Colloids and Surfaces B: Biointerfaces 141 (2016) 291–300

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

The importance of extracellular speciation and corrosion of copper nanoparticles on lung cell membrane integrity

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ARTICLE INFO

Article history:
Received 16 September 2015
Received in revised form 26 January 2016
Accepted 27 January 2016
Available online 4 February 2016

Keywords:
Copper nanoparticles
Nanotoxicity
Lung cells
Speciation
Corrosion
Membrane damage
DMEM
Equilibrium modeling
UV–vis spectroscopy
Polarography

ABSTRACT

Copper nanoparticles (Cu NPs) are increasingly used in various biologically relevant applications and products, *e.g.*, due to their antimicrobial and catalytic properties. This inevitably demands for an improved understanding on their interactions and potential toxic effects on humans. The aim of this study was to investigate the corrosion of copper nanoparticles in various biological media and to elucidate the speciation of released copper in solution. Furthermore, reactive oxygen species (ROS) generation and lung cell (A549 type II) membrane damage induced by Cu NPs in the various media were studied. The used biological media of different complexity are of relevance for nanotoxicological studies: Dulbecco's modified eagle medium (DMEM), DMEM$^+$ (includes fetal bovine serum), phosphate buffered saline (PBS), and PBS + histidine. The results show that both copper release and corrosion are enhanced in DMEM$^+$, DMEM, and PBS + histidine compared with PBS alone. Speciation results show that essentially no free copper ions are present in the released fraction of Cu NPs in neither DMEM$^+$, DMEM nor histidine, while labile Cu complexes form in PBS. The Cu NPs were substantially more membrane reactive in PBS compared to the other media and the NPs caused larger effects compared to the same mass of Cu ions. Similarly, the Cu NPs caused much more ROS generation compared to the released fraction only. Taken together, the results suggest that membrane damage and ROS formation are stronger induced by Cu NPs and by free or labile Cu ions/complexes compared with Cu bound to biomolecules.

1. Introduction

The physico-chemical properties of nanoparticles (NPs), including copper nanoparticles (Cu NPs), can be tailored and controlled on the nanoscale by changing their characteristics, *e.g.*, size, surface capping, or shape [1,2]. As a consequence, the use of Cu NPs and their areas of applications rapidly increase. This increasing use demands for an improved understanding of potential adverse effects that may be an inevitable consequence of their interactions and transformations in biologically relevant settings. Such fundamental mechanistic knowledge is further essential from a human health and environmental risk perspective [3]. Adverse effects induced by the dispersion of Cu NPs have for example been shown on both human cells and on the environment [4,5].

Cu-containing NPs can induce toxicity *via* several different mechanisms [6–8], creating a situation more complicated compared with, *e.g.*, aqueous Cu species [9]. In addition to the particle effect, the chemical speciation of Cu species in solution (*e.g.*, released from Cu NPs) has been shown to significantly influence the observed toxicity. Free Cu ions and labile Cu complexes have been shown more potent compared with more strongly bound Cu complexes [10–13]. An additional mechanism that enhances the toxic effect of Cu NPs [6,14,15] is *via* the formation of reactive oxygen species (ROS) *via* electrochemical (oxidation) reactions [16,17], catalytic surface reactions [15,18] or *via* Fenton reactions of aqueous Cu species released from Cu NPs [19–21]. An important distinction between NPs of Cu oxides and Cu metal is hence the ability of Cu NPs to corrode forming ROS as intermediate products (cathodic reactions) [17,22]. Cell uptake of Cu-containing NPs can in addition induce damage through ROS production and via released Cu species within the cell (often denoted the Trojan horse-mechanism) [23,24].

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http://dx.doi.org/10.1016/j.colsurfb.2016.01.052

Some investigations have identified released Cu species as the main reason for observed toxicity of Cu NPs, [25,26] whereas other studies have concluded such species to be of minor importance [18,27,28]. These observations may not be in direct conflict with each other, as both fluid specifics and loading (mass Cu/volume) will largely influence the speciation and amount of released Cu, and hence, their individual importance. Despite the significance of the chemical speciation of released Cu on the observed toxic response, detailed understanding of this matter is mostly lacking in commonly used cell media, such as the Dulbecco's Modified Eagle Medium (DMEM).

This paper aims to fill this evident knowledge gap by: (i) providing chemical speciation information on Cu released from Cu NPs at extracellular conditions (in cell media—DMEM), (ii) deducing the correlation between chemical speciation of released Cu (possible labile Cu species) and membrane damage, ROS production, and corrosion of Cu NPs, and (iii) suggesting a model that elucidates Cu NPs-induced membrane damage in dependence of chemical speciation of released Cu in media of different Cu complexation capacity. A multi-analytical approach using polarography, UV–vis spectroscopy, and chemical equilibrium modeling (JESS, Joint Expert speciation system) has been employed to gain insight on the chemical speciation of Cu released from Cu NPs. Experimental studies have been performed in $DMEM^+$ (DMEM with added fetal bovine serum (FBS), penicillin, streptomycin, and pyruvate) to investigate the influence of protein-containing serum on the Cu speciation. Histidine solutions were selected as this amino acid has been reported to induce more Cu release from CuO NPs compared with other amino acids [21]. Phosphate buffered saline (PBS), a medium without any biomolecule constituents, was investigated for comparative reasons. In parallel, the membrane integrity (Trypan blue assay) of A549 (type II) lung cells was investigated following exposures to Cu NPs or Cu ions (from $Cu(NO_3)_2$), and the production of acellular ROS was monitored using the dichlorodihydrofluorescin diacetate (DCFH-DA) assay.

A previous investigation speculates that the reason for the higher cell membrane damage observed for Cu NPs compared with NPs of CuO is related to corrosion reactions taking place at the surface of the Cu NPs [16]. The corrosion behavior of the Cu NPs was therefore investigated using a carbon paste electrode (CPE) [29], monitoring the open circuit potential (OCP) to provide additional insights on the correlation between corrosion, induced toxicity, and the release of Cu species. It is, to the knowledge of the authors, the first time that CPE has been used in nanotoxicological investigations.

2. Materials and methods

2.1. Solutions and chemicals

DMEM (Dulbecco's modified eagle medium) was purchased from Life Technologies (Sweden, Lot# 1644395). For $DMEM^+$, 10 vol.% of fetal bovine serum (Gibco®, Life Technologies, Lot# 07F2235 K), 1 mM sodium puryvate (Life Technologies), 100 units/mL penicillin and 100 μg/mL streptomycin (Pen Strep, Gibco® Life Technologies) were added to DMEM. The pH was set at 7.4 ± 0.1, adjusted by adding appropriate amounts of 5 vol.% NaOH, when necessary.

Histidine (L-histidine monohydrochloride monohydrate, puriss p.a.) and copper nitrate ($Cu(NO_3)_2$, puriss p.a.) were obtained from Sigma–Aldrich, Sweden.

Phosphate buffered saline (PBS, pH 7.4) was prepared by mixing 8.77 g/L NaCl, 1.28 g/L Na_2HPO_4, 1.36 g/L KH_2PO_4, 370 μL/L 50% NaOH, pH 7.2–7.4 (all chemicals of analytical grade, Sigma–Aldrich, Sweden) in ultrapure water (18.2 MΩ cm, Millipore, Sweden).

All solution vessels were acid-cleaned in 10% HNO_3 for at least 24 h, and thereafter rinsed four times with ultrapure water.

2.2. Compositional analysis of the surface oxide

A Horiba Yvon Jobin HR800 Raman spectrometer was employed to study the surface oxide composition of the Cu NPs using a laser wavelength of 785 nm and a 50× objective. The laser beam was focused softly on a particle layer of Cu NPs to avoid beam damage. The layer was checked by optical microscopy before and after the measurements to assure no laser-induced damage. Three different areas (each with a diameter of approximately 10 μm) were investigated.

2.3. Particle size measurements

Particle size distribution measurements in solution were performed with a photon cross correlation spectroscopy instrument (Nanophox, Sympatec GmbH, Germany). The sample volume was 1 mL. All measurements were conducted at room temperature (20 ± 2 °C) in Eppendorf cuvettes (Eppendorf AG, Germany, UVette Routine pack, LOT no. C153896Q). A non-negative least square (NNLS) algorithm with a robust filter was used to obtain the intensity-weighed size distributions from the correlation functions. The sample solutions were prepared by adding 1 mg Cu NPs to 10 mL $DMEM^+$, where after the particle dispersion was sonicated for 3 min (Branson Sonifier 250, 30% duty cycle, output 4).

2.4. Zeta potential

The zeta potential (apparent surface charge) of the Cu NPs was studied using a Malvern Zetasizer nano Z instrument. The sample solutions were prepared by adding 1 mg Cu NPs to 10 mL NaCl (10 mM). The particle solution was there after sonicated for 3 min (Branson Sonifier 250, 30% duty cycle, output 4). The Smoluchowski approximation was used to calculate the zeta potential from the electrophoretic mobility of the particles in solution. A representative intensity distribution curve is given in Supporting information (Fig. S1).

2.5. Copper nanoparticles

Cu NPs, produced via wire explosion [30] were kindly provided by Ass. Prof. A. Yu. Godymchuk, Tomsk Polytechnic University, Russia. Detailed information on the particle characteristics is published elsewhere [16,28]. Briefly, the primary particle size is in the order of 50–200 nm and the surface oxide consists of a mixture of Cu_2O and CuO. These and additional characteristics are compiled in Fig. 1.

2.6. Copper nanoparticle exposures

5 mg Cu NPs were loaded into 50 mL solution and exposed at dark conditions at 37 °C for different time periods in a Stuart S180 incubator at bilinear shaking conditions (12°, 25 cycles/min). Triplicate samples and one blank sample without added Cu NPs were exposed. Directly after exposure, the samples were acidified to pH 2 using 65% HNO_3.

Ultracentrifugation (RCF 52900 *g*, Beckman Optima L-90K, SW-28 rotor) was employed for 1 h to separate the particle and the aqueous (released metals in solution) Cu-fractions. This procedure should remove all NPs sized approx. >20 nm from solution [31], which is larger than the primary size of the studied NPs [16]. A centrifugation time of 1 h exceeds the nominal exposure time, which means that some of the NPs were in solution and released Cu for a longer time period.