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Bacterial inactivation using silver-coated magnetic nanoparticles as functional antimicrobial agents

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Abstract

The ability for silver nanoparticles to function as an antibacterial agent while being separable from the target fluids is important for bacterial inactivation in biological fluids. This report describes the analysis of the antimicrobial activities of silver-coated magnetic nanoparticles synthesized by wet chemical methods. The bacterial inactivation of several types of bacteria was analyzed, including Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and Gram-negative bacteria (*Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Escherichia coli*). The results have demonstrated the viability of the silver-coated magnetic nanoparticles for achieving effective bacterial inactivation efficiency comparable to and better than silver nanoparticles conventionally used. The bacteria inactivation efficiency of our MZF@Ag nanoparticles were also determined for blood platelets samples, demonstrating the potential of utilization in inactivating bacterial growth in platelets prior to transfusion to ensure blood product safety, which also has important implications for enabling the capability of effective separation, delivery and targeting of the antibacterial agents.

Bacterial contamination of platelets is the leading cause of morbidity and mortality (particularly among cancer patients whose immune systems are already compromised) from a transfusion-transmitted infection, due to the storage of platelets at room temperature allowing for rapid growth of bacteria. Platelet transfusion safety is a major concern for the general public. In addition to bacterial contamination detection, bacterial inactivation is an option to reduce infection risk. Conventional disinfection techniques such as solvent-detergent treatment or UV irradiation may be effective, but they also induce damage to the blood products and are not applicable to the cellular components of blood platelets. The development of methods that can inactivate bacteria in platelets will have great medical value as it can reduce transfusion risk and increase the shelf life of platelets. Since the wall of bacteria consists of an outer membrane which is usually the first interactive structure with inanimate surfaces, the adhesive properties are primarily responsible for biofilm formation^{1–3}. The mechanistic understanding of the cell interactions has attracted a great deal of research interests, especially in the development of antimicrobial agents that kill or inhibit the growth of microorganisms^{4–7}. Studies have shown that the surface capping or coating plays an important role in controlling the interactions between solid (or nanoparticle) surfaces and bacteria^{8–11}. One recent example involves the use of poly(L-lysine) for surface attachment of bacteria¹⁰ or other biomolecules such as DNAs¹¹.

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There is an increased interest recently in using silver and other metal nanoparticles in recent years for applications in blood platelet decontamination. The binding of silver ions to bacterial DNA may inhibit a number of important transport processes, such as phosphate and succinate uptake, and can interact with cellular oxidation processes as well as the respiratory chain. The presence of large amounts of silver ions and the counterion may cause electrolyte imbalances in patients and due to high amount of absorption, silver toxicity may be of concern. Metallic silver (known to be antimicrobial)¹² provides improvement over silver ions. A few reports have shown that silver nanoparticles have enhanced antibacterial properties even at low concentrations and are size dependent^{13,14}. The higher surface to volume ratio provided by these nanoparticles allow for a more efficient bacterial disinfection. There is a growing interest in the investigation of nanoscale metal particles such as silver nanoparticles in antimicrobial applications^{15–18}. The nanoscale sizes of the metal particles are expected to show better antimicrobial characteristics because of their larger specific surface area¹⁷. Bacterial interaction with the nanoparticles starts from the surface layer of the nanoparticles. The antibacterial activity of silver-based nanoparticles involves releasing silver ions to the biological binding sites upon adhesion onto the surfaces of the target^{18–21}. This initial adhesion is important for achieving an effective targeting.

The ability for silver nanoparticles to function as an antibacterial agent while being separable from the target fluids is important for bacterial inactivation in biological fluids. Magnetic nanoparticles display high saturation magnetization and high magnetic susceptibility, which have generated a great deal of interest for biological separation and targeting. This report explores the use of magnetic particles as a vehicle of silver nanoparticles for effective inactivation of bacteria in platelets in which the particles could be completely removed from the platelet medium prior to transfusion, eliminating the possibility of side effects from residual materials. Although pathogen contamination can occur in both cellular and non-cellular blood product, it is the requirement of having to store platelets at room temperature to keep their functionality that makes bacterial contamination of platelets the high risk of infection associated with transfusion. The antibacterial silver coated magnetic particles can be introduced into the platelets to inactivate the bacteria. A few reports have recently shown that silver nanoparticles can enhance antibacterial properties even at low concentrations and are size-dependent^{4,16,17}, which may provide a pathway for a more efficient bacterial disinfection with minimized presence of silver ions. While viability of synthesis and fabrication of metal-coated magnetic nanoparticles have been demonstrated using different methods^{17,22–34}, the capabilities for engineering the nanoscale size, composition, magnetic and surface properties towards core-shell magnetic nanomaterials for antibacterial applications remain challenging for specific applications. We report herein the results of an investigation of silver-coated magnetic nanoparticles for bacterial inactivation of blood platelets (Scheme 1). The introduction of magnetic cores in the metal nanoparticles enables the capabilities of effective separation, delivery and targeting of the antibacterial agents^{35–38}. In our previous work, we established the synthesis of gold-coated magnetic nanoparticles for catalysis and protein binding applications^{22–27,39}. In this work, we describe the synthesis of silver-coated magnetic nanoparticles for bacterial inactivation, and the quantitative analysis of the bacteria inactivation efficiency.

Experimental Section

Chemicals

The chemicals used in this work include zinc sulfate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 99.7%), manganous sulfate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 99.2%), ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 99.5%), sodium hydroxide, hydrogen peroxide (30%), oleylamine (OAM, 70%), benzyl ether ($\text{C}_{14}\text{H}_{14}\text{O}$, 99%), dicarboxylic acid (DCA, $\text{HOOC}(\text{CH}_2)_n\text{COOH}$, $n = 6\sim 18$), silver nitrate (>99%), sodium citrate (99%), sodium borohydride (NaBH_4 , 99%), 4-mercaptobenzoic acid (MBA), and 11-

mercaptopundecanoic acid (MUA, 97%), which were purchased from Aldrich and used as received. Iron(III) acetylacetonate ($\text{Fe}(\text{acac})_3$, 99%, Lancaster), manganese(II) acetylacetonate ($\text{Mn}(\text{acac})_2$, 95% Stream), zinc acetylacetonate ($\text{Zn}(\text{acac})_2$, 98%, Stream), and oleic acid (OAC, 99%, Alfa Aesar) were also used for the synthesis. The solvents included hexane (99.9%) and toluene (99.8%) from Fisher. Water was purified with a Millipore Milli-Q water system.

Nanoparticle synthesis

Silver nanoparticles were synthesized using a modified method^{15,40}, which involved the reduction of AgNO_3 in the presence of sodium citrate as capping and NaBH_4 as reducing agent. 5 mL of 2 mM sodium citrate and 5 mL of 2 mM of NaBH_4 were mixed with 40 mL deionized water, which was kept sonicating in ice bath. Adding 5 mL of 0.2 mM AgNO_3 into this solution in every 5 minutes until a total volume of 50 mL was added. The silver nanoparticles were capped with a monolayer of citrate molecules.

MnZn Ferrite (MZF) nanoparticles were synthesized in both aqueous and organic solutions^{23,41}. The synthesis in aqueous solutions was based on a modified protocol using H_2O_2 as oxidizing agent and FeSO_4 , ZnSO_4 and MnSO_4 as metal precursors²³. The ratio of Fe:Mn:Zn was kept at 2.1:0.7:0.2. Briefly, H_2O_2 (0.3 mL, 30% wt.) was used as oxidizing agent. It was added into a solution of FeSO_4 (25 mL, 0.24 M) at pH = 9. Ice bath was used to cool down temperature during this reaction. After stirring for 30 minutes, a solution of NaOH (30% wt.) was used to adjust pH = 13. ZnSO_4 (2.857 mL, 0.2 M) and MnSO_4 (10 mL, 0.2 M) was added into the mixture with stirring. The solution was refluxed for 8 hours. The black precipitation was collected, and washed at least three times using deionized water. The synthesis of MZF nanoparticles in organic solution was based on a protocol described recently⁴¹, which involved thermal decomposition of metal acetylacetonate compounds, e.g., 0.469 g $\text{Fe}(\text{acac})_3$, 0.081 g $\text{Mn}(\text{acac})_2$, and 0.087 g $\text{Zn}(\text{acac})_2$ in 20 mL benzyl ether with 2 mL oleic acid and 2 mL oleylamine. The mixture was refluxed for 60 minutes. The product was collected using a magnet. MZF nanoparticles were transferred from the organic solution to aqueous solution by ligand exchange reaction using dicarboxylic acid (DCA, $\text{HOOC}(\text{CH}_2)_n\text{COOH}$, $n = 6\sim 18$), which displaced the hydrophobic capping agents (OAC/OAM). The binding of DCA involves an interaction between the carboxylic acid and iron oxide particle surface. The degree of exchange can be controlled by the chain length and concentration of the DCA.

Silver-coated MZF (MZF@Ag) nanoparticles were synthesized in both aqueous and organic solutions using pre-synthesized MZF nanoparticles as cores for the growth of Ag shells. MZF@Ag nanoparticles were synthesized by reducing Ag acetate in organic solution using a modified method⁴². The precursors included: 10 mL MZF nanoparticle solution, 2.2 mmol Ag acetate, 12 mmol 1,2-dodecanediol, 6 mmol oleylamine, and 1.5 mmol oleic acid, which were mixed in a 30 mL benzyl ether. The reaction temperature was 100 °C and reaction time was 60 minutes. For the aqueous synthesis, the as-synthesized MZF nanoparticles were made water-soluble by ligand exchange reaction, which were then used as seeds for the synthesis of Ag shell in aqueous solution⁴⁰. Briefly, 0.5 mg DCA capped MnZn ferrite seeds were dispersed in 65 mL of pH=12 water followed by adding 5 mL of 2 mM sodium citrate and 5 mL of 2 mM of sodium borohydride. Then, 5 mL of 0.2 mM AgNO_3 was added into the mixture solution in each 5 minutes until a total volume of 50 mL was added. The as-synthesized MZF@Ag nanoparticles were stable in aqueous solutions, with an approximate concentration of 5×10^{10} /mL.

Bacteria and blood platelet samples

Several bacteria were examined in this study, including Gram-positive bacteria (*Staphylococcus aureus* (ATCC: 6538), and *Bacillus cereus* (ATCC: 7064) and Gram-negative bacteria (*Pseudomonas aeruginosa* (ATCC: 19960), *Enterobacter cloacae* (ATCC: 35030), and *Escherichia coli* (ATCC: 29181)). The platelet samples used in this work were provided by the Blood Bank at the MD Anderson Cancer Center, (Houston, TX). These are expiring anonymized platelets.

Bacteria inactivation measurements

Bacteria inactivation measurement was carried out by adding nanoparticles into buffer or platelets samples containing bacteria. The initial concentrations of the bacteria were platelets using the appropriate agar plates. The bacterial counts of the samples over a period of incubation time were monitored by removing a fixed volume of the sample for plating. The residual silver in the samples was analyzed using inductive coupled plasma emission optical emission spectrometry (ICP-OES).

Inactivation of microorganisms growth in saline solution—Typically, a Gram-positive bacteria, *Staphylococcus aureus* and Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC#19960) were streaked for isolation on agar plates and allowed to grow overnight at 37°C. Two colonies of each bacterium were picked from the plate and each was put into 0.9 mL of sterile saline solution (0.9%) yielding an approximate 10^7 CFU/mL concentration. The 10^7 CFU/mL bacterial suspensions were then further diluted into a volume of 2 mL to yield a final concentration of 10^6 CFU/mL. 500 μ L of the 10^6 CFU/mL bacterial suspension was then added to 4.5 mL of each of the following: PBS, Ag nanoparticles (Ag NPs) in PBS, and silver-coated magnetic nanoparticles (MZF@Ag) in PBS. The samples were then incubated in the dark for 5 hours at 37 °C. Each sample was then plated in triplicate for enumeration on their respective media and incubated overnight at 37 °C. In monitoring the growth of the bacterial and the action of antimicrobial nanoparticles over a 24-hour period, *Bacillus cereus* and *Enterobacter cloacae* prepared as 10^6 CFU/mL suspensions. 0.5 mL of the 10^6 CFU/mL bacterial suspensions (*Bacillus cereus* and *Enterobacter cloacae*) were added to 4.5 mL of each of the following: PBS, Ag NPs in PBS (50:50) and MZF@Ag in PBS (50:50). Samples were stored in the dark at 37 °C and a portion of the mixture was removed at different times for enumeration on their respective media.

Inactivation of bacteria in blood platelets with MZF@Ag—Typically, *Escherichia coli* bacteria were incubated at 37 °C to reach log phase growth and then diluted in saline to the target concentration of 10 CFU/mL. Three different samples were assembled in large glass test tubes by first adding 500 μ L of the bacterial suspension (10 CFU/mL) to 2.25 mL of human blood platelets. 2.25 mL of the Ag-coated magnetic nanoparticles (MZF@Ag) solution was added to the first tube. 2.25 mL of water and DCA capped magnetic nanoparticles without silver coating (MZF) were added to the second and third tubes as controls. This brought the three samples in the final volume of 5 mL. The samples were incubated at 37°C with mild agitation for 96 h during which aliquots were removed for bacterial plating. During each aliquot removal, a magnet was applied to pull the MZF, MZF@Ag aside so that they were not removed along with the aliquots. At the end of the 96 hours incubation, the magnet was once again applied to collect the magnetic nanoparticles. 1 mL of solution was pulled out and placed in a 1.5 mL microcentrifuge tube for testing of residual silver in the solution.

Silver analysis—After the completion of the inhibition, the sample was subjected to field of a magnet again for 15 minutes. After the particles had collected on the side of the tube, 1

mL of solution was pulled out and placed in a 1.5 mL microcentrifuge tube for silver iron analysis. The resulting supernatant (1 mL) was quantitatively transferred to 50-mL Falcon tubes (Polypropylene) and digested with concentrated HNO_3 . 2 mL of H_2O_2 was then added and the digestion continued at 100°C for approximately 2.5 hours. Following the digestion, the solution was further diluted with 5 mL of deionized water. The samples were analyzed using ICP-OES method. Proper silver solution standards were used in the analysis.

Instrumentation and Measurements

Ultraviolet - Visible (UV-Vis) spectra were acquired with a HP 8453 spectrophotometer. The spectra were collected over the range of 200 – 1100 nm. Transmission electron microscopy (TEM) was performed at 100 kV from a Hitachi H-7000. The nanoparticle samples dissolved in water were drop cast onto a carbon-coated copper grid sample holder followed by natural evaporation at room temperature. Surface enhanced Raman scattering (SERS) spectra were recorded using Advantage 200A Raman instrument (DeltaNu). The laser power was 5 mW, and the wavelength of the laser was 632.8 nm. The instrument collects data over the range of $200\text{--}3400\text{ cm}^{-1}$. Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) was used to analyze the composition, which was performed using a Liberty RL Sequential ICP-OES.

Results and Discussion

1. Characterization of MZF@Ag Nanoparticles

Morphology of MnZn Ferrite nanoparticles (MZF) and Ag-coated MnZn Ferrite Nanoparticles (MZF@Ag)—MZF nanoparticles were synthesized in both aqueous and nonaqueous solutions. The MZF nanoparticles synthesized in aqueous solution showed an average size is $39.5 \pm 7.1\text{ nm}$. However the particles displayed a propensity of aggregation, making the subsequent coating with silver relatively difficult. MZF nanoparticles without the propensity of aggregation were synthesized in organic solution by controlling the temperature ($210\text{--}275^\circ\text{C}$). In general, low temperature ($< 250^\circ\text{C}$) produced small spherical particles ($5\text{--}8\text{ nm}$), whereas higher temperature ($> 250^\circ\text{C}$) produced large cubic particles ($12\text{--}20\text{ nm}$). Figure 1A shows a representative TEM image for a sample of the MZF nanoparticles synthesized in organic solution. The average size is $12.4 \pm 0.9\text{ nm}$.

To achieve water solubility, MZF nanoparticles were transferred from hexane solution to aqueous solution using ligand exchange reaction. The magnetic nanoparticles were capped with a mixed monolayer of OAC and OAM, i.e., MZF /OAC-OAM. After the exchange reaction, OAC/OAM was displaced by DCA. In comparison with the MZF nanoparticles before ligand-exchange reaction (Figure 1A), the particle size after ligand exchange reaction (Figure 1B) remained unchanged ($12.5 \pm 1.0\text{ nm}$). The particles were dispersible in aqueous solution.

MZF nanoparticles were coated with Ag shells by chemical reduction routes in both aqueous and non-aqueous solutions. Figure 1C shows a representative TEM image for MnZn Ferrite nanoparticles after Ag coating (MZF@Ag) synthesized by reducing Ag acetate in the presence of MnZn Ferrite nanoparticles in organic solvent. The particle size ($26.0 \pm 13.0\text{ nm}$) was found to increase after Ag coating in comparison the MnZn Ferrite nanoparticles ($12.5 \pm 1.0\text{ nm}$), indicating likely the formation of MZF@Ag nanoparticles. The particle size distribution is rather poor after coating silver, reflecting a certain degree of aggregation. The MZF@Ag nanoparticles can also be made water-soluble by ligand exchange reaction.

Figure 1D shows a representative set of TEM micrographs for a sample of MZF@Ag nanoparticles derived from coating silver onto the surface of the MZF nanoparticles (Figure

1B) in aqueous solutions. The average size of the resulting MZF@Ag nanoparticles is 28.0 ± 2.0 nm. The average Ag-shell thickness was estimated to be about 5 nm. In comparison, Ag nanoparticles synthesized under the same condition were found to be much smaller (5.0 ± 0.8 nm) than MZF@Ag nanoparticles.

Surface Plasmon Resonance Properties of MZF@Ag Nanoparticles—The surface plasmonic characteristic of silver shells of the MZF@Ag nanoparticles were characterized using UV-Vis spectroscopy. Figure 2A shows a representative set of UV-Vis spectra characterizing the surface plasmon (SP) resonance band for samples of MZF, mixed MZF and Ag, and MZF@Ag nanoparticles. In contrast to the largely silent feature in the visible region for MZF nanoparticles, the MZF@Ag nanoparticles show a clear SP band at 420 nm, characteristic of the unique optical property of Ag shell on the MZF nanoparticles. This band is shifted to a higher wavelength in comparison with Ag nanoparticles which are physically mixed with MZF nanoparticles. Similar spectral characteristics were observed for gold-coated iron oxide nanoparticles in our previous work^{26,27}.

In order to assess the magnetic separation characteristic of the MZF@Ag nanoparticles in the aqueous solution, samples were dispersed in a solution and a magnetic field was applied to the side of the solution container. In this process, the SP band was monitored (Figure 2A insert). There is a gradual decrease of the SP band absorbance (Abs) for the MZF@Ag nanoparticles as a function of time upon applying magnet, demonstrating that the MZF@Ag nanoparticles are magnetically active. Based on first order kinetic model for the decay of the SP band (Figure 2B), i.e., $\text{Abs} = a + b \exp(-kt)$, where k stands for the apparent rate constant, the absorbance data were fitted by the model. The rate constant was extracted from the fitting. The k -values were found to be 0.06, 0.63, and 0.63 for MZF@Ag, mixed MZF and Ag, and MZF, respectively. The differences were indeed as expected for these samples by design.

The magnetization properties of the MZF@Ag nanoparticles could also be observed from the movement of the materials in a solution upon applying a magnet and the corresponding colorimetric changes (Figure 2B insert). A clear diminishing of the color for the MZF@Ag nanoparticles in aqueous solutions as a function of time upon applying the magnet is evident. This is in contrast to the lack of significant color change for the mixture of uncoated MZF and Ag nanoparticles. The incomplete removal of the magnetic nanoparticles within the 1-hour time frame is likely due to the presence of small-sized MZF or thicker silver coating on the MZF cores which render them less magnetically active than the larger-sized or thinner silver coated MZF particles. Note that these MZF@Ag nanoparticles could be attracted completely from solution after a prolonged time under magnetic attraction (e.g., > 2.5 hours).

SERS Characteristics of MZF@Ag Nanoparticles—The surface properties of silver were characterized using SERS technique^{25,39,43}. MBA was used as a Raman label for SERS detection of its immobilization on the surface of the nanoparticles. Briefly, a solution of the nanoparticles was mixed with an aqueous solution of 0.03 mM MBA for 10 hours for the adsorption of MBA on the particle surfaces. The resulting solution had a pH $\approx$ 11. Figure 3 shows a representative set of SERS spectra for the comparison of the different nanoparticles in aqueous solutions or gold thin-film substrates.

There are two findings from SERS data. First, in contrast to the largely silent feature in the region of 1000 to 2000 cm^{-1} for MnZn ferrite particles, the MZF@Ag particles showed clearly enhanced bands corresponding to MBA adsorbed on Ag or Ag shell on the MZF@Ag nanoparticles. For MBAs adsorbed on silver and silver-coated MZF nanoparticles in the solution, the MBA peaks are observed at 1077 and 1586 cm^{-1} . These two peaks

correspond to $\nu(\text{CC})$ ring-breathing modes of MBA. The other weaker/less intense bands correspond to $\delta(\text{CH})$ ($1130\text{--}1180\text{ cm}^{-1}$) and $\nu_s(\text{COO}^-)$ ($1380\text{--}1400\text{ cm}^{-1}$) modes. In the SERS measurement, the solution was basic ($\text{pH} \sim 11$) under which the $-\text{CO}_2\text{H}$ groups were deprotonated. Secondly, the MBA-labeled MZF@Ag in the solution redispersed after magnetic attraction showed some difference from a sample of the MBA-labeled MZF@Ag collected by magnetic attraction on a gold thin film slide. Moreover, there are some extra peaks at 1149 , 1272 , 1451 and 1502 cm^{-1} in the re-dispersed solution of MBA-labeled MZF@Ag after magnetic attraction. The origin of these peaks was possibly associated with additional ligands on the surface, including carboxylic groups on oleic acid and citrate.

2. Determination of Bacterial Inactivation Efficiency

The bacterial inactivation efficiency of our MZF@Ag nanoparticles was determined for several types of bacteria, including Gram-positive *Staphylococcus aureus* and *Bacillus cereus*, and Gram-negative *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Escherichia coli*.

Inactivation of microorganisms growth in saline solution—To demonstrate the antimicrobial property of the nanoparticles, we first used MZF@Ag nanoparticles to treat bacteria inoculated in saline. The results were then compared with those using silver nanoparticles. To illustrate the inactivation of bacterial growth, the solutions were plated and incubated overnight at 37°C . Figure 4 shows the plates from the 10^{-3} dilution of the stock solution ($5 \times 10^{10}/\text{mL}$) used in the plate count. As shown in Figure 4, a clear reduction in bacterial growth was observed in the samples that contained silver nanoparticles or silver coated magnetic nanoparticles.

The quantitative data for the inactivation of bacterial growth by the nanoparticles are shown in Table 1. After a 5-hour exposure time a 2.96 Log_{10} reduction was observed in the *S. aureus* sample treated with silver coated magnetic nanoparticles. Similarly, the use of silver nanoparticles led to a 3.59 Log_{10} growth reduction in *S. aureus*. The data also suggested that Gram-negative *P. aeruginosa* was more susceptible to the silver-coated magnetic particles and silver nanoparticles than the Gram-positive *S. aureus*.

The observation that the Gram-negative *P. aeruginosa* was more susceptible to the nanoparticles than the Gram-positive *S. aureus* is interesting. It has been reported that Gram-negative bacteria exhibit only a thin peptidoglycan layer ($2\text{--}3\text{ nm}$) between the cytoplasmic membrane and the outer membrane⁴⁴. In contrast, Gram-positive bacteria lack the outer membrane but have a peptidoglycan layer of about 30 nm thick⁴⁵. There are two explanations as to why Gram-positive bacteria are less susceptible to Ag^+ than Gram-negative bacteria⁴⁶. One involves the charge of peptidoglycan molecules in the bacterial cell wall. Gram-positive bacteria have more peptidoglycan than Gram-negative bacteria because of their thicker cell walls. In addition, peptidoglycan is negatively charged and silver ions are positively charged. This electrostatic effect could lead to more silver being trapped by peptidoglycan in Gram-positive bacteria than in Gram-negative bacteria. The decreased susceptibility of Gram-positive bacteria can also simply be explained by the fact that the cell wall of Gram-positive bacteria is thicker than that of Gram-negative bacteria. It has been shown that silver nanoparticles can be more effective as antimicrobial agent against certain Gram-positive bacteria⁴⁷. In this case, silver nanoparticles could anchor to or penetrate the bacterial cell wall.

Based on the above 5-hour exposure time data, the bactericidal effects of silver using silver coated magnetic nanoparticles are evident. Silver nanoparticles and silver coated magnetic nanoparticles are both effective in killing bacteria. More importantly, the effectiveness of inactivation by the use of silver on magnetic nanoparticles is not very different from silver

nanoparticles alone. The ability to remove the silver after the treatment through the use of magnetic nanoparticles is crucial to the use of MZF@Ag in bacterial inactivation where the residual silver is not desired.

We next monitored the bacterial growth and the action of antimicrobial nanoparticles over a 24-hour period. As shown in Figure 5(A), the MZF@Ag nanoparticles seemed to be more efficient at killing *Bacillus cereus* when compared to the silver nanoparticles. At the 6 hours sampling the silver magnetic particle sample was below a detectable level and remained there for the duration of the experiment. After 3 hours of exposure, the silver nanoparticles had an effect on the bacteria causing a 3 Log₁₀ reduction from the 3 to 6 hours sampling time points. The bacterial concentration further decreased as time elapsed.

Figure 5(B) illustrates the results obtained from the Gram-negative bacteria, *Enterobacter cloacae*. The results were highly similar to those of the *Bacillus cereus* sample shown in Figure 5(A) with a slight difference with the silver nanoparticle sample. The nanoparticles seemed to work more rapidly on the *E. cloacae*, which could be attributed to it being Gram-negative; however, between the 6 hours and 24 hours sampling times the bacteria incubated with silver nanoparticles seemed to recover from the silver inactivation as shown by their concentration increasing from the 6 hours to 24 hours sampling time points. The extent of aggregation for silver nanoparticles aggregate is greater than that for silver coated magnetic nanoparticles.

Inactivation of bacteria in Blood Platelets—Having successfully demonstrated that the MZF@Ag nanoparticles can inactivate bacterial growth similarly to plain silver nanoparticles in PBS, we performed measurements to test the viability of the MZF@Ag nanoparticles for inactivating bacteria in human blood platelet samples. MZF nanoparticles were also tested to confirm that the bacterial activation function comes from the silver and not the MZF component. Note that we did not test Ag nanoparticles considering that the platelet solution was too viscous for the much smaller nanoparticles. Figure 6 illustrates the inactivation of *E. coli* with MZF@Ag. After only 6 hours of exposure the bacterial concentration fell below a detectable level. The control samples (MZF and *E. coli* alone) behaved almost identically, reaching their growth peak at 48 hours. The bacterial concentration was below a detectable level by the 72 hours sampling time point. This could be explained by the exhaustion of nutrients in the culture media. A reduction of at least 5 Log₁₀ was observed between MZF@Ag and MZF particles in this experiment.

We analyzed the total silver (ionic silver and suspended silver nanoparticles that could not be removed by a magnet field) in the sample. The sample with *E. coli* showed a concentration of 0.117 ppm, whereas the sample containing *E. coli* and MZF nanoparticles (after magnet application) showed a concentration of 0.132 ppm. In both cases, the Ag concentrations were <0.15 ppm. While the sample containing the MZF@Ag (after magnet application) showed a silver concentration 5 times (0.662 ppm) that of the control samples, it was still less than 1 ppm. Although the MZF@Ag treated samples showed slightly more silver found than the controls, it provided significant bacterial inactivation. This finding is an important piece of evidence supporting that the inactivation is mainly caused by the direct contact of Ag on nanoparticles rather than Ag ions leached into the liquid phase. The concentrations of silver found in platelets (including ionic silver or silver nanoparticles came off the MZF@Ag) after the incubation period were well below 1 parts per million (ppm). This concentration is very low compared to other silver based antimicrobial products such as those in medical bandages. Also note that the level of <1 ppm found in our samples is significantly lower than the concentrations (20–40 ppm) found in Tryptic Soy Broth from silver coated wound dressing in a silver ion release study using SilverIon®⁴⁸. The amount of silver found in the platelets was also less than the level usually causing a reaction in the

human body, suggesting that the idea of using MZF@Ag to inactive bacteria in blood product will be safe after the removal of MZF@Ag nanoparticles.

While studies of silver microbial susceptibility have been performed, most of these studies have shown different data in terms of minimal inhibitory concentration (MIC) for AgNO₃. For instance, MIC values for *Staphylococcus aureus* (around 100 strains) have been reported to range from 8 to 80 mg/L (ppm) from the results from the two previous studies^{49,50}. Similarly, in the two latest studies examining silver ions for approximately 100 strains of *Pseudomonas aeruginosa*, the MIC values were reported to range from 8 to 70 mg/L^{51,52}. The low concentration of silver found (<1ppm) suggested that the presence of Ag ions is not the main mechanism for the inactivation by the MZF@Ag, and instead the direct contact between metallic silver on magnetic core and the bacteria is the major mechanism.

To confirm the assessment, we carried out an experiment that collected the MZF@Ag particles after a few hours of incubation between bacteria and platelets had started. The collection of MZF@Ag particles to the side of the container (without removing them from the sample) led to an increase of bacterial growth, reversing the trend of pathogen inactivation. If the pathogen-inactivation mechanism is caused by leached ionic silver from these nanoparticles, the bacterial growth should have still been inhibited by the ionic silver over time and not affected by the collection of MZF@Ag nanoparticles.

Summary

In summary, the analysis of the bacterial inactivation efficiency has demonstrated that the MZF@Ag nanoparticles are highly effective for inactivating bacterial growth in both saline solutions and blood platelets. As silver nanoparticles are becoming more and more popular as antimicrobial agent in commercial products and new findings regarding toxicity of silver nanoparticles become available⁵³, our approach offers the ability to recover the used silver nanoparticles and reduce the unwanted toxicity towards the patients (if they are used in treating blood platelets prior to transfusion) or the environment.

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Figure 1.

TEM micrographs: MZF nanoparticles synthesized from an organic solution (A); The MZF particles (i.e., A) after ligand exchange reaction (aqueous dispersible) (B); MZF@Ag nanoparticles synthesized by reducing Ag(I) acetate in the presence of MZF particles (i.e., A) in an organic solvent (C); and MZF@Ag nanoparticles synthesized by reducing Ag⁺ in the presence of water-soluble MZF (i.e., B) in an aqueous solution (D).

Figure 2.

(A) UV-Vis spectra comparing samples of MZF (a), MZF@Ag (b), and mixed MZF and Ag (c) nanoparticles in aqueous solutions (insert: UV-visible spectra showing the corresponding changes for MZF@Ag nanoparticles (i.e., solution-a) as a function of the magnet time ($t = 0 - 60$ mins.)). (B) Plots showing the magnetic separation kinetics in terms of the change in absorbance (Abs) of the SP band at ~ 420 nm for solutions of MZF (a), a solution of MZF@Ag (b), and a mixed MZF and Ag NP solution (c). (Fitting: $Abs = 0.04 + 0.96 \exp(-0.63 t)$ (a); $A = 0.37 + 0.64 \exp(-0.06 t)$ (b); and $A = 0.64 + 0.36 \exp(-0.63 t)$ (c)) (insert: Photos showing color changes for an aqueous solution of MZF@Ag nanoparticles (a) in comparison with a solution of mixed MZF and Ag nanoparticles (c) upon application of a magnet (b) for different time lengths. $t_0 = 0$ min; $t_1 = 30$ mins; $t_2 = 60$ min).

Figure 3.

SERS spectra comparing samples of different nanoparticles containing MBA: MZF NPs in solution (a), Ag NPs in solution (b), MZF@Ag NPs after magnetic attraction and being re-dispersed in the solution (c), MZF@Ag NPs collected by magnetic attraction on the gold coated glass slide (d), and supernatant left behind after the magnetic attraction of the MZF@Ag NPs in the solution (e).

Figure 4.

Photos showing samples of bacteria growth plated after 5-hours incubation with or without the nanoparticles. (A) *Staphylococcus aureus*; (B) *Pseudomonas aeruginosa*, in a solution containing Ag nanoparticles (a), a phosphate buffer saline (PBS) solution (b), and a solution containing MZF@Ag nanoparticles (c).

Figure 5. The inactivation efficiency data for the inactivation of two different types of bacteria: (A) *Bacillus cereus* and (B) *Enterobacter cloacae*, in a PBS buffer solution (a), in a solution containing with MZF@Ag nanoparticles (b), and in a solution containing with Ag nanoparticles (c).

Figure 6.

A comparison of the inactivation efficiency data for the inactivation of *E. coli* with water (a), *E. coli* with MZF nanoparticles (b), and *E. coli* with MZF@Ag nanoparticles (c) in blood platelets.

Scheme 1.

An illustration of silver-coated magnetic core-shell nanoparticles (M@Ag) as a functional antimicrobial agent for inactivation of bacteria in blood platelets, and the subsequent magnetic separation to remove M@Ag from the platelets (drawn not to scale). Image in right: an AFM image of bacteria, e.g., *Pseudomonas aeruginosa*, adsorbed on a mica surface.

Table 1

A summary of data the inactivation of Gram-positive and Gram-negative microorganisms (5 hours exposure time)

Bacteria	Diluent	AVERAGE CFU/mL	AVERAGE Log ₁₀
<i>S. aureus</i>	PBS	2.63×10^6	6.42
	MZF@Ag	3.25×10^3	3.51
	Ag	6.75×10^2	2.83
<i>P. aeruginosa</i>	PBS	1.77×10^7	7.25
	MZF@Ag	5.67	0.75
	Ag	4.00	0.60

(CFU/mL: Colony Forming Units/mL)