Biomaterials 35 (2014) 6707–6715

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The influence of lysosomal stability of silver nanomaterials on their toxicity to human cells

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ARTICLE INFO

Article history:
Received 27 March 2014
Accepted 1 May 2014
Available online 28 May 2014

Keywords:
Nanotoxicity
Silver nanoclusters
Reactive oxygen species
Oxidative dissolution
Lysosome degradation
Safety redesign of nanoparticles

ABSTRACT

How silver nanomaterials (Ag NMs) could induce toxicity has been debated heatedly by many researchers. We utilized Ag nanoclusters (Ag NCs) with the same size and ligand protection but different core surface speciation. Ag^+-rich NCs (Ag^+-R NCs) and their counterpart, the reduced Ag^0-rich NCs (Ag^0-R NCs) are synthesized to represent possible dichotomous stages in silver nanomaterial degradation process. Here we show Ag^0-R NCs induce higher cellular toxicity when compared to Ag^+-R NCs. This cellular toxicity is brought about *via* the modulation of reactive oxygen species (ROS) in cells as a result of the more rapid release of Ag species from Ag^0-R NCs and subsequent oxidation into Ag^+ in the lysosomal compartment. The weaker Ag^0-R bond greatly potentiated the release of Ag species in the acidic and enzymatic processes within the lysosomes. Since lysosomes are absent in bacteria, increasing silver nanomaterials stability may lower toxicity in mammalian cells whilst not reducing their efficacy to fight bacteria; this redesign can result in a safer silver nanomaterial.

1. Introduction

Humans are gradually being exposed to nanomaterials through consumer products that contain nanomaterials [1]. Many of these nanomaterials which were considered as benign in their bulk phase have displayed unusual interactions with the cells [2–5]. Considering the potential hazards of nanomaterials, more research efforts have been focused on understanding these nano-bio interactions to assess their potential toxicity [6–8] and later based on these understanding of the mechanisms involved; rational redesign of nanomaterials becomes possible. Silver nanomaterials (Ag NMs) could be found in common consumer products due to their excellent anti-microbial property [3,9,10]. However, Ag NMs have been reported to induce developmental abnormalities in zebra fish embryo [11], disrupt the cell membrane [12], and induce genotoxic and cytotoxic damage to human lung fibroblast (IMR90) and glioblastoma cells (U251) [13] in addition to a system-wide suppression of the immune system [14]. In order to produce a safe functional Ag NM, it is important to understand how its mechanism of toxicity might arise.

It has been suggested that the biological response to Ag NMs, ranging from simple life forms like bacteria to higher order organisms, could be induced by Ag NMs following the canonical toxicity mechanism of nanoparticle dissolution [15–19]. Specifically in bacterial toxicity, Ag^+ release from Ag NMs in aerobic condition has been suggested to induce cell toxicity [20], though this remains as an assumption. Mounting evidence suggests discrepancies on which form of Ag takes to induce toxicity in the eukaryotic cells. Dissolution of Ag NMs to Ag^+ species has been counteracted by adding cysteine and thus eliminating the toxicity in freshwater algae, *Chlamydomonas reinhardtii* [21]. Similarly, Ag^+ ions induced significantly higher cell death both in human alveolar epithelial cells (A549) and human bronchial epithelial cells (BEAS-2B) when compared to added Ag NMs [22]. In contrast, Ag NMs (5 nm) elicited more toxic response from A549, HepG2, MCF7 and

Abbreviations: ALDH4A1, aldehyde dehydrogenase 4 family member A1; BAD, Bcl-2-associated death promoter; BAX, Bcl-2-associated X protein; BCL2, B-cell lymphoma 2; CAT, catalase; DWC, dry cell weight; GPX1, glutathione peroxidase 1; LD_{50}, lethal dose 50% population killed; NAC, N-acetyl cysteine; NADH, nicotinamide adenine dinucleotide; NCs, nanoclusters; NMs, nanomaterials; ROS, reactive oxygen species; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2.

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http://dx.doi.org/10.1016/j.biomaterials.2014.05.007
0142-9612/

SGC 7901 cells compared with Ag^+ ions. The notion is supported in *Lolium multiforum* where again Ag NMs induced higher toxicity than Ag^+ ions of the same Ag basis concentration [23]. Kawata et al. suggested that both Ag NMs and their ion counterpart were responsible for the cellular toxicity observed in hepatocarcinoma cells (HepG2) [24]. Due to the more complex sub-cellular organization, intracellular processes and other major differences between eukaryotic and prokaryotic cells, it appeared that the Ag NMs toxicity mechanism in the bacterial system may not be fully translatable to explain the toxicity in eukaryotic and certainly mammalian systems. There is however, a coherent thread stringing the seemingly contradictory findings about Ag^+ ions or Ag NMs toxicity. It appears that the highest toxicity must have lain within the evolutionary processing of Ag species from its nanoparticulate state to a partially disintegrated state and finally to its oxidized ionic form. Since the oxidized ionic Ag^+ species, it would be logical to predict that within the evolutionary mechanism, silver must have been oxidized. Therefore, we attempted to concentrate on the currently unknown part of partially disintegrated Ag NMs and Ag either must have taken on an Ag^+ or an Ag^0state on the surface of this partially degraded form. As this state is a kinetic transition which would be extremely difficult to capture experimentally, we modeled this "transient state" by introducing ligand-protected Ag nanoclusters (or Ag NCs with core sizes below 2 nm) which fit snugly between the relatively large Ag NMs (6–20 nm, with well-characterized toxicity profiles in other papers) and the singlet Ag^+ ions. Furthermore, Ag NCs utilization, with a well-controlled Ag^+ or Ag^0 core, could help to clarify whether the charge itself plays a role in the often reported toxic outcome of Ag NMs on mammalian cells.

2. Experimental details

2.1. Materials

Ultrapure water (18.2 MΩ cm; Milli-Q ultrapure water system, Millipore, USA) was used throughout the study. All glassware were washed with *aqua regia*, and rinsed with abundant water and ethanol before drying in an oven. Ammonium chloride (NH_4Cl), nitric acid (HNO_3), silver nitrate ($AgNO_3$) and sodium hydroxide (NaOH) from Merck; acetic acid, sodium borohydride ($NaBH_4$), L-glutathione reduced (GSH), Dulbecco's Modified Eagle's Medium (DMEM), protease inhibitor, propidium iodide (PI), 2′, 7′-dichlorodihydrofluorescein (DCFH-DA), and *N*-acetyl cysteine (NAC) from Sigma–Aldrich; Fetal Bovine Serum (FBS) from Gibco; Penicillin/Streptomycin cocktail from GE Healthcare; Hoechst 33342 from Invitrogen; Nucleospin RNA from Macherey–Nagel; RevertAid™ H Minus Reverse Transcriptase kit from Fermentas; KAPA SYBR® FAST qPCR kit from KAPA Biosystem; were used as received.

2.2. Methods

2.2.1. Synthesis and characterization of GSH-Ag^+ NCs and GSH-Ag^0 NCs

Silver nanocluster (Ag NCs) employed in this study was synthesized according to previously reported cyclic $NaBH_4$ reduction-decomposition method [3,25,26]. Typically, aqueous solutions of GSH (150 μL, 50 mM), $AgNO_3$ (125 μL, 20 mM), and $NaBH_4$ [50 μL, 112 mM, prepared by introducing 43 mg $NaBH_4$ powder into a diluted NaOH solution (10 mL, 0.2 M)] were separately introduced into a glass vial containing 4.7 mL water under stirring condition to form a deep-red solution of Ag NCs (~5 mL) in 5 min. After incubation of ~3 h at room temperature, the deep-red solution was changed to colorless due to the thiolate-etching-induced NC decomposition. A $NaBH_4$ solution (50 μL) was then added into the colorless solution under stirring condition, and a light-brown Ag NC solution was obtained after ~20 min. Without stirring, the light-brown Ag NC solution was further incubated for 20 h at room temperature, and a strong red luminescence could be emitted from the solution. The as-synthesized highly luminescent GSH-Ag^+-R NCs were collected and purified by utilizing a Millipore 8010 ultrafiltration system (with the use of a membrane of 3000 Da MWCO). The GSH-Ag^0-R NCs could be obtained by introducing the $NaBH_4$ solution (15 μL) into the solution of GSH-Ag^+-R NCs (5 mL) and stirring for 5 min at room temperature.

UV–vis and luminescence spectra were acquired on a Shimadzu UV-1800 spectrometer and a PerkinElmer LS55 fluorescence spectrometer, respectively. Transmission electron microscopy (TEM) images were obtained on a JEOL JEM 2010 microscope operating at 200 kV. X-Ray photoelectron spectroscopy (XPS) measurements were conducted on a Kratos AXIS Ultra^{DLD} spectrometer (Kratos Analytical Ltd.) with a mono Al Kα radiation source ($h\nu$ = 1486.71 eV) operating at 15 kV and 5 mA.

2.2.2. Cell culture and cell exposure to Ag NCs treatment

Human neonatal foreskin fibroblast cells (BJ) were grown in cell culture medium comprises of DMEM, 10% FBS, and 1% Penicillin/Streptomycin cocktail. Standard cell culture condition (37 °C, 5% CO_2) was employed at all time during the study. In this study, BJ cells were seeded overnight at initial density of 3×10^4 cells/cm^2 before treatment.

Ag NCs stock solution (4 mM) was added into cell culture medium to yield treatment solution of 62.5, 250 and 1000 μM. Following that the treatment solution was sonicated with probe sonicator (Qsonica, USA) for 1 min to ensure dispersion of Ag NCs in the cell culture medium.

In rescue studies which involve the addition of NAC or NH_4Cl, the cells were pre-equilibrated with the said chemicals for 1 h prior to Ag NCs exposure.

2.2.3. Cell viability measurement

Cell viability following Ag NCs exposure was determined with Tali™ image based cytometer (Invitrogen, USA). Briefly, floating cells and adherent cells were collected, washed thrice with 1× PBS, and stained with PI for 5 min in dark before assaying with Tali™ image based cytometer.

2.2.4. ROS level measurement

Measurement of ROS was carried out by monitoring 2′, 7′-dichlorofluorescein (DCF) signals with microplate reader (Tecan, Switzerland). Following exposure to Ag NCs, the cells were washed thrice and were stained with DCFH-DA (final concentration of 10 μM) and Hoechst 33342 (final concentration of 1 μg/mL) for 30 min. Upon reaction with intercellular ROS, the DCFH-DA was cleaved to produce fluorescent species, DCF (Ex 488 nm/Em 535 nm). The ROS level was normalized to cell number which could be correlated to Hoechst 33342 signal (Ex 350 nm/Em 461 nm).

For acellular measurement of ROS, the cells were lysed in ultrapure water with high frequency sonication for 1 min in ice bath. Afterwards, the cell debris was separated by centrifugation (10,000× *g*, 10 min, 4 °C). Protein lysate was divided into 2 equal parts, whereby one was adjusted with acetic acid to mimic the lysosome environment (pH 4.8) and the other was adjusted to mimic the cytosolic environment (pH 6.9). Afterward, the lysate was added with Ag NCs stock solution to make up a solution with final concentration of 1 mM Ag NCs. In addition, the lysate–Ag NCs mix was further added with DCFH-DA (final concentration of 10 μM) and Hoechst 33342 (final concentration of 1 μg/mL). Separate blanks were prepared without the addition of DCFH-DA and Hoechst 33342. After 30 min exposure time, DCF and Hoechst 33342 signals were detected with microplate reader. The reading of each variable was corrected with its respective blank.

2.2.5. Ag NCs uptake measurement

The cells were treated with Ag NCs for 6 h and then were washed thrice with 1× PBS to remove any residual Ag NCs which hadn't been uptaken by the cells. Following that the cells were collected and freeze dried. The freeze dried cell pellet then was acid digested with 5% HNO_3 and analyzed for its Ag content using ICP-MS (Agilent 7500, Agilent, USA). The measured Ag content then was normalized back to the dry weight of the cell pellet (DWC).

2.2.6. Acellular Ag NCs stability

For acellular determination of Ag NCs' stability, Ag^+-R and Ag^0-R NCs were incubated either in the lysosome mimic solution (pH 4.8) or cytosolic mimic solution (pH 6.9) at 37 °C for 12 h. The free Ag^+ ions derived from the decomposition of Ag NCs were separated by ultrafiltration process using membranes with molecular weight cut-off (MWCO) of 3000 Da and consequently remained in the filtrates. The corresponding filtrates were analyzed by using inductively coupled plasma mass spectrometry (ICP-MS). Ag NCs stability was measured by the amount of Ag ions being released in reference to the initial Ag NCs concentration.

2.2.7. RNA extraction and quantitative RT-PCR

RNA was isolated with Nucleospin RNA kit according to the manufacturer's instructions. First strand cDNA was synthesized using RevertAid™ H Minus Reverse Transcriptase kit as per manufacturer's instruction. Differences in gene expression were detected using KAPA SYBR® FAST qPCR kit on a CFX96 system (Bio-Rad, USA). Actin expression was used for normalization. Sequences for qPCR primers used are listed below: