Size-dependent antibacterial activity for laser-generated silver nanoparticles

Peri Korshed,1 Lin Li,2* Zhu Liu,3 Aleksandr Mironov4 & Tao Wang1*

1 School of Biological Sciences, Faculty of Biology, Medicine and Health, The University of Manchester, Manchester, UK
2 Laser Processing Research Centre, School of Mechanical, Aerospace and Civil Engineering, The University of Manchester, Manchester, UK
3 School of Materials, The University of Manchester, Manchester, UK
4 Core Research Facilities, Faculty of Biology, The University of Manchester, Manchester, UK

Keywords
Antibacterial activity, E. coli, Nanoparticle size, ROS generation, Silver nanoparticles, Sucrose gradient centrifugation.

Abstract
Silver nanoparticles (Ag NPs) have been used widely for antibacterial applications; however, the effects of their sizes on antibacterial activities and toxicities to human cells, particularly for the laser-generated Ag NPs, are not fully understood. In this study, sucrose gradient centrifugation was used to separate laser-generated Ag NPs into different fractions by size. Transmission electron microscopy was used to analyze the size distribution of the Ag NPs, and well diffusion method was used to evaluate the antibacterial activity of the Ag NP fractions against the Escherichia coli. Results showed that the antibacterial effects of laser-generated Ag NPs inversely correlated to the particle size. Among Ag NP fractions with average sizes ranging 19–47 nm, the 19-nm Ag NPs presented the highest bactericidal effect. The smaller sized laser Ag NPs also significantly induced the generation of reactive oxygen species when applied to E. coli, compared with that of the larger sized laser Ag NPs. Cytotoxicity analysis revealed that the different sized laser-generated Ag NPs were not significantly toxic to the human fibroblasts and lung epithelial cells in a 72-h in vitro cell culture period. Understanding the size-dependent functional properties of the laser-generated Ag NPs helps informing the designs for future applications of the laser-generated Ag NPs.

© 2019 The Authors. Journal of Interdisciplinary Nanomedicine published by British Society for Nanomedicine and John Wiley & Sons Ltd. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
Introduction

Silver nanoparticles (Ag NPs) are commonly used in daily life including areas of textile, household antiseptics, cosmetic products, and medical devices (El-Nour et al., 2010). The wide applications of Ag NPs attribute to their broad spectrum of antibacterial properties. Ag NPs are among the first commercialized nanomaterials and account for 55.4% of the whole nanomaterial consumer market (Xiu et al., 2012; Center, 2011; Asghari et al., 2012). A number of studies reported that the antibacterial effects of Ag NPs were related to the particle size (Morones et al., 2005; Sotiriou and Pratsinis, 2010; Panacek et al., 2006; Carlson et al., 2008), shape (Pal et al., 2007), and surface charge (El Badawy et al., 2010). The physical and functional properties of NPs with a smaller size are different from those with bulk sizes (Ashe, 2011). Characterization of NPs, such as Ag NPs, ZnO NPs, and TiO2 NPs, revealed that any change to the NPs size led to changes of their chemical, morphological, structural, electrical, and optical properties. Modifications to NPs sizes could alter the interaction of NPs with cellular biomolecules (Rasmussen et al., 2010). These size-related properties are important for designing NPs applications in areas including nanomedicine, nanotechnology, and biosensors (Ashe, 2011).

Numerous studies have documented the relationship between NPs sizes and their antimicrobial activities. For example, the study by Morones et al. (2005) used Ag NPs with concentrations of 0, 25, 50, 75, and 100 μg/mL and size ranged from 1 to 100 nm against gram-negative bacteria Escherichia coli. Results showed that the excellent antibacterial activity came from the size of 16-nm Ag NPs at a concentration of 75 μg/mL (Morones et al., 2005). Choi and Hu (2008) demonstrated a better antibacterial activity for the 5-nm Ag NPs compared with other sized Ag NPs (10, 15, and 20 nm) used in the study. Espinosa-Cristobal et al. (2009) examined the antibacterial activity of Ag NPs with three different sizes (8.4, 16.1, and 98 nm) against Staphylococcus mutans and revealed that the size of 8.4 nm showed the highest antibacterial activity.

In recent years, we have employed laser technology for the generation of Ag NPs. Laser is a clean technology. The advantage for the laser-generated NPs is that they are highly pure, free from contaminations by reagents that are carried over from other chemically based reactions. In spite of studies on the correlation of antibacterial activities to the Ag NP size, little is known regarding the size-related antibacterial activity.

Size-related bactericidal effects of laser Ag nanoparticles

for the laser-generated Ag NPs. Limited information is available for the size-related antibacterial mechanisms and human cell toxicity for the laser-generated Ag NPs. In this study, we produced Ag NPs using picosecond laser ablation and employed sucrose gradient centrifugation to separate different sized NPs in order to evaluate size-dependent antibacterial activities of the laser NPs. We found that the bactericidal effects were inversely correlated to the nanoparticle size. Accordingly, the smaller sized Ag NPs induced more reactive oxygen species (ROS) generation than the larger sized Ag NPs, but with low human cell cytotoxicity. Results from this study could contribute to understanding and future biomedical applications of the laser-generated Ag NPs.

Materials and Methods

Nanoparticles production

Nanoparticle production by pulsed laser ablation was described in previous work by the authors (Korshed et al., 2018; Korshed et al., 2016). Briefly, an Ag plate (dimensions of 25 mm × 25 mm × 2 mm, purity 99.99%) was sterilized by immersing into ethanol and then autoclaved in deionized water (dH2O). The plates were then placed in a glass vessel separately, which contains 20 mL of dH2O with a level of 2 mm above the Ag samples. A picosecond pulsed Nd: YVO4 laser with a wavelength of 1064 nm was used to ablate the Ag plate at a high scanning speed v = 250 mm/sec at a pulse repetition rate of 200 kHz and an average power of 9.12 W.

Nanoparticle-sized separation by sucrose gradient centrifugation

To make 30% sucrose solution, 60 gm of pure sucrose (Fisher Scientific, UK) was dissolved in 100 mL of dH2O with heating and then topped up to 200 mL with dH2O. The 35%, 40%, 45%, 50, and 60% sucrose solutions were made using the same method. After cooling down at room temperature, 2 mL of each sucrose solution with different concentrations in an order of 60%, 50%, 45%, 40%, 35%, and 30% was added layer by layer to a Beckman centrifuge tube (polycarbonate, inner diameter 15 mm, length 90 mm) to create a density gradient with 30% on the top and 60% on the bottom of the tube (Li et al., 2011; Xiong et al., 2011). Five hundred microliters of Ag NPs solution were added slowly to the top of the sucrose gradient prepared previously and centrifuged at a speed of 150,000 g for 60 min, using Beckman.
ultracentrifuge Optima L-90 K and rotor SW40, to separate different sized NPs into different layers of the sucrose gradient. Different layers of the sucrose solution were then carefully collected using a pipette and redispersed in 500-μL deionized water for further characterizations (Li et al., 2011).

Characterization of separated nanoparticles
Fractions of Ag NPs collected from different layers of the sucrose gradient were centrifuged at 12,000 g for 10 min. The NPs were then washed by re-suspending the pellets in dH2O and centrifugation for two to five times. The final NPs pellets were suspended in 250 μL of dH2O. Samples of each fraction were added in quartz cuvettes (Fisher Scientific), and the absorbance spectrum was measured using Synergy HTX Multi-Mode Reader (BioTek, Swindon, UK).

Antibacterial activity examination
The well diffusion method was used to test the antibacterial activity of the laser Ag NPs against E. coli as we described previously (Korshed et al., 2016). A single colony of bacterial cells was inoculated in 10 mL of autoclaved Muller-Hinton broth media (Sigma) and incubated at 37°C overnight with shaking at 225 rpm. The bacteria suspension was diluted to give 10⁴ CFU/mL. A thin layer of the bacterial culture was spread uniformly on the Muller-Hinton agar plates using sterile cotton swabs and left for 10 min for culture absorption. Multiple 6-mm wells were made by punching the bacteria-coated Muller-Hinton agar plates using a cylinder glass tube. The concentrations of NP fractions obtained from different sucrose gradient were normalized by adding dH2O to make the NP suspension to an identical optical density value. Fifty microliters of normalized or non-normalized NP samples of each fraction were added into each well in triplicate and incubated overnight. Then the laser-generated Ag NPs were added to the culture to give a final concentration of 20 μg/mL and incubated at 37°C overnight. The cells were fixed with 4% formaldehyde containing 2.5% glutaraldehyde in 0.1 M Hepes buffer (pH 7.2) for 1 h and then treated sequentially with 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer (ph 7.2) for 1 h and then treated sequentially with 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer (ph 7.2) for 1 h, 1% tannic acid in 0.1 M cacodylate buffer (pH 7.2) for 1 h, and finally, 1% uranyl acetate for 1 h. The samples were dehydrated in ethanol series infiltrated with TAAB low viscosity resin and polymerized for 24 h at 60°C. Sections of 70 nm were prepared with Reichert Ultracut ultramicrotome and observed with FEI Tecnai 12 Biotwin microscope at 100-kV accelerating voltage with Gatan Orius SC1000 CCD camera (Gatan, Inc., Pleasanton, CA).

Bacterial oxidative stress reactive oxygen species test
The 2,7-dichlorofluorescin diacetate (DCFH-DA, Sigma-Aldrich) was used to test ROS generation in E. coli bacterial cells. First of all, 100 μL of laser-generated Ag NPs with different sizes was incubated with 0.9 mL of bacterial culture suspension (10⁴ CFU/mL) for 5 h at 37°C in triplicate with shaking at 225 rpm. The bacterial cells were then pelleted by centrifugation at 1000 g for 5-10 min. Hydrogen peroxide (H2O2, 20 μg/mL) was used as a positive control. The bacterial cell pellet was suspended in 1-mL LB broth, and DCFH-DA reagent was added to the cell suspension to give a final concentration of 100 μM and then incubated for 30 min and in a shaking incubator at 37°C in dark. Two hundred microliters of the cell suspension were transferred to a 96-well opaque white microplate, and the fluorescence of each sample was measured at a wavelength of 530 nm and excitation of 515 nm using the fluorescence spectrophotometer (Omega) (Korshed et al., 2016).

Human cell cytotoxicity test
Human lung adenocarcinoma cell line (A549) (Goel et al., 2007) was obtained from American Type Culture Collection (ATCC-LGC, UK) and cultured in Dulbecco's modified Eagle medium (DMEM) that was supplemented with 10% FBS and 1% of penicillin/streptomycin. Primary human dermal fibroblast cells (HDFc) C0135C were purchased from Invitrogen (Ramachandran et al., 2010) and cultured in DMEM/F12 media. The cell viability was tested using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mosmann, 1983) to test the toxicity of three different sizes of laser-generated Ag NPs (average size of 19, 21, and 42 nm, respectively) as described in our previous publications (Korshed et al., 2018; Korshed et al., 2016).

Transmission electron microscopy
For the imaging of nanoparticles interactions with the HDFc, the cells were first seeded in a 10-cm Petri dish and incubated for overnight. Then the laser-generated Ag NPs were added to the culture to give a final concentration of 20 μg/mL and incubated at 37°C overnight. The cells were fixed with 4% formaldehyde containing 2.5% glutaraldehyde in 0.1 M Hepes buffer (pH 7.2) for 1 h and then treated sequentially with 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer (ph 7.2) for 1 h, 1% tannic acid in 0.1 M cacodylate buffer (pH 7.2) for 1 h, and finally, 1% uranyl acetate for 1 h. The samples were dehydrated in ethanol series infiltrated with TAAB low viscosity resin and polymerized for 24 h at 60°C. Sections of 70 nm were prepared with Reichert Ultracut ultramicrotome and observed with FEI Tecnai 12 Biotwin microscope at 100-kV accelerating voltage with Gatan Orius SC1000 CCD camera (Gatan, Inc., Pleasanton, CA).
For transmission electron microscopy (TEM) imaging of the fractions of Ag NPs obtained from sucrose gradient centrifugation, a copper microgrid mesh/200 was incubated on the sample solution droplets and blotted with filter paper until a thin layer of the solution left on the grid, which then was air dried.

Statistical analysis
Data in this study were presented as mean ± SE. One-way ANOVA with post hoc Tukey HSD test was conducted for all data to evaluate the statistical differences between samples. $P \leq 0.05$ was considered as statistically significant.

Results
Separation of laser-generated silver nanoparticles using sucrose gradient centrifugation
Following sucrose gradient centrifugation, samples of the laser Ag NPs were separated into six distinct bands, which displayed different colors (Fig. 1), suggesting successful separation of NPs by sizes and possibly shapes in the preparation. The top band (Fraction 1) looked clear, indicating that minimum amount of NPs exists in this fraction. The three bands below (Fractions 2-4) were darker, suggesting that majority of the NPs were accumulated in these three fractions (Fig. 1). Each fraction was collected into different tubes and subjected to ultraviolet spectrometry analysis.

Ultraviolet-visible absorption spectra for each fraction of the laser Ag NP samples was shown in Figure 2. The measurement indirectly reflects the amount of NPs in each layer. The peak absorbance for the six fractions of the laser Ag NPs recovered from the sucrose gradient were 0.02, 2.50, 1.00, 0.80, 0.40, and 0.20, respectively (Fig. 2).

Samples of each fraction of the laser Ag NPs were then subjected to TEM imaging in order to determine the size of NPs in each fraction. Prior to gradient centrifugation, the laser Ag NPs had mixed sizes ranging 10-70 nm. After centrifugation, the NPs were clearly separated into different fractions according to their size (Fig. 3, Table 1). In the original sample before gradient centrifugation, the laser Ag NPs were mainly spherical. After gradient centrifugation, the upper layers (Fractions 1 and 2) of the gradient almost consisted of small spheres, and the bottom layer (Fraction 6) consisted of irregular larger spheres, while the layer in between (Fractions 3, 4, and 5) mainly consisted of nanospheres with fewer large spherical Ag NPs. The amount of Ag NPs in the first layer was very low (Fig. 2), for which we could not generate an accurate measurement by TEM, but the NP sizes were less than 15 nm.

Antibacterial test of laser silver nanoparticles
Equal volumes of each fraction of Ag NPs recovered from the sucrose gradient centrifugation were subject to antibacterial test against \textit{E. coli}. Results showed that each fraction had distinctively different antibacterial
The effects of Fractions 1–4 with an average NP size <32 nm were much significant as compared with Fractions 5 and 6 that contained larger Ag NPs (Fig. 4). Strikingly, the antibacterial effect of Fraction 1 with NP size ≤15 nm was also highly effective (Fig. 4), regardless of the very low amount of Ag NPs in the sample, demonstrating the effectiveness of the smaller Ag NPs in killing bacteria.

Although the laser Ag NPs in each sucrose fraction were reconstituted in dH2O to give the same volume, the absolute amounts of Ag NPs in each fraction were different as shown in Figure 2. To more accurately compare the size-dependent bactericidal effects of the laser Ag NPs, all fractions of the Ag NPs from the gradient centrifugation were normalized to the lowest peak absorbance value 0.2 by diluting the Ag NPs with dH2O, except for Fraction 1 where the amount of the NPs were too low to be included in the normalization experiment. Equal volume of the normalized fractions was then evaluated for their antibacterial effect. Results showed that the normalized Fraction 2 that had the smallest average NP size (19 nm) showed the best antibacterial effect among the all fractions (Fig. 5). Fraction 3 also had significantly higher antibacterial effect as compared with Fractions 4, 5, and 6 (Fig. 5). The results reinforced the notion that antibacterial activities were inversely correlated to the size of the NPs.

Generation of reactive oxygen species
Reactive oxygen species generation was measured using DCFH-DA assay. We found that Ag NPs from Fractions 1–4 with NP sizes below 32 nm produced significant amount of ROS from the bacterial cells as compared with the NP-free control (Fig. 6). Fraction 2 with an average particle size of 19 nm induced the highest ROS generation, while Fractions 5 and 6 with larger average sizes showed no significant increase in ROS generation compared with the control (Fig. 6). Intestinally, Fraction 1, which contained a minimum amount of Ag NPs, also induced significant ROS generation. This observation is in line with the significant antibacterial effect of Fraction 1 as shown in Figure 4, suggesting the importance of ROS in contributing to the antibacterial effect for the smaller sized Ag NPs.

Toxicity test of laser silver nanoparticles
To determine if there is any size-dependent NP toxicity to human cells, we measured the cytotoxicity of the laser Ag NPs against HDFc and human lung adenocarcinoma cell line (A459). Three fractions of the laser Ag NPs obtained from gradient centrifugation with average sizes of 19 nm (Fraction 2), 21 nm (Fraction 3), and 42 nm (Fraction 5) were added to the HDF and A459 cultures for 72 h. Results showed that laser Ag NPs had no significant toxicity compared with the NPs-free control, suggesting a lack of clear size-dependent toxicity to the two types of human cells (Fig. 7).

Figure 8 shows the TEM images of HDFc treated with smaller sized (19 nm, Fraction 2) or larger sized (42 nm, Fraction 5) laser Ag NPs from sucrose gradient centrifugation. There was no obvious invasion of the laser NPs into the dermal fibroblast cells for both Fractions, which agreed with the results obtained from the cytotoxicity analysis earlier, suggesting a low cytotoxicity of the laser Ag NPs and lack of size-dependent toxicity to human dermal fibroblasts in this experiment.

Discussions
In this study, we describe the use of sucrose gradient centrifugation to separate laser-generated Ag NPs into different sized fractions and demonstrate size-related antibacterial effects and ROS generation in E. coli by the laser Ag NPs. We also show a low cytotoxicity to the human cells for different sized laser Ag NPs.

In this work, laser Ag NPs were prepared using picosecond laser ablation of solid targets in deionized water and subjected to the sucrose density gradient centrifugation to obtain different sized Ag NPs. Gradient centrifugation of NPs is considered as an effective method in separation of NPs based on their diameters (Xiong et al., 2011). Different methods have been exploited to separate NPs according to their sizes and shapes,
Figure 3. Transmission electron microscopy (TEM) images of sucrose gradient fractions of laser-generated silver nanoparticles (Ag NPs). Laser-generated Ag NPs were separated by sucrose gradient centrifugation. Fractions of the laser Ag NPs obtained from the gradient centrifugation as well as the stock sample prior to fractionation were imaged using the FEI Tecnai 12 Biotwin TEM.

Table 1. Laser NPs size and shape obtained from TEM analysis.

| NPs layers          | Ag NPs size (nm) | Size range (nm) | Morphology                        |
|---------------------|------------------|-----------------|-----------------------------------|
| Fraction 1          | ≤15              | -               | Very small nanospheres            |
| Fraction 2          | 19               | 15-30           | Small nanospheres                 |
| Fraction 3          | 21               | 15-40           | Nanospheres                       |
| Fraction 4          | 32               | 20-45           | Mixed spheres                     |
| Fraction 5          | 42               | 35-55           | Mixed spheres                     |
| Fraction 6          | 47               | 30-70           | Irregular spheres                  |
| Stock solution      | 27               | 10-70           | Mixture of big and small nanospheres |

Ag NPs, silver nanoparticles; TEM, transmission electron microscopy.
such as filtration, centrifugation, selective precipitation, size exclusion chromatography, selective oxidation or etching, and electrophoresis, among which the density gradient centrifugation is one of the most convenient methods for this purpose. This method does not use the liquid-solid phase interaction, and there is no need for chemical reactions (Xiong et al., 2011). Examples of using gradient centrifugation include iodixanol gradient sorting of FeCo@C nanocomposites; sucrose gradient separation of chemically modified graphene (Xiong et al., 2011); CsCl2 density separation of gold NPs (Chen et al., 2009); and nonhydroxylic organic density gradient separation of CdSe NPs (Bai et al., 2010). Our current experiment showed that the sucrose density gradient centrifugation method is viable to be used for the separation of different sized Ag NPs. Results agreed with previous studies using sucrose as a density gradient and demonstrated that NPs with large diameters sedimented faster than the smaller ones. This could be explained by the fact that NPs sedimentation is diameters-dependent rather than mass-dependent (Li et al., 2011).

Results in the study demonstrated that laser Ag NPs with an average size of 19 nm displayed the most effective antibacterial activity against *E. coli*. Together with the subsequent fractions with size ranges of 19-47 nm, the bactericidal activities were inversely correlated to the particle sizes. Our results highlighted...
the ability of smaller sized laser produced Ag NPs in achieving best bacterial effects. Therefore, the antibacterial ability of laser Ag NPs is size-dependent, which agrees with what was reported in the previous study for Ag NPs generated using non-laser methods (Liu et al., 2010).

The reason for the size-dependent antibacterial effects of NPs could be explained by the fact that smaller NPs had larger surface areas, enabling the particles to release more Ag ions (Ag⁺), in addition to having better contacts with the bacterial cell membranes. This could explain the relatively high antibacterial activity of Fraction 1 that contained a very low amount of small-sized laser Ag NPs.

Ag⁺ was considered as the key factor responsible for the toxicity of Ag NPs to bacterial cells (Guggenbichler et al., 1999; Roe et al., 2008). Ag⁺ attacks bacterial respiratory chain and binds to sulfhydryl group within the cellular membrane and essential enzymes within the cell, leading to bacterial damage (Guggenbichler et al., 1999; Roe et al., 2008). In the current study, the antibacterial activity of laser-generated NPs was determined using the well diffusion method. This method is excellent in determination of the antibacterial activity of Ag⁺ due to the free movement of Ag⁺ to diffuse through the agar matrices in the bacterial culture plate. However, it could underestimate the bactericidal effect caused by direct interactions of NPs with the bacteria.

We found that the laser-generated Ag NPs elevated ROS generation in a size-dependent manner. Ag NPs with an average size of 19 nm had the highest ROS generation, and there was no significant ROS generation for the larger sized laser Ag NPs such as Fractions 5 and 6. This could be due to the larger surface area of the smaller sized Ag NPs that are available for interactions with bacterial cells as mentioned earlier (Liu et al., 2010; Samberg et al., 2011) and also the fact that smaller Ag NPs tend to release more Ag⁺, which could cause further ROS generation, DNA damages, and then cell death (Oberdorster et al., 2005; Navarro et al., 2008). This result is in line with the relatively high antibacterial effect of the laser Ag NPs in Fraction 1 and could explain why this Fraction showed significant ROS generation.
In this study, we investigated the toxicity of three different sized laser Ag NPs (19, 21, and 42 nm) on two human cell models, human lung adenocarcinoma cell line A459 and HDFc, using previously established method and conditions in our research group (Korshed et al., 2016). The reason for choosing the lung and skin cell types was because inhalation or skin contact are common routes for nanoparticle exposure to human. As shown in Figures 7 and 8, the human cell toxicity by the smaller average sized Ag NPs (19 nm) was not significantly different from that of the larger sized Ag NPs (42 nm). This indicates that toxicity of the laser Ag NPs to human cells is unlikely size-related in this study, which is in line with studies reported previously (Connor et al., 2005; Goodman et al., 2004). Additionally, we did not observe obvious uptake of the laser-generated Ag NPs by the cultured fibroblasts, although we found in our previous study that a very low amount of non-fractionated laser Ag NPs entered the same type of cells (Korshed et al., 2016). It could be because that the amount of fractionated Ag NPs used in this study was low or the Ag NPs aggregated to a certain degree after being added to the cell culture. In either case, the toxicity of the laser Ag NPs was low to human cells.

One phenomenon that we could not entirely explain in this study was the ultraviolet spectra peak position for Fractions 4 and 6 as shown in Figure 2. Generally speaking, ultraviolet spectra are NP size-dependent with blue shift for smaller particle sizes. The ultraviolet spectrum of Fractions 1, 2, 3, and 5 agreed well with the expectation, but Fractions 4 and 6 were exceptional. It is possible that residue sucrose in the sample could influence the position of peak absorbance. Aggregation could also be an impact factor, but we did not observe significant aggregation on TEM images. Nevertheless, Fractions 4 and 6 agreed well with size-dependent antibacterial effect for the laser-generated Ag NPs, and these two fractions were not used for the cytotoxicity experiments.

Conclusions

We have successfully separated the laser-generated Ag NPs into different sized fractions using sucrose density gradient centrifugation. Smaller laser Ag NPs, in particular the ones with an average size below 19 nm, had better antibacterial activities compared with the larger Ag NPs, suggesting an inverse correlation between the antibacterial effect and the NP sizes. ROS generation that was induced in bacterial cells by the laser Ag NPs was also NP size-dependent and agreed well with the antibacterial effect for the corresponding Ag NP fractions. The laser-generated Ag NPs with different average sizes (19, 20, and 42 nm) displayed low toxicity to human dermal fibroblasts and lung epithelial cells after co-culture for 72 h in vitro.
Acknowledgments

We would like to thank Dr. Caroline Ridley from the School of Biological Sciences, The University of Manchester, for her assistance in the sucrose gradient centrifugation. P. K was supported by a Ph.D. studentship from Kurdistan Regional Government.

Conflict of Interest

The authors report no conflicts of interest in this work.

REFERENCES

Asghari, S., Johari, S. A., Lee, J. H., Kim, Y. S., Jeon, Y. B., Choi, H. J., Moon, M. C., and Yu, I. J. 2012. Toxicity of various silver nanoparticles compared to silver ions in Daphnia magna. J. Nanobiotechnol. 10(14):1-14.

Ashe, B. (2011). A detail investigation to observe the effect of zinc oxide and silver nanoparticles in biological system.

Bai, L., Ma, X., Liu, J., Sun, X., Zhao, D., and Evans, D. G. 2010. Rapid separation and purification of nanoparticles in organic density gradients. Journal of the American Chemical Society. 132(7):2333-2337.

Carlson, C., Hussain, S. M., Schrand, A. M., Braydich-Stolle, L. K., Hess, K. L., Jones, R. L., and Schlager, J. J. 2008. Unique cellular interaction of silver nanoparticiles: size-dependent generation of reactive oxygen species. J. Phys. Chem. B 112(43):13608-13619.

Center, W. W. 2011. An inventory of nanotechnology-based consumer products currently on the market. The Project on Emerging Nanotechnologies.

Chen, G., Wang, Y., Tan, L. H., Yang, M., Tan, L. S., Chen, Y., and Chen, H. 2009. High-Purity separation of gold nanoparticle dimers and trimers. Journal of the American Chemical Society. 131(12):4218-4219.

Choi, O., and Hu, Z. 2008. Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria. Environ. Sci. Technol. 42(12):4583-4588.

Connor, E. E., Mwamuka, J., Gole, A., Murphy, C. J., and Wyatt, M. D. 2005. Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity. Small 1(3):325-327.

El Badawy, A. M., Silva, R. G., Morris, B., Scheckel, K. G., Suidan, M. T., and Tolaymat, T. M. 2011. Surface charge-dependent toxicity of silver nanoparticles. Environ. Sci. Technol. 45(1):283-287.

El-Nour, K. M. A., Eftaiha, A. a., Al-Warthan, A., and Ammar, R. A. 2010. Synthesis and applications of silver nanoparticles. Arab. J. Chem. 3(3):135-140.

Espinosa-Cristobal, L. F., Martinez-Castanon, G. A., Martinez-Martinez, R. E., Loyola-Rodriguez, J. P., Patino-Marin, N., Reyes-Macias, J. F., and Ruiz, F. 2009. Antibacterial effect of silver nanoparticles against Streptococcus mutans. Mater. Lett. 63(29):2603-2606.

Goel, A., Prasad, A. K., Parmar, V. S., Ghosh, B., and Saini, N. 2007. 2,4-Dihydroxy-4-methylcinnamol induces apoptosis of human lung adenocarcinoma cells by ROS-independent mitochondrial pathway through partial inhibition of ERK/MAPK signaling. FEBS Lett. 581(13):2447-2454.

Goodman, C. M., McCusker, C. D., Yilmaz, T., and Rotello, V. M. 2004. Toxicity of gold nanoparticles functionalized with cationic and anionic side chains. Bioconjug. Chem. 15(4):897-900.

Guggenbichler, J. P., Boswald, M., Lugauer, S., and Krall, T. 1999. A new technology of microdispersed silver in polyurethane induces antimicrobial activity in central venous catheters. Infection 27(1):516-523.

Korshed, P., Li, L., Liu, Z., Monrov, A., and Wang, T. 2018. Antibacterial mechanisms of a novel type picosecond laser-generated silver-titanium nanoparticles and their toxicity to human cells. Int. J. Nanomedicine 13:89.

Korshed, P., Li, L., Liu, Z., and Wang, T. 2016. The molecular mechanisms of the antibacterial effect of picosecond laser generated silver nanoparticles and their toxicity to human cells. Plos One 11(8):e0160078.

Li, S. A., Chang, Z., Liu, J. F., Bai, L., Luo, L., and Sun, X. M. 2011. Separation of gold nanorods using density gradient ultracentrifugation. Nano Res. 4(8):723-728.

Liu, W., Wu, Y., Wang, C., Li, H. C., Wang, T., Liao, C. Y., Cui, L., Zhou, Q. F., Yan, B., and Jiang, G. B. 2010. Impact of silver nanoparticles on human cells: effect of particle size. Nanotoxicology 4(3):319-330.

Morones, J. R., Elicheguerra, J. L., Camacho, A., Holt, K., Kouri, J. B., Ramirez, J. T., and Yacaman, M. J. 2005. The bactericidal effect of silver nanoparticles. Nanotechnology 16(10):2346-2353.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65(1-2):55-63.

Navarro, E., Picciapieta, F., Wagner, B., Marconi, F., Kaegi, R., Ozdak, N., Sigg, L., and Behra, R. 2008. Toxicity of silver nanoparticles to Chlamydomonas reinhardtii. Environ. Sci. Technol. 42(23):8909-8914.

Ober dorster, G., Ober dorster, E., and Ober dorster, J. 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ. Health Perspect. 113(7):823-839.

Pal, S., Tak, Y. K., and Song, J. M. 2007. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium Escherichia coli. Appl. Environ. Microbiol. 73(6):1712-1720.

Panacek, A., Kvitek, L., Prucek, R., Kolar, M., Vecerova, R., Pizurova, N., Sharma, V. K., Nevecna, T., and Zboril, R. 2006. Silver colloid nanoparticles: synthesis, characterization, and their antibacterial activity. J. Phys. Chem. B 110(33):16248-16253.

Ramachandran, S., Rajendra Prasad, N., and Karthikeyan, S. 2010. Sesamol inhibits UVB-induced ROS generation and subsequent oxidative damage in cultured human skin dermal fibroblasts. Arch. Dermatol. Res. 302(10):733-744.

Rasmussen, J. W., Martinez, E., Louka, P., and Wingett, D. G. 2010. Zinc oxide nanoparticles for selective destruction of tumor cells and potential for drug delivery applications. Expert Opin. Drug Deliv. 7(9):1063-1077.

Roe, D., Karandikar, B., Bonin-Savage, N., Gibbins, B., and Roullet, J. B. 2008. Antimicrobial surface functionalization of plastic catheters by silver nanoparticles. J. Antimicrob. Chemother. 61(4):869-876.

Samberg, M. E., Orndorff, P. E., and Monteiro-Riviere, N. A. 2011. Antibacterial efficacy of silver nanoparticles of different sizes, surface conditions and synthesis methods. Nanotoxicology 5(2):244-253.

Sotiriou, G. A., and Pratsinis, S. E. 2010. Antibacterial activity of nanosilver ions and particles. Environ. Sci. Technol. 44(14):5649-5654.

Xiong, B., Cheng, J., Qiao, Y., Zhou, R., He, Y., and Yeung, E. S. 2011. Separation of nanorods by density gradient centrifugation. J. Chromatogr. A 1218(25):3823-3829.

Xu, Z. M., Zhang, Q. B., Puppala, H. L., Colvin, V. L., and Alvarez, P. J. 2012. Negligible particle-specific antibacterial activity of silver nanoparticles. Nano Lett. 12(8):4271-4275.

Size-related bactericidal effects of laser Ag nanoparticles