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Effects of broth composition and light condition on antimicrobial susceptibility testing of ionic silver

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Abstract

Silver has widely been used as antimicrobial agent, but there is a long-standing debate over the microbiocidal potency of ionic silver versus colloidal silver. In this study, the minimum inhibitory concentrations (MICs) of $AgNO_3$ against Gram-positive and Gram-negative bacteria were investigated in a variety of microorganism culture broths. Broth- and light-dependent MIC values were observed across the present Ag^+ antimicrobial studies. Meanwhile, a negative correlation was observed between MICs of Ag^+ and nano-Ag speciation development. Time-course measurements by UV-Vis spectroscopy was used to follow the particle evolution. Transmission electron microscopy (TEM), energy-dispersive spectroscopy (EDS), X-ray diffraction (XRD) and dynamic light scattering (DLS) results indicate the formation of AgCl, silver nanoparticle decorated silver chloride (Ag@AgCl) nanohybrids and Ag nanoparticles in the size range of 20-40 nm. The results of this study straightforwardly show that Ag antimicrobial activity relies more on Ag^+ rather than on the particulate species of silver. We also advocate here the importance of broth and light standardization in Ag antimicrobial test.

Keywords: ionic silver, silver chloride/silver nanoparticle, antimicrobial susceptibility, MIC, broth

1. Introduction

In the past, the antimicrobial activities of silver-containing materials have been intensively studied due to the strong and broad-spectrum activity of Ag against bacteria, viruses, and fungi (Banerjee et al., 2011; Liu et al., 2013; Agnihotri et al., 2013). To evaluate the potency of different types of Ag biocides, typical antimicrobial susceptibility testing methods have been employed like the Kirby-Bauer method (Guzman et al; 2012; Dar et al., 2012) and the agar and broth dilution methods for minimum inhibitory concentration (MIC) determination (Mohanty et al., 2012). The MIC is defined as the lowest concentration of an antimicrobial agent that completely inhibits the visible growth of microorganism after overnight incubation (Andrew, 2001). It is generally regarded as the most basic laboratory measurement of the efficacy of an antimicrobial agent against a microorganism (Turnidge et al., 2003). More specifically, the microbroth dilution method is often employed to determine MIC and quantitatively evaluate the bactericidal activity of silver-based materials (Berger et al., 1976; Egger et al., 2009).

Despite the fact that most researchers follow the protocol provided by the Clinical and Laboratory Standards Institute (CLSI), large variation in MIC values was found across Ag biocidal studies. The MIC values of common clinical antibiotics may vary significantly in terms of incubation time, density of the inoculum and age of the batch of antibiotics (Schuurmans et al, 2009). The susceptibility test results of silver are even more vulnerable to changes in test conditions. For example, the MIC data of Ag^+ against *Staphylococcus aureus* varied up to 10-fold (Ug and Ceylan, 2003; Hamilton-Miller et al., 1993). The components of test medium have been recognized to influence silver ion availability (due to formation of various silver-halide anionic complexes (Gupta et al., 1998; Levard et al., 2013; Zhang et al., 2013). However, the effects of various medium on Ag^+ MIC measurements remain uncertain (Chopra, 2007). In addition, there is a great debate regarding ionic or colloidal silver is more

potent in antimicrobial application. Recent studies suggest that nano-Ag confers their antimicrobial activities via Ag^+ release (Xiu et al., 2012). We hypothesize that the in situ silver nanoparticulates formation may affect the bioavailability of Ag^+ therefore greatly contribute to MIC variation.

The first objective of this study was to determine the effects of microorganism culture media contents in Ag^+ MIC test. Herein, we study the MICs of $AgNO_3$ against typical bacteria in a variety of culture broths include Lysogeny broth, Mueller–Hinton broth and tryptic soy broth. Secondly, we will show that the antibacterial activity of Ag^+ is superior to Ag nanoparticulates since an increased MIC is related to *in situ* silver chloride or silver nanoparticle formation in these broths. Among many studies aimed to judge the potence of Ag^+ and nano-Ag (Sotiriou and Pracsinis, 2010; Sintubin et al., 2011) our experimental design provides direct comparison by excluding the effect of stabilizing surfactant and particle aggregation.

2. Materials and methods

2.1 Materials

Silver nitrate ($AgNO_3$) from Sigma Aldrich was used as received. Lysogeny broth, Mueller-Hinton broth, nutrient broth, tryptic soy broth were prepared following the manufacturer's instructions. Deionized (DI) water processed by the Milli-Q plus system (Millipore Co.).

2.2 Antimicrobial test

Bacterial strains of *Escherichia coli* (ATCC 25922) and *Bacillus subtilis* (ATCC 23857) were obtained from the American type culture collection (Rockville, MD). All bacteria were

stored in a - 80 °C refrigerator. The bacterial cultures were maintained in tryptic soy broth as recommended by ATCC. The inoculum was prepared by suspending the frozen cells in a fresh TSB medium and grown at 37 °C overnight. The suspension of each microorganism was diluted to an OD_{600} of ~ 0.1 on a microplate reader. Minimal inhibitory concentrations (MICs) were determined by microdilution assay. Typically, 100 µL microbial solutions (containing 2-10 $\times 10^7$ cells /mL) were added to 100 µL of sterilized DI water containing silver nitrate (ranging from 2 to 0.015 mM in serial two-fold dilutions) in each well of the 96-well microtiter plate. The plates were incubated at 37 ºC for 24 h with shaking at 100 rpm. The reported MICs are the minimum concentration necessary to inhibit visible cell growth. Experiments were run in triplicate. To test the MICs of Ag^+ in other media, bacteria grown in TSB after overnight were centrifuged and washed with PBS then re-suspended in LB and MHB respectively, at the same dilution factor. To test the effect of visible light on MICs, all other experimental conditions were kept the same except that the plates were incubated in the dark box covered with aluminium foil.

2.3 In situ formation of nanoparticulate silver in various broths

Silver nitrate was dissolved in sterilized DI water and the concentrations were adjusted from 2 to 0.015 mM in serial two-fold dilutions. 100 µL Ag^+ solutions were added to 100 µL of different media and the mixture was shaken at 37 °C for 24 or 48 h. To test the effect of visible light on nanoparticle formation, parallel experiments were carried out by wrapping the plates in aluminium foil.

The course of the AgCl, Ag@AgCl and Ag nanoparticle formation in the broth was monitored by UV-visible spectroscopy (GBC Scientific Equipment). The synthesized nanoparticles were characterized by a dynamic light scattering (DLS) using Nano-ZS90 analyser (Malvern). The particle size and polydispersity were determined from the DLS

measurements. DLS is a spectroscopic common technique used to determine the radius of nanoparticles, colloids, and molecules in Brownian motion in a solution (Gliga et al., 2014). Zeta potential measurements of nano-Ag were obtained with a ZETAPALS zeta potential analyser (Brookhaven Instruments Corporation). For transmission electron microscopy (TEM), a drop of solution was placed on carbon-coated copper grids and air dried. X-ray diffraction analysis was performed using a Bruker D8 Advance X-ray Diffractometer with Cu Kα (λ = 1.54Å) radiation. The diffracted intensities were recorded from 20° to 80° 2θ angles.

2.4 Comparative antimicrobial study of silver in ionic and colloidal form

The MICs of fresh $AgNO_3$ in TSB or $AgNO_3$ incubated with TSB for 24 h were determined against *B. subtilis* as described before in *Antimicrobial Test* section.

For transmission electron microscopy studies, a 1.5 mL of *E. coli* suspension was incubated with $AgNO_3$ in MHB or TSB broth at a concentration of 0.03 mM for 3 h. The control was prepared from the same batch of bacterial suspension as the treated one. The mixture was centrifuged at 1000 g for 5 min and washed with PBS (pH 7.4) for three times. The bacterial pellet was fixed in 2.5% glutaraldehyde in PBS overnight at 4 ºC, followed by centrifugation and wash with PBS 2 times and 3 times with deionized water. The post-fixation with 1% $OsO_4$was performed in the PBS for 1 h. The fixed sample was then dehydrated in a graded series of concentrations of ethanol. The sample was infiltrated with the mixture of acetone and Spurr resin (from 1:1 to 1:3 in volume ratio) for 1 h and 3 h, respectively, at room temperature. The pellet was finally embedded in pure Spurr resin for overnight. Ultrathin sections (~ 80 nm) were obtained using a Leica ultramicrotome, mounted on formvar-carbon-coated Cu grids prior to TEM (JEOL, Japan) observation at an acceleration voltage of 30 keV.

3. Results and discussion

3.1 Moderate to good growth of bacteria in three media

To assess the growth of the bacteria in LB, MHB and TSB, the cells were inoculated in the broth to a cell density corresponding to optical density 0.1 at 600 nm. The turbidity of the suspension was monitored by microplate reader. As shown in Figure S1, whereas *B. subtilis* showed moderate growth in LB and MHB media, both *B. subtilis* and *E. coli* showed good growth in TSB. Such growth results were considered adequate for susceptibility testing (Mättö et al., 2006).

3.2 MICs variations in three media and under different light conditions

Incubation of Gram-positive *B. subtilis* and Gram-negative *E. coli* with Ag^+ at concentrations from 0.0075 to 1.0 mM gradually inhibited the growth of bacteria in LB, MHB and TSB broths, as shown in Figure 1. To enhance the clarity of the chart, we opt to hide the vertical axis. The symbols on the lines represent bacterial optical density (OD) reading at 600 nm. An arbitrary unit is used in Y-axis to express approximate OD_{600} trend. The MICs were determined as the lowest concentrations of Ag^+ with which no apparent increase in OD_{600}. When experiments were carried out under ambient light, the MICs were found highest in TSB (MIC = 0.25 mM against both *B. subtilis* and *E. coli*) and lowest in MHB (MIC = 0.015 mM against *B. subtilis* and MIC = 0.06 mM against *E. coli*). MICs in LB were same against both *B. subtilis* and *E. coli* as detected to be 0.06 mM.

As with MIC assay performed in the dark (no light, NL), Ag^+ exhibited stronger inhibition against bacteria *B. subtilis* showed reduced MIC value from 0.06 mM to 0.03 mM in LB and from 0.015 mM to 0.0075 mM in MHB. The most striking differences being detected for Ag^+ against *B. subtilis* and *E. coli* was 0.25 mM versus 0.03 mM, under light and in the dark, respectively.

Numerous silver-based materials are being developed as environmental and clinical antimicrobial agents. An *in vitro* MIC test is critical in assessing the bactericidal efficacy of Ag compounds. Although MHB has been recommended by experienced researchers (Randall et al., 2013; NCCLS, 2000; Tkachenko and Karas, 2012) for silver MIC test, literature reviews show that other broths like LB (Mohanty et al., 2012; Gupta et al., 1998; Sotiriou and Pratsinis, 2010; Petrus et al., 2011), NB (Said et al., 2014), TSB (Liu et al., 2013; Brandt et al., 2012; Gao et al., 2013) and BHIB (brain heart infusion broth, Tkachenko and Karas, 2012; Kawahara et al., 2000) have been employed frequently. It is well known that susceptibility test results are highly dependent on medium-related factors due to silver binding components such as microbial biomass, proteins and chloride. The lowest MICs of Ag^+ obtained in MHB indicated that most of $AgNO_3$ in MHB were still in ionic or soluble complex form. In other words, there is less Ag nanoparticulates formed in MHB. This is confirmed by the very small amount of precipitate after centrifugation of the MHB broth. Note that the characterization on such small amount of particles is difficult, we focus on silver nanoparticulate study in LB, NB, TSB and yeast mold broth (YMB).

Previously, there are no data available regarding Ag^+ MICs tested in different broths and under different light conditions, hindering the accurate interpretation and comparison of silver antimicrobial studies. The present study disclosed that the variation is related to colloidal Ag formation, the nanoparticulates can be either silver decorated silver chloride (Ag@AgCl) hybrid or pure nano-Ag, which will be discussed in the following section.

3.3 In situ nanoparticulate silver formation in different bacterial culture media

Incubation of $AgNO_3$ solution with microorganism culture broths induced gradual colour change of the media from light yellow to brown, indicating the formation of Ag nanoparticulates. The colloids formed in Lysogeny broth, nutrient broth, tryptic soy broth and

yeast mold broth were labelled as AgLB, AgNB, AgTSB and AgYM, respectively. The inset of Figure S2 shows pictures of the corresponding medium containing 0.5 mM $AgNO_3$ after 24 h reaction at 37 °C. Figure S2 shows the UV-Vis extinction spectra of Ag NPs formed in different media. The intensity of surface plasmon resonance (SPR) band is related to the size and content of Ag NPs. According to the absorbance intensity, less silver nanoparticles were formed in Lysogeny broth as compared to other broths. AgLB, AgTSB and AgYM showed bands peaks around 420 nm. For AgNB sample, the Ag band red shifted and exhibited absorption maximum at about 460 nm.

The detailed morphology of Ag NPs in different media is demonstrated in Figure 2. All nanoparticles were found to be nearly spherical in shape. The observed zeta potential values for the AgLB, AgNB, AgTSB and AgYM were in the range of –15 and -20 mV. AgLB and AgTSB exhibited more uniform particle size distribution than AgNB and AgYM samples.

The crystallographic structure of the Ag NPs was examined by X-ray powder diffraction (XRD). When incubation was carried out in the dark, except for AgYM, all other samples displayed XRD pattern corresponding to planes (111), (200), (220), (311), (222), (400), (331), (420) revealed the crystalline nature of the AgCl (JCPDS file: 31-1238) as shown in Figure S3. However, when Ag^+ containing broths exposed to lab light, there were peaks emerging at $2\theta = 38.2°$ correspond to the (111) Miller indices of face-entered cubic Ag for AgNB and AgYM samples.

3.4 Antimicrobial performance of Ag^+ versus Ag nanoparticulate

To confirm that Ag@AgCl nanohybrid formation has a negative effect on antimicrobial performance of Ag^+, we firstly exposure various concentrations of silver nitrate in TSB at 37°C for 24 h under lab light. After that, both Ag@AgCl containing and freshly prepared $AgNO_3$ solution were inoculated with *B. subtilis* to determine their MICs under light

exposure. As can be seen in Figure 3, although the apparent MICs were same as 0.25 mM after 20 h incubation, 0.06 mM fresh $AgNO_3$ inhibited the growth of *B. subtilis* up to 4 h; in contrast, the organism multiplied quickly at this time point when Ag@AgCl containing solution was used.

The cell ultrastructure TEM results correspond well with the stronger Ag^+ antimicrobial performance as revealed by above MIC studies. Using Gram-negative *E. coli* as a model, untreated cells (Fig. 4C) had a smooth surface and a uniform interior distribution of proteins and DNA molecules. *E. coli* cells treated by $AgNO_3$ dissolved in MHB and TSB exhibited distinguished different morphologies. For example, Ag^+ (as the major silver speciation) in MHB, caused complete damage of cell membrane and severe leaking of cytoplasm from the cells as a remarkable electron-light centre was observed in most cells (Figure 4A). These results are in agreement with previous reports by Feng et al. (2000) and Jung et al. (2008); instead, once most Ag^+ were transformed into Ag@AgCl, the treated cell still maintained uniform cell microstructure although the integrity of cell membrane was destroyed as compared to control cells. We inferred such mild cell membrane damage is caused by oxidative damage from superoxide or hydroxyl radicals (Dong et al., 2012; Zhang et al., 2013). It is interesting to note that there are some electron-dense black granules distributed inside Ag@AgCl treated cells. Apart from Ag as the major content of these black dots, EDS results indicate the existence of elements of sulphur. The intra-cellular formation of Ag-nanoparticulates may be related to detoxification strategies of *E. coli* in responding to toxic environment (Klaus et al., 1999; Tran et al., 2013).

Conclusions

To the best of our knowledge this is the first description of Ag@AgCl nanohybrids formation in bacterial culture broths and discloses their influence on MIC study of ionic

silver. The work described herein establishes the necessity of harmonization of silver antimicrobial susceptibility testing medium for ensuring high agreement in MICs. Based on our results, we recommend testing silver MIC using MHB in the dark. Only by harmonizing of MIC test conditions, the reliable results will guarantee the interpretive accuracy necessary for drawing conclusions relevant to antimicrobial research.

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Table and Figure Captions

Figure 1 Inhibited growth of *B. subtilis* (A, B and C) and *E. coli* (D, E and F) by Ag^+ under light (L) or in the dark (NL). The symbols on the lines represent bacterial optical density (OD) reading at 600 nm. An arbitrary unit is used in Y-axis to express approximate OD_{600} trend. The MIC is defined as the lowest concentration of Ag^+ that completely inhibits the visible growth of microorganism after 24 h incubation. Data are mean ± SD of 3 independent experiments and the results are considered statistically different if p-value < 0.05.

Figure 2 SEM images of silver nanoparticulates formed in LB, NB, TSB and YMB broths after incubation of 1 mM $AgNO_3$ solutions at 37 °C for 24 h under ambient light.

Figure 3 Inhibited growths of *B. subtilis* by the mixture of $AgNO_3$ and Ag@AgCl in TSB broth (A) or freshly prepared $AgNO_3$ in TSB broth (B).

Figure 4 Electron micrographs of *E. coli* after treatment with 0.03 mM $AgNO_3$ in MHB (A) and in TSB (B). The control cells cultured in TSB (C) displayed distinct cell membranes and compact cytoplasm. Scale bars = 1 µm.

Figure S1 Growth curves *B. subtilis* (A) and *E. coli* (B) in Lysogeny broth (LB), Mueller-Hinton broth (MHB) and Tryptic Soy broth (TSB). Results are presented as mean ± SD (n = 3).

Figure S2 Absorbance of Ag@AgCl (in LB, NB and TSB broths) and Ag nanoparticles (in YM broth) prepared with 1.0 mM $AgNO_3$ in various microorganism culture broths recorded up to 48 h after exposure to light. Inset: Photographs of broths containing 0.5 mM $AgNO_3$ before (A) and (B) after incubation at 37 °C for 24 h under ambient light.

Figure S3 XRD patterns of silver nanoparticulates formed in different broths: under light (A) or kept in the dark (B).

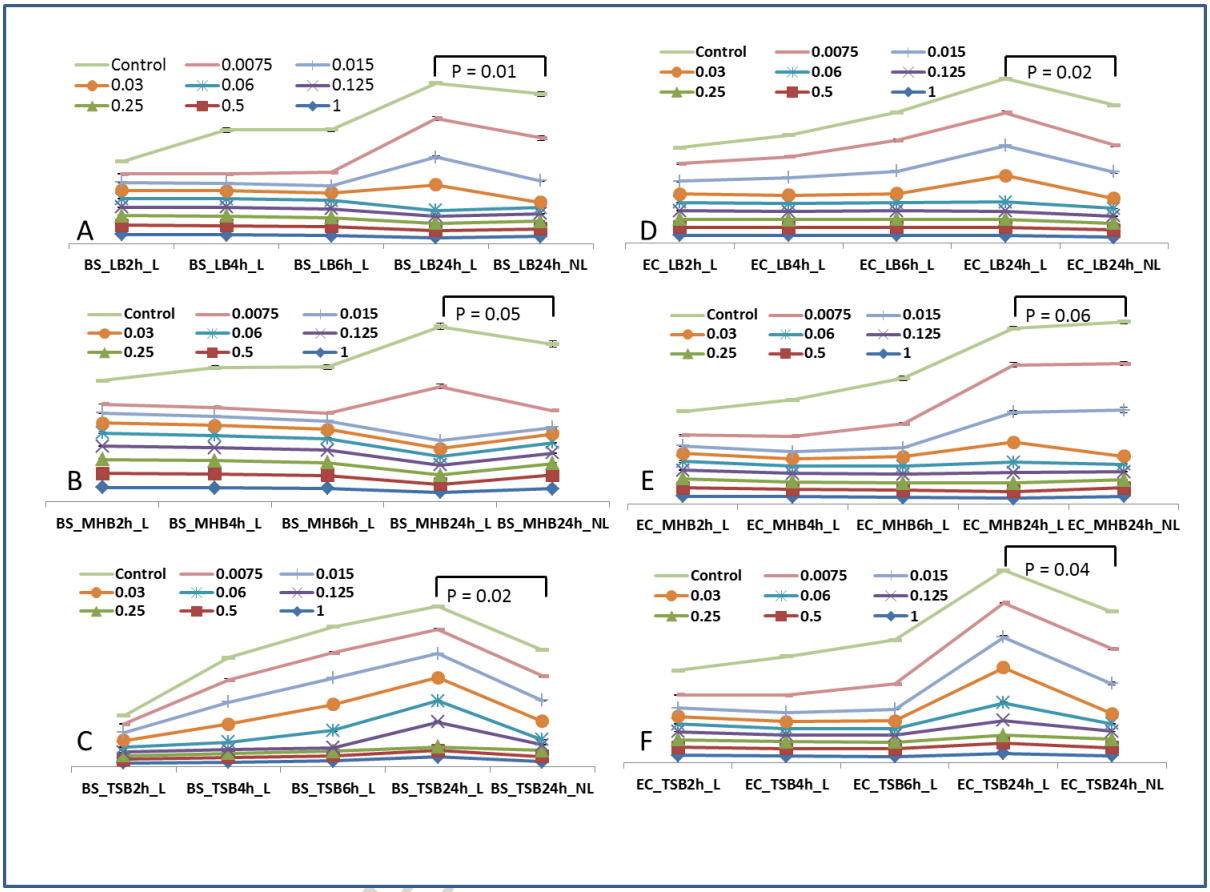


Figure 1

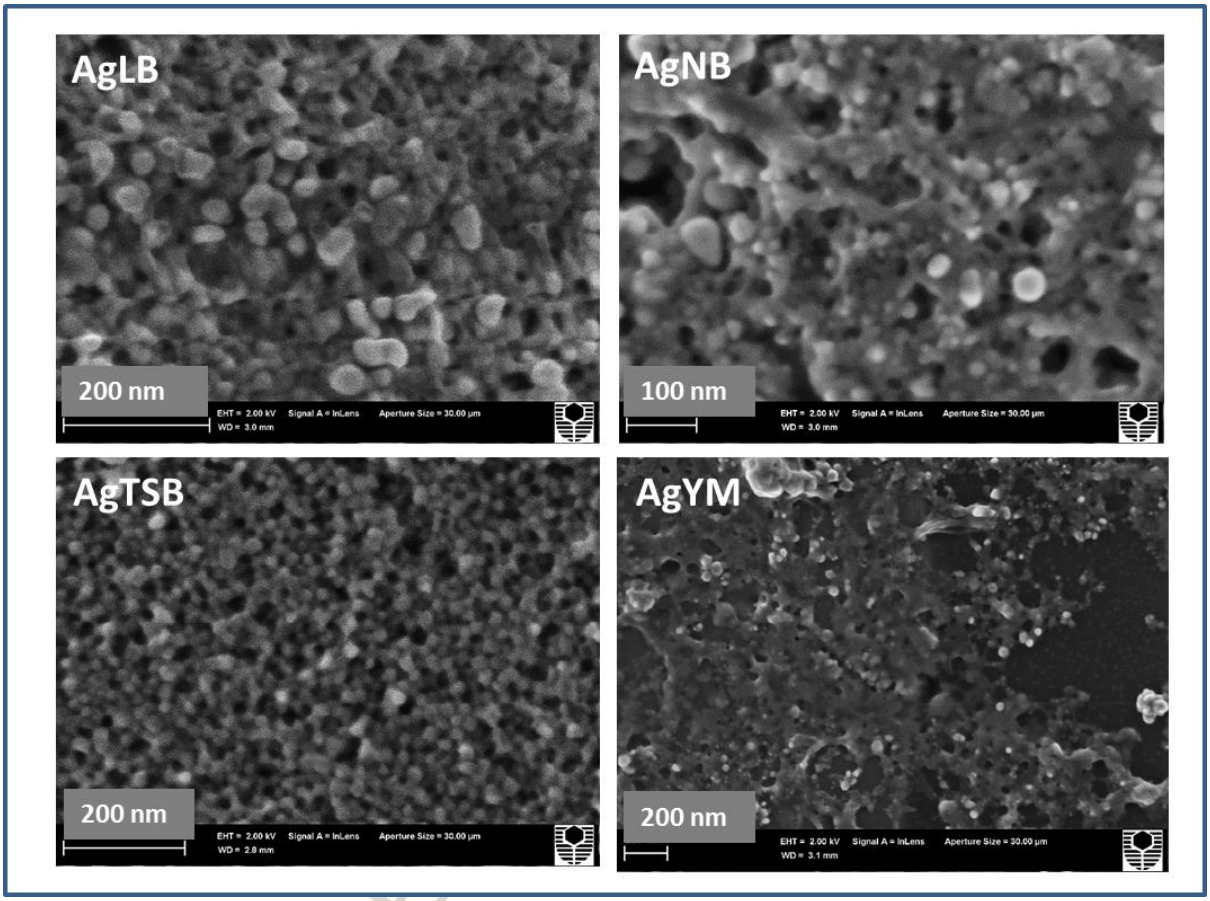


Figure 2

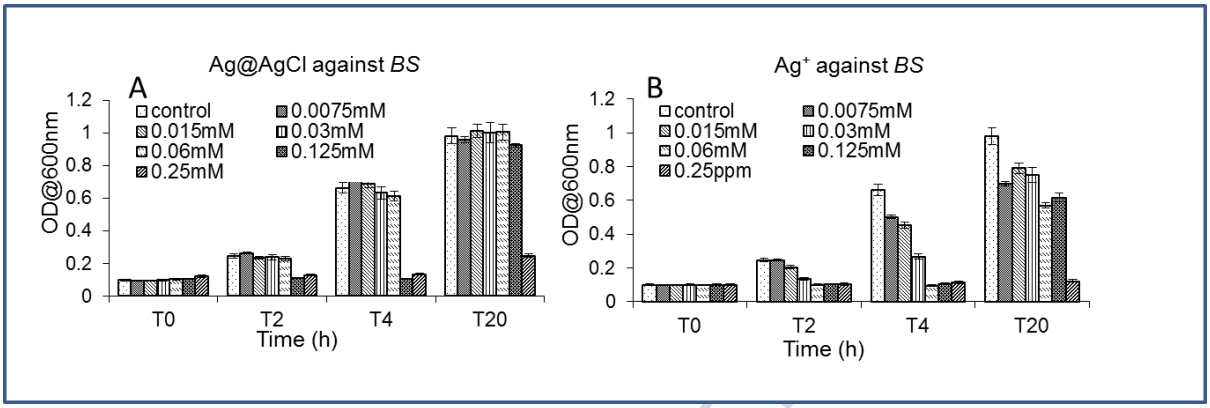


Figure 3

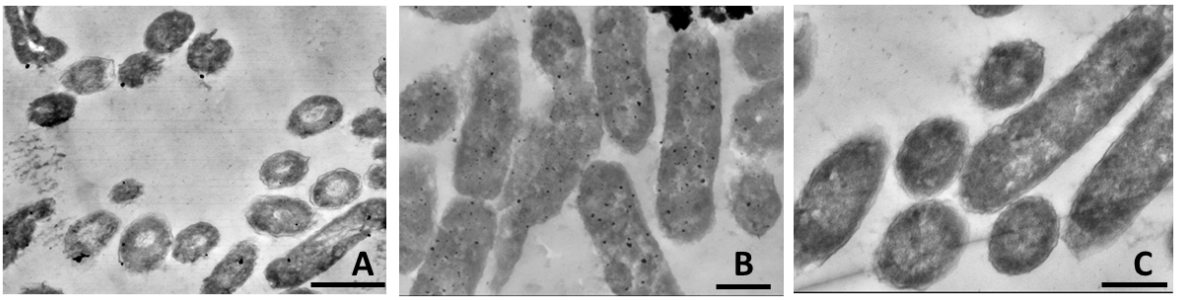
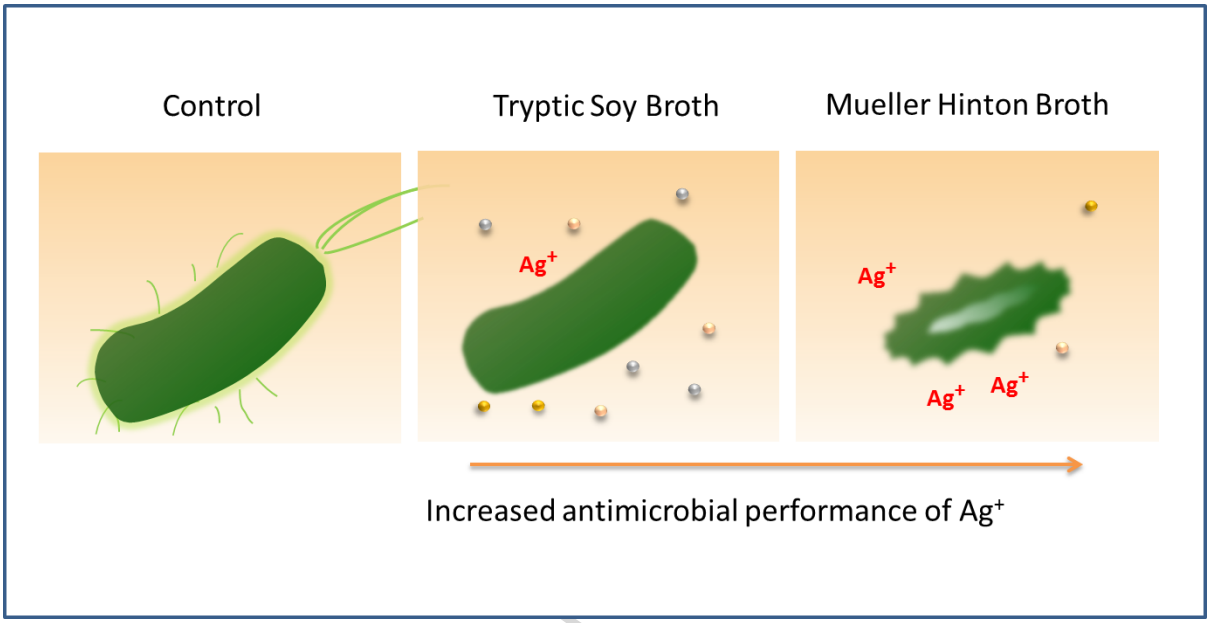


Figure 4



Graphical abstract

Highlights

- Ag^+ MICs varied with broth compositions and light conditions
- AgCl and Ag@AgCl colloid formation reduced biocidal effect of Ag^+
- Ag^+ has superior microbiocidal activity than Ag nanoparticulates
- Ag^+ MIC test condition should be unified