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Measuring *in vitro* cellular uptake of nanoparticles by transmission electron microscopy

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Abstract. Biomedical application of engineered nanoparticles (NPs) is a growing area of research and development. Uncertainty remains as to the mode of action of many NP types and TEM is a tool capable of addressing this if used in conjunction with standard cellular response assays. We will demonstrate imaging of thin sections of fixed, plastic embedded cells by analytical TEM to identify: superparamagnetic iron oxide NP translocation into cell compartments such as endosomes; amorphous silica NP penetration through a cell membrane without membrane encapsulation and zinc oxide NP degradation in cell compartments. We will then discuss how the *in vitro* cellular responses to a dose of NPs exposed to cell lines can be correlated to the internalized dose per cell section noting however that quantification of the latter requires random sampling procedures or correlation to higher throughout techniques to measure a population of whole cells. Similarly, analytical TEM measures of NP degradation within intracellular compartments will require a more appropriate sample preparation such as cryo-fixation.

1. Introduction

Engineered nano-particles (NPs) have been the focus of much recent research with a particular emphasis on application in the field of medicine as imaging probes for cells and tissues, for drug delivery and as sensors of target molecules [1-3]. Efficacious and safe implementation of NPs requires a complete understanding of cellular response to NP exposure. Knowing precisely how an exposure dose relates to the response is therefore critical to the development of both NP toxicology and medicinal applications. Electron microscopy has sufficient spatial resolution to achieve this correlation and has recently been used to monitor and quantify NP dispersion and dose internalised by *in vitro* cell lines [4,5].

In this work we will draw on the imaging and spectroscopic capabilities of (scanning) transmission electron microscopes (S/TEMs) to identify cellular uptake pathways and the final location of internalised particles. The key challenges for this work are representative sample preparation (that does not detrimentally alter the cells or NPs, or obscure NP location with contrast enhancing heavy metal stains) and suitable control of the electron fluence applied during imaging and, more importantly, spectroscopic analysis such that the particle structure (and ideally the cellular material) remains unaltered during investigation [6]. We will demonstrate the use of analytical TEM to identify: superparamagnetic iron oxide NP translocation into cell compartments such as endosomes; amorphous

silica NP penetration through a cell membrane without membrane encapsulation and zinc oxide NP degradation in cell compartments.

2. Experimental

Cells from the human lymphoblastoid cell line (MCL-5) were exposed to dextran coated iron oxide nanoparticles (Liquids Research, Wales) as described in [7]. Cells from the human lung alveolar carcinoma cell line (A549) were exposed to amorphous silica nanoparticles (Ludox SM-30) and aliphatic polyether, polymer-coated zinc oxide nanoparticles (Nanotek, Alfa Aesar) as described in [8] and [9] respectively.

Preparation of NP exposed cells for TEM followed the conventional wet chemical approach to view cellular ultrastructure: aldehyde and then osmium tetroxide fixation, dehydration in ethanol and infiltration with and polymerization of epoxy or acrylic resins. After embedding, thin sections (70 – 90 nm) were cut by an ultramicrotome, floated on a water bath and mounted on TEM grids [7-9]. No bulk and post embedding, heavy-metal staining steps were applied. TEM was conducted on two microscopes; an FEI CM200 field emission gun (FEG-)TEM running at 197 kV equipped with an Oxford Instruments energy dispersive X-ray (EDX) spectrometer (X-Max, silicon drift detector) and a Gatan Imaging Filter (GIF-200) and an FEI Tecnai F20 FEG TEM operating at 200 kV fitted with a Gatan Orius SC600A camera and an Oxford Instruments energy dispersive X-ray (EDX) spectrometer (X-Max, silicon drift detector). Tilt series were collected through a tilt range of +46 to -46 degrees with an image recorded every one degree on the Gatan Orius SC600A camera and processed using the software ImageJ, with the TomoJ plugin. Images in the tilt series were defocused (underfocus) by 180 μm from minimum contrast to enhance the contrast of the NPs in the unstained cell sections.

3. Results and Discussion

Dextran coated superparamagnetic iron oxide NPs are used as MRI contrast enhancement agents however partial oxidation of these particles has been shown to dramatically alter cellular internalization, with the more oxidised form exhibiting the most uptake and inducing significant DNA damage [7]. TEM confirms cellular internalization of these iron oxide NPs and suggests that uptake is dominated by encapsulation within vesicles, indicative of endocytosis (figure 1). The cellular response (including DNA damage) has been correlated to the uptake and the probable release of Fe^{2+} following dissolution of the NPs in lysosomes or late endosomes.

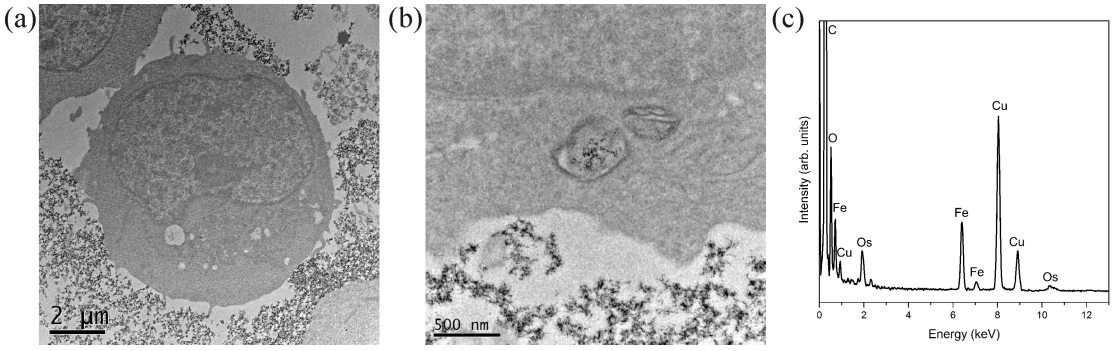

Figure 1 a) BF TEM image of MCL-5 cells exposed to iron oxide NPs, b) BF TEM image of an agglomerate of NPs in a vesicle within a cell (confirmed by tilting, [10]), and c) EDX spectrum from particles in the cell confirming the presence of Fe (the Cu signal is generated by the support grid) [6].

Amorphous silica NPs have potential for drug delivery however significant levels of induced DNA damage and reduction in cell viability (at higher exposure doses) are observed upon *in vitro* exposure

to these NPs [8]. A TEM tilt-series indicates that cellular internalization of NPs has occurred without membrane encapsulation and the observation of irreversible binding to model lipid membranes suggest this is driven by an adhesive interaction with cell membranes. NP internalization without membrane encapsulation is confirmed following cellular exposure at 4 °C, when endocytic uptake pathways are suppressed (figure 2). The identification of a non-active mode of entry directly into the cytoplasm supports the idea that these particles might be suitable for drug delivery and gene therapy, at relatively low exposure doses.

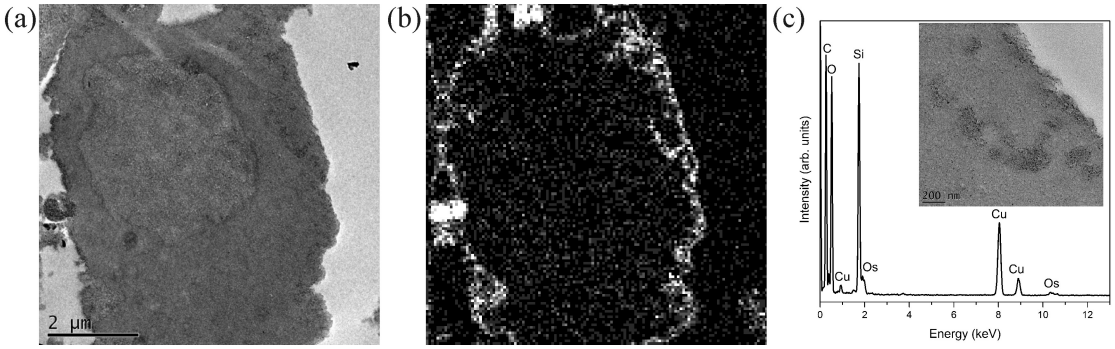

Figure 2 a) BF TEM image of A549 cells exposed to silica NPs at 4 °C, b) STEM Si-X-ray map showing membrane adsorption *and* localized penetration of silica NPs into the cell, without membrane encapsulation (BF TEM image inset in (c)) and c) EDX spectrum from particles in the cell confirming the presence of Si [6].

Zinc oxide (ZnO) NPs are used extensively in sun creams and whilst it is now generally accepted that these NPs do not penetrate beyond the stratum corneum of skin, the risks to human health through inhalation and ingestion at the manufacturing stage are still a concern [9]. Potential toxicity of ZnO is due to the compound's solubility in aqueous media, releasing Zn^{2+}. *In vitro*, exposure of cells to polymer-coated ZnO NPs results in reductions in cell viability that are more acute than exposure to uncoated ZnO NPs and almost comparable to the response upon exposure to soluble zinc salt solutions at similar Zn concentration. Monitoring of intracellular Zn^{2+} concentrations and non-reversible interactions with model membranes suggests the polymer-coated NPs are internalized before dissolution of the NPs is complete. TEM confirms NP internalization in membrane encapsulated compartments with clear evidence of porosity within the NPs, indicating partial dissolution (figure 3). Thus to limit toxicity, ZnO NPs with coatings that only weakly interact with cellular membranes and that inhibit pH driven dissolution of the ZnO in aqueous media are recommended.

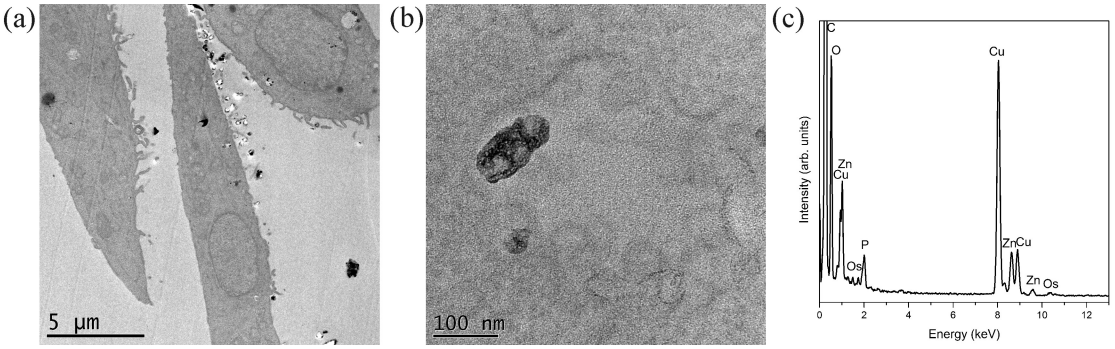

Figure 3 a) BF TEM image of A549 cells exposed to zinc oxide NPs, b) BF TEM image of ZnO NP in a vesicle within the cell, particle porosity suggests partial dissolution of the ZnO, and c) EDX spectrum from the particle in the vesicle confirming the presence of Zn [6].

In general, employing TEM sample preparation by resin embedding with only chemical-fixation (including osmium tetroxide) does enable visualization of the main cellular structure without contrast from the NPs themselves being obscured by heavy metal stains. This approach enables the easy localization of NPs within a cell and the use of a corresponding analytical capability is essential to confirm the elemental identity of the NPs [11]. Looking forward, quantification of NP uptake within thin sections of cells is also possible, however random sampling procedures or correlation to higher throughout techniques are required to measure a population of whole cells [5, 6]. Also quantification, by analytical TEM, of potential degradation of NPs within intracellular compartments (such as in figure 3), requires a more appropriate sample preparation to avoid re-diffusion of ionic species during fixing and processing. Analytical measures of the ionic content of whole cells prepared for EM by cryo-fixation and freeze drying have been shown to be a sensitive measure of cell viability [12]. Therefore, thin sections of cells prepared in this way should enable quantification of NP composition and ionic content within intracellular compartments.

4. Conclusions

Biomedical application of engineered nanoparticles is a growing research field in which some uncertainty remains as to the mode of action of many nanoparticle types. To eliminate this, *in vitro* cellular responses to a dose of NPs exposed to cell lines need to be correlated to the internalized dose per cell. In addition to the position and number of NPs within a cell, identification of any degradation of the NPs within intracellular compartments and its impact on cell viability is required. TEM is capable of identifying and quantifying cellular uptake of NPs however appropriate sample preparation and additional elemental analysis are required.

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