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Disinfection action of electrostatic versus steric-stabilized silver nanoparticles on *E. coli* under different water chemistries

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ABSTRACT

The capping layer stabilizing silver nanoparticles (AgNPs) affects its aggregation, dissolution, and net disinfection action, especially under conditions of varying water composition, such as, pH, ionic strength and organic matter content. Herein, we correlate the silver ion (Ag^+) release and reactive oxygen species (ROS) generation rates for AgNPs of varying functionalization to their net disinfection coefficient on *Escherichia coli*, under conditions of differing water chemistries. For electrostatically stabilized citrate-capped AgNPs, the rate of ROS generation, as measured using a fluorescent dye, is found to dominate over that of Ag^+ release, especially for smaller sized AgNP suspensions (~10 nm) at low pH (~6.2). For these AgNPs, the ROS disinfection mechanism is confirmed to dominate net disinfection action, as measured by the live/dead assay, especially at low levels of organic matter. Steric stabilization of AgNPs by protein or starch-capped layers enables disinfection through reducing AgNP aggregation and promoting silver dissolution over ROS generation. We suggest the involvement of protons and dissolved oxygen in causing the independent formation of Ag^+ and ROS, regardless of the AgNP capping layer. While protein-capping layers effectively stabilize AgNPs, the generated ROS is likely dissipated by interference with the bulky capping layer, whereas the interference is lower with citrate-capping layers. Steric stabilization of AgNPs enables disinfection within a wide range of water chemistries, whereas effective disinfection can occur under electrostatic stabilization, only at low NaCl (<1 mmol/L) and organic matter (<5 mg/L) levels.

1. Introduction

The risks from infections caused by ingestion of water containing pathogenic microbial species can be significantly reduced through "point-of-use" treatments [1]. Based on recent studies that have demonstrated an enhanced level of antimicrobial activity for silver nanoparticles (AgNPs) [2], there has been increased interest in applying AgNPs towards water disinfection [3–6]. In order to optimize AgNP formulations for disinfection, as well as to reduce the potential negative impacts on the environment [7–10], it is necessary to explore how variations in surface functionalization of AgNPs within water samples of various compositions (i.e., pH, ionic strength, and natural organic matter content) could affect the fundamental disinfection mechanisms [11].

There is some debate in literature on the disinfection mechanism of AgNPs. Some have proposed disinfection based solely on the release of silver (I) ions (henceforth, Ag^+) [12,13], while others suggest that the antimicrobial activity of AgNPs cannot be fully explained solely by Ag^+ release [14,15]. This is especially the case for the dependence of disinfection action on dissolved oxygen [16,17], suggesting the involvement of reactive oxygen species (ROS), such as, hydroxyl radicals ($^\bullet OH$), superoxide ($(O_2^\bullet)^-$) and singlet oxygen in cell death due to impairment of bacterial membranes [18]. Another recent study reports on the pH-dependent oxidation of AgNPs by H_2O_2 to Ag^+, to subsequently reform AgNP in situ [19]. Hence, given the highly linked mechanisms of Ag^+ release and ROS generation, there is a need to monitor the rates of both mechanisms, for correlation to net disinfection. While several studies have measured Ag^+ release rates for varying AgNP conditions [20,21], and water chemistry conditions [22,23], similar direct measurements of ROS generation rates have proved to be substantially more difficult, due to the short lifetime of ROS. Most prior studies have focused on characterizing intracellular ROS generation [24–28]. Some recent studies have measured exogenous ROS over the time-scale of hours to days, by monitoring the production of hydrogen peroxide (H_2O_2) [29,30], a downstream product, while others have used electron

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spin resonance spectroscopy [31]. Studies on the continuous monitoring of ROS formation and dissipation on AgNPs are limited [32], and have not been correlated to their disinfection action on model micro-organisms.

The capping layer applied to stabilize AgNPs can influence the nanoparticle dissolution, aggregation [33,34], and disinfection action [35–39], especially under varying water chemistries [40]. Hence, continuous monitoring of Ag^+ and ROS released from AgNP suspensions, measured in parallel to their disinfection activity on the target microorganism can enable identification of the dominant disinfection mechanism (Ag^+ release versus ROS generation) for each surface functionalization and water chemistry. This approach could eventually be applied to optimize AgNP formulations for specific water conditions within the proposed disinfection applications. In this current study, the rates of Ag^+ release and net ROS generation were monitored for AgNP suspensions with three distinct surface functionalizations: "citrate-capped" (nominal sizes of 10 nm, 50 nm and 100 nm), dextrin "starch-capped" (nominal size of 10 nm), and "protein-capped" (nominal size of 15 nm) using micelles of casein, which is an abundant milk protein. Citrate is a common capping layer for stabilizing AgNPs through electrostatic means. Non-polar "starch capping layers" and amphiphilic casein-micellar "protein capping layers" are larger than citrate-capping layers. They provide colloidal stability to AgNPs through steric means, similar to non-polar polymeric capping layers [41]. Using confocal fluorescence microscopy to track ROS-induced conversion of di-hydro-rhodamine 6G (DHR-6G) to rhodamine 6G (R6G) and a silver ion specific probe to monitor Ag^+ release, the ROS generation versus Ag^+ release rate for each AgNP surface functionalization and size was measured as a function of water conditions for subsequent comparison to their disinfection action on *Escherichia coli*.

2. Materials and methods

Certain trade names and company products are mentioned in order to adequately specify the experimental procedures and equipment used. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology (NIST), nor does it imply that the products are necessarily the best available for the purpose.

2.1. Nanoparticle synthesis and characterization

"Citrate-capped" NanoXact AgNPs were obtained from NanoComposix (San Diego, CA), which provided spherical and monodisperse AgNP solutions with mean diameters of 10 nm, 50 nm, and 100 nm. The 20 mg/L nanoparticle solutions were stored for up to one month in the dark, at 4 °C, before use. Casein micellar "protein-capped" AgNPs were obtained from Laboratorios Argenol, Spain (CAS # 9008-42-8) as a powder, which was dissolved in deionized (DI) organic-free water to achieve a total silver concentration of 1 mg/L. Dextrin "starch-capped" AgNPs were synthesized as per prior work [41]. Un-reacted constituents were removed and the AgNP solution was concentrated to 1 mg/L by pressurized stirred cell ultra-filtration with 10 kDa molecular weight cut-off (MWCO) Millipore membranes (Billerica, MA). AgNP suspensions in DI water were characterized by transmission electron spectroscopy (TEM), dynamic light scattering (DLS), and UV–vis spectroscopy, as detailed within Fig. S1, Supplementary Information. The measured diameter of the particles (mean ± one standard deviation, for $N > 100$ particles) by TEM (JEOL 2000FX TEM) are: (NanoXact 10 nm) 10.2 nm ± 1.7 nm, (NanoXact 50 nm) 53.1 nm ± 4.1 nm, (NanoXact 100 nm) 102 nm ± 9.4 nm, (Argenol Proteinate 15 nm) 14 nm ± 6.44 nm, and (Starch 10 nm) 21 nm ± 11.39 nm. A PhotoCor Complex dynamic light scattering (DLS) system (College Park, MD) with a helium–neon (HeNe) laser (623.8 nm) was used to measure the average hydrodynamic size of the AgNP suspensions at a concentration of 20 mg/L of total silver, to determine changes in particle size due to aggregation and surface hydration. UV–vis spectrophotometry (Cary® 100, Agilent, Palo Alto, CA) was used to determine the absorbance of the AgNP suspensions (Fig. S1). The total silver concentration of the AgNP suspension was measured after filtration, using atomic absorption (AA) spectrometry. A 1% (v/v) nitric acid (HNO_3) solution was used to digest all the silver particles into the solution, before the AA measurement. To study the pH dependence of ROS flux, Ag^+ flux, and the disinfection coefficient for each AgNP, the start pH of the suspension was adjusted in the range of pH 5.5 to pH 7.5, by using concentrated nitric acid or sodium hydroxide (Thermo-Scientific ROSS sure-flow KCl pH probe). In each case, an excess level of disinfectant (total silver > 1 mg/L) was used, as confirmed by invariance of the measurements to increased levels of total silver, at a constant pH.

2.2. Measurement of Ag^+ release

The set-up to measure Ag^+ release from the AgNP suspensions is shown in Fig. 1a. The AgNP suspension was stirred at 200 rpm in a polycarbonate flask within a water bath at 20 °C, as recommended by the EPA for Ag^+ monitoring with reduced Ag^+ [42]. O_2 and N_2 gases were passed through a bubble column with glass beads and water, to control dissolved oxygen levels and humidity. All experiments were carried out under dark conditions to minimize the effect of light on the AgNP suspensions. An Orion combination silver/sulfide electrode (Model: 249031-A01, Cole-Parmer, Vernon Hills, IL) was used to measure the concentration of Ag^+. The voltage was acquired from the probe using a 16-bit National Instruments Labview (Austin, TX) system at a rate of 1 Hz. The silver ion probe was calibrated before each experiment using stock solutions of silver nitrate at known concentrations (i.e., known mass of silver added to known volume of water). Fig. 1b illustrates the change in silver ion concentration as a function of time for a representative experiment.

2.3. Measurement of ROS generation

ROS generation from the AgNP suspensions was measured as per the set-up in Fig. 1c. The degradation of a ROS-sensitive probe, di-hydro-rhodamine 6G, DHR6G (Invitrogen), upon contact with the ROS continuously generated by the AgNP suspension was measured over time. DHR6G is a well-established standard fluorescence dye (xanthene family) that has been utilized extensively to study ROS in cellular systems [43,44]. One unit of DHR6G reacts with two units of ROS to form the fluorescent rhodamine 6G (R6G) species. Samples of AgNP and DHR6G were pipetted into a micro-well for imaging by confocal microscopy, composed of a Leica SP5 X imaging system coupled to DMI6000 epifluorescence microscope with a HCX PL APO CS 20x 0.7NA air lens. Fig. S2 (Supporting Information) shows: (a) the schematic methodology used for detecting R6G; and (b) settings for the imaging (R6G was excited at 514 nm with an Argon-ion laser at 70% full power and the emissions were detected with a photo-multiplier tube between 533 nm and 738 nm). Fig. 1d illustrates the change in ROS concentration as a function of time for a representative experiment. The necessary precautions were taken to prevent dimerization of R6G, and ensure that R6G monomers exhibit an absorbance peak of 527 nm (at 22 °C, equilibrium constant = 5.9×10^{-4} mol/L) at a quantum efficiency of 95% (under 488 nm excitation). Oxygen quenching of the xanthene dye is minimal [45]; hence, varying levels of ROS only