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BRIEF COMMUNICATION

Augmented antibacterial activity of ampicillin with silver nanoparticles against methicillin-resistant *Staphylococcus aureus* (MRSA)

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Abstract

At present, including failed attempts, it takes about 15 years and costs totaling up to $2.6 billion to take a promising new compound from laboratory to the market. Increasing drug resistance among microbial pathogens has led to a growing interest in exploring novel methods to enhance the efficacy of existing drugs. Combination therapies involving two or more known antimicrobial methods, particularly those involving nanoparticles for combating the clinical problems associated with antibiotic resistance, have been garnering interest. In the current study, we determined whether a combination therapy involving silver nanoparticles, which are known for their antimicrobial activity, and the widely used antibiotic ampicillin can be effective against methicillin-resistant *Staphylococcus aureus* (MRSA). In the presence of sub-lethal dose of silver nanoparticles, ampicillin was found to be effective against MRSA. Indeed, the results show that silver nanoparticles and ampicillin act synergistically, with the effect being more pronounced when a lower concentration of ampicillin is present. When present at a higher concentration, ampicillin coats the silver nanoparticle, preventing the direct interaction of nanoparticles and bacteria. This study discusses the possible applications of combination antimicrobial therapies involving silver nanoparticles for therapeutic treatments.

Introduction

The emergence of drug resistance in fungi, parasites and bacteria is a global concern, and significant efforts are being dedicated toward developing new or improved antimicrobial compounds. For the past three decades pharmaceutical industries have focused on creating new antibiotics that possess the ability to effectively inhibit DNA replication, protein synthesis and bacterial cell wall synthesis [1]. Despite these advancements, a high fatality rate persists due to the rise of antimicrobial resistance in bacterial infections. Bacterial resistance toward the traditional antibiotics is an important health care issue with serious impact on communities globally [2]. Ampicillin, an antibiotic of choice, is used to prevent and treat many bacterial infections, such as respiratory tract infections, urinary tract infections, meningitis, salmonellosis, and endocarditis [3]. After having been widely effective for many decades, the drug is losing its effectiveness in treating infections owing to bacterial resistance [4].

Staphylococccus aureus is a major human pathogen around the world and is responsible for various infections. Resistant strains of *S. aureus*, including methicillin-resistant *S. aureus* (MRSA), have been identified by use of antibiotics. The MRSA strain gains resistance due to the presence of the *mecA* gene that is integrated in the staphylococcal cassette chromosome mec. This *mecA* gene encodes a 78 kD penicillin-binding protein that lessens the inhibitory effect of β-lactam antibiotics on bacterial cell

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wall synthesis [5]. Antibiotic resistant bacteria, like MRSA, could also spread and result in broader infection across communities outside the hospital [6, 7]. Although community-acquired MRSA strains are less resistant to antimicrobial agents compared to those associated with nosocomial strains, they are more likely to produce toxins such as Panton–Valentine leucocidin [8]. This continuous spread of resistant bacteria within the community poses additional problems for infection control, not only in health care facilities but also among sports teams, military recruits, child day care centers, and schools placing an increasing burden on health care cost [9].

Utilizing combination therapy to battle bacterial infection is one the proposed approaches to treat infections with antibiotic resistant bacterial strains [10]. In combination therapy two existing antibiotics that either have an additive or synergistic effect against a target organism are employed [10]. Nanoscale materials have emerged as novel antimicrobial agents [11]. While a variety of nanomaterials with promising antimicrobial activities have been reported in the literature, silver nanoparticles have shown to be the most effective in vitro in recent literature [12, 13].

Numerous studies have shown the effectiveness of silver nanoparticles against methicillin sensitive *Staphylococcus aureus* (e.g., [14, 15]). To our knowledge, however, there is limited research on the combination of silver nanoparticles and antibiotics as a therapeutic method in treating antibiotic resistant bacterial infections. Previously, Ruden et al. showed the synergistic effect between antimicrobial peptides and silver nanoparticles in targeting Gram-negative bacteria [16]. In 2013, Kora and Rastogi demonstrated that polyvinylpyrrolidone (PVP) capped silver nanoparticles have produced the highest antimicrobial activity in combination with known antibiotics as compared to citrate or SDS capped silver nanoparticles [2]. More recently, Thirumurugan et al. reported the potential benefits of combining silver nanoparticles and topical antibiotics for treating bacterial infections [17]. Das et al. and Rahim and Mohammed suggested that ampicillin in combination with silver nanoparticles may have the potential to increase antibiotic efficacy [18, 19].

The objective of the current research was to determine if the antibiotic concentration is a variable impacting potential synergistic effects that exist between silver nanoparticles and ampicillin. The relevance of the concentration/dosage and the time to the antibacterial activity were determined by estimating and comparing the minimal inhibitory concentration (MIC) of ampicillin and silver nanoparticles in combination and alone against MRSA.

The study was performed with spherical 10 nm silver particles, purchased from nano-Composix, San Diego, USA. The nanoparticles were capped with PVP to reduce agglomeration and release of silver ions in the medium. The size of nanoparticles and detailed properties were independently characterized in a previous study [16]. In brief, the average diameter of the Ag nanoparticles was found to be 10.9 ± 1.2 nm, and the zeta potential of -25.2 mV. TEM analysis indicated absence of any agglomeration of nanoparticles [18]. MRSA strain (ATCC 33591) was used for the antimicrobial assay. MRSA was grown overnight in tryptic soy broth (TSB) at 37 °C, 200 rpm. After incubation, the culture was centrifuged at 10,000 rpm for 1 min. The supernatant was discarded, and the cells were washed twice with sterile distilled water to remove any traces of protein left over from the media. The cells were then resuspended in sterile distilled water and the optical density adjusted to 0.1 at 600 nm. It was important to carry out the study without any protein from the culture media since proteins and other organic molecules present in the media could interact with the nanoparticles and form a dynamic nanoparticle–protein corona [20, 21]. In a 96-well microtiter plate, 50 µl of cell suspension was mixed with varying concentrations of antibiotics and nanoparticle suspensions. The final volume was adjusted to 200 µl, and the plate was incubated at room temperature without shaking for 1 h. Aliquots of 10 µl were taken out at 0, 30, and 60 min and serially diluted. 10 µl of the diluted sample were plated on tryptic soy agar. The plates were incubated overnight at 37 °C and colony forming units (CFU) recorded [22, 23]. A similar protocol was followed for antimicrobial assay with silver nitrate. At each time point, three samples were taken, and each sample was plated and duplicated. Data analysis was performed based on the average values, and the standard deviation for the results did not exceed 10% for any result. The concentration that inhibited more than 90% growth of the organism was record as the MIC_{90}. Positive control (cells plus water) and negative control (water only) were included in each experiment. Percent inhibition was calculated by comparing the experimental readings with the positive control. A plot of log CFU/ml vs. time was generated in Microsoft Excel and the slope of the curve (death rate, k_d) obtained. It is worth noting that the values are negative as a death rate, and lower values indicate higher death rates.

The hydrodynamic diameter (HDD) of the PVP-coated silver nanoparticles was evaluated by using a Zeta-Sizer Nano Series instrument (Malvern, Worcestershire, UK). The reported HDD were measured by using the average intensity of three independent experiments, with each one consisting of a minimum of 12 measurements. The HDD of the silver nanoparticles alone was determined by dilution of the stock solution (1:100) in distilled deionized water and monitored for aggregation over a 6 h period. Additional measurements of the silver nanoparticle HDD were conducted in the presence of ampicillin where 6.25 µg/ml of silver nanoparticles were mixed with 12.5 µg/ml of ampicillin. Subsequent measurements of the HDD were recorded over 12 h.

The study tested the hypothesis that the synergistic activity of silver nanoparticles and ampicillin could be more effective against MRSA at higher concentrations of antibiotic. The first step toward testing the proposed hypothesis was to evaluate the sensitivity of MRSA strain ATCC 33591 to silver nanoparticles. Silver nanoparticles showed high levels of bactericidal activity against the MRSA strain (Fig. 1). After 30 min of incubation, 100% cell death was observed at concentrations of 12.5 µg/ml and above. At 6.25 µg/ml, 95% cell death was identified (Fig. 1). Even at concentrations as low as 0.78 µg/ml, silver nanoparticles showed efficacy of over 55% cell death. MIC_{90} was calculated to be 12.5 µg/ml. The results observed in this study are close to those reported in the literature with drug sensitive strains of *Staphylococcus aureus* [19, 24, 25]. Differences in the actual MIC values could be attributed to differences in the properties of nanoparticles and the strains of bacteria used. Bacterial killing was seen in much lesser concentrations of silver nanoparticles than reported by Chien et al. [26]. When one compares the results reported here to antimicrobial activity of selenium nanoparticles or chitosan/silver nanoparticles against MRSA, silver nanoparticles coated in PVP are shown to be active at lower concentrations [27, 28].

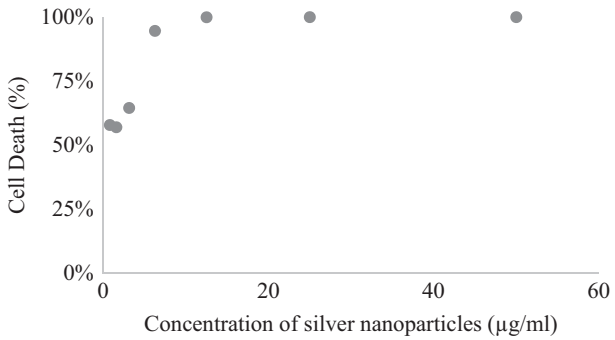

Fig. 1 Percentage MRSA cell death in presence of varying concentrations of silver nanoparticles after 30 min of incubation

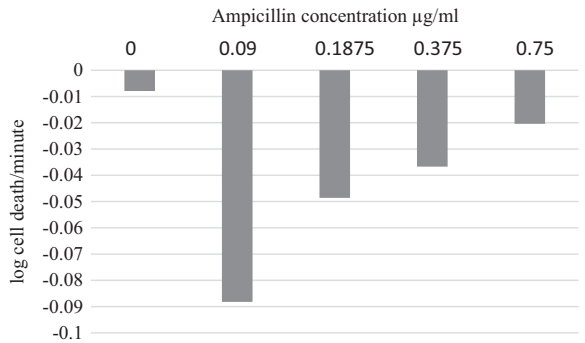

Fig. 2 Influence of varying concentration of ampicillin on death rate (k_d) of MRSA in presence of 6.25 µg/ml silver nanoparticles

Comparison of death rate (k_d) obtained from experiments performed under identical experimental conditions allows one to assess if the combination of silver nanoparticles and ampicillin is synergistic. As shown in Fig. 2, the k_d for MRSA when incubated with 6.25 µg/ml nanoparticles in the absence of ampicillin was −0.0079. At the lowest ampicillin concentration of 0.09 µg/ml, over an 11-fold increase in k_d was observed in comparison to that observed in death curve with silver nanoparticles alone. k_d decreases sharply with increasing ampicillin concentration; a value of −0.0204 was observed for death curve in the presence of 0.75 µg/ml ampicillin and 6.25 µg/ml silver nanoparticles (Fig. 2). It needs to be noted that the k_d for MRSA in the presence of 12 µg/ml ampicillin and no nanoparticle was −0.0016. No decrease in cell viability was observed (k_d, 0.010) when cells were incubated in water without ampicillin and nanoparticles. Regression models developed from individual death curves for calculating k_d had R^2 values higher than 0.9. This result shows that the variation in data is minimal and that the slopes are obtained from linear death curves.

Regardless of the concentration of ampicillin, the k_d observed for MRSA while exposed to a combination of ampicillin and silver nanoparticles was higher than when exposed to individual antimicrobial agents. This observation supports the hypothesis that ampicillin and silver nanoparticles indeed act synergistically in targeting MRSA. Results also show that the synergistic effect is more pronounced at lower ampicillin concentrations. To test if high concentrations of ampicillin induced the aggregation of silver nanoparticles, the HDD of silver nanoparticles was measured as a function of time after the addition of ampicillin. The HDD of the silver nanoparticles used in this study was 110 nm. The large HDD comprises the light scattered by the nanoparticles and the surface coating, PVP. The suspension was allowed to equilibrate over a 6 h period with nominal changes in the measured HDD, indicating little to no aggregation of particles and thus making a stable suspension. The addition of ampicillin to the suspension followed by gentle mixing did not result in increasing the measured HDD. The suspension was monitored for 12 h, and significant aggregation of the Ag NPs in the presence of ampicillin was observed.

To rule out the possibility that the observed result was due to the interaction between silver ions and ampicillin, antimicrobial assays were performed with $AgNO_3$. In the absence of ampicillin in the suspension, k_d value of −0.010 was observed with 1 µg/ml $AgNO_3$. When 6 µg/ml and 12 µg/ml ampicillin were added to the suspension, the k_d values increased to −0.015 and −0.016, respectively. The observed effect of the death rate increasing with the rising concentration of ampicillin is opposite to the observed effect with silver nanoparticles, indicating a differential

mechanism of action between silver ions and silver nanoparticles when combined with ampicillin against MRSA.

The results indicate that our hypothesis was false and synergistic activity of silver nanoparticles and ampicillin is more effective at lower concentration of ampicillin. While further investigation is required to better understand how ampicillin and silver nanoparticles interact, we hypothesize that at higher concentrations, ampicillin completely coats the silver nanoparticle to form a corona. This corona provides a protective layer against direct interaction with the MRSA cell surface, hence decreasing antimicrobial activity. Das et al. observed a similar concentration effect against the human gut ecosystem [18]. Nevertheless, our results clearly indicate that the combination of silver nanoparticles and a low concentration of ampicillin has potential in treating infections with drug resistant bacteria. These results will need to be validated in future studies conducted in the presence of protein in biological systems. The ampicillin concentration used in this study is significantly lower than those used clinically, and combination therapy could potentially lower the use of ampicillin in treating bacterial infections in the future.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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