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Synergistic Effect of Metal Nanoparticles on the Antimicrobial Activities of Antibiotics against Biorecycling Microbes

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Biorecycling microbes, which have critical functionalities in natural cycles, are essential to sustain ecosystem of the earth. Any alterations in these cycles caused by the mutations of microbes could be a potential threat to life on earth. Antibiotics leached from pharmaceutical waste, animal food and agribusiness products are accumulating in the environment. Metal nanoparticles are also accumulating in environment because of their extensive use as biocidal agent in domestic products. Interaction of antibiotics and metal nanoparticles with eco-friendly microorganisms has a potential to alter the ecosystem of the earth. In this article, we have studied the antibacterial activities of silver and copper nanoparticles and their formulations with antibiotics, tetracycline, and kanamycin against biorecycling microbes, *Bacillus subtilis* and *Pseudomonas fluorescens*. Strong synergistic effect of metal nanoparticles on the antimicrobial activities of commercial antibiotics has been observed. Antimicrobial activity of tetracycline improves by 286%–346% and 0%–28% when being tested in the presence of 250 ppm of silver and copper nanoparticles, respectively. For kanamycin, the improvement is 154%–289% for silver and 3%–20% for copper nanoparticles. Irrespective of the antibiotics and tested organisms, synergy is more prominent for silver nanoparticles even at their minimum active concentration (100 ppm). This study demonstrates that the combination of metal nanoparticles with antibiotics could be more fatal to ecosystem than either the metal nanoparticles or the antibiotics alone.

1. Introduction

Metal nanostructures have superior biocidal activities against all forms of microorganisms, so they are getting increasingly popular as anti-infective agents in products like bandages, antiseptic skin care and personal hygiene products[1]. Leaching of nanoparticles from these products will lead to their accumulation in land fields and water bodies. Use of conventional antibiotics in pest control and dairy products is also on surge, which will eventually accumulate in ecosystem[2–4]. Considering the critical role of microbial communities in organic matter and nutrient recycling in ecosystems, environmental exposure of nano and conventional antibiotics can alter ecosystem productivity[5]. Whether environmental accumulation of these antibiotics poses a threat to microbes essential for recycling in natural or engineered systems is an outstanding question of great relevance to ecosystem health and sustainable nanotechnology[6,7].

Antimicrobial activities of metal nanoparticles are size dependent. Biocidal activities of metal nanostructures increase with the decrease in their hydrodynamic size[8]. Most of the studies found in literature on the biocidal activities of metal nanostructures are restricted to strains of pathogenic microorganisms and rarely extend to non-pathogenic eco-friendly microorganisms[9,10]. Hence, the impacts of environmental accumulation of nanoparticles, especially on microbial communities residing in land fields and water bodies, which are critical to environmental recycling, are unknown. Further, there are isolated reports on synergistic effect of metal nanoparticles on the biocidal activities of conventional antibiotics[11,12]. How far reaching the consequences of such synergies is not clear. It is absolutely essential to understand any synergistic effects of metal nanostructures on the biocidal activities of conventional antibiotics against microbes that are essential for environmental recycling. In the absence of this information, it will be hard to formulate regulatory protocols for the environmental pollution that is going to be originating from the simultaneous exposure of ecosystem to conventional and nanoantibiotics. Hence, detailed investigation on synergistic effect of metal nanoparticles on the antimicrobial activities of antibiotics against biorecycling microbes is vital.

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Tetracycline and kanamycin are broad spectrum first-line antibiotics used to control spread of infectious microorganisms. Because of increasing use of these antibiotics in husbandry and dairy farming, substantial amount of these antibiotics are already accumulated in environment. In this work, we aimed to investigate synergistic effects of silver and copper nanoparticles on antibacterial activities of these antibiotics. Antibacterial activities of silver nanoparticles (SNPs), copper nanoparticles (CNPs) and antibiotics tetracycline and kanamycin adsorbed silver and copper nanoparticles have been evaluated by micro-dilution and disk diffusion tests on biorecycling microbes, *Bacillus subtilis* (*B. subtilis*) and *Pseudomonas fluorescens* (*P. fluorescens*). These microbes play a critical role in elemental recycling, bioremediation of pollutants and plant growth[13,14]. Hence, it is absolutely essential to understand harmful effects of antibiotic pollutants on these microbes.

For this study, high quality aqueous colloidal dispersion of monodisperse silver and copper nanoparticles was prepared by chemical reduction technique. Antibiotics tetracycline and kanamycin were adsorbed on the surface of these nanoparticles and their antimicrobial activities have been tested on *B. subtilis* and *P. fluorescens*. Results of antibacterial tests are analyzed to understand the synergy between conventional antibiotics (tetracycline and kanamycin) and nanoantibiotics (silver and copper nanoparticles).

2. Experimental

2.1. Materials

Silver nitrate ($AgNO_3$) (99.8%) and diphenyl ether were procured from S.D. Fine-Chem. Ltd. Oleylamine (OA) (70%), pluronic F-127, copper (II) chloride dihydrate (≥99%), polyvinylpyrrolidone (PVP) (average mol. wt. 10,000), sodium borohydride ($NaBH_4$) (≥98%), tetracycline (hydrochloride salt) and kanamycin (sulphate salt) were procured from Sigma-Aldrich. Absolute ethanol, n-hexane (95%) and L-ascorbic acid (AA) were purchased from Merck. Mueller Hinton Agar (MM019) and nutrient broth (NM019) were purchased from Sisco Research Laboratories. Antibacterial activities have been investigated on *B. subtilis* (MTCC No. 441) and *P. fluorescens* (MTCC No. 1749), which were obtained from IMTECH, Chandigarh. Solutions were prepared in ultrapure Milli-Q water ($\rho = 18.2$ MΩ). All the chemicals were used as received without any further purification.

2.2. Synthesis of SNPs and CNPs

Synthesis of uniform, monodisperse SNPs was carried out by chemical reduction technique by using oleylamine as capping and reducing agent[15]. It is a two-step process. In the first step, oleylamine capped hydrophobic SNPs were prepared by reducing $AgNO_3$ with OA[15]. In the second step, hydrophobic SNPs were phase transferred into water by ligand exchange reaction using block copolymer, pluronic F-127[8]. In brief, 20 mL diphenyl ether was mixed with 15 mmol/L OA. Temperature was raised to 200 °C and 3 mmol/L $AgNO_3$ was added to it. Upon addition, OA immediately reduced $AgNO_3$ to Ag^0. The nucleation time was optimized to be 30 min, following which the reaction temperature was lowered to 150 °C to have better control on the growth of individual crystallites. The nuclei were allowed to ripen at 150 °C for 4 h. The colloid was subsequently cooled to room temperature and purified by precipitation–redispersion[15]. Water dispersible SNPs were obtained by facile phase transfer protocols[8]. Aqueous solution of 20 mL, 0.2 mol/L pluronic F-127 was mixed with equal volume of stock solution of SNPs in n-hexane. It was covered with a perforated aluminum foil. The mixture was magnetically stirred till the n-hexane evaporated completely. Phase transferred aqueous dispersion of SNPs was preserved at 4 °C. The ligand exchange reaction was presented as a schematic in Fig. 1 along with photographs of colloidal SNPs before and after the phase transfer.

CNPs were synthesized by chemical reduction of copper chloride under mild reaction conditions. To achieve fast and homogenous nucleation of CNPs, aqueous solution of copper (II) chloride was added into the preheated mixture of reducing ($NaBH_4$ + L-ascorbic acid) and capping (PVP) agents. In brief, 10 mmol/L each AA, PVP and $NaBH_4$ were mixed and heated to 80 °C. Aqueous solution of copper chloride (1.16 mmol/L) was added dropwise to this preheated solution of reducing and capping agents. Heating was continued till the color of the mixture turned bright red. The mixture was then cooled to 25 °C. CNPs were collected by centrifugation @ 15471 × g. Well dispersed CNPs were obtained as supernatant and larger cluster of CNPs, if any, were discarded as sediment.

Chemical reactions that lead to the formation of CNPs are presented in Scheme 1. It is a multistep process. Precise control is essential at every step to obtain monodisperse, uniform, single phase CNPs. The first step in the reduction process of Cu^{2+} is the formation of PVP-Cu^{2+} complex[16–18]. This complex formation between PVP and Cu^{2+} is proposed on the basis of the structural features of PVP. It has polyvinyl skeleton with nitrogen and oxygen polar groups[16–18]. These polar groups can form coordinative bonds between PVP and Cu^{2+} ions by donating their lone pair electrons to Cu^{2+}. In the second step, $NaBH_4$ ionizes and forms a $(BH4)^-$ ligand, which then reacted with hydroxyl anions. During this reaction (step-II), eight electrons are liberated from the hydroxyl ions. These electrons react with Cu^{2+} in the PVP–Cu^{2+} complex and reduce it to Cu^0. The execution of reduction reaction (Step-III) is evidenced from the change in the solution color from light yellow to dark red. In the oxidizing environment, Cu^0 is not stable and oxidizes back to Cu^{2+}. In the fourth step, AA oxidizes into dehydroascorbic acid with a release of two electrons[19]. These electrons react with the oxidized product of step-III and reduce Cu^{2+} back into Cu^0. The oxidation by-product of AA, i.e. "dehydroascorbic acid" simulates a dynamic equilibrium (Step-V) around the Cu^0, which stabilizes CNPs against oxidation[19].

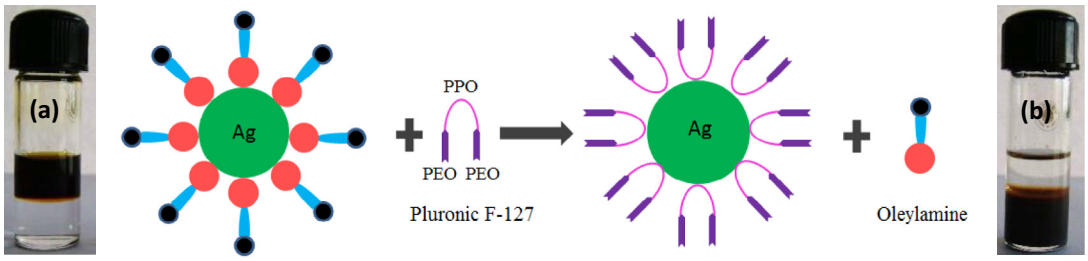

Fig. 1. Schematic representation of legend exchange reaction used for the hydrophobic to hydrophilic conversion of SNPs by using block-copolymer, pluronic F-127. Photograph in bottle (a) represents dispersion of SNPs in n-hexane before phase transfer and bottle (b) represents SNPs dispersion in water after the phase transfer.