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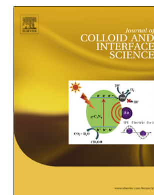

Enhanced stability and activity of an antimicrobial peptide in conjugation with silver nanoparticle

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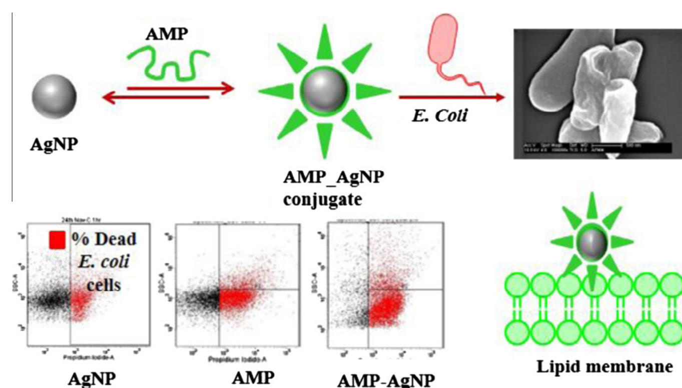
GRAPHICAL ABSTRACT



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ABSTRACT

The conjugation of nanoparticles with antimicrobial peptides (AMP) is emerging as a promising route to achieve superior antimicrobial activity. However, the nature of peptide-nanoparticle interactions in these systems remains unclear. This study describes a system consisting of a cysteine containing antimicrobial peptide conjugated with silver nanoparticles, in which the two components exhibit a dynamic interaction resulting in a significantly enhanced stability and biological activity compared to that of the individual components. This was investigated using NMR spectroscopy in conjunction with other biophysical techniques. Using fluorescence assisted cell sorting and membrane mimics we carried out a quantitative comparison of the activity of the AMP-nanoparticle system and the free peptide. Taken together, the study provides new insights into nanoparticle-AMP interactions at a molecular level and brings out the factors that will be useful for consideration while designing new conjugates with enhanced functionality.

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1. Introduction

Antimicrobial peptides (AMPs) are naturally abundant and diverse group of molecules [1], which are increasingly being considered as useful alternatives to conventional antibiotics

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owing to the emergence of drug-resistant bacterial strains [2]. On the one hand, design of AMPs with enhanced activity such as small AMPs containing highly concentrated positive charge and hydrophobic residues have opened up new avenues because of their superior microbial cell membrane rupturing capability [3,4]. On the other hand, it is being increasingly recognized that nanoparticles, especially silver nanoparticles (AgNPs), have good antimicrobial properties [5,6]. Thus, conjugation of AMPs with nanoparticles have been proposed to enhance the activity [7,8]. However, two important parameters have not been considered hitherto, namely, the nature of interaction between AgNP and the peptides, and the conjugation of cysteine containing AMPs. The nature of interaction is closely associated with stability of the system, which is an important parameter for practical utility of the conjugates. Unfortunately, AgNPs are generally unstable in solution [9] and hence a AgNP-AMP system having long-term stability with enhanced activity is preferable. Nuclear magnetic resonance (NMR) spectroscopy is a unique tool for probing such molecular interactions [9,10]. The other aspect concerns cysteine containing peptides, which are highly abundant in several classes of AMPs and are usually present in di-sulfide bridged forms playing an important role in structure and function of AMPs [11]. Cysteines are also known to interact directly with AgNPs [12]. Thus, conjugating a cysteine containing peptide with AgNP is expected to have a favorable effect on the strength and nature of nanoparticle-peptide interactions. In this study a cationic peptide Odorranain-A-OA1 (abbreviated as OA1) was chosen from a recently reported set of AMPs extracted from the skin of Chinese odorous frogs [13] and was conjugated with AgNPs having a diameter of 10 nm. The peptide (OA1) had a minimum inhibitory concentration (MIC) of 35 μM with two positively charged residues K3, R7 and two cysteine residues (C4 and C15) [13]. The resulting silver nanoparticle-antimicrobial peptide (AgNP-OA1) conjugate was investigated using one-dimensional (1D) and two-dimensional (2D) heteronuclear NMR spectroscopy, along with transmission electron microscopy (TEM), UV–Vis spectroscopy and zeta potential measurements. Scanning electron microscopy (SEM) helped in observing the changes in the bacterial cell morphology [14], while fluorescence activated cell sorting (FACS) was used for quantifying the percentage of dead cells [15]. Furthermore, the membrane disrupting activity of the peptide and its conjugate (AMP and AgNP-OA1) was elucidated using the dye leakage assay, consisting of 6-carboxy-fluorescein dye entrapped within the bacterial membrane mimicking vesicles [16]. Taken together, the studies helped in elucidating factors, which will be useful for enhancing the activity of antimicrobial peptides attached to silver nanoparticles, opening new avenues for designing antibiotics with high efficiency.

2. Materials and methods

2.1. Preparation of AgNP

Citrate capped AgNPs having a diameter of ~10 nm were synthesized from silver nitrate using sodium borohydride as a primary reducing agent and trisodium citrate acting as both a reducing and capping agent as described previously [9]. The required amount (2 mM) of sodium citrate and sodium borohydride (2 mM) were mixed thoroughly for 30 min at 60 °C. Silver nitrate (1.17 mM) was added to this solution with vigorous stirring and temperature was increased to 90 °C. Yellow colored colloidal nanoparticles were obtained and excess citrate was removed and solution concentrated using a 10 kDa centricon membrane [17].

2.2. Preparation of OA1

Chemically synthesized Odorranain-A-OA1 (OA1) (VVKCSYRLGSPDSQCN) with >95% purity was purchased from Genscript (Genscript USA Inc., NJ).

2.3. Preparation of AgNP-OA1 conjugate

The conjugate was prepared by mixing 400 μL of 22.5 μg/mL OA1 dissolved in H_2O (pH 7) with 50 μL of 50 μg/mL AgNP at 298 K and the solution was made up to 500 μL in H_2O. The conjugate formed spontaneously as verified by NMR and UV–Visible spectroscopy. These concentrations were chosen based on the sensitivity observed in the NMR spectrum. No specific incubation time was required. The excess of peptide was removed by passing the solution through a 10 kDa membrane. For biological studies, a buffer exchange was carried out with phosphate buffer saline pH 7.4.

2.4. UV–Visible spectroscopy

The AgNP-OA1 conjugate was monitored by UV–Visible spectroscopy on a Shimadzu UV-1800 UV–Vis spectrophotometer with slit width of 1 nm using a quartz cuvette having a path length of 1 cm. Spectra were acquired at different time intervals to monitor the stability of the conjugate and the wavelength range was scanned from 200 to 800 nm.

2.5. Zeta potential measurements

The zeta potential of AgNP and AgNP-OA1 conjugates were measured on a Nanozetasizer (Brookhaven Zeta PALS). Measurements were carried out using samples containing final concentration of 22.5 μg/mL of the peptides and 5 μg/mL of AgNP with a scattering angle of 90° and acquisition time of 3 min. The "Zetasizer" software was used for processing and analyzing the data.

2.6. Transmission electron microscopy (TEM)

The TEM images were obtained with a Technai T-20 machine operating at a voltage of 200 kV. AgNPs were incubated at room temperature in deionized water (18 MΩ cm) at neutral pH and were drop casted on 300 Carbon coated copper Grids prior to all measurements.

2.7. Scanning electron microscopy (SEM)

The morphological changes occurring during the bacterial cell death upon treatment of AgNP, OA1 and AgNP-OA1 conjugate were monitored using scanning electron microscopy. Bacterial cells (Gram-negative BL21 (DE3) *Escherichia coli* cells) starting from single colonies were grown in different flasks each with 500 μL of Luria-Broth (LB) medium until the cells reached an optical density of 0.3 as measured at 600 nm and treated separately with AgNPs (which had a concentration of 5 μg/mL after adding to the medium), OA1 alone (22.5 μg/mL) and the AgNP-OA1 conjugate (having a final concentration of 5 μg/mL of AgNP and 22.5 μg/mL of OA1 in the medium after addition). One flask served as a control, which was grown without addition of AgNP or the OA1/conjugates. After addition of OA1, AgNP or AgNP-OA1 conjugates, the cells were grown further for 1 h and centrifuged at 10,000 rpm for 2 min. The pellets were washed with phosphate buffer saline three times and pre-fixed with 2.5% glutaraldehyde. The pre-fixed cells were washed with PBS and about 2–4 μL of samples were taken on a silicon wafer and kept for vacuum drying. The cells were then gold-coated using an ion sputter. The samples were observed on a

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