

# Avoiding artefacts during electron microscopy of silver nanomaterials exposed to biological environments

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## Abstract

Electron microscopy has been applied widely to study the interaction of nanomaterials with proteins, cells and tissues at nanometre scale. Biological material is most commonly embedded in thermoset resins to make it compatible with the high vacuum in the electron microscope. Room temperature sample preparation protocols developed over decades provide contrast by staining cell organelles, and aim to preserve the native cell structure. However, the effect of these complex protocols on the nanomaterials in the system is seldom considered. Any artefacts generated during sample preparation may ultimately interfere with the accurate prediction of the stability and reactivity of the nanomaterials. As a case study, we review steps in the room temperature preparation of cells exposed to silver nanomaterials (AgNMs) for transmission electron microscopy imaging and analysis. In particular, embedding and staining protocols, which can alter the physicochemical properties of AgNMs and introduce artefacts thereby leading to a misinterpretation of silver bioreactivity, are scrutinised. Recommendations are given for the application of cryogenic sample preparation protocols, which simultaneously fix both particles and diffusible ions. By being aware of the advantages and limitations of different sample preparation methods, compromises or selection of different correlative techniques can be made to draw more accurate conclusions about the data.

Keywords: electron microscopy, biological sample preparation, resin embedding, silver nanomaterials, toxicity, artefacts, staining, sulfidation

## Introduction

Silver nanomaterials (AgNMs) are being incorporated into many consumer products such as wound-dressings, anti-bacterial sprays and anti-odour-control fabrics due to its efficient, broad-spectrum antimicrobial properties.(Chen & Schluesener, 2008, Marambio-Jones & Hoek, 2010, Hansen & Baun, 2012, Wijnhoven *et al.*, 2009) Additionally, the optoelectronic properties of AgNMs are increasingly being exploited in a range of biosensors (Haes & Van Duyne, 2002, Ren *et al.*, 2005) and electrical devices.(Zhang *et al.*, 2011, Shipway *et al.*, 2000) The market for silver (Ag) products is already significant and growing rapidly; the potential for exposure of AgNMs is therefore high. For this reason, the impact of AgNMs on human health and the environment has been the focus of intensive investigation. Many biological assay-based studies have shown that exposure of AgNMs to cells leads to oxidative stress, lipid peroxidation, inhibition of mitochondrial activity, DNA damage and cell apoptosis.(Foldbjerg *et al.*, 2011, Park *et al.*, 2011, Costa *et al.*, 2010, Suresh *et al.*, 2012, Kim *et al.*, 2011, Stoehr *et al.*, 2011, Arora

*et al.*, 2008, Kang *et al.*, 2012, Piao *et al.*, 2011, AshaRani *et al.*, 2009) There has been much discussion in the literature as to whether the reactivity of AgNPs arises due to an ionic (Yang *et al.*, 2012, Xiu *et al.*, 2012b) or particulate effect (Xiu, Z.M. *et al.*, 2012), or both. Attempts have been made to resolve this question by incubating cells with silver nitrate (AgNO<sub>3</sub>) or by culturing cells in anaerobic conditions (Xiu *et al.*, 2012b)). However, several discrepancies still exist between the published results. This is most likely caused by the lack of sufficient controls over the particles used and the cellular systems investigated, making it hard to compare between experiments performed by different groups.

Electron microscopy (EM) images provide spatially resolved information about interfaces between nanomaterials and proteins (Chen *et al.*, 2012, Mahmoudi *et al.*, 2014) (Figure 1a), lipids (Leo *et al.*, 2013) (Figure 1b), cells (Chen *et al.*, 2013a)(Figure 1c-d), and tissues (Loeschner *et al.*, 2011). When applied in combination with analytical and diffraction techniques, changes in the physicochemistry of AgNMs can be mapped at the sub-nano- to micro-meter length scales.(Chen *et al.*, 2013a) However, conventional biological sample preparation protocols for the transmission electron microscope (TEM) can be extremely complex. Cells exposed to AgNMs can be processed in many different chemicals to fix the proteinaceous constituents of the cells. Heavy metal staining can also be applied to increase contrast from cell organelles. The effect of these chemicals, and other sample preparation processes, on the nanomaterials in the system must be carefully considered otherwise undesirable artefacts may be created leading to misinterpretation of the data. To avoid these problems, samples must be prepared by at least two methods to fully appreciate artefacts generated during sample preparation. Although silver is a noble metal, it is far from being chemically inert. Atmospheric corrosion and oxidation/reduction during chemical fixation or staining will likely change its chemistry. Here, we provide a comprehensive step-by-step analysis of potential artefacts which could be produced during conventional TEM sample preparation of the AgNMs-cell interface. Artefacts generated during both room temperature fixation and staining, and also during *in vitro* exposure of cells to the AgNMs, will be discussed. More generally, we intend to highlight challenges involved in preserving the physicochemistry of labile particles in complex biological environments. Pitfalls in interpretation that can be made by failing to appreciate artefacts generated during TEM sample preparation will be considered.

## **Overview of processing of resin-embedded samples for TEM**

Conventional sample preparation would involve the following steps. After culturing, cells are exposed to AgNMs dispersed in cell culture medium, for a desired period of time. Fixatives, like glutaraldehyde, are added for at least 1 h at 4 °C to arrest cellular processes and stabilize ultrastructure. Cells are rinsed with a buffer solution, for example hydroxyethyl piperazineethanesulfonic acid (HEPES) or piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), scraped down and centrifuged to generate a cell pellet. Cell pellets are treated with aqueous 1% osmium tetroxide solution for the purpose of staining and further fixation. Ferricyanide and electrolytes, e.g. CaCl<sub>2</sub> are often added together with the aqueous osmium tetroxide solution to enhance cell contrast and stabilize the cells, respectively. The specimens are dehydrated in graded concentrations of organic solvents, and embedded in polymer resin. Thin sections (50-100 nm) are prepared using a diamond knife and placed on grids for TEM imaging and analysis. Uranyl acetate and lead citrate staining can be applied to ultramicrotomed sections, in order to increase the contrast of the cell ultrastructure. A more detailed resin embedding sample preparation protocol can be found elsewhere.(Graham & Orenstein, 2007, Schrand *et al.*, 2010)

TEM sample preparation processes can produce artefacts which are misleading in predicting the processes by which dissolution of AgNMs (and subsequent release of Ag<sup>+</sup> ions) leads to cell reactivity. For example, Figure 2a shows an intact morphology of as-synthesized silver nanowires (AgNWs). Figure 2b shows the morphology change of AgNWs inside cells, which have been processed using methods which do not alter their physicochemical properties.(Chen *et al.*, 2013a, Chen *et al.*, 2013c) It is important to highlight that the resin embedding and curing does not create any artefacts during this preparation procedure. The

AgNWs have lost their original intact structures with small particles surrounding and decorating the AgNWs surface, after 24 hours incubation with transformed human alveolar type 1-like (TT1) cells. These images show that the AgNWs dissolve and  $\text{Ag}^+$  ions reprecipitate as silver sulfide ( $\text{Ag}_2\text{S}$ ) inside the cells, which could act as a detoxification mechanism due to the very low solubility of  $\text{Ag}_2\text{S}$  (Solubility product constant  $K_{\text{sp}}=5.92 \times 10^{-51}$ ) which is thus not reactive.(Levard *et al.*, 2012) However, when DCCM-1 cell culture media and osmium tetroxide/ferricyanide staining steps were used (Figure 2c), marked differences in the structure of the AgNWs were seen. The AgNWs were present inside the cell cytoplasm and interacting with the nuclear membrane, and had a hollowed-out structure surrounded by very small particles. These artefacts were caused by sulfidation of AgNWs in DCCM-1 cell culture media (discussed further below) and oxidative dissolution of AgNWs during the osmium tetroxide/ferricyanide staining steps, a protocol which is traditionally used to enhanced contrast from cell organelles. Indeed, AgNWs with a similar hollowed-out morphology were ‘created’ in a control experiment *without* cells (Figure 2d). This example demonstrates that the changes in the AgNWs morphology can be markedly altered as a result of TEM sample preparation. Consequently, it is not possible to conclude that the changes in the structure of the intracellular silver is due to intracellular processes, even though increased intracellular oxidative stress might contribute, as silver is sensitive to oxidative dissolution in the preparation media. The possibility that the preparation procedure itself induced some oxidative dissolution should not be ruled out (discussed further below).

#### **The stability of AgNMs in biological solutions: the effects of tissue culture media and proteins**

Understanding the stability of AgNMs in biologically-relevant solutions is important in the interpretation of their mechanisms of bioreactivity. Nanomaterials are usually administered to cells dispersed in tissue culture medium. The behaviour of AgNMs in media containing varying concentrations of salt and protein can be significantly different to AgNMs in distilled water.(MacCuspie, 2011, Chinnapongse *et al.*, 2011) In particular, changes to the agglomeration state, the release of ions, and alterations to surface chemistry in tissue culture media, are all factors which may affect cellular responses and AgNM toxicity.(Jiang *et al.*, 2009)

The agglomeration state of a nanomaterial alters the surface area available to the surrounding environment, and therefore can modulate dissolution, or adsorption of biological molecules. Agglomeration also changes the effective size of the particles presented to cells, which may result in differences in cellular uptake mechanisms and subsequent intracellular localisation.(Lankoff *et al.*, 2012, Albanese & Chan, 2011) For example, MacCuspie *et al.*(MacCuspie, 2011) investigated the colloidal stabilities of 20 nm Ag NPs with different surface coatings (bovine serum albumin (BSA), citrate, starch) in a range of tissue culture media. They found that silver nanoparticles (AgNPs) that had been sterically-stabilized with bulky biocompatible coatings remained dispersed in a wider range of electrolyte concentrations and pH conditions than electrostatically-stabilized AgNPs. Epple *et al.*(Kittler *et al.*, 2010) reported aggregation of 50 nm AgNPs in RPMI medium containing BSA, whereas they remained dispersed if the BSA was replaced by fetal calf serum. The presence of both proteins was reported to reduce AgNP toxicity by binding  $\text{Ag}^+$  ions.

Recently, we reported that the physicochemical properties of AgNWs incubated in DCCM-1 tissue culture medium were altered, while no changes were observed in DMEM or RPMI media.(Chen *et al.*, 2013d) AgNWs were found to sulfidise within 15 minutes of incubation in DCCM-1, due to the presence of small molecule solutes and salts, which was observed by TEM as the formation of  $\text{Ag}_2\text{S}$  nanocrystals, and eventually a sulfidized shell at the AgNW surface (Figure 3a).  $\text{Ag}_2\text{S}$  NPs themselves have been reported to have negligible effects in altering cell proliferation, initiating apoptosis and necrosis, generating reactive oxygen species (ROS), and causing DNA damage.(Zhang *et al.*, 2012) We suggested that sulfidation in such systems can act as a potential detoxification mechanism by trapping free  $\text{Ag}^+$  ions.(Chen *et al.*, 2013a)

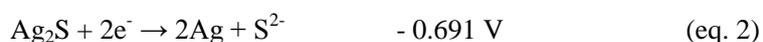
## Reduction of Ag<sup>+</sup> ions during tissue fixation

After exposure of cells to NMs, aldehydes are often used as primary fixatives to halt cellular activities and preserve the cell fine structure. Glutaraldehyde (CH<sub>2</sub>(CH<sub>2</sub>CHO)<sub>2</sub>), one of the most effective fixing agents, has two aldehyde groups linked by a short hydrocarbon chain. Through nucleophilic addition on aldehyde groups, glutaraldehyde reacts primarily with amine groups and other nucleophilic functional groups such as thiols, phenols, and imidazoles.(Migneault *et al.*, 2004) Therefore, this dialdehyde fixative can efficiently crosslink proteins, nucleic acids, carbohydrates and lipids containing primary amines, e.g. phosphatidylserine and phosphatidylethanolamine. A detailed discussion regarding glutaraldehyde crosslinking mechanisms can be found in a review published by Migneault *et al.*(Migneault *et al.*, 2004)

As well as functioning as fixatives, aldehydes are known reducing agents of Ag. For example, aqueous diamminesilver (I) complex known as Tollen's reagent is commonly used to distinguish between aldehydes and ketones. A positive test results in precipitation of metallic silver. Equation 1 below shows the reaction mechanism, in which silver ions are reduced by the aldehyde to form metallic silver precipitates, and the aldehyde is oxidised to carboxylate.

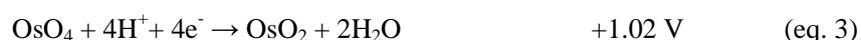


Therefore, any silver ions released from AgNMs intracellularly can be expected to be reduced by aldehydes used during the fixation process. Indeed, we have observed the transformation of silver ions to metallic silver particles during fixation for TEM. Figure 3b shows TEM analysis of embedded human lung airway smooth muscle cells (ASM) exposed to silver nitrate solution at an incubation concentration of 5 µg/mL. Exposure of ASM cells to silver nitrate solution was used as a control experiment designed to demonstrate that the reduction of Ag<sup>+</sup> ions is possible during cell fixation. Cells were fixed using 2.5 % glutaraldehyde for 1 h at 4 °C, but neither osmium tetroxide nor uranium and lead staining were applied. The cell ultrastructure appeared necrotic which could be attributed to the toxicity of Ag<sup>+</sup> ions.(Greulich *et al.*, 2012, Xiu *et al.*, 2012a) High resolution high angle annular dark field-scanning transmission electron microscopy (HAADF-STEM) revealed that some cell organelle structures, especially the nucleus, nuclear membrane and cell membrane fragments, were closely associated with small, high contrast particles (Figure S1a-c). Scanning transmission electron microscopy-energy-dispersive X-ray spectroscopy (STEM-EDX) and TEM-EDX indicated that these particles were silver-rich in composition (Figure S1b-c). This indicates that the silver ions had been reduced, and as a consequence also function as a staining agent, allowing the plasma and nuclear membranes to be visualized. High resolution transmission electron microscopy (HRTEM) images revealed that the particles are crystalline (Figure S1d). In contrast, there was no formation of high contrast small particles in ASM cell without AgNO<sub>3</sub> exposure (Figure S1e). A possible mechanism is that during AgNO<sub>3</sub> exposure, silver ions diffuse into cells and bind to the phosphate heads of lipids, proteins or nucleic acids and then are reduced to metallic silver during the fixation step. The reduction of silver ions by aldehyde fixatives alters the oxidative state and morphology of silver, however, this step does not affect the evaluation of the amount of silver dissolved from the original AgNMs. Furthermore, in contrast to cells exposed directly to silver salts like AgNO<sub>3</sub>, the exposure of cells to AgNMs often gives enough time to allow transformation of dissolved Ag<sup>+</sup> ions into other formats of silver compounds/complexes, due to the relatively slow dissolution rate of silver. For example, recently we have reported a silver transformation process within TT1 cells, during which silver dissolves and precipitates as silver sulfide.(Chen *et al.*, 2013a) This process is thermodynamically favourable, as silver sulfide is a highly insoluble silver compound with a solubility product constant of  $K_{sp} = 5.92 \times 10^{-51}$  and reduction potential of -0.691 V (eq.2). The reduction process during fixation will likely have no significant effect on such highly stable silver species.



## Oxidative dissolution of Ag during osmium tetroxide staining for enhanced contrast of cell organelles

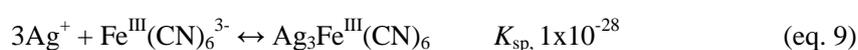
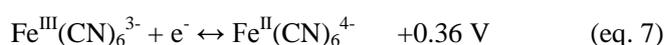
Osmium tetroxide ( $\text{OsO}_4$ ) is often applied as a secondary fixative and staining agent.  $\text{OsO}_4$  reacts with C=C double bonds on unsaturated lipids and various functional groups on proteins such as thiol, disulphide, phenolic, hydroxyl, carboxyl, amino and certain heterocyclic groups. Therefore,  $\text{OsO}_4$  functions as an inter- or intra- molecular crosslinking agent between proteins and unsaturated lipids. After reaction,  $\text{OsO}_4$  is often reduced and precipitates as  $(\text{OsO}_2 \cdot n\text{H}_2\text{O})^-$  or metallic osmium, imparting contrast for electron microscopy.  $\text{OsO}_4$  is a strong oxidant which has a reduction potential ( $E^0$ ) of 1.02 V (eq. 3) to be reduced to  $\text{OsO}_2$ , as well as a lower reduction potential of being reduced to metallic osmium ( $E^0 = 0.85$  V, eq. 4).  $\text{OsO}_4$  can oxidise metallic silver and therefore accelerate the silver oxidation dissolution rate (eq. 5). Figure 3c-left, shows significant changes to the morphology of AgNWs following incubation with 1 wt% osmium tetroxide solution (buffered to pH 7.4 with 0.1M sodium cacodylate) for 30 seconds. It was further confirmed that no substantial morphology and chemistry change of AgNWs incubated with 0.1M sodium cacodylate (pH 7.4) for 30 seconds was observed (Figure S2).



In practice, ferricyanide ( $[\text{Fe}(\text{CN})_6]^{3-}$ ) or ferrocyanide ( $[\text{Fe}(\text{CN})_6]^{4-}$ ) are frequently used together with  $\text{OsO}_4$  to enhance staining contrast of various cellular components including lipid membranes and glycogen. (White *et al.*, 1979, Rivlin & Raymond, 1987) When  $\text{OsO}_4$  and ferricyanide or ferrocyanide are used simultaneously, a mixture of intermediate compounds are formed, which contain  $\text{Os}^{\text{VIII}}\text{O}_4$ ,  $\text{Fe}^{\text{II}}(\text{CN})_6^{4-}$ ,  $\text{Fe}^{\text{III}}(\text{CN})_6^{3-}$ ,  $\text{Os}^{\text{VI}}\text{O}_2(\text{OH})_4^{2-}$  and cyano-bridged Os-Fe species containing Os in a range of oxidation states (VIII, VII and VI). It has been suggested that these osmium complexes are chelated by donor atoms in the macromolecules, resulting in the reduction of osmium to lower oxidation states. This mixture of Os (VII and VI) intermediates has a greater reactivity and leads to more Os deposition than Os(VIII) alone. (White *et al.*, 1979, Hayat, 2000) However, ferricyanide is known to react with silver, and thus holds the potential to create artefacts if ferricyanide is used during staining process. The reaction of Ag with ferricyanide likely involves a silver cyanidation process. This reaction has been used in the extraction of silver and gold from the Earth's core for over one hundred years. The silver cyanidation reaction can be described by the well-known Elsner equation below (eq. 6). (Xie & Dresinger, 2009)



Ferricyanide is also an oxidising agent (eq. 7), with a reduction potential slightly lower than oxygen (eq. 8). Possible reactions involve 1) the oxidation of  $\text{Ag}^0$  to  $\text{Ag}^+$  (eq. 5), and reduction of ferricyanide to ferrocyanide (eq. 7) 2) the formation of insoluble silver ferricyanide/ferrocyanide compounds (eq. 9-10) and 3) the formation of the insoluble blue compound ferric ferrocyanide (known as Prussian blue, eq. 11). An overall reaction could be written as (eq. 12). The formation of insoluble silver ferricyanide/ferrocyanide compounds significantly reduces the half-cell redox potential of  $\text{Ag}/\text{Ag}^+$ , allowing this reaction to occur spontaneously (the detailed calculation is presented in SI).





A control experiment was carried out by incubating AgNWs in potassium ferricyanide solution. The formation of blue precipitates indicates the reduction of ferricyanide to ferrocyanide and then formation of insoluble blue ferric ferrocyanide compound. Figure 3c-right shows the morphology of AgNWs after incubation with potassium ferricyanide for 30 min at RT. Significant AgNW morphology changes, and the formation of diffuse materials containing Fe, Ag, C and N and silver rich particles around the AgNWs were detected (Figure S3).

Due to the reaction of osmium and ferrocyanide/ferricyanide with silver, the use of osmium and cyanide compounds should be avoided if researchers are interested in preserving the biologically-relevant morphology and chemistry of AgNMs in biological tissues. However, if the main aim is to observe the fine structure of biological components, the use of osmium, combined with cyanide, can be still applied.

### Post-staining effects for further enhancement of cell contrast

Double staining with uranyl acetate ( $\text{UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2$ ) and lead citrate ( $\text{Pb}(\text{C}_5\text{H}_5\text{O}_7)_2$ ) can be applied to tissues fixed with aldehydes followed by  $\text{OsO}_4$  staining, as multiple staining can significantly enhance the electron density of all cell components which cannot be obtained with any single stain alone. The double staining process can be applied either straight after osmium staining but before dehydration (*en* block staining), or on ultramicrotomed thin sections supported on a TEM grid (post-staining). A 1-2 wt% uranyl acetate water solution is often used, which has a pH of ~3-5. In this pH range, uranyl ions ( $\text{UO}_2^{2+}$ ) and different forms of uranyl acetate complexes, i.e.  $\text{UO}_2\text{Ac}^+$ ,  $\text{UO}_2\text{Ac}_2$ ,  $\text{UO}_2\text{Ac}^{3-}$ ,  $\text{UO}_2(\text{OH}_2)\text{UO}_2\text{Ac}_3$ , co-exist in the solution. These uranyl ions and ionic complexes of different charges bind primarily to the negatively charged carboxyl and phosphate groups, or to positively charged amine groups. Enhanced contrast can be achieved through lead staining after uranyl staining, as more carboxylic acid groups become deprotonated in alkaline lead solution (pH ~ 12), which are available for binding with lead ions.

Precipitates will form if uranyl acetate solution is exposed to light, which may confound the determination of AgNP morphology if elemental analysis is not employed. In addition, lead citrate solution is sensitive to carbon dioxide, which forms lead carbonate precipitates. Therefore, precautions need to be taken to ensure that precipitation does not occur during post-staining. Figure 3d, shows a comparison of 'dirty' staining sample vs. a 'clean' staining sample. The formation of precipitates with similar contrast to Ag particles under bright field transmission electron microscopy (BFTEM) imaging makes it difficult to distinguish AgNWs (labelled as arrows) and precipitated stain (Figure 3d left). In contrast, degraded or transformed Ag species showing weaker electron contrast (circled) can be seen in the 'clean' post-staining section (Figure 3d right). Furthermore, the high acidity of the uranyl acetate solution may accelerate the dissolution of Ag particles during *en* block staining. However, this side effect may not be significant within the normal staining times of ~ 30 min at room temperature. As an alternative to *en* block staining, methanolic uranyl acetate (1-2 wt% uranyl acetate in 70 % methanol water solution) is often used. Methanolic uranyl acetate can penetrate the hydrophobic resin sections faster and deeper than pure water solution. This methanolic ost-staining procedure likely has fewer side effects on AgNMs, due to the lower dielectric constant of the solvent and short standing time (3-5 min). Indeed, no substantial morphology and chemistry change of AgNWs incubated in uranyl acetate and lead citrate staining solution for 30 min was observed (FigureS5).

### The importance of avoiding atmospheric corrosion during sample storage

Atmospheric silver corrosion alters the chemistry and morphology of AgNMs. Even the presence of atmospheric gaseous hydrogen sulfide ( $\text{H}_2\text{S}$ ), carbonyl sulfide (OCS) and carbon disulphide ( $\text{CS}_2$ ), will

cause sulfidation upon exposure to air.(Elechiguerra *et al.*, 2005) As compared to silver oxides, the formation of silver sulfide is thermodynamically favoured. The growth of an Ag<sub>2</sub>S layer on AgNMs was detected after just one hour in dry laboratory air containing a H<sub>2</sub>S concentration of less than 0.2 ppb.(Bennett *et al.*, 1969) Figure 3e shows the morphology of AgNWs deposited on a TEM grid which had been exposed to the ambient environment for 2 months. Significant corroded AgNW surface, including hollowed-out morphologies were observed, which will change Ag ions dissolution kinetics and therefore bioreactivity. The 2 month AgNW sample was further analysed by acquiring STEM-EDX maps, showing the presence of both sulfur and silver within the shell, and a silver rich core. More details about silver atmospheric corrosion can be found in other reports.(Elechiguerra *et al.*, 2005). The embedding of AgNPs in polymer resins prevents the direct contact of AgNMs with atmosphere. However, once microtomed, the Ag on the surface of the cell section will be exposed to air, which may lead to changes to the chemistry and morphology of the particles. Therefore, we recommend storing ultrathin resin embedded TEM sections containing AgNMs in an inert atmosphere or under vacuum to prevent atmospheric sulfidation.

## Conclusions and future perspectives

In summary, our paper highlights that the effects of exposure of AgNMs to the environment, and during TEM sample preparation should be considered to ensure that the AgNMs do not transform in these environments and therefore must be considered in the analysis of toxicity assays assessing their safety to human health and the environment.

This article discusses possible alterations to the physicochemical state of AgNMs exposed to biological environments during TEM sample preparation. The use of Ag-reactive cell culture media and chemical reagents during cell fixation and staining, e.g. aldehydes, osmium tetroxide/ferricyanide can change the oxidative state and morphology of Ag species, which can lead to incorrect the prediction of AgNMs bioreactivity. The “well established” room temperature biological TEM sample preparation protocols developed 30-40 years ago were designed and optimised to study cell ultrastructure. These methods are often treated as a standard protocol for preparation of nanomaterials inside cells and tissues. However, the physicochemical properties of redox active and labile materials are often altered during fixation and staining processes. Here we provide a comprehensive step-by-step analysis of potential artefacts which could be produced during conventional TEM sample preparation of the AgNM-cell interface. These protocols can be applied to guide the study of interactions between other redox sensitive and labile materials and cells. With the explosion of literature concerning the toxicity of engineered nanomaterials, this article provides new insight into the importance of appreciating artefacts induced during sample preparation to fully interpret TEM data.

Our recent study on uptake of AgNWs by TT1 cells demonstrates how, by omitting steps such as osmium tetroxide/ferricyanide staining, and choosing suitable cell culture media and sample storage to minimise extracellular transformation of AgNWs, it is possible to avoid artefacts which alter the physicochemical properties of AgNWs in cell samples.(Chen *et al.*, 2013b) This work concluded that AgNWs were observed in the cytoplasm and membrane-bound vesicles of TT1 cells, and partial dissolution of AgNWs and re-precipitation as Ag<sub>2</sub>S within the cell occurred after 1 h exposure. Cell viability studies showed no evidence of cytotoxicity and ROS generation on cells exposed to AgNWs. We suggested that the Ag to Ag<sub>2</sub>S transformation mechanism acts as a ‘trap’ for free Ag<sup>+</sup>, significantly limiting short-term toxicological effects – with important consequences for the safety of AgNMs to human health. In contrast, the artefacts seen in Figure 2c, d and Figure 3 would have lead us to an entirely different conclusion, *i.e.* that the AgNWs dissolve and reprecipitate as Ag<sub>2</sub>S much more significantly in both the extra- and intra-cellular environments, and that the AgNWs can interact with the cytoplasm (and constituent organelles) and the

nuclear membrane of the cells. Similarly, use of cell culture media which change the chemistry and morphology of the AgNMs (Chen et al., 2013c) and any form of environmental transformation AgNMs (Levard et al., 2012) such as silver sulfidation in ambient air, could have altered the bioreactivity of the AgNMs and changed their transport, fate and toxicity. This will lead to misinterpretation of the impact of consumer product containing AgNMs on human health and the environment. Therefore choice of cell culture medium and sample storage must be carefully considered in the biological toxicity analysis. The results suggest appropriate media and material controls must be in place to allow accurate predictions about the toxicity, and ultimately, the health risk of this commercially relevant class of nanomaterial.

An outstanding question in this field is whether the toxicity of AgNWs arises due to an ionic or particulate effect. Conventional TEM preparation protocols cannot answer this question as they will not preserve the distribution of ions within the cell cytosol as Ag<sup>+</sup> ions could potentially be lost or redistributed during the relatively slow chemical fixation process, and ultramicrotomy when the thin section is floated on a water bath. An alternative sample preparation method is rapid freezing to arrest cellular processes, followed by analysis of samples in the frozen hydrated state, which is now used in our laboratory to study cellular distribution of Ag<sup>+</sup> ions. In a recent study of AgNPs in vitrified cells, Drescher *et al.* (Drescher *et al.*, 2013) used soft X-ray microscopy to map a ring-like distribution of nanoparticles within endosomes in fibroblasts and macrophages; similar results, with much higher spatial resolution, can be expected in a cryo-TEM. Cryo-TEM methods provide a route to generate spatially resolved maps of ion distributions in cells. However, electron microscopy of vitrified samples will place considerable constraints on elemental analysis of tissues, due to problems with radiation damage. This constraint may be overcome by preparing samples by high pressure freezing (HPF) followed by freeze substitution (FS) (Boonrungsiman *et al.*, 2012), since cryofixed samples are stabilised in an embedding resin during HPF/FS. In our recent work, we successfully used electron energy loss spectroscopy (EELS) to map the distribution of calcium in vesicles containing calcium phosphate (CaP) containing mitochondria inside osteoblast cells, which had been prepared by HPF/FS. (Boonrungsiman *et al.*, 2012)

In conclusion, conventional embedding TEM sample preparation allows sub-nanometer resolution TEM imaging and analysis of AgNMs in tissues which can be used to provide mechanistic insights into how Ag transforms in complex biological environments. However, artefacts generated during sample preparation must be considered with caution or results are liable to misinterpretation. In future, these limitations may be overcome by the application of cryogenic methods. By being aware of all the advantages and limitations of different sample preparation methods and imaging techniques, compromises or combination of different correlative techniques; for example utilising both electron and X-ray microscopies, can be made to achieve research goals.

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## Reference

- Albanese, A. & Chan, W. C. W. (2011) Effect of Gold Nanoparticle Aggregation on Cell Uptake and Toxicity. *ACS Nano*, **5**, 5478-5489.
- Arora, S., Jain, J., Rajwade, J. M. & Paknikar, K. M. (2008) Cellular responses induced by silver nanoparticles: In vitro studies. *Toxicol Lett*, **179**, 93-100.
- AshaRani, P. V., Mun, G. L. K., Hande, M. P. & Valiyaveetil, S. (2009) Cytotoxicity and Genotoxicity of Silver Nanoparticles in Human Cells. *ACS Nano*, **3**, 279-290.
- Bennett, H. E., Peck, R. L., Burge, D. K. & Bennett, J. M. (1969) Formation and Growth of Tarnish on Evaporated Silver Films. *J Appl Phys*, **40**, 3351-3360.
- Boonrungsiman, S., Gentleman, E., Carzaniga, R., Evans, N. D., McComb, D. W., Porter, A. E. & Stevens, M. M. (2012) The role of intracellular calcium phosphate in osteoblast-mediated bone apatite formation. *P Natl Acad Sci USA*, **109**, 14170-14175.
- Chen, R., Choudhary, P., Schurr, R. N., Bhattacharya, P., Brown, J. M. & Chun Ke, P. (2012) Interaction of lipid vesicle with silver nanoparticle-serum albumin protein corona. *Appl Phys Lett*, **100**, 13703-137034.
- Chen, S., Goode, A. E., Sweeney, S., Theodorou, I. G., Thorley, A. J., Ruenraroengsak, P., Chang, Y., Gow, A., Schwander, S., Skepper, J., Zhang, J. F., Shaffer, M. S., Chung, K. F., Tetley, T. D., Ryan, M. P. & Porter, A. E. (2013a) Sulfidation of silver nanowires inside human alveolar epithelial cells: a potential detoxification mechanism. *Nanoscale*, **5**, 9839-9847.
- Chen, S., Goode, A. E., Sweeney, S., Theodorou, I. G., Thorley, A. J., Ruenraroengsak, P., Chang, Y., Gow, A., Schwander, S., Skepper, J., Zhang, J. J., Shaffer, M. S., Chung, K. F., Tetley, T. D., Ryan, M. P. & Porter, A. E. (2013b) Sulfidation of silver nanowires inside human alveolar epithelial cells: a potential detoxification mechanism. *Nanoscale*, **5**, 9839-9847.
- Chen, S., Theodorou, I. G., Goode, A., Gow, A., Schwander, S., Zhang, J., Chung, K. F., Tetley, T., Shaffer, M. S., Ryan, M. P. & Porter, A. E. (2013c) High resolution analytical electron microscopy reveals cell culture media induced changes to the chemistry of silver nanowires. *Environ Sci Technol*.
- Chen, S., Theodorou, I. G., Goode, A. E., Gow, A., Schwander, S., Zhang, J. F., Chung, K. F., Tetley, T. D., Shaffer, M. S., Ryan, M. P. & Porter, A. E. (2013d) High-Resolution Analytical Electron Microscopy Reveals Cell Culture Media-Induced Changes to the Chemistry of Silver Nanowires. *Environ Sci Technol*, **47**, 13813-13821.
- Chen, X. & Schluesener, H. J. (2008) Nanosilver: A nanoparticle in medical application. *Toxicol Lett*, **176**, 1-12.
- Chinnapongse, S. L., MacCuspie, R. I. & Hackley, V. A. (2011) Persistence of singly dispersed silver nanoparticles in natural freshwaters, synthetic seawater, and simulated estuarine waters. *Sci Total Environ*, **409**, 2443-2450.
- Costa, C., Ronconi, J. V. V., Daufenbach, J. F., Goncalves, C. L., Rezin, G. T., Streck, E. L. & Paula, M. M. D. (2010) In vitro effects of silver nanoparticles on the mitochondrial respiratory chain. *Mol Cell Biochem*, **342**, 51-56.
- Drescher, D., Guttman, P., Buchner, T., Werner, S., Laube, G., Hornemann, A., Tarek, B., Schneider, G. & Kneipp, J. (2013) Specific biomolecule corona is associated with ring-shaped organization of silver nanoparticles in cells. *Nanoscale*, **5**, 9193-9198.
- Elechiguerra, J. L., Larios-Lopez, L., Liu, C., Garcia-Gutierrez, D., Camacho-Bragado, A. & Yacaman, M. J. (2005) Corrosion at the nanoscale: The case of silver nanowires and nanoparticles. *Chem Mat*, **17**, 6042-6052.
- Foldbjerg, R., Dang, D. A. & Autrup, H. (2011) Cytotoxicity and genotoxicity of silver nanoparticles in the human lung cancer cell line, A549. *Arch Toxicol*, **85**, 743-750.
- Graham, L. & Orenstein, J. M. (2007) Processing tissue and cells for transmission electron microscopy in diagnostic pathology and research. *Nat Protoc*, **2**, 2439-2450.
- Greulich, C., Braun, D., Peetsch, A., Diendorf, J., Siebers, B., Epple, M. & Koller, M. (2012) The toxic effect of silver ions and silver nanoparticles towards bacteria and human cells occurs in the same concentration range. *Rsc Adv*, **2**, 6981-6987.

Haes, A. J. & Van Duyne, R. P. (2002) A nanoscale optical biosensor: Sensitivity and selectivity of an approach based on the localized surface plasmon resonance spectroscopy of triangular silver nanoparticles. *J Am Chem Soc*, **124**, 10596-10604.

Hansen, S. F. & Baun, A. (2012) When enough is enough. *Nature nanotechnology*, **7**, 409-411.

Hayat, M. A. (2000) *Principles and Techniques of Electron Microscopy: Biological Applications, Fourth Edition*, Cambridge University Press.

Jiang, J. K., Oberdorster, G. & Biswas, P. (2009) Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. *J Nanopart Res*, **11**, 77-89.

Kang, K., Jung, H. & Lim, J. S. (2012) Cell Death by Polyvinylpyrrolidone-Coated Silver Nanoparticles is Mediated by ROS-Dependent Signaling. *Biomol Ther*, **20**, 399-405.

Kim, H. R., Kim, M. J., Lee, S. Y., Oh, S. M. & Chung, K. H. (2011) Genotoxic effects of silver nanoparticles stimulated by oxidative stress in human normal bronchial epithelial (BEAS-2B) cells. *Mutatres-Gen Tox En*, **726**, 129-135.

Kittler, S., Greulich, C., Gebauer, J. S., Diendorf, J., Treuel, L., Ruiz, L., Gonzalez-Calbet, J. M., Vallet-Regi, M., Zellner, R., Koller, M. & Epple, M. (2010) The influence of proteins on the dispersability and cell-biological activity of silver nanoparticles. *J Mat Chem*, **20**, 512-518.

Lankoff, A., Sandberg, W. J., Wegierek-Ciuk, A., Lisowska, H., Refsnes, M., Sartowska, B., Schwarze, P. E., Meczynska-Wielgosz, S., Wojewodzka, M. & Kruszewski, M. (2012) The effect of agglomeration state of silver and titanium dioxide nanoparticles on cellular response of HepG2, A549 and THP-1 cells. *Toxicol Lett*, **208**, 197-213.

Leo, B. F., Chen, S., Kyo, Y., Herpoldt, K. L., Terrill, N. J., Dunlop, I. E., McPhail, D. S., Shaffer, M. S., Schwander, S., Gow, A., Zhang, J. F., Chung, K. F., Tetley, T. D., Porter, A. E. & Ryan, M. P. (2013) The Stability of Silver Nanoparticles in a Model of Pulmonary Surfactant. *Environ Sci Technol*, **47**, 11232-11240.

Levard, C., Hotze, E. M., Lowry, G. V. & Brown, G. E. (2012) Environmental Transformations of Silver Nanoparticles: Impact on Stability and Toxicity. *Environ Sci Technol*, **46**, 6900-6914.

Loeschner, K., Hadrup, N., Qvortrup, K., Larsen, A., Gao, X., Vogel, U., Mortensen, A., Lam, H. R. & Larsen, E. H. (2011) Distribution of silver in rats following 28 days of repeated oral exposure to silver nanoparticles or silver acetate. *Part Fibre Toxicol*, **8**, 18.

MacCuspie, R. I. (2011) Colloidal stability of silver nanoparticles in biologically relevant conditions. *J Nanopart Res*, **13**, 2893-2908.

Mahmoudi, M., Lohse, S. E., Murphy, C. J., Fathizadeh, A., Montazeri, A. & Suslick, K. S. (2014) Variation of protein corona composition of gold nanoparticles following plasmonic heating. *Nano Lett*, **14**, 6-12.

Marambio-Jones, C. & Hoek, E. M. V. (2010) A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment. *J Nanopart Res*, **12**, 1531-1551.

Migneault, I., Dartiguenave, C., Bertrand, M. J. & Waldron, K. C. (2004) Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *Biotechniques*, **37**, 790-+.

Park, M., Neigh, A. M., Vermeulen, J. P., de la Fonteyne, L. J. J., Verharen, H. W., Briede, J. J., van Loveren, H. & de Jong, W. H. (2011) The effect of particle size on the cytotoxicity, inflammation, developmental toxicity and genotoxicity of silver nanoparticles. *Biomaterials*, **32**, 9810-9817.

Piao, M. J., Kang, K. A., Lee, I. K., Kim, H. S., Kim, S., Choi, J. Y., Choi, J. & Hyun, J. W. (2011) Silver nanoparticles induce oxidative cell damage in human liver cells through inhibition of reduced glutathione and induction of mitochondria-involved apoptosis. *Toxicol Lett*, **201**, 92-100.

Ren, M. L., Meng, X. W., Chen, D., Tang, F. Q. & Jiao, J. (2005) Using silver nanoparticle to enhance current response of biosensor. *Biosens Bioelectron*, **21**, 433-437.

Rivlin, P. K. & Raymond, P. A. (1987) Use of osmium tetroxide-potassium ferricyanide in reconstructing cells from serial ultrathin sections. *J Neurosci Meth*, **20**, 23-33.

- Schrand, A. M., Schlager, J. J., Dai, L. M. & Hussain, S. M. (2010) Preparation of cells for assessing ultrastructural localization of nanoparticles with transmission electron microscopy. *Nat Protoc*, **5**, 744-757.
- Shipway, A. N., Katz, E. & Willner, I. (2000) Nanoparticle arrays on surfaces for electronic, optical, and sensor applications. *Chemphyschem*, **1**, 18-52.
- Stoehr, L. C., Gonzalez, E., Stampfl, A., Casals, E., Duschl, A., Puentes, V. & Oostingh, G. J. (2011) Shape matters: effects of silver nanospheres and wires on human alveolar epithelial cells. *Part Fibre Toxicol*, **8**, 36.
- Suresh, A. K., Pelletier, D. A., Wang, W., Morrell-Falvey, J. L., Gu, B. H. & Doktycz, M. J. (2012) Cytotoxicity Induced by Engineered Silver Nanocrystallites Is Dependent on Surface Coatings and Cell Types. *Langmuir*, **28**, 2727-2735.
- White, D. L., Mazurkiewicz, J. E. & Barnett, R. J. (1979) A chemical mechanism for tissue staining by osmium tetroxide-ferrocyanide mixtures. *J Histochem Cytochem*, **27**, 1084-1091.
- Wijnhoven, S. W. P., Peijnenburg, W., Herberts, C. A., Hagens, W. I., Oomen, A. G., Heugens, E. H. W., Roszek, B., Bisschops, J., Gosens, I., Van de Meent, D., Dekkers, S., De Jong, W. H., Van Zijverden, M., Sips, A. & Geertsma, R. E. (2009) Nano-silver - a review of available data and knowledge gaps in human and environmental risk assessment. *Nanotoxicology*, **3**, 109-U178.
- Xie, F. & Dresinger, D. B. (2009) Use of ferricyanide for gold and silver cyanidation. *T Nonferr Metal Soc*, **19**, 714-718.
- Xiu, Z. M., Zhang, Q. B., Puppala, H. L., Colvin, V. L. & Alvarez, P. J. (2012a) Negligible particle-specific antibacterial activity of silver nanoparticles. *Nano Lett*, **12**, 4271-4275.
- Xiu, Z. M., Zhang, Q. B., Puppala, H. L., Colvin, V. L. & Alvarez, P. J. J. (2012b) Negligible Particle-Specific Antibacterial Activity of Silver Nanoparticles. *Nano Lett*, **12**, 4271-4275.
- Yang, X., Gondikas, A. P., Marinakos, S. M., Auffan, M., Liu, J., Hsu-Kim, H. & Meyer, J. N. (2012) Mechanism of silver nanoparticle toxicity is dependent on dissolved silver and surface coating in *Caenorhabditis elegans*. *Environ Sci Technol*, **46**, 1119-1127.
- Zhang, Y., Hong, G. S., Zhang, Y. J., Chen, G. C., Li, F., Dai, H. J. & Wang, Q. B. (2012) Ag<sub>2</sub>S Quantum Dot: A Bright and Biocompatible Fluorescent Nanoprobe in the Second Near-Infrared Window. *Acs Nano*, **6**, 3695-3702.
- Zhang, Z. L., Zhang, X. Y., Xin, Z. Q., Deng, M. M., Wen, Y. Q. & Song, Y. L. (2011) Synthesis of monodisperse silver nanoparticles for ink-jet printed flexible electronics. *Nanotechnology*, **22**.

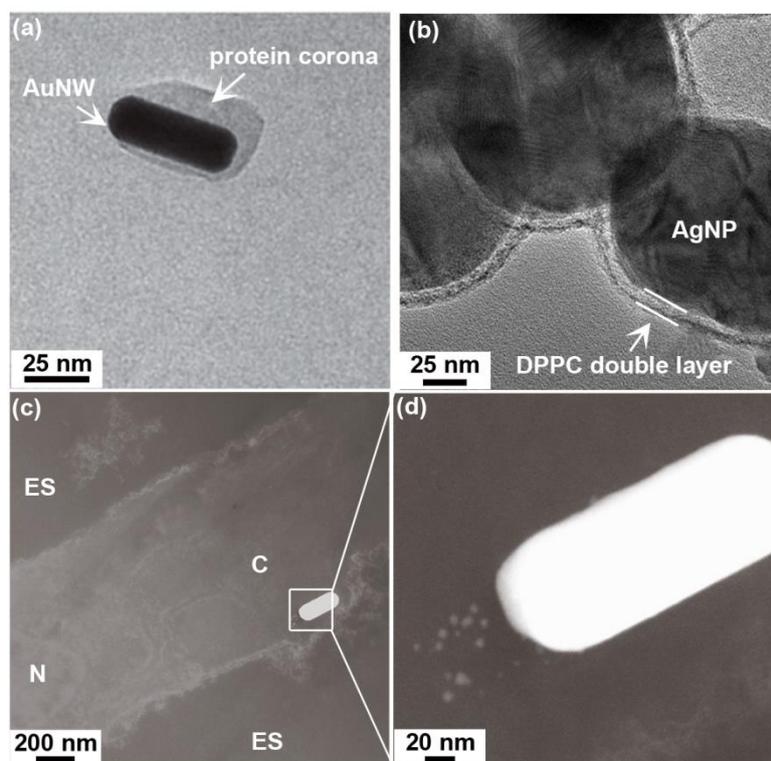


Figure 1. EM characterization showing interfaces between gold (Au) and Ag metallic nanomaterials and different biological *milieu*, including proteins, phospholipids and cells. (a) Bright field TEM (BFTEM) image of a Au nanowire (AuNW) fetal bovine serum complex. Adapted with permission from (Mahmoudi et al., 2014). Copyright (2014) American Chemical Society, (b) BFTEM image shows the interface of AgNPs and dipalmitoylphosphatidylcholine (DPPC). Uranyl acetate was used to enhance the contrast of the DPPC corona. Adapted with permission from (Leo et al., 2013). Copyright (2013) American Chemical Society, (c) high angle annular dark field-scanning transmission electron microscope (HAADF-STEM) image of a silver nanowire (AgNW) inside the cytoplasm of a transformed alveolar type I-like epithelial cell line (TT1) and (d) a corresponding higher magnification HAADF-STEM image which shows formation of particles surrounding the tip of the AgNWs.(Chen et al., 2013a) Adapted by permission of The Royal Society of Chemistry. No staining was applied. ES: extracellular space; C: cytoplasm; N: nucleus.

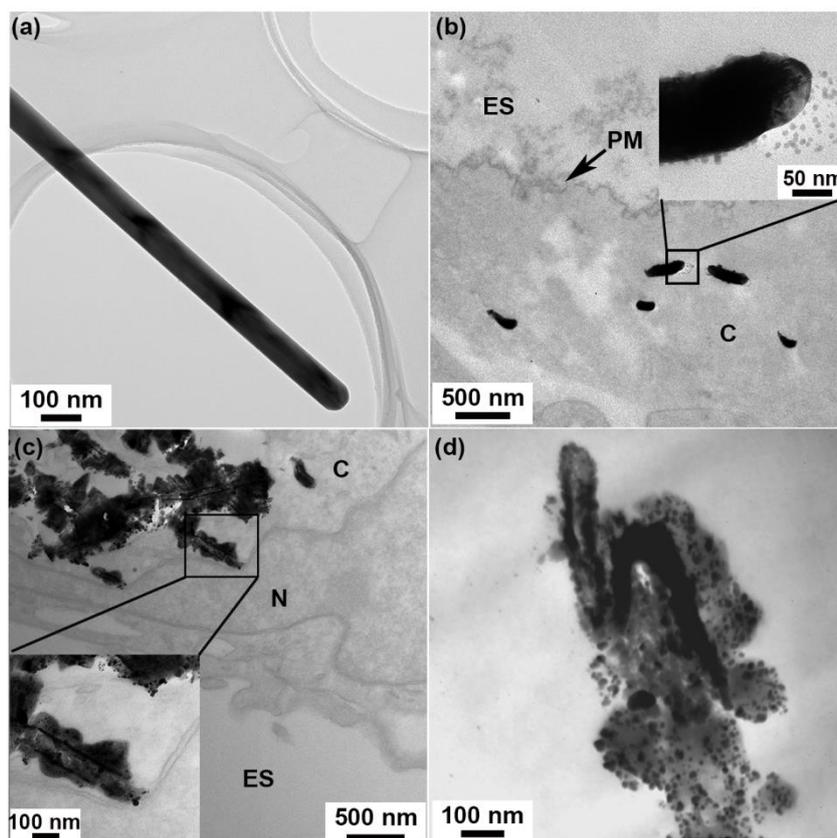
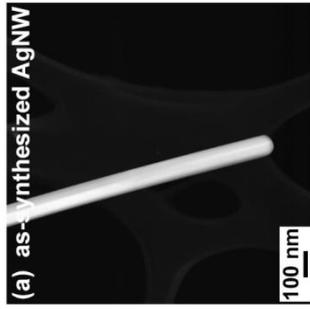
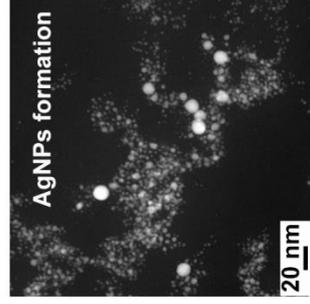
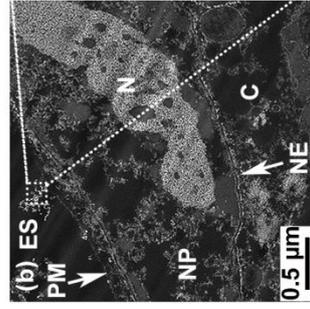
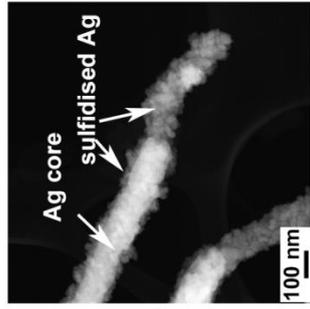


Figure 2. TEM images showing the morphology of (a) as-synthesized AgNWs (b) AgNWs inside TT1 cells after 24 hours incubation. RPMI cell medium was used for the cell exposures. This medium does not cause silver sulfidation. No heavy metal staining was applied. (c) AgNWs inside TT1 cells after 24 hours incubation, showing significant dissolution of AgNWs within the cell cytoplasm and at the nuclear membrane (inset). No cytotoxicity or changes to cell ultrastructure were observed following exposure of the TT1 cells to AgNWs. The cells were cultured in DCCM-1 cell media which sulfides silver.(Chen et al., 2013d) The cells in (c) were stained with osmium tetroxide/ferricyanide. (d) AgNWs treated with the same experimental conditions as (c), but without the involvement of any cells. C: cytoplasm, ES: extracellular space and N: nucleus.

(1) NMs solution preparation → (2) Expose cells to NMs → (3) Cell processing: rinse, fixation, pellet



DCCM-1  
37 °C, 6 h



Potential artefact: transformation of AgNMs in biological media, e.g. sulfidation

Potential artefact: reduction of Ag ions by aldehydes

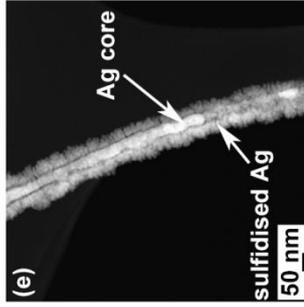
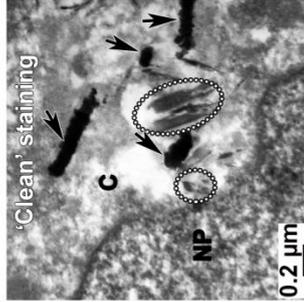
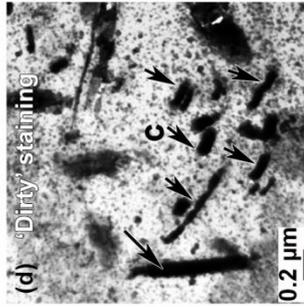
(7) Thin sectioning ← (6) Resin embedding and curing → (5) Dehydration ← (4) Post-fixation process (Os staining)

Potential artefacts:  
1) loss of Ag ions  
2) holes  
3) dragging

No potential artefact \*

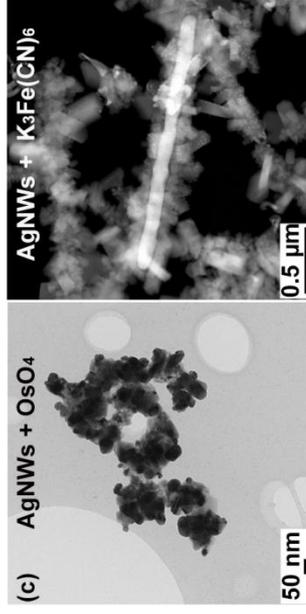
Potential artefact:  
loss of Ag ions

(8) Post-staining (U and Pb staining) → (9) Sample storage



Potential artefact: precipitation formation during uranyl acetate and lead citrate staining

Potential artefact:  
atmosphere corrosion of AgNMs



Potential artefact: transformation of AgNMs during osmium fixation/staining process

Figure 3. Overview of sample preparation processes (1-9) and potential artefacts. (a) HAADF-STEM image of as-synthesized AgNW (left) and the morphology of transformed AgNWs incubated in DCCM-1 tissue culture medium for 6 h at 37 °C (right)(Chen et al., 2013d) (b) HAADF-STEM image of ASM cells exposed to AgNO<sub>3</sub> for 72 h (left) and a corresponding higher resolution HAADF-STEM of the boxed area in (b-left) showing the formation of nanoparticles (right). (c) BFTEM image showing morphology changes of AgNWs incubated to 1wt% osmium tetroxide buffered to pH 7.4 with 0.1M sodium cacodylate for 30 seconds at room temperature (RT) (left). Adapted by permission of The Royal Society of Chemistry.(Chen et al., 2013a) and a HAADF-STEM image of AgNWs incubated with 0.15 wt% potassium ferricyanide for 30 min at RT. (d) BFTEM images showing an example of dirty (left) and clean (right, adapted by permission of The Royal Society of Chemistry.(Chen et al., 2013a)) post-staining with methanolic uranyl acetate and lead citrate. Some typical AgNWs are marked by arrows. Degraded or transformed Ag species (circled, showing weaker electron contrast) can be seen in the 'clean' section (Fig 3d right). (e) HAADF-STEM image showing the core-shell structure of AgNWs sitting on a carbon film supported by a TEM grid which was exposed to the atmosphere for 2 months (Chen et al., 2013a), adapted by permission of The Royal Society of Chemistry. \*A control experiment demonstrated that the resin embedding and curing process does not alter the morphology or the chemistry of the AgNWs (Figure S4). Adapted by permission of The Royal Society of Chemistry.(Chen et al., 2013a) ES = extracellular space; C = cytoplasm; PM = plasma membrane; NE = nuclear envelope; NP = nucleoplasm and N = nucleolus.