Antibacterial activity of 3,6-di(pyridin-2-yl)-1,2,4,5-s-tetrazine capped Pd(0) nanoparticles against Gram-positive \textit{Bacillus subtilis} bacteria

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Antibacterial activity of 3,6-di(pyridin-2-yl)-1,2,4,5-s-tetrazine capped Pd(0) nanoparticles against Gram-positive *Bacillus subtilis* bacteria

Sutapa Joardar¹,²*, Shounak Ray³, Suvendu Samanta³ and Paramita Bhattacharjee²*

**Abstract:** In this work, we report antibacterial activity of 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine (pytz) capped Pd(0) nanoparticles (TzPdNPs). The TzPdNPs were characterized by transmission electron microscopy (TEM) and powder X-ray diffraction techniques (PXRD). The antibacterial properties of TzPdNPs were studied using Gram-positive *B. subtilis* and Gram-negative *E. coli* by the plate count method. The antimicrobial activity of this TzPdNPs shows that the microbial growth has been fully inhibited only for Gram-positive *B. subtilis* bacteria. Morphological changes obtained by transmission electron microscope observation show that TzPdNPs can cause leakage and chaos of intracellular contents.

**Subjects:** Microbiology; Biology; Biotechnology

**Keywords:** palladium nanoparticles; antimicrobial activity; antibacterial growth kinetics; transmission electron microscopy

1. Introduction

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. In this regard, nanoparticles are particularly effective, due to their enhanced efficacy compared to traditional antibiotics. This is facilitated by their small size, which allows them to easily penetrate bacterial cell membranes, leading to increased intracellular drug concentration and thus enhanced antibacterial activity. These properties can also render the bacteria more susceptible to other antimicrobial agents, thereby increasing the overall efficacy of the treatment. Additionally, nanoparticles can be engineered to have a longer residence time in the body, which can further enhance their therapeutic effects.

**ABOUT THE AUTHORS**

Sutapa Joardar (SJ) is currently working as an assistant professor in the Department of Biotechnology, Neotia Institute of Technology, Management and Science, West Bengal, India. The focus of her research is application of nanostructured materials as antimicrobial agents. She is particularly interested in the antimicrobial properties of metallic nanoparticles immobilized on carrier such as zeolites, silicates, and polymer. Paramita Bhattacharjee (PB) is currently working as an assistant professor in the Department of Food Technology & Bio-Chemical Engineering, Jadavpur University, West Bengal, India. The focus of her research is supercritical fluid extraction, microencapsulation, gamma irradiation technology, technology of fats and oils, and technology of herbs and spices.

**PUBLIC INTEREST STATEMENT**

Infectious diseases remain among the top five causes of mortality in countries of all socioeconomic classes worldwide, resulting in 9.2 million deaths in 2013 (about 17% of all deaths). At any given time, an estimated 1.4 million people are affected by healthcare-associated infections (HAIs) in developed countries, around 5–10% of patients in hospitals get HAIs, but the risk is 200–2,000% higher in undeveloped countries. Therefore, antibacterial agents play a significant role in combating microbial infections. However, due to the wide use of antibiotics and the emergence of antibiotic-resistant bacterial strains, conventional antibiotics are becoming less effective, resulting in poor treatment efficacy and significantly increased cost of healthcare. Each year, in the United States alone, more than 2 million people with antibiotic-resistant infections are reported, leading to at least 23,000 deaths. Therefore, there is a continuous demand to discover new antimicrobial agents to control microbial infections.
a high surface-to-volume ratio, which provides a large active surface in the contact with microorganisms. It has been demonstrated that nanoparticles (NPs) have a wide variety of unique applications, including cell targeting (Manolova et al., 2008; Weissleder, Kelly, Sun, Shtatland, & Josephson, 2005), intravenous nucleic acid delivery (Dobson, 2006; Li & Szoka, 2007; Neuberger, Schöpf, Hofmann, Hofmann, & von Rechenberg, 2005), environmental remediation (Kamat & Meisel, 2003; Tratnyek & Johnson, 2006; Zhang et al., 2010), and catalysis (Witham et al., 2010). The antimicrobial function of metal ions and NPs has also long been recognized and exploited industrially for several purposes, including amendments to textiles and cosmetics, food processing, water treatment, and so on (Ilić et al., 2009; Mueller & Nowack, 2008). Commonly used antimicrobial nanocomposite materials include metal ions or NPs (silver (Tamboli et al., 2012; Vukoje et al., 2014), copper (Cady et al., 2011; Gioffi et al., 2005; Liang et al., 2012), gold (Boomi & Prabu, 2013; Mei et al., 2014), platinum (Ma et al., 2012), metal oxide (titanium dioxide (Zhang & Chen, 2009), zinc oxide (Liang et al., 2012), copper oxide (Ojas, Bhagat, Gopalakrishnan, & Arunachalam, 2008)), and organically modified nanoclay (Rhim, Hong, Park, & Ng, 2006), to name a few.

So far, the antimicrobial property of silver, copper, gold, and platinum NPs has been investigated thoroughly; however, potential of palladium nanoparticles (PdNPs) as antimicrobial agent remains a relatively undeveloped area. Pd is one of the most widely used transition metals for carbon–carbon and carbon–heteroatom cross-coupling reactions such as the Suzuki–Miyaura reaction (1995), Heck reaction (Heck, 1968a, 1968b, 1968c, 1968d; Heck & Nolley, 1972), Kumada reaction (Jana, Pathak, & Sigman, 2011), Sonogashira reaction (Sonogashira, 2002), Negishi reaction (Astruc, 2007), Stille reaction (Milstein & Stille, 1978), and Buchwald–Hartwig reaction (Widenhoefer & Buchwald, 1996). Pd has also been used as a catalyst to manufacture pharmaceuticals (Malleron, Fiaud, & Legros, 2000), degrade harmful environmental pollutants (Nutt, Hughes, & Wong, 2005), and as sensors for the detection of various analytes (Chang et al., 2008; Favier, Walter, Zach, Benter, & Penner, 2001; Yu et al., 2005). Additionally, Pd and Pd²⁺ ions also play fundamental roles in several biotechnological processes (Baccar, Adams, Abdelghani, & Obare, 2013). Baccar et al. developed a non-enzymatic biosensor using various sizes of PdNPs to detect hydrogen peroxide in milk (2013). The anti-tumor, -viral, -malarial, -fungal and -bacterial activities of various Pd(II) complexes of N, O, S donor ligands, and drugs are long known (Baccar et al., 2013; Garoufis, Hadjikakou, & Hadjikakou, 2009). Size-dependent antimicrobial effects of PdNPs have also been reported recently (Adams, Walker, Obare, Docherty, & van Raaij, 2014; Garoufis, Hadjikakou, & Hadjilaidis, 2005).

In the present study, we report the activity of 3,6-di-(pyridin-2-y1)-1,2,4,5-tetrazine (pytz) capped PdNPs (TzPdNPs) against bacterial growth. To investigate the antibacterial activity of TzPdNPs, the viability of Gram-positive Bacillus subtilis was investigated in the presence of TzPdNPs.

2. Experimental

2.1. Materials and methods

The synthesis of 3,6-di(pyridin-2-y1)-1,2,4,5-tetrazine (pytz) capped Pd(0) nanoparticles (TzPdNPs) was carried out according to the method reported earlier (Das, Samanta, Ray, & Biswas, 2015). The antimicrobial activities were investigated against bacterial strains Gram-positive Bacillus subtilis (MTCC 441) and Gram-negative Escherichia coli (MTCC 2939).

2.2. Instrumentation

Powder X-ray diffraction (PXRD) patterns were obtained on a Philips PW 1140 parallel beam X-ray diffractometer with Bragg–Bretano focusing geometry and monochromatic CuKα radiation (\(\lambda = 1.540598 \text{ Å}\)). Transmission electron micrographs (TEM) images were collected using JEOL JEM-2100 microscope working at 200 kV. TEM samples were prepared by sonication of an aliquot of the sample in ethanol for 15 min and a single drop of this suspension was drop-casted onto carbon-coated 300 mesh copper grids. Grids were allowed to air-dry prior to imaging.
2.3. Antimicrobial test for TzPdNPs
The antibacterial activities of TzPdNPs were measured by serial dilution method. The 50 mg of TzPdNPs was suspended in 6 mL of Muller-Hinton broth and inoculated with 1 mL of 18-h stock culture of *Bacillus subtilis*. Aliquots of each sample were diluted and 200 μL were plated on sterile Petri dishes, 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 TzPdNPs.

2.4. Minimum inhibitory concentration (MIC)
*Bacillus subtilis* was cultured in Muller-Hinton broth at 37°C on a shaker incubator at 200 rpm for 6 h. Then the concentration of micro-organism of 1 × 10⁸ CFU mL⁻¹ dilution was fixed at 0.1, optical density at 600 nm with addition of medium and then again diluted to 1 × 10⁶ CFU mL⁻¹ with medium. We mixed bacterial suspension (1 × 10⁶ CFU mL⁻¹, 100 μL) with the solutions of TzPdNPs with different concentrations in medium and the final volume of the solutions was 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 (OD₆₀₀nm). 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:

\[
R = (C_0 - C)/C_0 \times 100
\]

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, and all experiments were repeated at least thrice in parallel.

2.5. Antibacterial growth kinetics
Bacterial suspension (~1 × 10⁷ CFU mL⁻¹) was inoculated with different concentrations (63–250 μg mL⁻¹) of TzPdNPs 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 TzPdNPs incubated with bacteria were plotted against time. Aliquots were drawn from each of the mixtures at definite time intervals and diluted further. Afterward, 200 μL of the final solutions were 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 (log₁₀ CFU mL⁻¹ vs time) was then plotted to determine the bactericidal kinetics.

2.6. Characterization of treated bacteria by TEM
The size and morphology of TzPdNPs treated and untreated bacteria were examined by TEM. Prior to microscopy analysis, bacterial samples were prepared using procedures described previously in literature (Hao, Jayawardana, Chen, & Yan, 2015). *Bacillus subtilis* was inoculated for 10 h in Muller-Hinton broth at 37°C and at 200 rpm until an OD₆₀₀ of 0.5 was attained. The bacteria cell suspension (30 mL) was then harvested, centrifuged, and redispersed in pH 7.4 PBS buffer. TzPdNPs (300 μg mL⁻¹) were added to bacteria and the mixture was incubated at 37°C for 10 h while shaking at 200 rpm. The mixture was then centrifuged at 2,000 rcf for 8 min, and the supernatant was discarded. A drop of bacteria cell suspension was placed onto a Cu grid followed by overnight drying.

3. Results and discussion

3.1. Synthesis and characterization of the TzPdNPs
Synthesis of 3,6-di(pyridin-2-yl)-1,2,4,5-s-tetrazine (pytz) capped Pd(0) nanoparticles (TzPdNPs) was achieved by reacting aqueous solution of Na₂PdCl₄ with 3,6-di(pyridin-2-yl)-1,4-dihydro-1,2,4,5-tetrazine (H₄pytz) in ethanol under sonication. The crystallinity and purity of TzPdNPs were examined (Figure 1(a))
Figure 1. (a) XRD pattern, (b) TEM image of TzPdNPs and (c) size distribution of 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine (pytz) capped Pd(0) nanoparticles (TzPdNPs).
by the PXRD technique. The XRD pattern of TzPdNPs shows the inter planar d-spacing of XRD peaks correspond to (111), (200), (220), and (311) planes of Pd(0) with fcc structure (JCPDS No. 01–1310), without any impurity phase. The morphology and size distribution of the prepared nanoparticles were elucidated from the TEM. Figure 1(b) exhibits morphological images of the prepared palladium nanoparticles. The shape of the particles is quasi-spherical and the size of the particles varies from 1.5 to 3.5 nm and the average size observed was 2.5 ± 0.2 nm (Figure 1(c)). It can also be noted that, despite the small size, the particles are separated from one another and no significant aggregation is observed.

3.2. Antibacterial activity
The antibacterial activities of these TzPdNPs nanoparticles have been evaluated toward Gram-positive *B. Subtilis* and Gram-negative *E. coli*. TzPdNPs (50 mg) were dispersed in 6 mL of Mueller-Hinton Broth and inoculated with 1 mL of 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. The Mueller-Hinton agar plates with the *B. subtilis* exhibited no growth of bacterial colonies for $10^9$–$10^7$ CFU mL$^{-1}$ concentrations of bacteria, indicating excellent antibacterial activity against Gram-positive bacteria (Figure 2). Antibacterial activities of TzPdNPs were then compared with pytz and a control solution was prepared with the bacteria but without any nanoparticles. Solutions of bacteria with TzPdNPs, 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 Figure 2, after 20 h of incubation, dense bacterial colonies were observed on the control and pytz Mueller-Hinton agar *B. subtilis* (Figures 3(a) and (b)). However, the Mueller-Hinton agar plates with the TzPdNPs exhibited no growth of bacterial colonies, indicating again excellent antibacterial activity of TzPdNPs against *B. subtilis* (Figure 3(c)).

MIC values of TzPdNPs were evaluated by growth inhibition of *B. subtilis* by varying the concentration of TzPdNPs and measuring their OD$_{600}$, after 24 h. The original bacterial concentration was $1 \times 10^6$ CFU mL$^{-1}$. Aliquots of each sample were diluted and plated on Mueller-Hinton agar, incubated for 24 h, and colonies were counted. The MIC of TzPdNPs to inhibit proliferation of *B. subtilis* was
Percentage of the cell growth reduction was calculated to be 95% for \textit{B. subtilis} after 24 h of incubation. The effect of TzPdNPs nanoparticles on the growth kinetics of \textit{B. subtilis} in liquid media was studied (Figure 4) further. Freshly cultured bacteria were added with various concentrations of TzPdNPs solution; the optical density at 600 nm (OD$_{600 \text{nm}}$) 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 both the bacteria was inhibited as the concentration of TzPdNPs increased, but the rates of bacterial growth inhibition were different. The growth of \textit{B. subtilis} started to decrease when the concentration of TzPdNPs was 62.5 μg mL$^{-1}$ and at a concentration of 250 μg mL$^{-1}$, the growth can be completely inhibited (Figure 4). It can also be observed that no colony was formed for bacteria in the plate until 36 h.

Next, we explored antibacterial mechanisms of TzPdNPs. The exact mechanisms of NPs toxicity against bacteria are not understood completely. Generally, NPs are able to bind to the membrane of bacteria by electrostatic interaction and disrupt the integrity of the bacterial membrane (Thill et al., 2006). Nanotoxicity is generally triggered by the induction of oxidative stress by formation of reactive oxygen species (ROS), following the administration of NPs (Nel et al., 2009; Soenen, Rivera-Gil, Montenegro, Parak, & De Smedt, 2011). On the other hand, rather different mechanism has been proposed particularly for silver nanoparticles. Previous studies suggest that silver nanoparticles (AgNPs) release Ag$^+$ ions which interact strongly with thiol groups of intracellular enzymes and proteins, leading to degeneration (Agnihotri, Mukherji, & Mukherji, 2013; Kim, Lee, Cha, Kim, & Kang, 2007; Xiu, Zhang, Puppala, Colvin, & Alvarez, 2012). The activities of enzymes and proteins are closely related to physiological functions of bacteria (Eckhardt et al., 2013; Xiu et al., 2012). Therefore, it may be suggested that Ag$^+$ could affect the physiological functions of intracellular enzymes and proteins, leading to inhibition of activity and cellular damage.
To clarify the antibacterial mechanism of the action of TzPdNPs, we investigated ultrastructural damage of \textit{B. subtilis} before and after incubation with TzPdNPs, examining TEM images. Untreated bacteria exhibited a smooth surface and distinct nucleoid structures (Figure 5(a)). In contrast, the bacterial cell surface became rough after incubation with TzPdNPs for 12 h. Moreover, numerous blebs were formed with cytoplasmic release (Figure 5(b)). The penetration of positively charged nanoparticles into negatively charged bacterial cytomembranes via electrostatic interactions accelerates cell division, leading to the formation of blebs. Literature reports have shown that electrostatic attraction between negatively charged bacterial cells and positively charged nanoparticles is crucial for the activity of nanoparticles as bactericidal materials (Hamouda & Baker, 2000; Stoimenov, Klinger, Marchin, & Klabunde, 2002). In addition, pytz molecules on nanoparticle probably provide a lipophilic segment compatible with the lipid bilayer of the bacterial cytoplasmic membrane (Beney & Gervais, 2001) and can cause decreased membrane fluidity and disrupt the cytoplasmic membrane (Silvestro, Weiser, & Axelsen, 2000). These two factors act together to damage the bacterial cytomembrane, resulting in leakage of intracellular content. It is also possible that the nanoparticles interact with the intracellular enzymes and proteins to inhibit their activity. Therefore, positively charged nanoparticles increase the permeability of the cytoplasmic bacterial membrane due to the presence of external pytz. Subsequently, these nanoparticles penetrate the cells and strongly associate with intracellular enzymes and proteins, resulting in cell death.

4. Conclusions
Synthesis of 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine (pytz) capped PdNPs was achieved by a simple method. Synthesized TzPdNPs have been characterized by powder X-ray diffraction and transmission electron microscope techniques. A systematic evaluation of the antibacterial activity of the 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine (pytz) capped Pd(0) nanoparticles (TzPdNPs) was carried out and showed excellent bactericidal properties against Gram positive \textit{B. subtilis} bacteria. TzPdNPs showed effective antimicrobial behavior, which caused 100% mortality against \textit{B. subtilis} bacterial strains within 24 h. This material reported here can be used for different applications in biomaterials.
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