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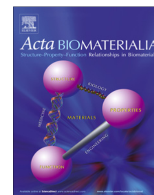

Designing nanogel carriers for antibacterial applications

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ABSTRACT

We have developed a novel and simple synthesis route to create nanosized (∼5 nm) silver nanoparticles (Ag NPs) embedded in a biocompatible nanogel (NG) comprising degradable, natural polymers, namely dextran and lysozyme. In this study, we prepared hybrid nanogels with varying lysozyme content, evaluated their potential to reduce Ag NPs in situ (using ultraviolet–visible spectroscopy, cryo-transmission electronic microscopy, thermogravimetric analysis and Fourier transform infrared spectroscopy) and determined their antibacterial properties against *Escherichia coli* and *Staphylococcus aureus*. Lysozyme was found to enhance nucleation and stabilization of Ag NPs while limiting their growth. As lysozyme concentration increased, larger nanogels with greater loading of smaller Ag NPs were obtained. The antibacterial properties of hybrid NGs were found to depend upon nanogel type and bacterial conditions. Hybrid nanogels with the largest Ag NPs showed the lowest minimum inhibition concentration. However, the greatest bacterial killing efficiency (up to 100%) occurred within 1 h if the bacteria were exposed to hybrid nanogels with smaller Ag NPs while agitating the medium. These results suggest that nanogel properties as well as antibacterial activity can be tuned by varying the lysozyme content. By targeting drug delivery (e.g. ligand grafted surface), these nanogels can be used to prevent biofilm formation and control infection without the complications (i.e. overexposure) associated with classical antibiotic delivery platforms.

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1. Introduction

Silver nanoparticles (Ag NPs) exhibit unique chemical, physical and biological properties, which include high surface-to-volume ratio, broad optical properties and ease of synthesis as well as facile surface chemistry and functionalization. Biologically, Ag NPs show antimicrobial and anti-inflammatory properties [1–3], which are the subject of intense research aimed at using Ag NPs in disease diagnosis, treatment of infection and imaging, among many other applications [4,5]. For instance, antibacterial, biocompatible coatings were developed by embedding Ag NPs (∼5 nm) in dextran [6,7] and Ag NPs (∼20 nm) stabilized with egg white were used to enhance efficacy in radiotherapy for 231 tumor cells [8]. Moreover, surface enhanced Raman spectroscopy was used to detect strains of the respiratory syncytial virus using substrates composed of silver nanorods [9]. More recently, folate silver–dendrimer composite nanodevices were targeted to human epidermoid cancer cell lines, which were subsequently destroyed by microbubbles generated through uptake of laser light energy by the Ag NPs [10].

Concerns regarding the cytotoxicity of Ag NPs have motivated the development of hybrid nanogels (NGs) to limit exposure of cells to Ag. Recently, we developed a novel and simple synthesis route for creating nanosized silver particles (∼5 nm) inside a relatively inert and biocompatible NG (∼160 nm) [7,11]. This NG consists of a lysozyme core and a dextran shell. In particular, the shell presents multiple hydroxyls that can be used for targeting specific biological applications, ensure stability of the NG over broad ranges of pH, ionic strength and temperatures, and impart a "stealth-like" property that prevents them from recognition by the mononuclear phagocyte system [12]. By encapsulating Ag NPs in a NG, we aim to increase their efficacy while limiting their cell uptake and possible cytotoxicity. In comparison to other hybrid NGs reported in the literature [13,14], our hybrid NGs are relatively green because they are prepared in aqueous solution without additional chemicals, other than the silver precursor agent, and they consist of the degradable and natural polymers, dextran and lysozyme.

In previous work, when a fixed concentration of dextran and lysozyme was used to prepare the NG (1:1, molar stoichiometry), we demonstrated that the loading of Ag in the NG can be tuned from 5 to 9 wt.% by varying silver precursor concentration from 2 to 25 mM [11]. In this work, we first examine the role of dextran and lysozyme in the in situ synthesis of Ag NPs and then test if

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the resulting hybrid NGs function as an antimicrobial agent. In contrast to previous studies, we vary the amount of dextran to lysozyme (1:1, 1:4 and 1:8, dextran:lysozyme stoichiometry) using a fixed silver precursor concentration (10 mM).

The in situ synthesis of Ag NPs in three distinct NGs of varying lysozyme concentration is characterized by dynamic light scattering (DLS), ultraviolet–visible spectroscopy (UV–vis), thermogravimetric analysis (TGA) and cryo-transmission electronic microscopy (cryo-TEM). Furthermore, we explore the antibacterial properties of the hybrid NGs against *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)*. The minimal inhibitory concentration (MIC) of the hybrid NG is evaluated using a broth microdilution method. Lastly, the effects of the hybrid NG on bacterial growth are determined as a function of time.

2. Materials and methods

2.1. Synthesis of hybrid NGs

The synthesis of NGs was carried out as previously described, via a Maillard heating reaction followed by heat-gelation at 80 °C [7]. This methodology leads to core–shell type NGs with a lysozyme-rich core and a dextran-rich shell [11]. Briefly, dextran (70 kDa) from *Leuconostoc* ssp. and lysozyme were dissolved (1:1, 1:4 and 1:8 M stoichiometry) in water, the pH was adjusted to 7–8 using 0.1 N sodium hydroxide and the solution was lyophilized. The lyophilized powder was reacted at 60 °C under 79% relative humidity in a desiccator containing saturated KBr solution for 24 h. The reacted powder was dissolved in water (5 mg ml^{-1}), the pH was adjusted to 10.7 using 0.1 N sodium hydroxide and the solution was further reacted at 80 °C for 30 min. The resulting NGs were purified by centrifugation using Amicon ultra 0.5 ml centrifugal filter devices with a 100 kDa molecular weight cutoff (MWCO; Millipore, Billerica, MA) and were stored in the dark at 4 °C. The final concentration of lysozyme in the NG was estimated by measuring the lysozyme concentration in the filtrate by UV–vis. The absence of dextran in the filtrate was confirmed by Molisch assay [15]. Following purification by filtration, the final stoichiometric ratio of dextran to lysozyme is 1:0.8, 1:3.4 and 1:7.6 for NG1:1, NG1:4 and NG1:8, respectively.

The hybrid NGs were prepared by mixing 2 ml of NG solution with 1 ml of 10 mM $AgNO_3$ and were autoclaved for 5 min using a Sterilimatic sterilizer (Market Forge Industries Inc., Everett, MA). The free Ag NPs were separated from the NGs by dialysis in deionized water (49 ml), using a semi-permeable standard regenerated cellulose membrane (MWCO12–14 kDa, Spectrum Laboratories, Rancho Dominguez, CA) for 3 days.

2.2. Characterization techniques

The particle size and size distribution of the hydrated nanogels were measured by DLS using a Malvern Zetasize Nano series instrument (ZS90) equipped with a 22 mW He–Ne laser operating at a wavelength of 633 nm and analyzed with a software package (Zetasizer Nano series software Version 7.01). UV spectra of NG solution and NGs cast on glass slides were recorded in transmission on a Varian spectrophotometer (Cary 5000 UV–vis–NIR) equipped with a software package (Cary WinU Version 4.10). Fourier transform infrared (FTIR) spectra of the drop cast solutions on cleaned silicon wafers were recorded using an attenuated total reflection accessory as a sampling system on a Perkin Elmer infrared spectrophotometer (Spectrum RX I FTIR system) at a resolution of 8 cm^{-1} averaging 256 scans. Data were analyzed using Omnic E.S.P v5.2 software. Nanogel morphology was imaged by cryo-TEM on a JEOL JEM 2010 at 80 kV. TEM micrographs were analyzed using ImageJ (NIH, Bethesda, MD). At least 600 Ag NPs were analyzed per sample. The diameter distribution of Ag NPs was fitted to a log-normal function. The amount of silver (wt.%) in the NGs was determined by TGA using a Universal V4.1D TA Instruments (SDT Q600) with 2–4 mg samples under air atmosphere. The NG solutions were first dried and then dispersed in ethanol. This solution was placed in a platinum pan and heated to 80 °C at 10 °C min^{-1}. The sample was held at 80 °C for 2 h for complete removal of the ethanol and then allowed to cool down to room temperature. Next, the samples were heated to 100 °C at 10 °C min^{-1} and held for 30 min to ensure complete removal of moisture. Then, the samples were heated to 675 °C at 10 °C min^{-1} and held for 120 min to ensure complete removal of organic matter. Data were analyzed using TA Universal Analysis 2000 v4.5A.

2.3. Bacterial culture and antibacterial tests

S. aureus (ATCC®25923™) and *E. coli* (ATCC®25922™) were cultured in trypticase soy broth (TSB) at 175 rpm and 37 °C for 12–16 h (overnight culture) and diluted to 10^8 colony-forming units (CFU) ml^{-1} using a 0.5× McFarland standard, a turbidity standard equivalent to 10^8 CFU ml^{-1}. To determine the MIC as indicated by lack of visible bacterial growth, the standard broth dilution technique in Costar V-bottom 96-well (corning, Life Sciences) was employed. This test assesses the bacteria susceptibility to the hybrid NGs, according to NCCLS M7-A4 (1997). The hybrid NGs were serially diluted (1:1-1:512) with 100 μl of TSB inoculated with bacteria (10^6 CFU ml^{-1}), and incubated overnight at 37 °C. For each nanogel and bacterial strain, each 96-well plate contained negative (broth only) and positive growth (bacteria only) controls. On the same plates, NG1:1-Ag and NG1:4-Ag were tested in triplicate and NG1:8-Ag in duplicate. Results presented are the average of three independent 96-well plates per bacteria type. In separate experiments, bacterial survival (10^5 CFU ml^{-1}) in the presence of the hybrid NGs (100 μg ml^{-1}) was assessed at 1 h, 5 h and 24 h by serial dilution and plating on 3M™ Petri films. Experiments were performed in triplicate.

2.4. Statistical analysis

The means and standard deviations (SDs) of the hydrodynamic diameter and diameter determined by cryo-TEM of the Ag nanoparticles, as well as the MIC and counts of viable bacteria, are presented as average ± SD.

3. Results

The size distribution and hydrodynamic diameter (D_H) of the NGs are given in Fig. 1. At the concentrations in this study, all NGs display Gaussian size distributions. The D_H of the NGs

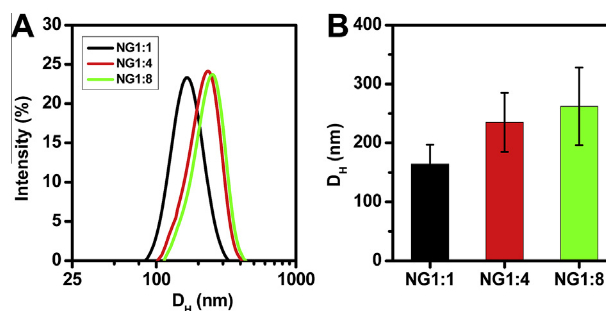

Fig. 1. NG diameters measured by DLS: (A) intensity distribution of NG diameter and (B) hydrodynamic diameter (D_H).

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