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Gold nanoparticles induce a reactive oxygen species-independent apoptotic pathway in *Escherichia coli*

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ABSTRACT

Gold nanoparticles (AuNPs) are a promising material for use in biological and biotechnological applications. While applications such as drug delivery, sensory probe, and organic photovoltaics have been widely evaluated, studies of the antimicrobial activity of AuNPs in therapeutic agents are lacking. In this study, the antibacterial activity and mode of action of AuNPs on *Escherichia coli* was focused. The membrane-impermeable dye SYTOX green was not taken up and membrane potential was depolarized by AuNPs. This demonstrated that AuNPs inhibit cell growth without directly causing membrane damage. Depolarization of membrane potential results in calcium uptake and processes such as bacterial apoptotic-like cell death. We confirmed that AuNPs induced DNA fragmentation resulting in apoptosis-like cell death in a TUNEL assay. FITC-VAD-FMK showed that caspase-like protein(s) such as RecA were activated, induced, and overexpressed. Additionally, elevated levels of intracellular reactive oxygen species (ROS) and decreased reduced glutathione were observed. In AuNP-treated cells, ROS elevation was not confirmed; however, glutathione was decreased. Based on these observations, AuNPs induce apoptotic-like death by severely damaging DNA and this was independent of ROS in *E. coli*.

1. Introduction

Infectious diseases induced by pathogenic bacteria are among the greatest health problems worldwide, as well as important causes of morbidity and mortality [1]. The continuously decreasing number of approved antibiotics in the past decade has exacerbated the situation, resulting in an urgent need for the discovery of novel antibacterial and treatment strategies for confronting increase frequency of resistance to conventional antibiotics [2,3]. Major target interaction by drug is inhibition of DNA replication and repair, inhibition of protein synthesis, inhibition of cell-wall turnover and cellular membrane damage [4–6]. Alternative strategies have been actively pursuing, including searching for new antimicrobials from natural products, modification of existing antibiotic classes, development of antimicrobial peptides, and drug delivery applications [2,3]. Bacterial apoptosis like cell death is proposed approach to be a possible antimicrobial mechanism to create a second generation of the existing antibiotics [7]. This antibacterial mechanism caused by serious DNA damage and it has specific characteristics of eukaryotic apoptosis such as DNA fragmentation and membrane depolarization [8,9].

Metals have been used as antimicrobial agents for a long time, but their modes of action remain unclear [10]. Currently, antimicrobial metal compounds including metallic surfaces and coatings, chelates, and nanomaterials have a multitude of applications in industry, agriculture, and healthcare [10]. Some metallurgic nanomaterials have been approved as bactericidal and bacteriostatic agents; silver, gold, and zinc each have different properties and spectrum activities [11]. These metals disrupt antibiotic-resistant biofilms, exert synergistic bactericidal activities with other biocides, inhibit metabolic pathways in a selective manner, and kill multidrug-resistant bacteria [10]. Nanoparticles (NPs) are increasingly utilized for biological and medical applications [12]. Simple or composite metal nanoparticles (also referred to as ultrafine particles) are composed of clusters of atoms and have a size range of 1–100 nm [10]. Their high surface to volume ratio allows incorporation of abundant functional ligands, enabling multivalency on NP surface to enhance interactions to target bacteria [2]. Gold nanoparticles (AuNPs) are important for biomedical applications in drug delivery, cellular and tissue imaging, and cancer therapy [12,13]. They have been widely employed in bionanotechnology because of their unique properties and multiple surface functionalities. The ease of AuNP functionalization provides a versatile platform

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for nanobiological assemblies with oligonucleotides, antibodies, and proteins [13]. AuNPs are potent biocides that can eliminate bacterial pathogen in a short time [14]. However, while AuNPs have important roles in biological applications, studies of their antibacterial activity are lacking. In this study, we determined the antibacterial effect of AuNP and suggested the mode of action, which involved targeting the bacterial membrane or apoptosis like death.

2. Material and methods

2.1. Bacterial condition and compound preparation

AuNPs (diameter = 30 nm) and norfloxacin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Melittin was purchased from Anygen (Gwangju, Korea). *E. coli* MG1655 cells (ATCC 700926) was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). *E. coli*, which was cultured at 37 °C in Luria-Bertani (LB) broth (Difco, Detroit, MI, USA).

2.2. Antimicrobial activity assay

The *E. coli* cell suspensions were adjusted to obtain standardized populations (1×10^6 cells/mL) by measuring the optical density at 600 nm. Minimum inhibitory concentrations (MICs) were determined using serial two-fold dilutions, based on the Clinical and Laboratory Standards Institute method [15]. *E. coli* cell suspensions were adjusted to obtain standardized populations (1×10^6 cells/mL) and dispensed into microtiter plates. After overnight incubation at 37 °C, the MIC was defined with a microtiter ELISA Reader (BioTek) by monitoring the optical density at 600 nm.

2.3. Analysis of SYTOX green influx

E. coli cells in the log phase (1×10^6 cells/mL LB) were resuspended in phosphate-buffered saline (PBS) (2.7 mM KCl, 137 mM NaCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), treated with 16 μg/ml AuNPs and 4 μg/ml melittin values, and incubated for 4 h at 37 °C. Melittin used a positive control due to having potent membrane damage. Cells were harvested by centrifugation and suspended in PBS. Subsequently, the cells were treated with 2 μg/ml SYTOX green nucleic stain and analyzed with a FACSverse flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) [16].

2.4. Detection of changes in membrane potential

Membrane depolarization was detected using bis-(1,3-dibutylbarbituric acid) trimethineoxonol [$DiBAC_4(3)$]. The cells were washed with PBS and incubated with 16 μg/ml AuNPs and 0.75 μg/ml norfloxacin for 4 h at 28 °C. To compare the antibacterial activity of AuNPs, norfloxacin was used. Norfloxacin was used as a positive control to compare with bacterial apoptosis like death. The cells were harvested by centrifugation and resuspended in 1 mL of PBS. The cells were then treated with 5 μg of $DiBAC_4(3)$ (Molecular Probes). The fluorescent intensity was measured using a FACSverse flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) [16].

2.5. Measurement of cytosolic Ca^{2+} levels

To measure changes in intracellular Ca^{2+} levels, the fluorescent dye Fura-2AM (Molecular Probes) was used. *E. coli* cells (1×10^6 cells/mL) were treated with 16 μg/ml AuNPs and 0.75 μg/ml norfloxacin for 4 h at 37 °C as described above [17]. After incubation, the cells were washed twice with Krebs buffer (pH 7.4) composed of 132 mM NaCl, 4 mM KCl, 1.4 mM $MgCl_2$, 6 mM glucose, 10 mM HEPES, 10 mM $NaHCO_3$, and 1 mM $CaCl_2$. The cells were loaded with Fura-2AM (5 μM) supplemented with 0.01% pluronic acid F-127 (Molecular Probes) and 1% bovine serum albumin in Krebs buffer for 40 min at 37 °C. After loading, the cells were washed twice with calcium-free Krebs buffer to completely hydrolyze the acetoxymethyl ester. Fluorescence intensity was assessed by spectrofluorophotometer (Shimadzu RF-5301PC, Shimadzu, Kyoto, Japan) at the wavelengths 340 nm (excitation) and 510 nm (emission).

2.6. Assessment of DNA fragmentation and condensation

Nucleic acid fragmentation and condensation were examined by 4′-6-diamidino-2-phenylindole (DAPI) staining [18]. After the incubated cells with 16 μg/ml AuNPs and 0.75 μg/ml norfloxacin as described above were washed in PBS, they were stained with 1 μg/ml DAPI for 20 min and examined under spectrofluorophotometer (Shimadzu RF-5301PC, Kyoto, Japan). DNA fragmentation was quantified by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Briefly, compound-treated cells and untreated cells were incubated and suspended in permeabilization solution consisting of 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. The cell suspensions were fixed in 2% paraformaldehyde for 1 h. Cells were then washed and DNA ends were labelled using an *in situ* cell death detection kit for 1 h at 37 °C [19]. The stained cells were analyzed fluorescence intensity of FITC-conjugated dUTP in individual cells with a FACSverse flow cytometer.

2.7. Assessment of morphological changes

To investigate morphological change, the alternation in side scatter (SS) and forward scatter (FS) were analyzed by flow cytometry. The cells were incubated with 16 μg/ml AuNPs and 0.75 μg/ml norfloxacin for 4 h at 37 °C. After incubation, the cells were harvested by centrifugation and resuspended in PBS. The unstained cells were measured in each sample by determining their on the FS and SS contour plots with a FACSVerse flow cytometer [20].

2.8. Caspase-like protein activation

Activation of caspase (cysteine-dependent aspartate-specific protease)-like protein was detected with the CaspACE FITC-VAD-FMK In Situ Marker (Promega, Madison, WI, USA). FITC-VAD-FMK is cell permeable and irreversibly binds to activated caspases [21]. VAD-FMK, a FITC-conjugated peptide pan-caspase inhibitor, is transported into cells and binds to the active site of the caspase to investigate bacterial caspase-like protein expression [19]. The cells were incubated with 16 μg/ml AuNPs and 0.75 μg/ml norfloxacin for 4 h at 37 °C. Next, the cells were washed twice and incubated with CaspACE FITC-VAD-FMK for 20 min. After the cells centrifuged, the cells were resuspended in PBS and fluorescence was measured using a FACSverse flow cytometer [19].

2.9. Western blot analysis

Isolation of RecA and western blot analysis were performed as follows: *E. coli* cells (1×10^6 cells/mL) were cultured in LB medium at 37 °C, collected by centrifugation, and washed twice with PBS. The cells were treated with 16 μg/ml AuNPs and 0.75 μg/ml norfloxacin and incubated for 4 h at 37 °C. The cells were lysed by sonication and centrifuged at 12,000 rpm for 20 min to remove intact cells and any cell debris. The supernatant was collected, and proteins were precipitated with trichloroacetic acid. These proteins were used to detect RecA by western blotting. The protein content of the precipitate was determined using the Bradford