of TzAuNPs (3.75−17 μgmL^{-1}) in Muller-Hinton Broth. The mixtures were shaken at 37 °C on a shaker incubator at 200 rpm for 0–36 h. The initial time of addition of the bacteria was taken as zero. The growth curves resulted from bacteria only, and pytz capped-gold nanoparticles incubated with bacteria were plotted against time. Aliquots were withdrawn from each of the mixtures at definite time intervals and diluted further. 200 μL of the final solutions was plated on Muller-Hinton agar plates immediately. The plates were incubated at 37 °C for 24 h, and bacterial colonies were counted. A plot of colony forming unit (CFU) in logarithmic scale versus time ($\log_{10}$ CFU mL^{-1} vs time) was then plotted to determine the bactericidal kinetics.

Characterization of treated bacteria by TEM

The sizes and morphologies of TzAuNPs treated and untreated bacteria were examined by TEM. Prior to microscopy analysis, bacteria samples were prepared using procedures described previously in

literature.[27] *Bacillus subtilis* was inoculated for 10 h in Muller-Hinton broth at 37 °C and at 200 rpm until an OD_{600} of 0.5 was attained. The bacteria cells (30 mL) were then harvested, centrifuged, and re-dispersed in pH 7.4 PBS buffer. Pytz capped-gold nanoparticles were added to bacteria and the mixture was incubated at 37 °C for 6 h while shaking at 200 rpm. The mixture was then centrifuged at 2000 G-force for 8 min, and the supernatant containing was discarded. 1 μL of bacteria cell suspension was placed onto a Cu grid followed by drying overnight in a desiccator.

Results and Discussion

Synthesis and Characterization of TzAuNPs

The synthesis of 3,6-di(pyridin-2-yl)-1,2,4,5-s-tetrazine (pytz)capped-gold nanoparticles (TzAuNPs) was carried following a method reported earlier for silver nanoparticles.[26] In this synthesis, 3, 6-di(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazine(H_2pytz) acts as reducing agent for $HAuCl_4 \cdot 3H_2O$ and H_2pytz oxidizes to (pytz)which eventually capped AuNPs to produce TzAuNPs. The present process does not need any external capping agent for stabilization of AuNPs. The capping of AuNPswith pytz not only controls the aggregation of AuNPs but also helps AuNPs to remain soluble in water.

The crystallinity and purity of TzAuNPs were examined by the XRD technique. Fig. 1 depicts the XRD pattern of TzAuNPs. The XRD pattern of TzAuNPs shows that the AuNPs are highly crystalline and XRD peaks correspond to (111), (200), (220), (311) and (222) planes of Au(0) with fcc structure (JCPDS No. 04-0784).

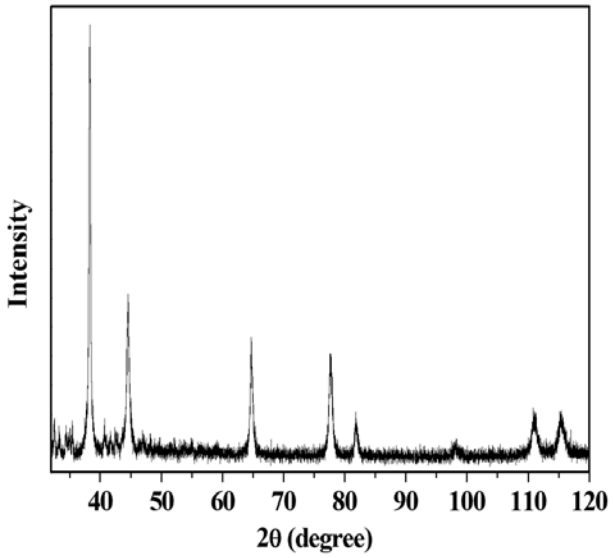

Fig. 1 — X-Ray diffraction pattern of 3,6-di(pyridin-2-yl)-1,2,4,5-*s*-tetrazinecapped gold nanoparticles (TzAuNPs)

The morphology and size distribution of the prepared AuNps were elucidated from the transmission electron micrographs. Fig. 2 exhibits morphological images of the prepared TzAuNPs. Typical TEM images in Fig. 2, illustrates that the TzAuNPs are spherical in shape with average diameter of about 12 nm, albeit, the particles have a broader size distribution ranging from 5 to 20 nm.

Antibacterial activity of TzAuNPs

The antibacterial activities of TzAuNPs are evaluated for Gram-negative *E. coli K12* and Gram-positive *B. subtilis*. TzAuNPs (50 mg) was dispersed in 6 mL of Mueller-Hinton broth and inoculated with 1 ml freshly cultured bacteria. After an incubation time of 18 h, sample was diluted and plated on Mueller-Hinton agar plates. The plates were incubated for 20 h. Colonies were counted and recorded for dilutions containing between 30 and 300 colonies. In presence of TzAuNPs, the Mueller-Hinton agar plates with *B. subtilis* exhibited no growth of bacterial colonies for 10^9–10^7 CFU mL^{-1} concentrations of bacteria, indicating excellent antibacterial activity of TzAuNPs against Gram-positive bacteria (Fig. 3). On the other hand, TzAuNPs exhibited very poor activity against Gram-negative *E. coli*. Antibacterial activities of TzAuNPs against *B. subtilis* were then compared with pytz and a control solution was prepared with the bacteria

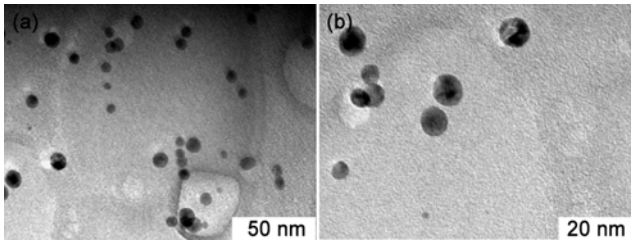

Fig. 2 — TEM images of 3,6-di(pyridin-2-yl)-1,2,4,5-*s*-tetrazinecapped-gold nanoparticles (TzAuNPs)

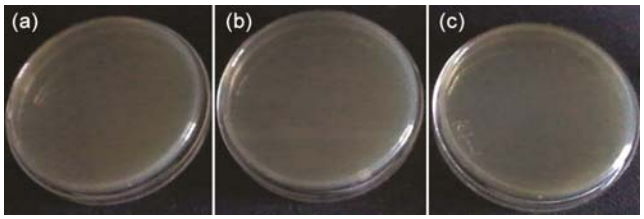

Fig. 3 — Antibacterial activities of TzAuNPs with 10^9, 10^8 and 10^7 CFU mL^{-1} *B. subtilis* (a–c)

without any nanoparticles. Solutions of *B. subtilis* with TzAuNPs, pytz and without any nanoparticles were diluted to 10^5 CFU mL^{-1} after 18 h incubation and plated on Mueller-Hinton agar plates. As shown in Fig. 4, after 20 h of incubation, dense bacterial colonies were observed on the control and pytz Mueller-Hinton agar *B. subtilis* (Fig. 4a and 4b). However, the Mueller-Hinton agar plates with the TzAuNPs nanoparticles exhibited no growth of bacterial colonies, indicating excellent antibacterial activity of TzAuNPs against *B. subtilis* (Fig. 4c).

The MIC value of TzAuNPs was evaluated by growth inhibition of *B. subtilis* by varying the concentration of TzAuNPs and measuring their optical density at 600 nm ($OD_{600\ nm}$) after 24 h. The original bacterial concentration was 1×10^6 CFU mL^{-1}. Aliquots of sample were diluted and plated on Mueller-Hinton agar, incubated for 24 h and colonies were counted. The minimal inhibition concentration (MIC) of TzAuNPs to inhibit the proliferation of *B. subtilis* was determined to be 3.75 μg mL^{-1}. The cell growth reduction percentage was calculated to be ~ 95% for *B. subtilis* after 24 h of incubation. The effect of TzAuNPs on the growth kinetics of *B. subtilis* in liquid media was studied (Fig. 5). Freshly cultured bacteria were added with various concentrations of TzAuNPs solution; the $OD_{600\ nm}$ based on the turbidity of the cell suspension was measured to monitor bacterial growth. Each solution was also diluted, plated on Mueller-Hinton agar, incubated for 24 h and bacterial colonies were counted. The growth of the bacteria was inhibited as the concentration of TzAuNPs increased. Fig. 5 shows the growth curves of *B. subtilis* (1×10^6 CFU mL^{-1}) obtained by culturing bacteria in broth containing different concentrations of TzAuNPs. The results show that the growth of *B. subtilis* was effectively inhibited when the concentration of TzAuNPs is 3.75 µg mL^{-1} within 10 h, compared with the curve obtained from culturing *B. subtilis* in TzAuNPs free broth. *B. subtilis* cell growth was completely inhibited within 24 h when the concentration of the TzAuNPs was 15 µg mL^{-1}.

To clarify the antibacterial mechanism of the action of TzAuNPs, ultra-structural damage of *B. subtilis* before and after incubation with nanoparticles were investigated by transmission electron microscopy. The representative TEM images are shown in Fig. 6. In the presence of TzAuNPs, apparently there is cell wall destruction leading to partial or whole lysis with

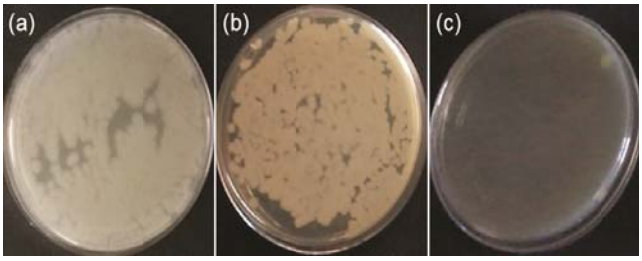

Fig. 4 — Photographs of colonies of *B. subtilis* (a) control, (b) after treatment with pytz and (c) treated with TzAuNPs

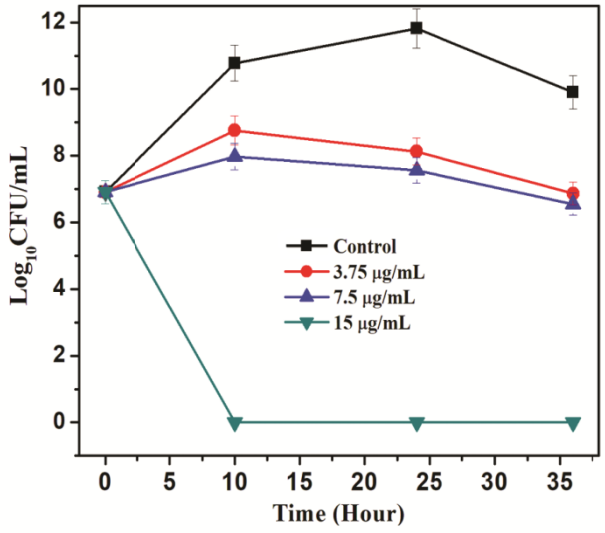

Fig. 5 — Concentration effects of TzAuNPs on the growth of *B. subtilis*

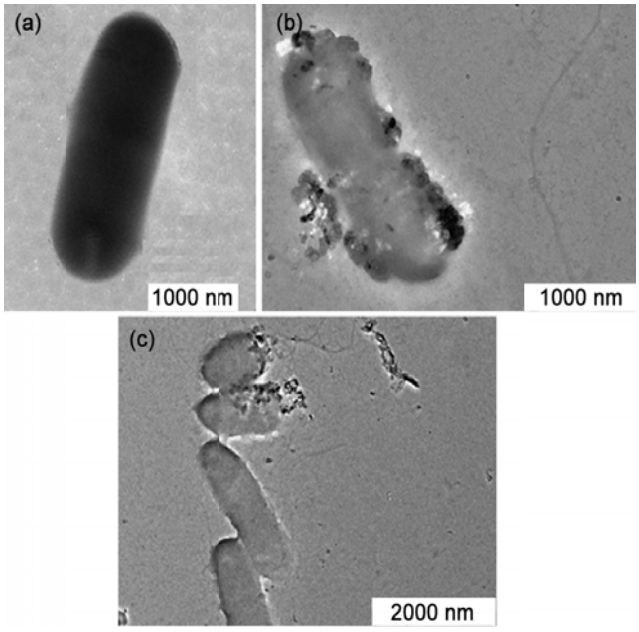

Fig. 6 — TEM images of *B. subtilis* (a) before and after inoculation with (b and c) TzAuNPs for 6h

formation of blebs and collapse of bacterial cells (Figs 6b and 6c) as compared to control where very smooth morphology was observed (Fig. 6a).

Conclusions

3,6-Di(pyridin-2-yl)-1,2,4,5-tetrazine (pytz) capped gold (TzAuNPs) was synthesized and characterized by powder XRD and TEM techniques. A systematic evaluation of the antibacterial activities of the pytz-capped Au(0) nanoparticle were carried out. TzAuNPs showed excellent bactericidal properties against Gram-positive *B. subtilis* bacteria. The MIC value for TzAuNPs against *B. subtilis* was obtained as 3.75 μg mL^{-1}. The material reported here can effectively be used for different applications in biomaterials.

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