

Research Article

A Ferritin Photochemical Synthesis of Monodispersed Silver Nanoparticles That Possess Antimicrobial Properties

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We previously reported that ferritin acts as a photocatalyst to form monodispersed gold nanoparticles. Because silver possesses a favorable reduction potential (+0.80 V), we postulated that the ferritin photochemical method could be used to reduce Ag(I) to Ag(0). Additionally, we postulated that similar to gold, the reduced silver should nucleate and form silver nanoparticles (AgNPs) on the external surface of ferritin. This study reports that Ag(I) can function as an electron acceptor to form monodispersed AgNPs using the ferritin photocatalytic method. The formation and growth of the AgNPs were monitored by following the surface plasmon resonance (SPR) peak at 420 nm by spectrophotometry. The resulting monodispersed AgNPs were analyzed by transmission electron microscopy (TEM), scanning transmission electron microscopy (STEM), and energy-dispersive X-ray spectroscopy (EDX). Gel filtration chromatography demonstrated that the AgNPs comigrated with ferritin through the column suggesting a strong association with ferritin. AgNPs are known to possess antimicrobial properties. The antimicrobial properties of Ferritin-AgNPs were compared to commercially available AgNPs by testing the minimal growth inhibition of *Staphylococcus aureus*. Commercially available AgNPs inhibited bacterial growth at 5 ppm AgNPs, and Ferritin-AgNPs inhibited growth at 20 ppm Ferritin-AgNPs.

1. Introduction

Silver nanoparticles have unique properties that differ from the bulk material and also differ from the individual atoms or ions [1–8]. The interesting properties of nanoparticles include quantum confinement effects and catalytic properties related to strained atoms or ions on the surface of the nanoparticle. Nanoparticles have been used for a variety of purposes and include catalysts, biosensing, nanoelectronics, drug deliver, and antimicrobial treatments [9].

A variety of characteristics govern the properties of the nanoparticles and depend on the size, shape, and stability of the nanoparticles [10–13]. Capping agents are often used to control the size, shape, and stability of the nanoparticles, but capping agents also influence the properties of the resulting nanoparticles [14–16]. Since many nanoparticles are synthesized for used in biological applications, the use of nontoxic biocompatible capping agents has become an

important priority in the synthesis of nanoparticles. Additionally, reducing agents used to prepare nanoparticles are expensive and can be toxic in the environment [7]. Therefore, green synthetic methods for preparing and stabilizing nanoparticles are an important priority. To identify capping and reducing agents that are biocompatible, nanoparticles have been synthesized in the presence of organic matter or fruit extracts to identify biocompatible capping agents [17–20].

Silver nanoparticles (AgNPs) possess many of the interesting nanoparticle properties discussed above, but the susceptibility of AgNPs to oxidative corrosion and aggregation has limited their use in many applications [21]. To overcome these problems, several groups have attempted to synthesize AgNPs inside hollow protein nanocages such as ferritin [21–25]. The theory is that the protein nanocage will replace the capping agents and protect the AgNPs from oxidation but will still allow the nanoparticles to retain their unique properties.

Ferritin is a large spherical protein measuring 12 nm in diameter with an 8 nm hollow interior occupied by a ferrihydrite core (FeOOH) that possesses semiconductor properties [26–30]. This protein, with or without its iron cargo, has been used as a scaffold for forming metal nanoparticles on both the inside and outside by a variety of techniques [31]. Early research focused on depositing metal oxides on the interior of ferritin as a size-constrained reactor to form nanoparticles with a tight size distribution by replacing iron with other metals [32]. Later studies produced elemental forms of the metals inside ferritin by using powerful reducing agents [22, 33]. Ferritin has also been used as a scaffold for the formation of metal nanoparticles on the external surface of ferritin [34–36].

Several groups have synthesized AgNPs inside ferritin [21–25]. Early studies used molecular biology to engineer the ferritin interior to possess silver-binding peptides or thiols to enhance silver accumulation inside ferritin [22, 23]. The bacterial ferritin from *Pyrococcus furiosus* was shown to possess unique silver-binding domains that bound and allowed the encapsulation AgNPs [25]. Other groups demonstrated the synthesis of size-controlled 1 nm or 4 nm diameter AgNPs inside ferritin [21] or demonstrated that the AgNPs deposited inside ferritin were catalytically active and increasing the rate of iron oxidation [24].

Recently, the photochemical methods for nanoparticle synthesis have been described and included using ferritin as a charge separation catalyst to oxidize organic acids and channel the liberated electrons to reduce metal ions in the solution [28–30, 35–37]. The photochemical reaction is represented in Scheme 1 and outlines one proposed reaction mechanism for forming AgNPs.

The ferritin photocatalyst functions because light can excite electrons from the valence band into the conduction band of the ferrihydrite semiconductor allowing ferritin to act as a charge separation catalyst [28]. The excited electrons are donated to target ions that bind to nucleation sites on the ferritin exterior surface, and the excited electrons are transferred through the redox active protein shell of ferritin [38], or by Fe(II) reduction and release from ferritin [39], to reduce these metal ions to the elemental form. To replenish the electron holes created by the charge separation event, ferritin is able to oxidize sacrificial electron donors present in the solution. Typically, citrate or oxalate is used as the electron donors in these reactions. This reaction allows a photochemical reduction of the metal ions without harsh reducing agents or byproducts and uses ferritin as the capping agent. The resulting AgNP product is associated with the external surface of ferritin so parts of the AgNP are “capped” and stabilized by ferritin. However, the AgNP surface is partially exposed allowing more solvent access than an AgNP completely encapsulated inside ferritin. This synthesis may mimic the work done by Zhai et al. where they deposited AgNPs on the surface of silica spheres to stabilize the AgNPs but preserve the nanoparticle catalytic properties [40]. Ferritin synthesized with other metal nanoparticles encapsulated inside ferritin are also capable of performing the photochemical charge separation reactions [41–45]. We chose to test the surface reactivity of

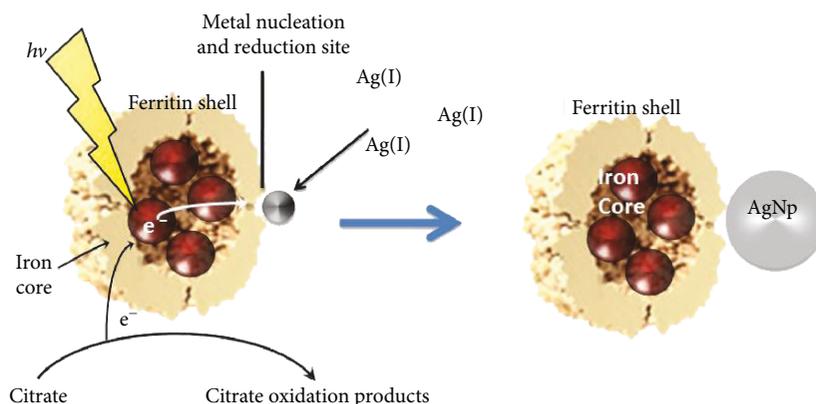
the Ferritin-AgNPs (Ftn-AgNPs) by analyzing the antimicrobial activity of these nanoparticles.

Metal nanoparticles have antimicrobial activity [46, 47]. AgNPs have antimicrobial properties but generally have very low toxicity to humans so they have been used in topical crèmes and to treat burn wounds [7, 48, 49]. Additionally, AgNPs and AuNPs have been deposited on the surfaces of medical devices and in consumer products as disinfectants [8, 49, 50]. The toxicity to bacteria is caused by the binding of Ag⁺ ions or AgNPs to the sulfhydryl groups, blocking respiration or generating reactive oxygen species (ROS). Additionally, the charge and nature of the capping agents has also been shown to contribute to bacterial toxicity. We desired to test the Ftn-AgNPs for their ability to inhibit bacterial growth as an evaluation of the reactivity of the AgNPs bound on the surface of ferritin.

This work describes the successful photoreduction of silver ions to form silver nanoparticles (AgNPs) on the surface of ferritin, using the ferritin photocatalytic reaction system. The synthesis reaction uses ferritin, citrate, and light to photoreduce Ag(I) ions to Ag(0) so the reaction has no hazardous reactants or products. The novelty is the photochemical reaction that drives the AgNP synthesis. The AgNP samples were characterized by UV-visible spectrophotometry, transmission electron microscopy (TEM), scanning transmission electron microscopy (STEM), and energy-dispersive X-ray spectroscopy (EDX). Size exclusion chromatography was used to confirm that the AgNPs are attached to the external surface of the ferritin protein. Finally, we report that Ferritin-AgNPs possess antimicrobial activity against *Staphylococcus aureus*.

2. Materials and Methods

We obtained the native horse spleen ferritin (Ftn) and silver chloride from Sigma-Aldrich. Ferritin protein concentrations were determined by the Lowry method [51]. The iron content of the native ferritin was 1800 Fe/Ftn as determined by the dithionite/bipyridyl method [38]. The silver nanoparticles (AgNPs) were formed in a solution containing Ftn (150 µg/mL) with TRIS buffer at pH 7.4 (20 mM), sodium citrate (30 mM), AgNO₃ (800 µM), and NaCl (50 mM). The temperature of the samples during illumination was maintained at 25°C using a water-jacketed cuvette holder connected to a temperature-controlled water bath in an Agilent 8453 UV-Vis spectrophotometer. 1 mL samples were placed in a quartz cuvette and exposed to a full spectrum Hg-halide floodlight (Integrated Dispensing Solutions Inc.), and the reaction progression was monitored in the 200–1100 nm range in kinetic mode for the length of the reaction in a UV-Vis spectrophotometer (Agilent 8453). AgNPs were formed and monitored at 420 nm during an 800-second irradiation time. The appearance and size of the NPs were evaluated and visualized by transmission electron microscopy (TEM) performed on a Tecnai F30 operating at 300 kV and by scanning electron microscopy (STEM) and energy-dispersive X-ray spectroscopy (EDX) on a Tecnai F20 operating at 200 kV. The preparation of the samples for TEM was performed using the method described by Petrucci et al.



SCHEME 1: Ferritin containing an iron mineral absorbs light and transfers the excited electrons to Ag(I) ions bound to the nucleation sites on the exterior of the ferritin protein shell. This process oxidizes citrate to replenish the holes and continue the electron flow for further reactions. Continued light exposure allows the AgNPs to grow to larger sizes.

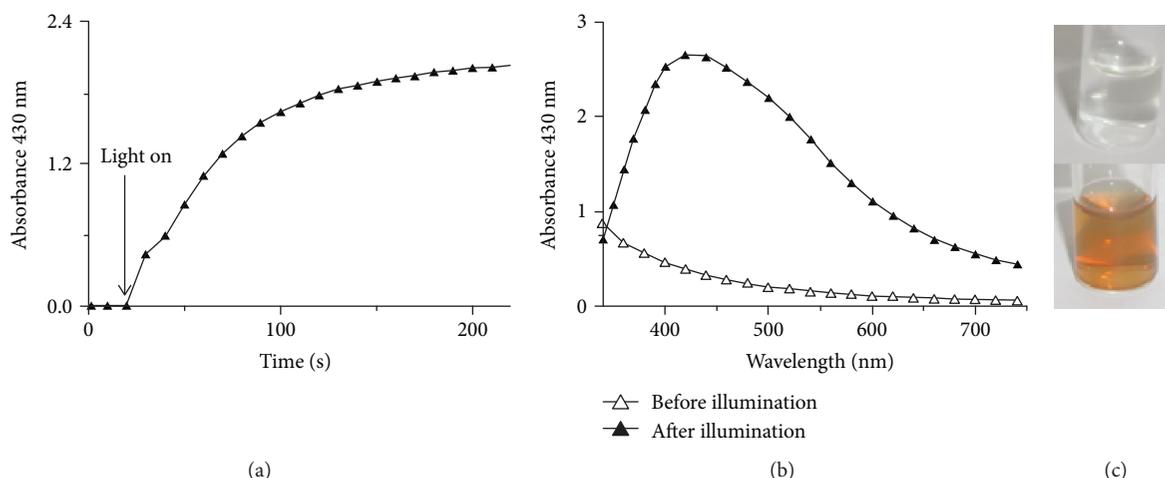


FIGURE 1: The synthesis of AgNPs using the ferritin photochemical system. Kinetic trace (a) of AgNPs formation and spectra (b) of the samples before (Δ) and after (\blacktriangle) illumination. Upon illumination, the absorbance increases at 420 nm due to the formation of AgNPs. This absorbance is due to the surface plasmon resonance properties of AgNPs. (c) The color of the solution changes from almost colorless before (top) to amber after (bottom) illumination.

[36]. Size exclusion chromatography was performed with a Superdex 200 10/300 GL column (GE Healthcare Life Sciences, cat. 17-5175-01) by injecting 0.5 mL of freshly prepared AgNPs or 0.5 mL of 150 $\mu\text{g}/\text{mL}$ Ftn. Control reactions were conducted in the dark or without ferritin.

2.1. Bacterial Growth Minimal Inhibitory Concentration Test. Fifteen 12 \times 75 mm sterile culture tubes were prepared by adding 1 mL of tryptic soy broth (TSB) containing 1×10^6 colony-forming units (CFU) of *Staphylococcus aureus* (ATCC 6538). Then 1 mL of a solution containing Ferritin-AgNPs (at 80, 40, 20, 10, 5, 2.5, or 1.25, 0.625 parts per million (ppm) silver) or commercial AgNPs (American Biotech Labs, at 10, 5, 2.5, or 1.25, 0.625 ppm) was added to each culture. The two remaining tubes, one containing only 2 mL of TSB and one containing 2 mL of TSB plus 1×10^6 CFU of *S. aureus*, were used as experimental controls. The experiment was performed in triplicates.

3. Results

The photocatalytic formation of the AgNPs was monitored by following the change in absorbance at 420 nm which corresponds to the surface plasmon resonance band of AgNPs (Figures 1(a) and 1(b)) [52]. A visible color change from colorless to amber, characteristic of silver nanoparticles in aqueous solution, was observed and provides further evidence supporting the conclusion that AgNPs were successfully synthesized by this method (Figure 1(c)). Control reactions conducted in the dark, as well as in the light but without ferritin, were not able to produce AgNPs.

After illumination the samples were placed on TEM grids and analyzed by TEM and STEM. Two populations of particles were observed, very dense particles and diffuse particles (Figure 2(a)). We hypothesized that the darker particles were AgNPs and that the lighter particles were the iron core of ferritin. The increased atomic density of AgNPs gives a better contrast than the iron cores of ferritin (Figure 2(a)).

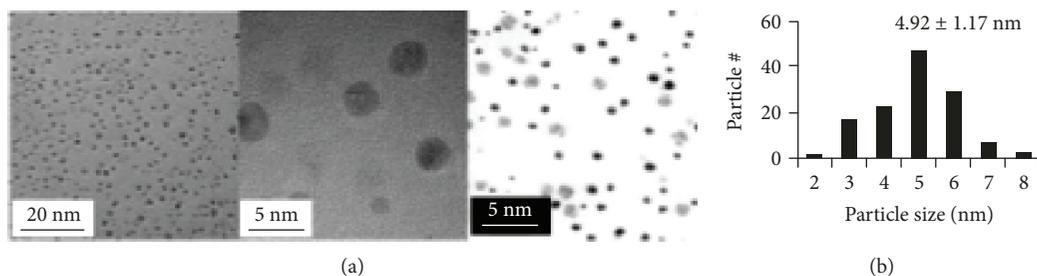


FIGURE 2: TEM and STEM of AgNPs. (a) TEM micrographs at lower magnification (left) and higher magnification (center); STEM image in negative mode (right). These images clearly show the presence of two types of particles: the less dense iron cores of ferritin and the more dense AgNPs (see Figure 3 for EDX analysis). (b) Size distribution of the AgNPs \pm S.D. (126 AgNPs analyzed).

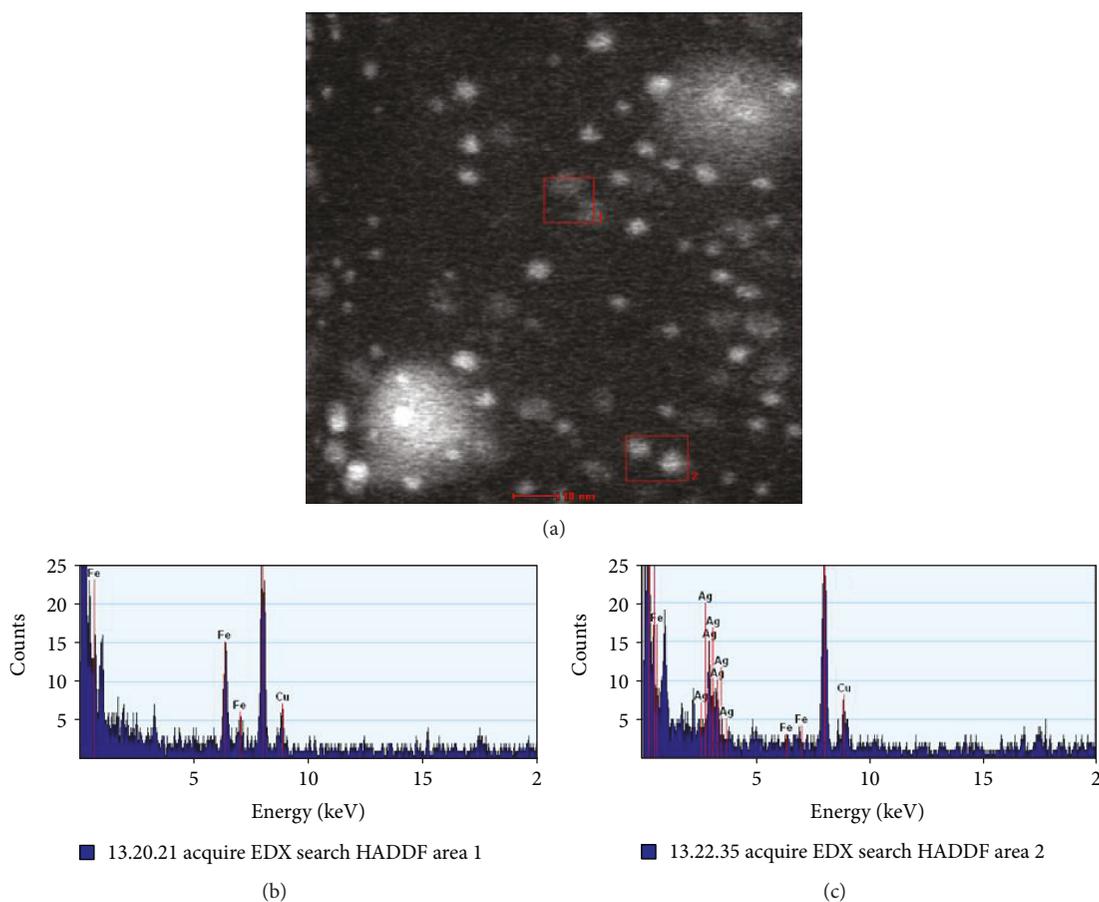


FIGURE 3: STEM and EDX elemental analysis of nanoparticles. STEM image (a) and EDX patterns (b, c). EDX confirms that the less dense particles in box 1 are the iron cores of ferritin (b), and box 2 shows that the more dense particles are AgNPs (c).

The dark and light particles were analyzed by STEM using the elemental analysis capability of EDX (Figure 3). In box 1, the electron beam was focused on two light particles, and in box 2, the electron beam was focused on two dark particles. The EDX analysis confirmed the diffuse particles (box 1) was iron and the dense particles (box 2) was silver (Figure 3). The size of the AgNPs is uniform with diameters of 4.92 ± 1.17 nm (Figure 2(b); calculated from 126 AgNPs).

To determine if the AgNPs were attached to or possessed a strong association with ferritin, the sample was passed over a gel filtration column to determine if the AgNPs comigrated with ferritin or if they could be separated from ferritin. A

ferritin standard was passed over the column, and the elution was detected by monitoring the ferritin protein at 280 nm (Figure 4). Note that ferritin exists as monomers and as dimers and produces two peaks when passed over a gel filtration column [53]. The sample containing AgNPs and ferritin had a similar two-peak 280 nm elution profile for ferritin monomers and dimers, but the sample eluted much earlier than the ferritin control. This indicates an increase in the ferritin cross-sectional diameter consistent with AgNPs bound on the ferritin exterior surface. Additionally, the elution of AgNPs was monitored following the 420 nm absorbance that corresponds AgNPs. The AgNPs

elution peak (~8 min) corresponds to the ferritin dimer peak indicating that AgNPs coelute with ferritin. Remarkably, the AgNPs appear to preferentially associate with ferritin dimers or may cause an increase in ferritin dimerization. This is consistent with the changes in peak height between the ferritin control and the Ferritin-AgNP (Ftn-AuNP) sample. The peaks corresponding to monomers and dimers in the control ferritin sample are found in an almost 1:1 ratio, but the dimer peak in the Ftn-AgNP sample is about double that of the monomer peak.

We propose the AgNPs associate with ferritin by the following interactions. First, the Ag(I) ions bind to the ferritin shell and are reduced to Ag(0) to start the nucleation of the AgNP on the exterior surface of ferritin (see Scheme 1). Continued illumination facilitates additional Ag(I) reduction to Ag(0) to allow AgNP growth. The AgNPs appear to grow to about 5 nm and then stop growing, but the reason of why growth termination occurs has not been determined. We postulate that the citrate caps the majority of the AgNP surface and the association between ferritin and the AgNPs is through ferritin lysine residues with positive charge and the citrate cap of the AgNP with negative charge.

AgNPs are known for their potent antimicrobial properties [7, 48, 54]; for this reason, we performed a minimal inhibitory concentration test using Ftn-AgNPs to inhibit the growth of *Staphylococcus aureus*. This would allow us to determine if AgNPs associated on the exterior surface of ferritin retained antimicrobial activity. For an experimental positive control for AgNP antimicrobial activity, we used a commercially available 10 ppm water solution of 5 nm diameter AgNPs to compare with the same size Ftn-AgNPs produced by the ferritin photochemical method. Figure 5 shows that the Ftn-AgNPs inhibit growth at 20 parts per million AgNPs. In contrast, commercial AgNPs inhibit at 5 parts per million. Although the Ftn-AgNPs are effective at inhibiting the growth of *S. aureus*, they are about 4-fold less effective than commercial AgNPs. It is possible that the interaction with ferritin blocks some of the surface of the AgNPs and prevents some of the available surface area for inhibiting the bacterial growth. The control tubes containing bacteria without antimicrobial agents or no bacteria showed growth or no growth, respectively.

The literature shows that metal NPs have their maximal antimicrobial reactivity in the 1-7 nm range, depending on the material. Our nanoparticles are in the 5 nm range, which explains why their antimicrobial activity is comparable to the commercial AgNP standard. The slight decrease in activity (less than 1 log unit) is seemingly due to the interference/stabilization of the AgNPs by the ferritin.

4. Conclusions

The purpose of this research was threefold: (1) to test whether the ferritin photocatalytic method could produce AgNPs, (2) to test if the AgNPs remained associated on the exterior surface of ferritin, and (3) to determine if ferritin AgNPs possessed antimicrobial properties even if part of the AgNP surface was passivated by ferritin. This work

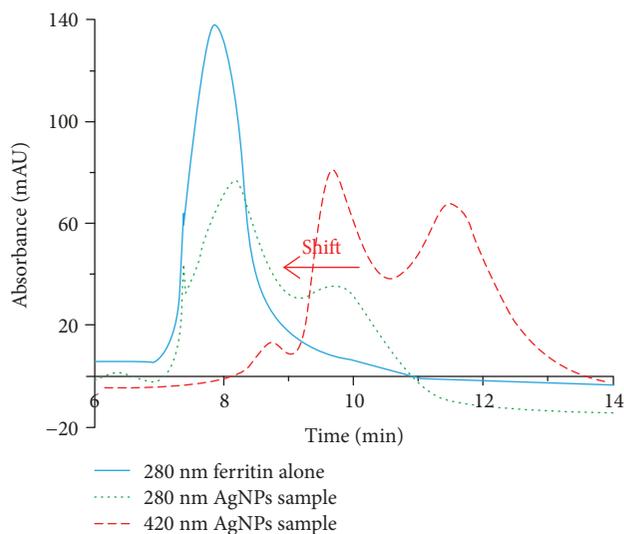


FIGURE 4: Size exclusion chromatography of the ferritin AgNP solution. A standard of ferritin was passed over the column, and the elution profile was monitored at 280 nm (red). Ferritin runs as a dimer and monomer so it has two peaks. A sample of AgNPs synthesized photochemically by ferritin was passed over the column, and the absorbance was monitored at 280 nm (detecting the protein) and 420 nm (detecting the AgNP plasmon resonance). The AgNPs (420 nm peak, blue) and the ferritin (280 nm peak, green) coelute (~8 min) and are significantly shifted to an earlier elution than the ferritin standard (red), indicating that the particles are attached to the external surface of the protein shell. The presence of AgNPs also appears to shift the ferritin into the dimer species. Note: the absorbance dropping below zero is due to the changes of buffer composition compared to the blank.

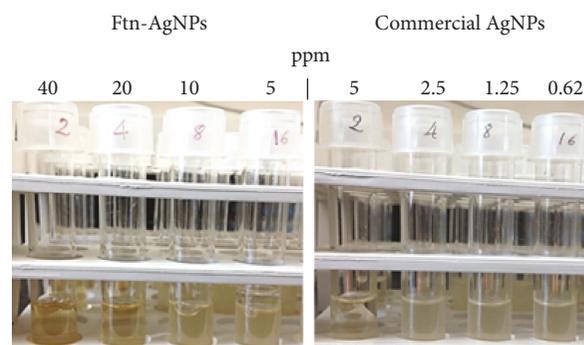


FIGURE 5: AgNP minimal inhibitory concentration tests. Bacterial growth is represented by a turbid solution. The inhibition of bacterial growth is demonstrated by a clear solution. The AgNP concentration added to the growth media is represented above each tube. The Ftn-AgNPs (left image) and control commercial AgNPs (right image) inhibit bacterial growth at 20 ppm and 5 ppm, respectively. The test was executed in triplicates.

demonstrates that we were able to photochemically synthesize ~4 nm diameter monodispersed AgNPs that remained associated on the surface of ferritin. Additionally, the resulting Ftn-AgNP solution possesses antimicrobial activity at a low ppm concentration range.

Finally, we suggest that the biocompatible reagents used to prepare the Ftn-AgNPs, particularly the absence of harsh reagents or surfactants, might allow the Ftn-AgNPs to be used as a mild topical disinfectant. In fact, the combined antimicrobial and iron-sequestering abilities (iron is an essential nutrient for all microorganisms [55, 56]) of the Ftn-AgNPs might represent an effective combined treatment to prevent the growth of human pathogens.

Data Availability

The kinetic, TEM, gel filtration, and bacterial growth inhibition data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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