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Mitochondrial dysfunction induced by ultra-small silver nanoclusters with a distinct toxic mechanism

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HIGHLIGHTS

- Effects of ultra-small nanoclusters (NCs) were first studied on rat liver mitochondria.
- Ag NCs induced *in vitro* mitochondrial dysfunction on energetic and structural levels.
- Ag NCs interacted with the phospholipid bilayer rather than with MPT pore proteins.
- A possible toxic mechanism of the mitochondrial dysfunction was proposed.

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ABSTRACT

As noble metal nanoclusters (NCs) are widely employed in nanotechnology, their potential threats to human and environment are relatively less understood. Herein, the biological effects of ultra-small silver NCs coated by bovine serum albumin (BSA) (Ag-BSA NCs) on isolated rat liver mitochondria were investigated by testing mitochondrial swelling, membrane permeability, ROS generation, lipid peroxidation and respiration. It was found that Ag-BSA NCs induced mitochondrial dysfunction via synergistic effects of two different ways: (1) inducing mitochondrial membrane permeability transition (MPT) by interacting with the phospholipid bilayer of the mitochondrial membrane (not with specific MPT pore proteins); (2) damaging mitochondrial respiration by the generation of reactive oxygen species (ROS). As far as we know, this is the first report on the biological effects of ultra-small size nanoparticles (~2 nm) at the subcellular level, which provides significant insights into the potential risks brought by the applications of NCs. It would inspire us to evaluate the potential threats of nanomaterials more comprehensively, even though they showed no obvious toxicity to cells or *in vivo* animal models. Noteworthy, a distinct toxic mechanism to mitochondria caused by Ag-BSA NCs was proposed and elucidated.

1. Introduction

Noble metal nanoclusters (NCs), as emerging nanomaterials, with ultra-small size (usually less than 2 nm), bridge the gap between isolated atoms and nanoparticles [1–3]. Due to their unique molecular-like properties, NCs have shown bright prospects in the fields of catalysis, bio-sensing, bio-labeling, bio-imaging, diagnostics and biomedicine [4–9]. Silver NCs are promising agents for next generation bioimaging agents because of their ultrafine size, high chemical stability, and unexceptionable photostability [10], however, their potential biological effects and environmental risks should be assessed comprehensively.

As known to all, the variations of different nanomaterials of physical (size, shape, crystallinity, surface charge) and chemical (surface coating, elemental composition and solubility) attributes will demonstrate significant distinctions to biological and environmental consequences [11,12]. The variety of available experimental protocols revealed that the potential cellular toxicity was usually caused by either their inherent chemical composition (*e.g.*, shedding heavy metal ions) or nanoscale properties (*e.g.*, high sur-

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face activity, extreme specific surface area) [13]. Thus far, most reported biological effects about nanoclusters have focused on protein adsorption, *in vitro* cell culture experiments and several *in vivo* animal model experiments, aiming at evaluating the biological response to Ag NCs exposure [14–20]. However, one should note that nanomaterials may have more or less impact on the sub-cellular level, such as organelles or biomacromolecules, even though they show no obviously adverse effects on cells or *in vivo* models in short term. Until now, there are few publications about the biological effects of nanoclusters at the sub-cellular level.

The isolated rat liver mitochondria, chosen as a sub-cellular level model in this work, are double-membrane organelles, which can be found in most eukaryotic cells. Besides known as energy producers and providers, mitochondria play a central role in cellular metabolism events such as cell derivation and cell-cycle control [21]. Due to their exquisite structures and implicated functions, mitochondria are potential target for xenobiotics. Human diseases (*e.g.*, cardiac disease, aging, Alzheimer disease, Parkinson disease, Huntington disease, Amyotrophic lateral sclerosis, myocardial infarction) were confirmed to be closely linked to mitochondrial dysfunctions [22–27]. Mitochondria have been considered as an important intracellular target for nanomaterials. For example, many evidences showed that nanoparticles (*e.g.*, Ag nanoparticles, carbon nanotubes, and TiO_2 nanoparticles, silica nanoparticles, quantum dots) could impair mitochondria and cause mitochondrial dysfunction with alterations of mitochondrial permeability or generation of reactive oxygen species (ROS) [28–34].

Herein, the biological effects of silver NCs coated with bovine serum albumin (BSA) (Ag-BSA NCs) on isolated rat liver mitochondria were investigated with spectroscopic, microscopic, polarographic (with an oxygen electrode) methods, *etc*. In order to stabilize nanoparticles and alleviate toxicity, we chose BSA as the synthetic template and stabilizer for NCs, which was anticipated to improve the biocompatibility of NCs at the cellular level and eliminate inflammatory response in the body. Our work provides a sub-cellular perspective to explore the biological effects of NCs, showing that nanotoxicity of NCs needed to be comprehensively considered at multiple levels. In addition, our work presented a distinct toxic mechanism of Ag NCs to mitochondrial dysfunction, namely mitochondrial permeability transition (MPT) induced by the interaction of Ag-BSA NCs specifically with phospholipids layer of mitochondrial membrane, not with specific MPT pore proteins. We attribute the mitochondrial toxicity of NCs to their extreme smaller size than common nanoparticles (which usually induce a mitochondria-independent damage by interacting with MPT pore protein and generating ROS) and their high surface activity. To the best of our knowledge, this is the first report on the biological effects of ultra-small nanoclusters at the sub-cellular level.

2. Experimental

2.1. chemicals

Bovine serum albumin (BSA, 66.435 kDa), silver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$), hematoporphyrin (HP), 1,6-diphenyl-1,3,5-hexatriene (DPH), cyclosporine A (CsA), dithiothreitol (DTT), Ruthenium Red (RR), oligomycin, 3-morpholinopropanesulfonic acid (MOPs), adenosine diphosphate (ADP), EDTA, ethylene glycol bis(2-aminoethyl) tetraacetic acid (EGTA), oligomycin, rhodamine 123 (Rh123), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), rotenone, and valinomycin were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO) and used without further purification. All other reagents (NaOH, mannitol, sucrose, Tris–HCl, succinate, KAc, HEPES, KNO_3, KCl and K_2HPO_4) were of analytical grade and purchased form Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All reagents were used as received and aqueous solutions were prepared with deionized water (18.2 MΩ cm^{-1}, Millipore).

2.2. Instrumentation

Centrifugal filter devices were purchased from Millipore (5000MWCO, 0.1 mL, catalog no. UFV5BCC00). Fluorescence emission spectra were recorded on a LS 55 spectrometer (PerkinElmer, USA). Absorption spectra were obtained with a UV-6100PC double beam spectrometer (MAPADA, China). High resolution transmission electron microscopy (HRTEM) images were recorded on a JEOL JEM-2100 (HR) electron microscope operating at 200 kV. X-ray photoelectron spectroscopy (XPS) was carried out on a KRATOS XSAM800 X-ray photoelectron spectrometer using Mg as the exciting source. The *zeta* potential of nanoclusters was determined in ultrapure water by Nano-ZSZEN3600 (Malvern Instruments) at 25 °C.

2.3. The preparation of BSA-confined Ag nanoclusters

Ag-BSA NCs were prepared by a reported method with a little modification [35]. Typically, 500 μL of 100 mM $AgNO_3$ solution was dropped into 10 mL of 50 mg mL^{-1} BSA solution with vigorous stirring (1000 rpm) at room temperature. Then, the pH of the mixture was adjusted to 11 with addition of 1 M NaOH, which turned out to be clear and transparent. After stirring for 30 min at room temperature, 100 μL $NaBH_4$ solution (11.2 mM, freshly prepared, containing 0.1 M NaOH) was added dropwise, along with the solution from colorless to reddish–brown in 2.5 h, indicating the formation of various amounts of clusters. The as-synthesized Ag-BSA NCs were concentrated to 1 mL with ultra-filtration (using a pair of 100 kDa ultra-filtration centrifuge tube), then dispersed in 15 mL ultrapure water and ultra-filtrated to 1 mL. By repeating this operation for five times, ions and excessive BSA can be removed completely. The Ag-BSA NCs solution was collected and kept at 4 °C in the dark for further use. The contents of silver element in Ag NCs solution were determined by inductively coupled plasma (ICP) spectrometry (ICPS-7500, Shimadzu, Kyoto, Japan).

2.4. Isolation of liver mitochondria

Rat liver mitochondria were isolated from Wistar rats (150–200 g) according to a typical method, standard differential centrifugation procedures with a little modification, of which the details were described in Section 1, Supplementary material [36]. The mitochondrial protein concentration was determined by the method described by Lowry et al., using bovine serum albumin as the standard [37].

To ensure reasonable activity for every experiment, respiratory control ratio (RCR) was measured. Oxygen consumption rates (state 3 and state 4) of normal isolated rat liver mitochondria were measured polarographically using a Clark-type oxygen electrode, as descried in Section 7, Supplementary material. Measured by electrode (Oxygraph, Hansatech, UK), the appropriate value of RCR (state 3/state 4) showed that mitochondrial membrane structure and function were kept sufficiently [38].

2.5. Mitochondrial swelling and inner membrane permeabilization to H^+ and K^+

Mitochondrial swelling can be investigated by recording the change of absorbance at 540 nm (A540) over 500 s with a UV-6100PC double beam spectrophotometer (MAPADA, China) equipped with 1.0 cm quartz cells at room temperature, as described in Section 2, Supplementary material.