

Supporting information

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

Shu Chen^{1*}, Angela E. Goode¹, Jeremy N. Skepper², Andrew J. Thorley³, Joanna M. Seiffert³, K. Fan Chung³, Teresa D. Tetley³, Milo S. P. Shaffer⁴, Mary P. Ryan¹, Alexandra E. Porter^{1*}

¹Department of Materials and London Centre for Nanotechnology, Imperial College London, Exhibition Road, London SW7 2AZ, UK

²Cambridge Advanced Imaging Centre, Department of Physiology, Development and Neuroscience, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK

³National Heart and Lung Institute, Imperial College London, SW3 6LY, UK

⁴Department of Chemistry and London Centre for Nanotechnology, Imperial College London, Exhibition Road, London SW7 2AZ, UK

Method

Cell sample processing for TEM

Transformed human alveolar type I-like epithelial cells (TT1)

TT1 cells were grown on six-well tissue culture plates, in complete DCCM-1 medium (containing 10% NCS, 5% PSG) to reach 100% confluence at 37°C and 5% CO₂. Cells were then starved of serum for 24h prior to particle exposure.

For Fig 2b sample, to avoid the AgNWs sulfidation in DCCM-1, cells were thoroughly washed and then incubated in RPMI serum free medium for an hour, before exposure to AgNWs. Cells were exposed to AgNWs, 25µg/ml in RPMI for 1 h pulse treatment then washed, cultured for a further hour in RPMI, washed and cultured for up to 24 h in DCCM-1 medium. Detailed AgNWs synthesis and TT1 cell culturing please refer to ref(Chen *et al.*, 2013b). For **Fig 2c** sample, cells were exposure to 25µg/ml in DCCM-1 for 24 h. After cell exposure, cells were rinsed briefly in saline (0.9 % NaCl) to remove any non-ingested particles and were then fixed in 2.5 % glutaraldehyde in 0.1 M PIPES buffer, pH 7.2 for 1 h at 4 °C. The fixatives were then removed by washing cells with 0.1 M PIPES buffer for 3 times. Cells were scraped and transferred into 1.5 mL Eppendorf tubes and cell pellets were obtained by centrifugation at 10,000 g for 20 min. For **Fig2b**, cells were embedded without bulk staining with osmium tetroxide. For **Fig 2c**, the cell pellet was osmicated (1% OsO₄, 0.15% potassium ferricyanide, and 2 mM CaCl₂ in DI-H₂O) for 1 h at room temperature, and then washed three times with dionized water. For **Fig 2d**, A 20 µL mixture of AgNWs (25 µg/mL, pre-incubated with DCCM-1 at 37°C for 24 h) 1 % Agarose (low gelling temperature, BioReagent, for molecular biology, Sigma) were prepared at 40 °C and allowed to gel at 4 °C. They were subsequently treated with the same osmication process as **Fig 2c** sample. Samples were dehydrated in a graded ethanol series of 50%, 70%, 95%, and 100% (volume ratio of ethanol to DI-H₂O) ethanol for 5 min each then rinsed three times in acetronitrile (Sigma) for an additional 10 min each, all at room temperature. After dehydration, samples were progressively infiltrated with a Quetol-based resin, created by combining 8.75 g quetol, 13.75 g nonenyl succinic anhydride, 2.5 g methyl acid anhydride, and 0.62 g benzyl dimethylamine (all from Agar Scientific). Samples were infiltrated in 50 % resin/acetonitrile solution (volume ratio of resin to acetonitrile) for 2 h, followed by infiltration in a 75 % resin/acetonitrile solution overnight and finally in 100 % resin for 4 days with fresh resin replaced daily. Embedded samples were cured at 60 °C for 24 h. Thin sections (70 nm) were cut directly into a water bath using an ultramicrotome with a diamond knife with a wedge an angle of 35°. Sections were immediately collected on bare, 300 mesh copper TEM grids (Agar Scientific), dried and kept under vacuum for TEM analysis. **Fig 3d** shows sections were post-stained with 2 wt% uranyl acetate 70 % methanolic water solution and Reynold's Lead Citrate solution for 5 min each before imaging. This enhances the contrast of cell structures. Extra care should be taken when handling uranium compounds (toxic and radioactive) and lead compounds (toxic). Extra precautions should be taken during post-staining. Uranium acetate is light sensivity, therefore the staining proces should be handled in dark, otherwise precipitates will form. On the other hand, lead citrate is carbon dioxide (CO₂) sensitive. To aviod lead forming precipitates with CO₂, the staining can be handled inside a closed container with sodium hydroxide pellets absorbing atmospheric CO₂. **Fig 3d right** shows a 'clean' post-staining example, while **Fig 3d left** shows a TEM section post-stained without taking extra precautions, precipitates formed consequently.

Primary ASM cells

Primary ASM cells were dissected from main or lobar bronchus removed from resection or transplant donor lungs and were cultured in DMEM cell culture medium supplemented (with 4 mM L-glutamine, 20 U/L penicillin, 20 mg/ml streptomycin, and 2.5 mg/ml amphotericin B and 10% FCS. Before exposure,

confluent cells were incubated for 24 h in serum-free DMEM medium supplemented with 1 mM sodium pyruvate, 4 mM L-glutamine, 1:100 nonessential amino acids, 0.1% BSA and antibiotics. Cells were exposed to AgNO₃ at a concentration of 5 µg/mL for 72 h, before fixation, dehydroation and embedding. No osmium was applied. The TEM results are presented in **Fig 3b** and **Fig S1**.

Control samples

Cell culture media effect on AgNWs stability (**Fig 3a**) was published in ref(Chen *et al.*, 2013c). AgNWs were incubated in a temperature-controlled incubator set at 37 °C for 6 h. After incubation, AgNWs were collected by centrifugation (16000 g, 20 min), washed with DI-H₂O three times and dispersed in DI-H₂O. Then the AgNWs were drop cast on to 300 mesh holey carbon film TEM grids and immediately placed under vacuum and in the dark for drying and storage, to avoid exposure to contamination or reactions induced by ambient atmosphere.

Effect of osmium tetroxide (**Fig 3c left**) and potassium ferricyanide (**Fig 3c right & Fig S3**) on AgNWs stability study was published in ref(Chen *et al.*, 2013b). AgNWs (25 µg/mL) were incubated with 0.15 % potassium ferricyanide water solution for 30 min at room temperature. AgNWs were collected by centrifugation and washed with DI-H₂O (repeated 3 times) before TEM analysis. For osmium tetroxide, AgNWs were incubated with 1% osmium tetroxide buffered to pH 7.4 with 0.1M sodium cacodylate for 30 seconds, rinsed briefly with DI-H₂O and mounted on holey carbon film grids. Extra care should be taken when handling osmium compounds (highly toxic).

Effect of sodium cacodylate (**Fig S2**) on AgNWs stability: AgNWs were incubated with 0.1M sodium cacodylate for 30 seconds, rinsed briefly with DI-H₂O and mounted on holey carbon film grids.

The embedding procedure on the stability of AgNWs (**Fig S3**) was published in ref(Chen *et al.*, 2013b). A 20 µL mixture of AgNWs (25 µg/mL) 1 % Agarose (low gelling temperature, BioReagent, for molecular biology, Sigma) were prepared at 40 °C and allowed to gel at 4 °C. They were subsequently dehydrated and embedded in Quetol without treatment with osmium tetroxide or bulk staining with uranyl acetate as described previously.

Effect of 2 wt% uranyl acetate 70 % methanolic solution (**Fig S4a-b**) and Reynold's Lead Citrate solution (**Fig S4c-d**) on AgNWs stability: AgNWs (25 µg/mL) were incubated with uranyl acetate and lead citrate solutions for 30 min at room temperature, respectively. AgNWs were collected by centrifugation and washed with DI-H₂O (repeated 3 times) before TEM analysis.

TEM imaging

TEM imaging analysis was performed on a JEOL 2000 microscope operated at 80 kV and an FEI Titan 80-300 scanning/transmission electron microscope (S/TEM) operated at 80 kV, fitted with Cs (image) corrector and SiLi EDX spectrometer (EDAX, Leicester UK). HRTEM and STEM-HAADF/EDX analyses were carried out on non-stained samples.

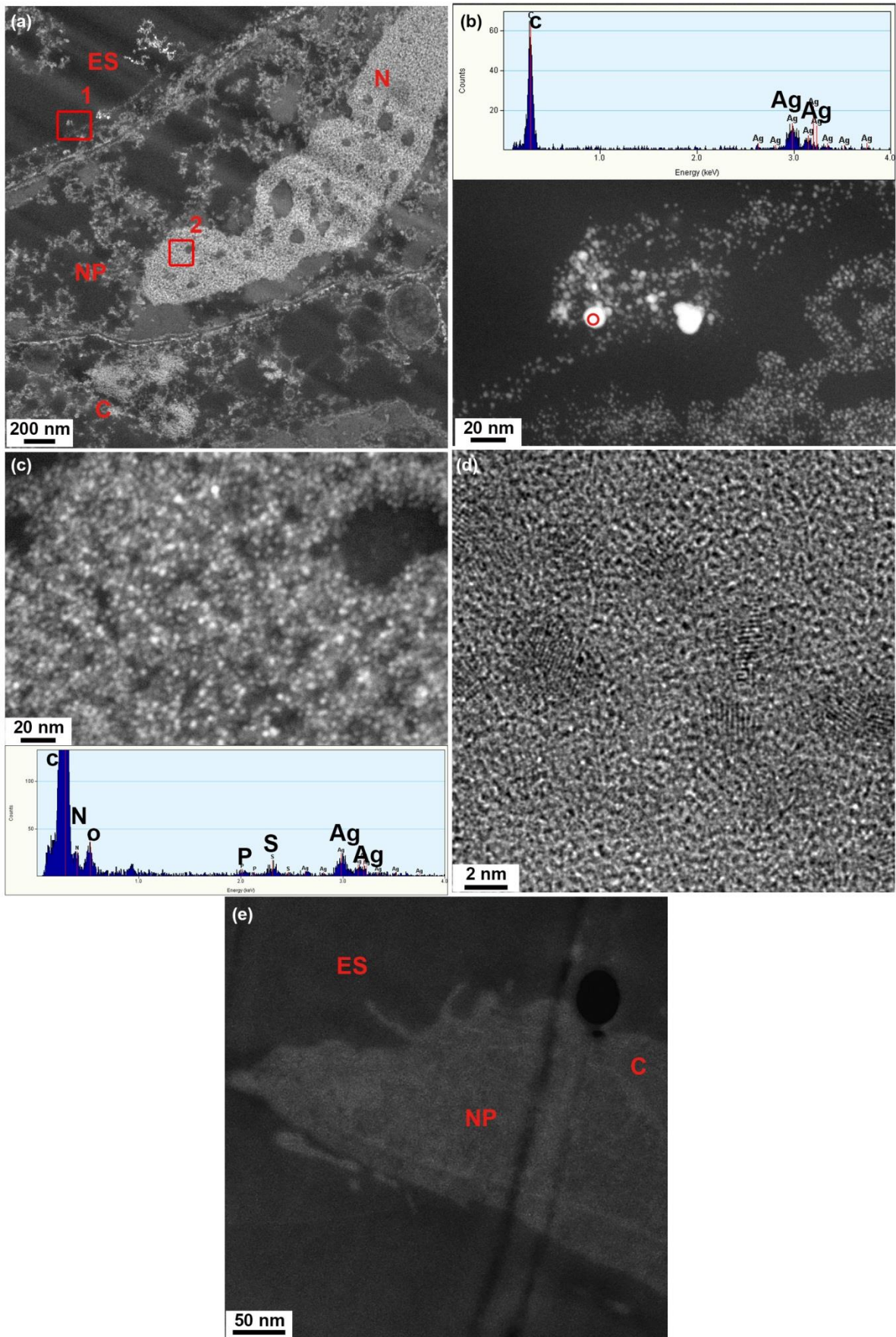


Figure S1. HAADF-STEM image of smooth muscle cells exposed to AgNO_3 , fixed with glutaraldehyde and embedded without staining. (a) Overview of a damaged cell showing electron dense nanoparticles lining cellular membranes and within the nuclear structures. (b) A higher magnification HAADF-STEM image (bottom) of particles associated with the damaged cell and a typical STEM-EDX spectrum (top) taken from one of the particles as marked by a circle, suggest these particles are likely formed by reduction from Ag^+ ions by the aldehydes used for fixation. (c) Particles present within the nucleus were analysed by STEM-EDX (inset) and found to contain Ag, O, and S. (d) HRTEM image of small particles within the nucleus showing their crystalline nature. (e) HAADF-STEM image of smooth muscle cell without AgNO_3 exposure shows there was no formation of small nanoparticles. ES = extracellular space; C = cytoplasm; NP = nucleoplasm and N = nucleolus.

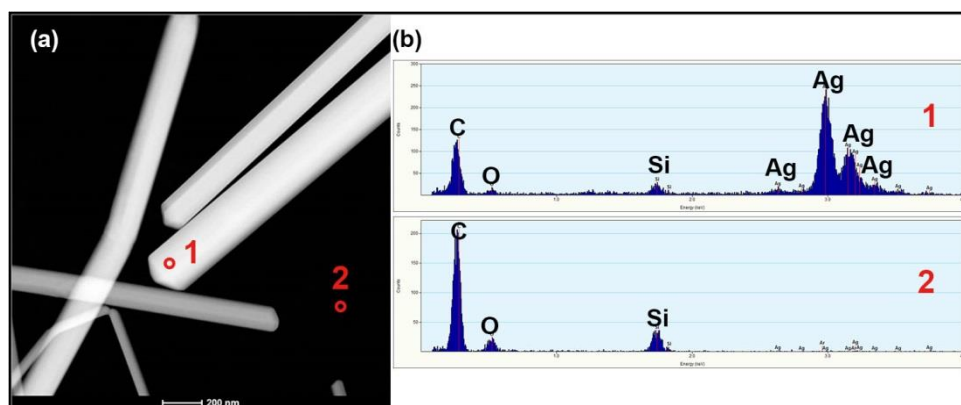


Figure S2. (a) HAADF-STEM image showing no significant changes in the AgNWs morphology exposed to 0.1 M sodium cacodylate for 30 s, (b) STEM-EDX spectra taken from corresponding areas 1-2 marked in (a), spectrum (1) confirms no significant chemistry change and spectrum (2) shows the contribution of C, O and Si are mainly from the background.

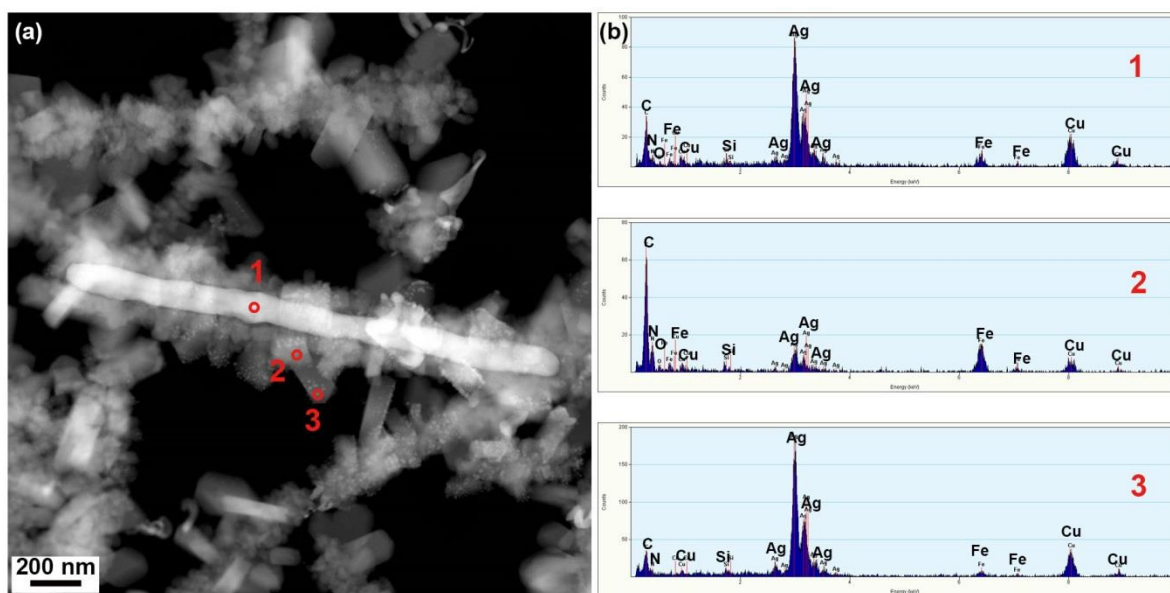
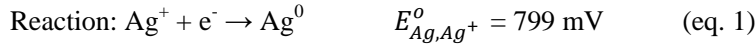


Figure S3. AgNWs after incubation with ferricyanide solution for 30 minutes at room temperature. (a) HAADF-STEM image showing changes in the NW morphology and (b) STEM-EDX spectra taken from

corresponding areas 1-3 marked in (a). Spectrum (1) shows the composition of the core of the NWs is Ag rich, Spectrum (2) indicates the presence of Fe, Ag, C and N in the diffuse surrounding material and Spectrum (3) showsthat the dense nanoparticles within the shell-structure are Ag-rich.

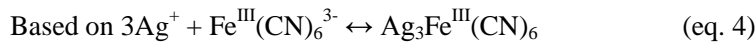


$$\text{Based on Nernst Equation(Stansbury \& Buchanan, 2000): } E'_{\text{Ag,Ag}^+} = E_{\text{Ag,Ag}^+}^o + \frac{RT}{zF} \ln \alpha_{\text{Ag}^+} \quad (\text{eq.2})$$

Where, $E'_{\text{Ag,Ag}^+}$ is the half-cell reduction potential of Ag/Ag^+ at the temperature of interest, $E_{\text{Ag,Ag}^+}^o$ is the standard half-cell reduction potential, R is the universal gas constant: $R = 8.314 \text{ J K}^{-1} \text{ mol}^{-1}$, T is the absolute temperature, z is the number of moles of electrons transferred in the cell reaction or half-reaction, F is the Faraday constant, the number of coulombs per mole of electrons: $F = 9.649 \times 10^4 \text{ C mol}^{-1}$ and α_{Ag^+} is the chemical activity for Ag^+ (can be replaced by molar concentration here).

After simplification, eq.2 becomes

$$E'_{\text{Ag,Ag}^+} = 799 + 59 \log \alpha_{\text{Ag}^+} \quad (\text{mV}) \quad (\text{eq. 3})$$



$$K_{\text{SP}} = (\alpha_{\text{Ag}^+})^3 (\alpha_{\text{ferricyanide}}) = 10^{-28} \quad (\text{eq. 5})$$

$$\text{Then } \alpha_{\text{Ag}^+} = \sqrt[3]{\frac{K_{\text{sp}}}{\alpha_{\text{ferricyanide}}}} \quad (\text{eq. 6})$$

Combine eq. 3 and 6,

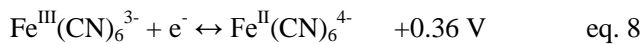
$$E'_{\text{Ag,Ag}^+} = 799 + 59 \log \sqrt[3]{\frac{K_{\text{sp}}}{\alpha_{\text{ferricyanide}}}} \quad (\text{eq. 7) can be obtained}$$

As $\alpha_{\text{ferricyanide}}$ is the chemical activity for ferricyanide ions (can be replaced by its molar concentration), which is about 0.046 mol/L (the concentration of potassium ferricyanide was 1.5 wt% used in this control experiment and the amount of ferricyanide ions react with Ag can be neglect here, due to the low total concentration of Ag in the system, i.e. $5 \mu\text{g/mL}$ ($4.5 \times 10^{-5} \text{ mol/L}$))

Put $\alpha_{\text{ferricyanide}}$ and K_{SP} values into eq.7,

half-cell potential value $E'_{\text{Ag,Ag}^+}$ of 294 mV for the reaction of $\text{Ag}^+ + \text{e}^- \rightarrow \text{Ag}^0$ in the presence of ferricyanide can be obtained.

Combine eq. 1 and eq. 8



the overall reaction eq. 9 can be obtained,



which has a cell reaction potential of +0.07 V, indicates this reaction can occur spontaneously.

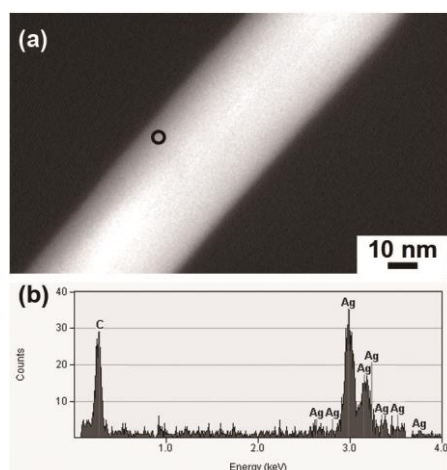


Figure S4. EM characterization illustrates the effect of the embedding procedure on the stability of AgNWs. No heavy metal staining was used. AgNWs were embedded with Quetol and thin sections (70 nm) were taken for TEM imaging and analysis. (a) The HAADF-STEM image shows the AgNW and (b) a typical EDX spectrum taken from a region over the AgNW. This analysis demonstrated the embedding and curing process does not alter the morphology or the chemistry of the AgNWs. Adapted by permission of The Royal Society of Chemistry.(Chen *et al.*, 2013a)

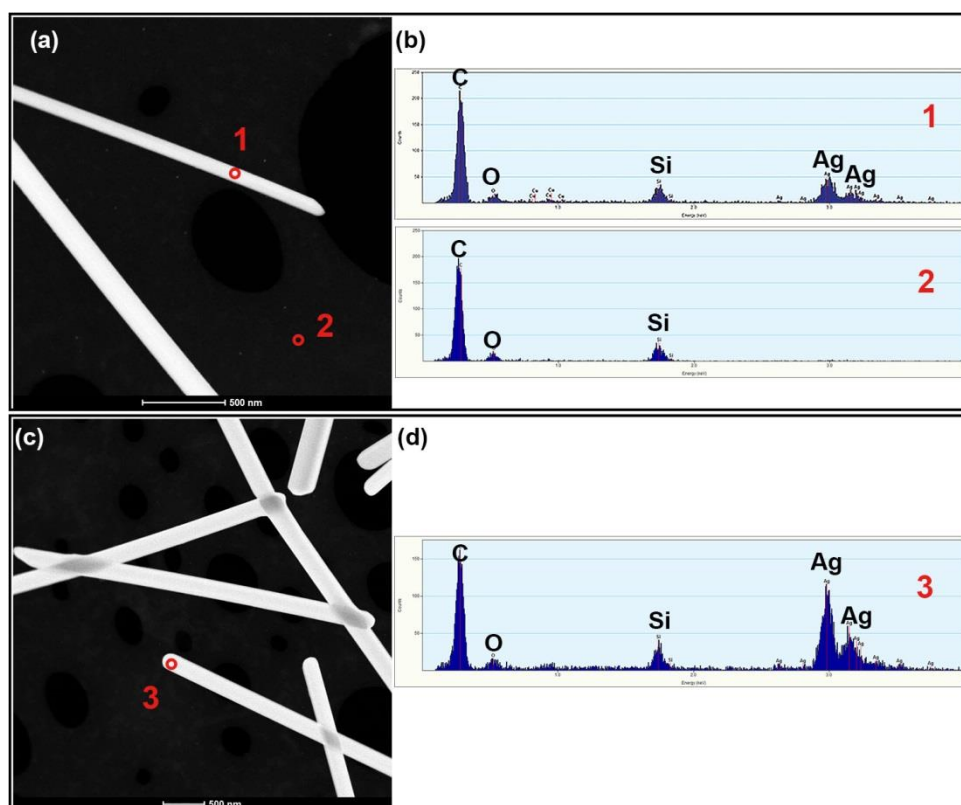


Figure S5. (a) HAADF-STEM image showing no significant changes in the AgNWs morphology incubated with Reynold's Lead Citrate solution for 30 min at room temperature, (b) corresponding STEM-EDX spectra from one of the NWs confirms no significant chemistry change and (c) STEM-EDX spectra shows the contribution of C, O and Si are mainly from the background. (c) HAADF-STEM image showing no significant changes in the AgNWs morphology incubated with uranyl acetate methanolic solution for 30 min at room temperature and (d) corresponding STEM-EDX spectra from one of the NWs confirms no significant chemistry change.

Reference

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