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Environmental scanning electron microscopy applied to visualize and quantify gold nanoparticle storage in whole, hydrated lung cancer cells

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The interaction of cells with nanoparticles (NPs) is a research field with strongly growing interest. Despite an increasing body of publications on NP uptake, transport, and storage, many fundamental aspects remain poorly understood. One of the main limitations is the difficulty to unite three important requirements for the imaging of NP-cell interactions: a) a resolution sufficient to image NPs, b) intact samples of whole cells, and c) a preparation and imaging protocol with a speed allowing to gain data of multiple regions on multiple cells as needed for quantitative and statistical analysis. The resolution requirement of several nanometers for these types of samples can only be achieved with electron microscopy (EM). Unfortunately, for conventional EM, samples have to be cut into thin sections, which cannot provide suitable data for NP quantifications, because the usual storage compartments of NPs, i.e. intracellular vesicles, are cut at random levels and not at their equatorial plane. In addition, the sample preparation is elaborate and carries the risk for artifacts. Gaining data from a high number of cells would therefore require an impractically long amount of time. Our approach for meeting the three above-mentioned requirements is to reduce the sample preparation to a minimum, similar to methods used in light microscopy, and to image NPs in whole, hydrated cells with a scanning transmission electron microscope (STEM) detector [1]. Imaging is done at 30 kV electron beam energy with an environmental scanning electron microscope (ESEM) equipped with a STEM detector [2]. The unique design of ESEM overcomes the usual EM associated need for a high vacuum and allows to directly image samples in hydrated state in a water vapor atmosphere. Alternatively, the cells can be imaged at 200 kV using a microfluidic chamber in a dedicated STEM [1].

We investigated the storage of uptaken gold nanoparticles (AuNPs) in a total of 176 intact and hydrated lung cancer cells (A549). The focus of the study was to examine if specific features of the AuNPs, such as their size and the presence of bound serum proteins, could affect the intracellular AuNP storage vesicles. Both parameters, AuNP size and presence or absence of serum proteins, have been shown to affect the early phases of AuNPs uptake, but for later periods in time, i.e. >24h after the AuNP uptake, their influence remained unclear [3]. Especially in view of the high potential of AuNPs to serve medical applications, quantifiable data are needed for assessing long-term intracellular storage and/or possible ways of elimination and removal from the cells.

The cells were grown on microchips with thin electron transparent SiN windows. For the AuNP uptake, the cells were incubated with citrate stabilized AuNPs of 10 or 30 nm diameter in cell medium without serum. For experiments with serum proteins, 10, 15 or 30 nm large, citrate stabilized AuNPs were first incubated for 2 h with cell medium supplemented with 10% serum in order to yield serum proteins coated AuNPs. These AuNPs were then diluted in serum containing medium and incubated with the cells. For both groups of AuNPs, with or without serum proteins, the AuNP incubation time was set to 2 h, followed by a 22 or 43 h incubation period under normal cell growth conditions, thus without AuNPs. Subsequently, the cells were fixed, cooled to 3 °C, rinsed with ultrapure water and placed in the ESEM (FEI Quanta 400 FEG). Two detectors were used synchronously: a STEM detector underneath the sample, for the recording of high-resolution images from the AuNPs, and a gaseous secondary electron (GSE) detector above the sample, serving the control of a thin water layer over the cells (see scheme in Figure 1A). The specimen chamber was kept at 3 °C, and was successively filled with saturated water vapor, whereby the chosen pressure settings avoided evaporation. During imaging the pressure was set to 740 Pa.

The image in Fig. 1B shows a group of confluently grown cells, fixed 24 h after they had been incubated with AuNPs of 30 nm diameter without serum proteins. The image was recorded with a low magnification of 2,000 × in order to provide an orienting overview. The randomly scattered distribution of round, electron dense structures in the cells can be seen. The dispersed character of the distribution was in contrast to findings from earlier, similar AuNP uptake studies in live COS7 cells (a monkey kidney fibroblast cell line), were the majority of AuNP storage compartments had gathered in a dense accumulation close to the nucleus [4]. Fig. 1C was taken with a magnification of 50,000 × at the indicated location in Fig. 1B. The image gives insight into the individual vesicles, revealing a mostly uniform density of AuNPs. This uniform intravesicluar distribution points to the adherence of AuNPs at the vesicle membrane, instead of a filling of the whole vesicle volume, and was in agreement with earlier results from COS7 cells [4]. Fig 1D is an exemplary image recorded with a magnification of 50,000 × from a cell that had been incubated under similar conditions but with serum coated AuNPs of 30 nm. The vesicles look very similar to those found with AuNPs without serum proteins. We found similar distributions of AuNPs in the

vesicles and similiar distributions of the vesicles throughout the cells for all images, also those with incubation times of 45 h. For further analysis, the sizes of 1,106 AuNP containing vesicles were determined. A statistical analysis of the size distribution found in each experimental group revealed a significant effect of the AuNP size on the size of the vesicles. The vesicles containing 30 nm AuNPs were on average 80 nm larger than those containing 10 or 15 nm AuNPs. Remarkably, this difference was not found for AuNPs without serum proteins.

These results show that the cellular storage vesicles for AuNPs found after >24 h of incubation can be influenced by the AuNPs themselves, i.e. their size, and this effect is triggered by the presence of serum proteins. Future studies aiming to examine cellular effects of different NP features should therefore also take account of a possible interplay of distinct NP features.

This study exemplifies how STEM detection in an ESEM opens new ways to study intracellular routing mechanisms for nanoparticles with nanometer precision in statistically relevant amounts of intact and minimally processed cells.

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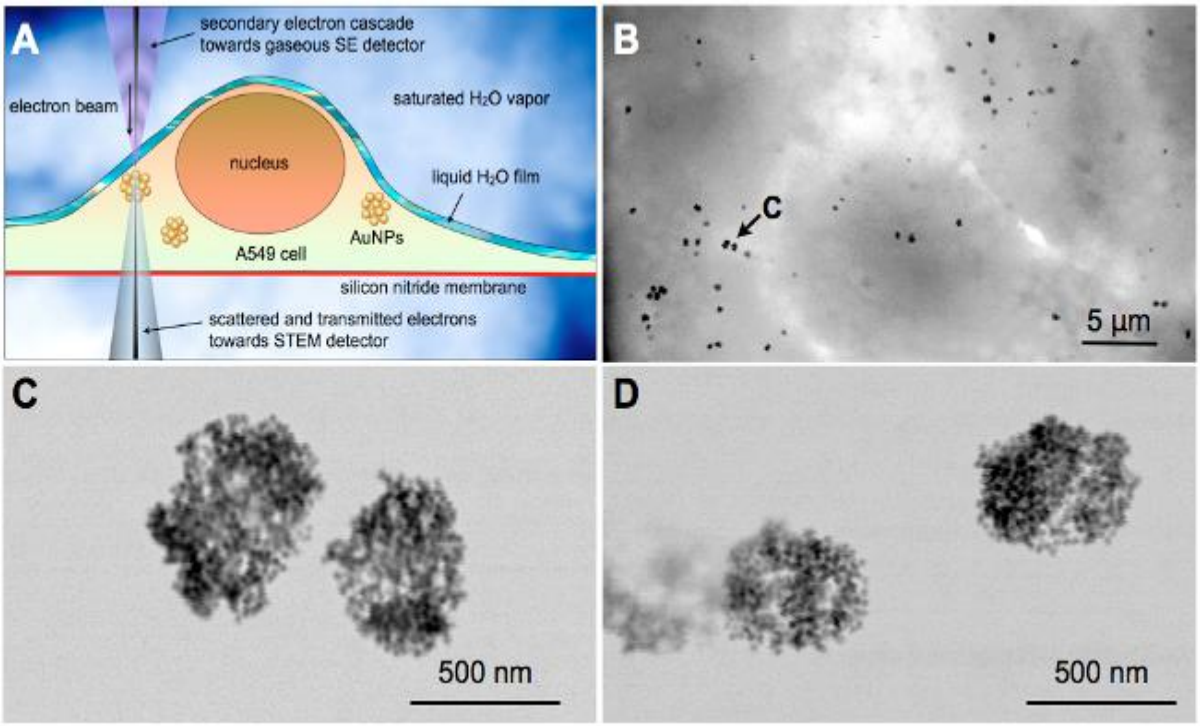

Figure 1. Whole A549 cells were imaged in hydrated state, 24 or 48 h after the uptake of differently sized AuNPs (10, 15 or 30 nm), with or without bound serum proteins A) Schematic representation of environmental scanning electron microscopy (ESEM) of whole cells in wet state. A focused electron beam (30 kV) was scanned over the cell, whereby a gaseous secondary electron detector (GSED), located above the sample, and a scanning transmission electron microscopy (STEM) detector, located beneath the sample, simultaneously collected the signals. B) STEM image showing several cells fixed 22 h after the 2 h during uptake period of 30 nm large AuNPs without serum proteins. The AuNPs concentrated in dark spots, diffusely scattered throughout the cell. C) Detail from a new image recorded with 50,000 × from the 2 vesicles indicated with an arrow in B. AuNPs were dispersed with an almost uniform density within the vesicles, pointing to the adherence of AuNPs at the vesicle membrane. D) Image recorded with a magnification of 50,000 × from a cell incubated under similar conditions as those shown in B and C, except for in the presence of serum proteins on the 30 nm AuNPs. The vesicles show a similar pattern of AuNP distribution.