Disinfection performance of nanosilver and impacts of environmental conditions on viral inactivation by nanosilver

Yang Xu, Xiaona Chu, Jiangyong Hu and Say Leong Ong

ABSTRACT

Three types of nanosilver materials, which were commercial, chemically-synthesized and biologically-synthesized, respectively, were compared in terms of the disinfection efficiencies against *Escherichia coli* and MS2 coliphage in order to pinpoint promising material with the best performance. Disinfection results showed biologically-synthesized silver nanoparticles (referred to hereafter as 'bio-AgNPs') had the best disinfection performance, 10 mg/L of which was able to inactivate all the *E. coli* in 1 min (>6 log removals) and achieved 4 log removals of MS2 coliphage. Bio-AgNPs were therefore selected for further study in terms of effects of the concentration and contact time as well as the impacts of environmental conditions on the viral inactivation. Given the viral inactivation profile of bio-AgNPs shown in this study, it could be concluded that viral inactivation by bio-AgNPs could be inhibited by total organic carbon (TOC) (10 mg/L as humic acid) and chloride ion (5 mg/L) to a large extent while Ca^{2+}/Mg^{2+}/ionic strength only had minor effects on the viral inactivation at high concentrations (188 mg/L as $CaCO_3$ of hardness or 5.6 mM of ionic strength, respectively). This part of the study may help enlighten further mechanism studies on viral inactivation by nanosilver.

Key words | disinfection, environmental conditions, *Escherichia coli*, MS2 coliphage, nanosilver

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INTRODUCTION

Drinking water treatment is always of great concern worldwide and there are many problems associated with lack of clean and safe water: 1.2 billion people lack safe drinking water, 2 billion have poor sanitation, millions of people die annually from waterborne diseases directly (Montgomery & Elimelech 2007) and many more are still suffering from water-related issues. However, conventional disinfection methods have many inherent drawbacks that can no longer be tolerated: chlorination and ozonation have disinfection by-products formation issues and ultraviolet irradiation leaves no disinfection residues along the distribution system. Therefore, there is an urgent need to develop a novel and robust disinfection strategy while avoiding those adverse effects.

Silver and silver-related materials have been used and recognized for centuries as a sustainable strategy against microbial growth (Silver *et al.* 2006). Now nanosilver has attracted attention from many researchers for its unique properties and potential application in many areas (Rai *et al.* 2009); and it has been developed as a novel disinfectant that could be applied in water treatment due to its high intrinsic toxicity to microbes and insignificant harmful effects on humans (Fabrega *et al.* 2009; Dankovich & Gray 2011; De Gusseme *et al.* 2011). Previous studies convincingly demonstrated the potential use of nanosilver and the feasibility of nanosilver disinfection in water treatment against both bacteria and viruses.

Apart from chemical synthesis, which has been intensively studied in the past decade, Sintubin and his coworkers recently explored an innovative way to produce nanosilver using *Lactobacillus fermentum* (Sintubin *et al.* 2009). Nanosilver obtained with this method was reduced and immobilized in the bacterial cell wall, which served as a carrier matrix for nanosilver, preventing nanoparticles

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from aggregating. Researchers from the same group further evaluated the disinfection capacity of this biogenic nanosilver against UZ1 bacteriophage and Murine Norovirus 1 (De Gusseme *et al.* 2009) and investigated the potential application in membrane technology (De Gusseme *et al.* 2011).

Although there are considerable studies on the synthesis and antimicrobial effects of nanosilver materials (Sondi *et al.* 2003; Wiley *et al.* 2007; Sintubin *et al.* 2009), few studies have compared different kinds of nanosilver in terms of antimicrobial ability (El Badawy *et al.* 2011; Zhang *et al.* 2012). Particularly, little direct comparison of nanosilver from various sources has been performed against *Escherichia coli* and MS2 coliphage, which are surrogates commonly used in the field of drinking water quality control. To fill this research gap, our study first compared nanosilver from three sources in terms of disinfection efficiencies. Subsequently we adopted the nanosilver material giving the best performance, which was bio-AgNPs, for further experiments in order to obtain the profile of inactivation against MS2 coliphage by bio-AgNPs. In order to do so, effects of contact time and concentration of bio-AgNPs were studied while typical environmental conditions were also assessed, including ionic strength, electrolytes (hardness and chloride ion) and total organic carbon (TOC) concentrations. To the best of our knowledge, although impacts of these conditions have been widely studied on bacterial growth (Fabrega *et al.* 2009; Jin *et al.* 2010), no systematic study on viral inactivation has been reported yet. Thus this study aims at filling this gap. Despite the fact that mechanisms remain not fully understood, nanosilver was believed to inactivate microorganisms in three ways: (1) Ag^+ ions released from nanosilver, which may interfere with DNA replication and protein function (Morones *et al.* 2005); (2) the adhesion of nanosilver to the microbes (Elechiguerra *et al.* 2005); and (3) ROS formation (Sintubin *et al.* 2011). Behaviours of nanosilver under different conditions in our study may enlighten further the study of mechanisms in this field.

MATERIAL AND METHODS

Production of nanosilver

Chemicals used were purchased from Sigma-Aldrich, USA, unless specified. Deionized water (DI water) used throughout this study was 18 mΩ pure water produced by MilliQ water purification system (Millipore, USA). Commercial silver nanoparticles (99.5% pure, referred to hereafter as 'com-AgNPs') were purchased directly from Sigma-Aldrich, USA and used without further purification. Chemically synthesized silver nanoparticles (referred to hereafter as 'chem-AgNPs') were synthesized based on a previous publication (Pal *et al.* 2007) with minor changes. Typically, 0.5 ml of 0.01 M $NaBH_4$ was rapidly injected into a solution containing 0.5 ml of 0.01 M $AgNO_3$ and 20 ml of 1 mM sodium citrate. The solution obtained was vigorously stirred for 5 min and aged for 1.5 hours to serve as Ag seed solution. Subsequently, after 100 ml of 1 mM $AgNO_3$ was boiled, 3 ml of Ag seed solution and 1.04 ml of 0.1 M sodium citrate were added and the solution was kept at boiling point until it turned greenish yellow. After being cooled down to room temperature, the obtained nanosilver colloids were washed with DI water three times using a centrifuge (13,000 × g, 15 min).

Biologically synthesized silver nanoparticles (referred to hereafter as 'bio-AgNPs') were synthesized according to Sintubin *et al.* (2009). Typically, some biomass of *L. fermentum* ATCC 11976 was harvested and washed three times by centrifuge (5,000 × g, 10 min) after 48-hour incubation in Man–Rogosa–Sharpe broth (Oxoid, UK) at 30 °C. After being diluted to 4.6 g (as cell dry weight (CDW)) per litre, the biomass was alkalified by adding 2.56 v% of 1 M NaOH solution. 16.5 mg/ml (as Ag) of $Ag(NH_3)_2NO_3$ solution was then added into the alkalified biomass to achieve a ratio of 1:4.6 for Ag:CDW. The mixture was carefully covered with aluminium foil and shaken at room temperature (28 °C) for 24 hours. Bio-AgNPs were then purified using a centrifuge (10,000 × g, 15 min).

A transmission electron microscope (TEM, JEM 3010, 300 kV, JEOL Ltd, Japan) was used to characterize AgNPs in this study, samples of which were prepared by depositing a drop of freshly prepared AgNPs suspension (aqueous) on a carbon coated copper grid and air-drying at room temperature overnight. The TEM test was associated with energy-dispersive X-ray spectroscopy (EDX, INCAx-Sight, Oxford Instruments) in order to further verify AgNPs. Inductively coupled plasma optical emission spectrometry (ICP-OES, PerkinElmer, USA) was used to quantify the concentration of AgNPs synthesized after being digested with HNO_3 and H_2O_2 according to US EPA 3050B.

Growth and detection of *E. coli* and MS2 coliphage

E. coli ATCC 15597 and MS2 coliphage ATCC 15597-B1 were chosen as models of bacteria and viruses in water and purchased directly from ATCC. All incubations were conducted at 37 °C and 120 rpm. A culture of *E. coli* ATCC 15597 was incubated in tryptic soy broth (TSB, Oxoid, UK) overnight. One millilitre of such *E. coli* overnight culture was transferred and incubated in 30 ml of TSB for 4 hours to obtain *E. coli* at exponential phase, which was washed three times using a centrifuge (2,500 × g, 10 min) before inactivation experiments.

MS2 coliphage ATCC 15597-B1 was mixed with *E. coli* of exponential phase and incubated for 20 hours. As-prepared MS2 suspension was centrifuged at 3,600 × g for 10 min and the obtained supernatant was passed through a 0.22 μm sterile filter. The filtrate was used as the MS2 stock. Detection of *E. coli* and MS2 coliphage relied on the plate culture method and the standard double agar overlay technique, respectively, this latter method is explicitly described elsewhere (Butkus *et al*. 2004).

Bacterial and virus inactivation by AgNPs

AgNPs suspension was mixed with washed *E. coli* (or MS2 coliphage stock) to render a 10 ml reaction system that included 10 mg/L of AgNPs and *ca.* 10^5 CFU/ml of *E. coli* (or *ca.* 10^6 PFU/ml of MS2 coliphage). Such reactions were continuously stirred at room temperature and covered with aluminium foil to minimize the impact of ambient light. Samples were taken after 5-, 10-, 30- and 60-min contact time. Inactivation was expressed as log values (base 10), which equals $\log_{10}(C_0/C_t)$. Each experiment was conducted in triplicate and error bars denote one standard deviation.

Study on effects of electrolyte and TOC

Sodium nitrate was selected to simulate ionic strength while calcium nitrate and magnesium nitrate were selected to simulate the hardness present in natural water. Sodium chloride was introduced as the source of Cl^- while humic acid was used as the source of TOC. These conditions were chosen because antimicrobial effects of nanosilver were reported to be affected by these conditions according to previous studies (Fabrega *et al*. 2009; El Badawy *et al*. 2010; Jin *et al*. 2010). The concentration of bio-AgNPs and contact time were fixed at 10 mg/L and 10 min, respectively, in this part of the study.

RESULTS AND DISCUSSION

TEM characterization of three selected nanosilver materials

TEM images of the nanosilver are shown in Figure 1. It should be noted the com-AgNPs had severe aggregation, with sizes ranging from 70 nm (inset of Figure 1(a)) to several hundred nanometres (Figure 1(a)) while chem-AgNPs were smaller in size, ranging from 20 to 100 nm (Figure 1(b)). Compared with the study of Pal *et al*. (2007), chem-AgNPs in this study were successfully synthesized as they had the same size and shape. Bio-AgNPs (Figures 1(c) and 1(d)) might have the best dispersion as the biomass served as a scaffold, thereby avoiding aggregation from occurring (Sintubin *et al*. 2009). Black dots in Figures 1(c) and 1(d) are silver nanoparticles proven by EDX (data not shown). The dark area in Figure 1(c) was simply the TEM copper grid

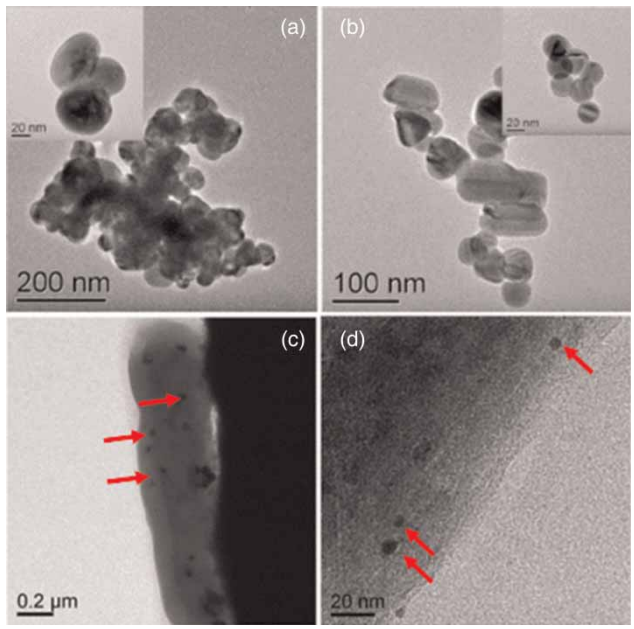

Figure 1 | TEM images of (a) com-AgNPs, (b) chem-AgNPs, and (c) and (d) bio-AgNPs. Insets are respective single particles with the same scale bar of 20 nm.

determined by EDX (data not shown). From Figure 1(c), it is clearly seen that most of the silver nanoparticles were located in the cytoplasm with some of them on the cell wall (Figure 1 (d)). Apart from better dispersion, the size of the bio-AgNPs was smaller (around 10 nm in Figure 1(d)) than that of either com-AgNPs or chem-AgNPs. These TEM images were consistent with those provided in the study of Sintubin *et al.* (2009).

Bacterial and viral inactivation

Comparison of com-AgNPs, chem-AgNPs and bio-AgNPs against *E. coli* is summarized in Figure 2. Performances of com-AgNPs and chem-AgNPs were gradually enhanced by increase of contact time from 5 to 60 min; however, no significant difference between these performances were detected ($P = 0.81$). One possible reason that chem-AgNPs were no better than com-AgNPs could be the severe aggregation of nanoparticles as denoted in the TEM images, which was in good agreement with a previous study regarding the size effect of nanosilver (Morones *et al.* 2005). On the other hand, astounding disinfection performances were achieved by bio-AgNPs. There were no colonies seen on the plates after overnight incubation of bio-AgNPs treated samples, even for those samples of 5-min contact time, which denoted greater than 6.3 log removals. In fact, experiments with shorter (1-min) and longer (10, 30 and 60-min) contact times were carried out with the same outcome gained. Considering this 6.3-log removal is the detection limit of this detection method, no error bar is given and data points only for 1 and 5-min contact time are shown.

The conclusion that bio-AgNPs have a better performance against *E. coli* is in good agreement with Sintubin *et al.* (2011). It was reported that bio-AgNPs had much lower values of minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC) against *E. coli* than chem-AgNPs had (Sintubin *et al.* 2011). In spite of the different quantification methods (MIC/MBC versus plate culture method), their findings could be supportive that bio-AgNPs are more likely to be the better disinfectant compared to com-AgNPs and chem-AgNPs.

Comparison of three types of nanosilver against MS2 coliphage is shown in Figure 3. Similar to the results for *E. coli*, bio-AgNPs achieved the best performance of all three, inactivating 3.0 and 4.3 log MS2 with contact times of 5 and 10 min, respectively. Extending the contact time to 30 min or even 60 min didn't increase the log removal much.

Inactivation of MS2 coliphage by bio-AgNPs with varying contact time and concentration of bio-AgNPs was profiled in order to optimize the conditions so as to fulfil the 4-log viral removal recommended in drinking water guidelines by USEPA (Figure 4). Five mg/L of bio-AgNPs was not capable of achieving 4 log removals even if the contact time was up to 120 min, while 10 mg/L of bio-AgNPs

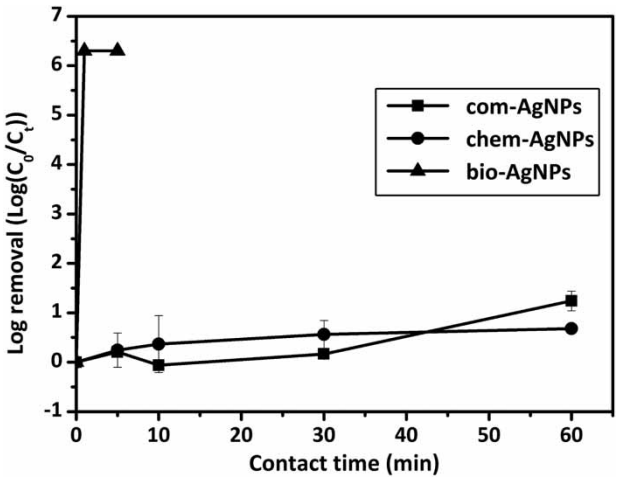

Figure 2 | Comparison of log removals of *E. coli* by three types of nanosilver. Concentration of AgNPs was 10 mg/L. Some error bars may be smaller than the depicted symbols.

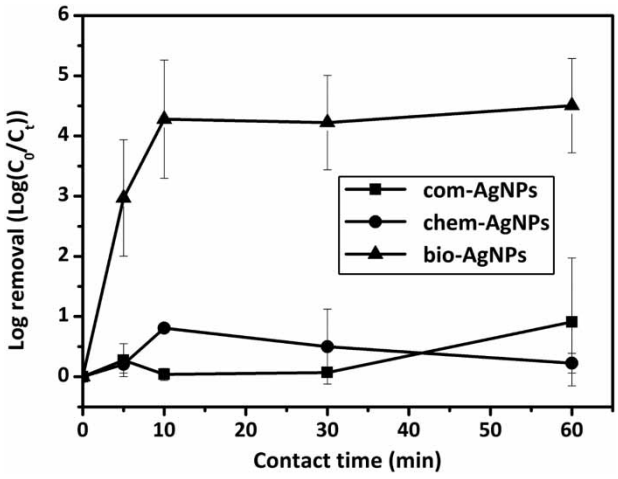

Figure 3 | Comparison of log removal of MS2 coliphage by three types of nanosilver. Concentration of AgNPs was 10 mg/L. Some error bars may be smaller than the depicted symbols.

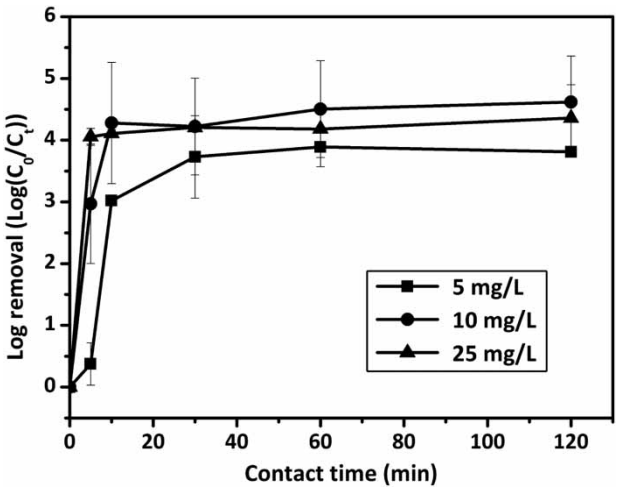

Figure 4 | Log removal of MS2 coliphage by bio-AgNPs.

was capable of fulfilling 4-log removal in only 10 min. On the other hand, increasing the dosage from 10 to 25 mg/L had no significant benefits ($P = 0.84$); and increasing the contact time did not result in an increase in log inactivation of MS2 after 10 min for 10 mg/L dosage. Such a phenomenon was also observed in De Gusseme *et al.* (2009), in which no increase of log inactivation was gained after 3 h. These two findings helped to determine that 10 mg/L of bio-AgNPs for 10 min was the recommended condition afterwards.

De Gusseme *et al.* (2009) reported viral removal of bio-AgNPs on UZ1 bacteriophage. That disinfection performance was slightly better than this study and the main reason might be the resistance difference between UZ1 and MS2. UZ1, a DNA phage, is rarely used as an indicator in drinking water quality control whilst a previous study has already suggested that only F-specific RNA phages (e.g. MS2 coliphage) would have a resistance high enough to be considered as an acceptable indicator (Havelaar *et al.* 1991). Therefore a study on removal of MS2 would be of greater significance in drinking water quality control.

Effects of ionic strength, calcium and magnesium ion concentrations

Ca^{2+} and Mg^{2+} were reported to be able to affect silver nanoparticles stability and, as a consequence, the bacterial inactivation as well (Jin *et al.* 2010). It was proposed by Jin *et al.* (2010) that Ca^{2+} and Mg^{2+} ions may serve as ion-bridges and enhance the contact between cell surface and silver nanoparticles, which were both negatively charged in their case. Such enhanced contact would apparently enhance the antibacterial effect of silver nanoparticles as observed by Jin and her co-workers. This is the initiative to examine the effect of divalent ions on nanosilver disinfection performance.

Effects of Ca^{2+} and Mg^{2+} concentrations on viral inactivation are summarized in Figure 5, which shows that log removal of MS2 coliphage decreased as concentration of hardness increased. Log inactivation by bio-AgNPs decreased incrementally from 4.0 log and 4.5 log to 1.5 log and 1.6 log for Ca^{2+} and Mg^{2+}, respectively, when the hardness (either Ca^{2+} or Mg^{2+}) increased from 0.94 to 188 mg/L as $CaCO_3$. Such findings were contradictory to Jin's study. Therefore, one thing that is certain is that the 'ion-bridge theory' proposed by Jin *et al.* (2010) may not be applicable to the case of the MS2, even if the MS2 coliphage and bio-AgNPs were both negatively charged.

To further examine this result, sodium nitrate was introduced to simulate the ionic strength in previous studies, with an attempt to understand whether the decreased log removals could be attributed to increased ionic strength. Concentrations of $NaNO_3$ were delicately tuned so as to

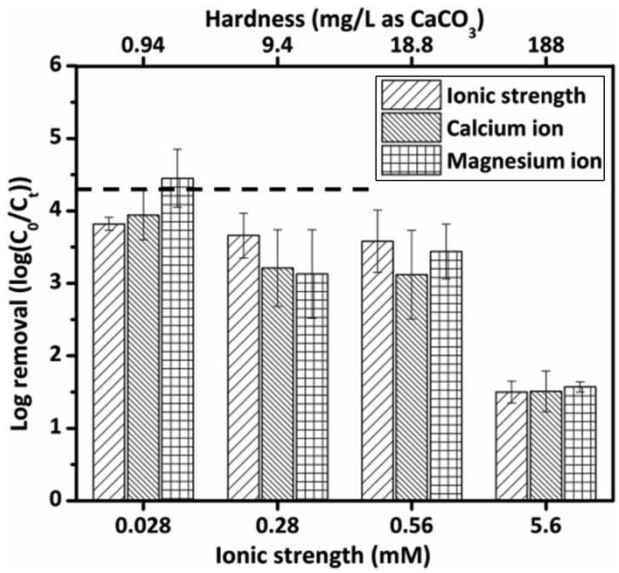

Figure 5 | Effects of ionic strength and hardness on log removal of MS2 coliphage by bio-AgNPs. Dashed line denotes log removal by bio-AgNPs in DI water.

be equivalent to the corresponding ionic strength of each hardness sample (either Ca^{2+} or Mg^{2+}). Results showed a similar trend was observed and these three arrays of results, furthermore, were not significantly different ($P > 0.5$). Therefore, the adverse impacts of Ca^{2+} and Mg^{2+} might be believed to simply result from the impacts of ionic strength and it is further suggested that divalent cations may not serve as an ion-bridge in the case of viral inactivation by bio-AgNPs, which was suggested in Jin *et al.* (2010). One possible reason for this decreased log removal is the enhanced aggregation of nanoparticles promoted by higher ionic strength (El Badawy *et al.* 2010), and subsequently such aggregation would inhibit the disinfection performance (Morones *et al.* 2005). However, this proposed 'aggregation' might be flawed since bio-AgNPs were believed to be fixed in and protected by biomass (dead cells) from aggregation. Future study is needed to further elaborate this result.

Effects of TOC and chloride ion concentrations

TOC and Cl^- revealed detrimental impacts on bio-AgNPs in terms of viral inactivation (Figure 6). Ten mg/L of TOC rendered a decrease of log removal from 3.3 to 0.4 logs while further increasing the concentration of TOC to 30 mg/L would totally inhibit the viral inactivation of bio-AgNPs (Figure 6(a)). Catastrophic results also occurred in the case of Cl^-, in which even 1 mg/L of Cl^- could decrease log removal from 4.3 to 2.5 and 5 mg/L or more of Cl^- would totally inhibit the bio-AgNPs' performance.

Although viral and bacterial inactivation are two different scenarios, hints might be found in the study of Fabrega *et al.* (2009), in which the effect of organic matter was studied on bacterial growth in the presence of silver nanoparticles. Fabrega and co-workers proposed that humic acid may present a physical barrier between AgNPs and bacteria, thereby impeding interaction between them. Thus our results (Figure 6(a)) were in good agreement with the proposed mechanism and may suggest viral inactivation of bio-AgNPs might be attributed to physical contact between AgNPs and viruses.

Results regarding effects of Cl^- could be well explained by the fact that the toxicity of silver largely depends on the presence of ligands (Ratte 1999). It is proposed that when Ag^+ is released from nanoparticles, Cl^- may precipitate Ag^+ *in situ* on the surface of nanoparticles. Such a precipitate may likely form an inert layer on the surface of nanosilver, thereby 'quenching' the disinfection performance of nanosilver. This proposed model was in good agreement with studies of Ho *et al.* (2010) and Levard *et al.* (2011).

CONCLUSION

Nanosilver from three sources, namely com-AgNPs, chem-AgNPs and bio-AgNPs, were compared in terms of log inactivation against *E. coli* and MS2 coliphage. Bio-AgNPs, which had a smaller particle size and less aggregation than the other two, achieved the best and formidable performance against both *E. coli* and MS2 coliphage, 10 mg/L of which was capable of inactivating 6-log *E. coli* and 4-log MS2 in 5 min. Bio-AgNPs disinfection against

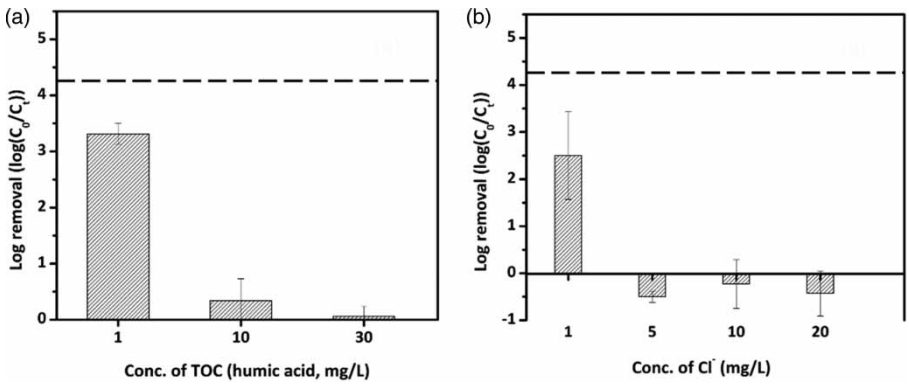

Figure 6 | (a) Effects of TOC and (b) effect of chloride ion on log removal of MS2 coliphage by bio-AgNPs. Dashed line denotes log removal by bio-AgNPs at the same conditions in DI water.

MS2 was optimized and 10 mg/L for 10-min contact time was the recommended condition if 4-log viral removal is required.

Effects of ionic strength, selective electrolyte and TOC were subsequently evaluated with bio-AgNPs at a concentration of 10 mg/L for a contact time of 10 min. Ionic strength and hardness would only affect the disinfection performance at a high concentration (5.6 mM, 188 mg/L as $CaCO_3$ for ionic strength and hardness, respectively). TOC and chloride were found to dramatically affect the viral inactivation of bio-AgNPs, in which 30 mg/L of TOC (as humic acid) and 5 mg/L of Cl^- totally quenched the antiviral effects of bio-AgNPs. Such findings may suggest viral inactivation be attributed to physical contact between nanosilver and viruses. Results showed that there should be a strict requirement for the source water if we intend to apply nanosilver in the drinking water disinfection process.

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