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Time and Space Resolved Uptake Study of Silica Nanoparticles by Human Cells

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A spatio-temporal mapping of the uptake of silica (SiO$_2$) nanoparticles of different sizes by lung epithelial cells has been obtained. Based on high control of nanoparticle dispersion in cell media and cell exposure, one obtains reproducible and quantitative time-resolved data using a combination of flow cytometry, fluorescence and electron microscopies. We are thereby able to give a rather detailed account the journey of SiO$_2$ nanoparticles from the early events of uptake to their final sub-cellular localization.

**Introduction**

Scientists, regulatory authorities and industry are currently considering whether living organisms interact differently with nanoparticles than with larger particles of similar substances, or small molecules.$^{1-4}$ The increased accessibility of nanoparticles to cellular machinery (combined with their enormous surface area which binds large quantities of proteins and other biomolecules) may lead to new biological impacts from nanoscale materials.$^{5-8}$ Significant experimental and data interpretation challenges can also result from the nanoscale nature of materials, such as biomolecule-induced particle agglomeration and non-linear dose-responses due to particle agglomeration at high concentrations,$^9$ or the presence of the nanomaterials interfering with biological assay readouts by adsorbing the reporter molecules to their surface.$^{10}$ Combined, these effects have lead to some significant uncertainty in the literature, and to the need for systematic and highly controlled studies to assess the potential health implications of nanomaterials.$^{11, 12}$ There are few examples where a complete account of the uptake pathway and final localization of engineered nanoparticles can be given, for this requires a full time resolved study using a variety of complementary techniques. Furthermore, if one is to compare the different methods, it is necessary to ensure that the uptake process itself can be accomplished reproducibly. A variety of different cell-interaction outcomes with silica nanoparticles have been reported recently, some citing export or degradation after particle accumulation.$^{13}$ The existing literature contains a number of results involving different materials (mesoporous silica as opposed to amorphous silica, or materials of
different surface charge, size and aspect ratio) presented to a range of different cell types, with different suggestions for the uptake and transport pathways.\textsuperscript{14-18} In part because of this diversity of results and outcomes in the literature, we seek to be as definitive as possible on the more limited question of the spatio-temporal description of nanoparticle uptake up until cell division. To clarify these issues in a direct manner, sufficient controls have been established in this work to ensure reproducibility of the various measurands including, for example flow cytometry data, so that the basic elements of cell-uptake/export, intracellular transport, and final nanoparticle localization can be settled with certainty. Much more extensive studies would be required to settle the functional impacts, and detailed entry mechanism than have been hitherto undertaken.

Here, using lung epithelial cells as a simple model system, we illustrate how cellular uptake, trafficking and localization of nanoparticles can be resolved in a spatio-temporal manner from early entry to their final sub-cellular localisation. Limited results from our lab, not yet reported, using other cell types such as astrocytes, HeLa and others lead to similar conclusions. Using complementary methodologies of flow cytometry, confocal and electron microscopy (EM) studies, we describe and explain the detailed time course of uptake and localization of SiO\textsubscript{2} nanoparticles of different sizes (50, 100 and 300 nm) by A549 cells. Using fluorescently labelled SiO\textsubscript{2} nanoparticles, all of these methods can be applied to replicate samples, giving complementary information on different length and time scales. A detailed gallery of high resolution EM images at different exposure times is given to illustrate the typical locations of the SiO\textsubscript{2} nanoparticles inside the cells as a function of time. To address the much cited issue of the special role of 100 nm as a cut off for uptake in biological interactions,\textsuperscript{19-21} we show some comparisons for 300 nm particles, and discuss the implications of the results. We find that the uptake process for all sizes of the SiO\textsubscript{2} nanoparticles is active (requiring energy), and EM indicates that nanoparticles enter one by one engulfed in a vesicle, without evident involvement of classical-clathrin-coated pits. The intracellular nanoparticle concentration grows linearly with increasing exposure time. At shorter exposure times, endosomal structures are occupied, and later lysosomes are populated. Extensive studies have shown no evidence of
SiO$_2$ nanoparticle export or particle degradation, and lysosomes appear to be the major final end destination, with no evident association of the particles with other organelles in the cells. In no case is there evidence of association of SiO$_2$ particles of these sizes with the nucleus.

**Results and Discussion**

SiO$_2$ nanoparticles have been characterized by EM, and in dispersion in relevant buffers (as shown in the Supplementary Information in Table S1 and Figure S1A-C). Although the fluorescent dye label is chemically linked and (largely) internal, particles have also been checked for the presence of residual labile dye which could affect the interpretation of the uptake studies, as described in Salvati et al.$^{22}$ A fluorescence image of a representative SDS gel is shown in Figure S1D, together with the excitation and emission spectra of the SiO$_2$ nanoparticles in PBS, as a reference (Figure S1E). These gels indicate the relative intensities of the fluorescence associated with the SiO$_2$ nanoparticles (top bands) and the residual labile dye (bottom bands), which could be released from the particles in the cellular environment, an issue that confounds uptake and localisation results in many samples.

The SiO$_2$ nanoparticle dispersions in cell culture medium containing serum have also been characterised by DLS over a period of 24 hours at 37 °C, in order to obtain insight regarding the stability of the nanoparticles over the duration of their exposure to cells. The results for the 50 and 100 nm SiO$_2$ nanoparticles are shown in Figure S1F-G; upon contact with serum, the particles are coated by proteins and some larger particle-protein complexes are formed.$^{23,24}$ These dispersions are quite stable (as are the particle protein coronas) for the duration of our experiments (see for example Walczyk et al.$^{25}$).

Using well-characterized standardised procedures for nanoparticle dispersion and exposure to cells, quantitatively reproducible uptake curves can be obtained. In Figure 1A, uptake profiles for the 50nm SiO$_2$ nanoparticles taken in independent experiments are shown. The data are the averaged mean of the distributions obtained by flow cytometry on at least 15000 individual cells (see Methods for details).

Reproducibility of the replicates is excellent, and permits comparisons between the different techniques used. Figure 1B shows the uptake profiles for the three SiO$_2$ nanoparticle sizes after
normalization to the total number of particles in the dispersion and the average fluorescence per particle for each size, as the fluorescence intensity per particle differs for the different particle sizes, as shown in Figure S1E (see Methods section for a detailed explanation). The corresponding un-normalized data are given in Figure S2A, while Figure S2B shows time profiles for early stages of uptake of the SiO\textsubscript{2} nanoparticles. It is important to notice that un-normalised data can lead to wrong interpretations of these kinetic profiles. Since we have made efforts to obtain quantitative data, we also interpret it in terms of absolute intracellular mass (see Methods for details, though the raw data is still given in S2A). Without this step, the size dependence of uptake is deceptive, and we believe that this approach may be of value in future. The results indicate that SiO\textsubscript{2} nanoparticle uptake is less rapid as particle size increases, but even particles as large as 300nm enter the cells, with little evidence of a dramatic change in the internalisation kinetics for sizes beyond 100nm. Nanoparticle uptake is linear for the first few hours, and the deviations from this at later times are discussed after the presentation of Figure 4.

Systematic imaging studies on identical samples are revealing regarding the SiO\textsubscript{2} particle location(s) during the different periods of the uptake. Since the cells are exposed continuously to excess particles, we discuss when particles first reach a given location, and, separately, the different events that can be observed at steady state. Confocal microscopy and EM imaging clearly show that the final localisation of both the 50 and the 100nm SiO\textsubscript{2} nanoparticles is in the lysosomes. This is particularly clear after long exposure times, as shown in Figure 2 after 24h of uptake.

Earlier events of uptake and trafficking in cells are more complex. Fluorescence microscopy shows that after 2h of exposure to SiO\textsubscript{2} nanoparticles of sizes 50 and 100nm, there is no clear evidence of co-localization in either EEA1 positive endosomal structures (Figures S3A and S3B for 50 and 100 nm particles respectively), or in LAMP1-positive lysosomes (Figure S3C and S3D). Naturally, objects are not accumulated in EEA1-positive endosomes but are instead sorted to different locations into cells, and one is thus dependent on fluorescence intensity limits. Co-localisation with these structures cannot be observed by simply increasing the exposure time. Still, we note that, even after 24h of exposure, there is
no evident co-localisation with EEA1-endosomes (Figure S3E and S3F for 50 and 100 nm SiO$_2$ nanoparticles).

The time course of the EM images is quite unambiguous and shows that in first tens of minutes (Figure S4) SiO$_2$ nanoparticles pass the cell membrane and enter early endosomal structures, but are still largely absent from the rest of the cell. After 4 hours, as shown in Figure 3, SiO$_2$ nanoparticles have reached the lysosomes in some numbers (Figure 3A and B for 50 and 100 nm particles) and at 24 hours lysosomes are highly populated with particles (Figure 3C-D for both particle sizes). These results essentially interpret the flow cytometry results over the relevant time period.

Detailed EM images, as shown in Figure 4, illustrate the events occurring during the SiO$_2$ nanoparticle uptake and trafficking processes. Early cell membrane crossing and transport events are shown (Figure 4A for 50 nm, and B for 100 nm SiO$_2$ nanoparticles), indicating that particles enter one by one via membrane invaginations and early stages of transport into the cells within endosomes. Enlarged views of the invaginations yield no evidence of the involvement of clathrin. The resulting vesicle has sizes slightly larger than the 100nm nanoparticles (see panel A and B). Deeper into the cytosol there is evidence of more complex endosomal fusion events (Figure 4C). Though uncommon, multi-vesicular and multilamellar bodies containing particles have also been identified (4D and E). Short-time immunostaining with EEA1, clathrin heavy chain and caveolin 1 antibodies give no clear evidence of co-localisation with any of these structures (Figure S5). Whilst not conclusive, such experiments highlight the ongoing uncertainty about the nature of nanoparticle uptake pathways, and we consider it necessary to exercise caution in this whole question and in interpretation of the experimental results for the moment.

Depletion of cellular energy using sodium azide (which inhibits the respiratory chain in the mitochondria, thus impairing the production of ATP in the cell) or lowered cell culture temperature reduces considerably the rate of intracellular fluorescence increase, corresponding to reduced particle uptake, as shown by flow cytometry in Figure 5A. EM images (see Figure S7) of cells after 4h of exposure to SiO$_2$ nanoparticles in the presence sodium azide or at 4°C confirm the idea that uptake
largely ceases with energy depletion: almost no particles could be found in cells under these conditions. Interestingly though, with depletion of energy at 4°C, the few particles that enter are stopped (even after 4 hours) at early events and no particles could be found in lysosomes. This could reflect the fact that at this temperature not only are active processes impaired, but also that the lipids of the cell and organelle membranes have an increased tendency to form a ‘gel-like’ phase which further inhibits trafficking inside the cell. On the other hand, with sodium azide depletion of cellular energy, the rare particles that do enter go to the lysosomes. Even though these subtle differences could be observed, we may conclude that the major outcome of both these treatments confirmed that uptake is a strongly energy dependent process. These results are in agreement with similar data from the literature regarding the interaction of mesoporous silica with pancreatic and hepatic cancer cells, and silica coated nanoparticles with HeLa cells, where the authors also found the uptake to be energy dependent.\textsuperscript{17,18}

It is also of interest to investigate whether the SiO\textsubscript{2} nanoparticles have exit pathways once they have been taken up into cells and if silica degradation can occur once SiO\textsubscript{2} particles accumulate in the lysosomes. Indeed, silica has been even been posed as a useful drug delivery material, and it is of some interest to appreciate its fate at the cellular level. Thus, after some hours of exposure to SiO\textsubscript{2} nanoparticles, cells are washed and incubated with medium (without particles) and the fluorescence intensity in the cells observed as a function of time. The flow cytometry studies in Figure 5B indicate that, in this scenario, once nanoparticles have reached the lysosomes (after 17 hours exposure) fluorescence decrease is extremely slow. Direct imaging observations indicate that, upon cell division, the intracellular nanoparticle load is split, more or less equally, between the 2 daughter cells (representative confocal images are given in Figure S6). One can show that uptake competes with cell division leading to the apparent long-time saturation of fluorescence, as shown in Figure 1B. Using upper and lower bounds to cell division time (16 to 22 hours as indicated for this cell line), these time profiles can be modelled as explained in ref.\textsuperscript{22} and the typical slow decrease of fluorescence observed in Figure 5B can be explained by the only cell division, without recourse to nanoparticle export. Time
resolved EM images (see Figure 3) are in agreement and quite convincing. They yield no evidence of loss of SiO₂ nanoparticles by export or degradation, at least on the time-scales investigated here.

More refined analysis has been carried out by additional EM studies on nanoparticle final localisation and export: after allowing uptake for 4 hours, followed by removal of excess external particles and incubation of the cells in medium without particles, the time course of the typical movements of the internalised SiO₂ nanoparticles can be followed by EM (see Figure 6). These image sequences are striking. They suggest that the SiO₂ nanoparticles continue to travel to the lysosomes (only rarely are particles still seen in intermediate locations), and after 24h of this chasing experiment, nanoparticles could still only be found in the lysosomes, where they appear to remain. Whilst one cannot fully exclude export processes (and indeed there is no real reason to expect lysosomes to release the particles), if they exist, they are rare events. That is, nanoparticles will tend to accumulate irreversibly in lysosomes.

Having summarized the main results, several more subtle aspects of the system are worth comment, especially for the larger SiO₂ particles. Detailed EM images for the uptake of the 300nm particles by A549 cells (Figures S8) show that even these larger particles enter as single objects. It is difficult, however, to visualise the presence of a vesicle around them during the early events of uptake, as was evident for the smaller particle sizes. This could be due to the increased contrast for these larger SiO₂ particles, which makes the proximate structures of the cell less visible, but it may also signal a different uptake process. As was the case for the smaller SiO₂ particle sizes, the final particle localization is in confined structures inside the cells (see Figure S9). These confined structures appear to be lysosomes (note that the ‘holes’ are likely due to the much bigger size of the particles in comparison to the ultramicrotomy slices of 80nm). In some cases, the 300nm SiO₂ particles seem to accumulate in organelles that resemble macropinosomes. Further studies are required to confirm these preliminary results. In light of what is reported here, efforts should be made to study the precise nature of the uptake mechanisms, without prejudice or a priori assumption that any of the well known uptake mechanisms are at work. Fluorescence immunostaining confirms co-localization of the 300nm SiO₂ particles with
lysosomes already after 4h (see Figure S9), but as for the other particle sizes these is no evidence of co-localization with EEA1 labeled early endosomes.

**Conclusions**

In this work, SiO$_2$ nanoparticles of different sizes have been exposed in a controlled and reproducible way to A549 lung epithelial cells. We establish that, if one fixes carefully all aspects of the system, from nanoparticle dispersion in cell media, to exposure to cells and sample preparation for the final measurements, dose control and quantitative reproducibility of these complex systems can be achieved. This allows us to ‘make a movie’ of the uptake process via the different, complementary, imaging approaches, so that they may be visualized and the process quantified from the early entry to the final sub-cellular destination of the particles. This clarifies rather well the nature of the processes, at least for silica nanoparticles, and illustrates how such studies may be planned in future.

Our results show that uptake of SiO$_2$ nanoparticles by A549 cells is energy dependent but with no evident involvement of classic pathways like clathrin or caveolin dependent mechanisms. Moreover, even particles larger than 100nm can easily enter the cells (300nm) although with lower efficiency of uptake. Competing nanoparticle export, if present at all, is insignificant, SiO$_2$ nanoparticles accumulate in the lysosomes, and there is no evidence of silica particles reaching the nucleus under the conditions studied. By making these statements we do not exclude the possibility of rarer events in which nanoparticles escape the uptake pathway and reach other organelles, and certainly fluorescence microscopy of this type is limited by the label intensity, while electron microscopy is limited by statistics.

Our hope is that these studies will frame the further investigations needed to obtain a full understanding of the biological pathways of uptake of nanoscale materials, which is still a topic of considerable uncertainty.
Experimental section

Nanoparticle characterization. Silica (SiO$_2$) nanoparticles were purchased from G. Kisker-Products for Biotechnology (Steinfurt, Germany) at sizes of 50, 100 and 300 nm with green or red fluorescent labels. Unless otherwise stated, the experiments were performed using the green fluorescent variants. To confirm that the size of the nanoparticles matched the size as stated by the manufacturers, EM pictures of the dried nanoparticles were taken. Particle dispersions were characterized at concentrations of 100 µg/ml in millipore water, PBS, and the cell culture media, using a Malvern Zetasizer Nano ZS90 (Worcestershire, UK) to measure the hydrodynamic radius by Dynamic light scattering (DLS) and the zeta potential (surface charge). The samples in cell culture media have been characterized for up to 24h of incubation at 37°C, in order to obtain a better description of the evolution of the protein corona formed upon contact with the serum and to study their stability against agglomeration during the full length of the exposure to cells. The emission and excitation spectra of the fluorescent SiO$_2$ nanoparticles were produced using a Perkin-Elmer LS 50B fluorimeter (Perkin-Elmer, Waltham, Massachusetts). The green-labelled SiO$_2$ nanoparticles were excited at their optimum wavelength of 485 (as specified by Kisker-Products) and at 488nm (to match the excitation wavelength used in the Flow Cytometer for cell uptake) and their emission spectra compared.

Cell Culture. A549 cells (passage 1-30 after defrosting from liquid nitrogen; original batches from ATCC, item number CCL-185, at passage number 105 or 82) were cultured at 37 °C in 5% CO$_2$ in Minimum Essential Medium (MEM, with additional L-Glutamine) supplemented with 10% Fetal Calf Serum (FCS, Gibco), 1% penicillin/streptomycin (Invitrogen Corp.), and 1% MEM non-essential amino acids (HyClone). Cells were confirmed to be mycoplasma negative using the mycoAlert kit (Lonza Inc. Allendale, NJ) and were tested monthly.

Cellular treatments and nanoparticle dispersion in cell medium. Cells were plated at a density of 2.5 x 10$^5$ cells in a 6cm plate and allowed to adhere for 24 hours before exposure to 100µg/ml SiO$_2$ nanoparticle dispersions. Nanoparticle dispersions were prepared by diluting the concentrated nanoparticle stock solutions into the complete medium used for cell culture at room temperature,
immediately prior to the experiments on cells, with an identical time delay between diluting and introducing the particles to the cells for all experiments. The medium was kept at room temperature and not pre-warmed to 37°C to ensure better nanoparticle dispersions.

Cells were incubated with nanoparticles for the required times, depending on the experiment, and then the particle-containing medium was discarded. In the case of export experiments, after exposure to particles performed as described above, the dispersion was discarded and after 3 washes with DPBS, medium without particles was added to the cells which were further incubated for the appropriate times. After the required import or export incubation time, medium was removed and the samples were washed thrice with DPBS and prepared for flow cytometry as described below. Uptake and export profiles of the different sized SiO_2 nanoparticles were investigated under several conditions. Energy dependence of the uptake of the SiO_2 nanoparticles was determined by pre-incubating cells for 60 minutes at either 4°C or in media containing 5mg/ml sodium azide (Invitrogen) prior to the introduction of the nanoparticles. These energy-depleting conditions were maintained for the duration of the uptake experiments.

**Flow Cytometry.** Flow cytometry was carried out in the Flow Cytometry Core Facility of the Conway Institute at University College Dublin. Fluorescence levels in the cells were measured using a CyAn™ ADP Analyzer by Beckman Coulter (Fullerton, CA). Following treatment with nanoparticles, cells were washed three times with PBS, to ensure particle removal from the outer cell membrane, and trypsinized for 3 minutes with 1ml 0.5% Trypsin-EDTA (GIBCO, Invitrogen). After deactivation of the trypsin by addition of 1 ml of complete MEM, cells were pelleted by centrifugation at 1500rpm for 3 minutes before being re-suspended in 4% Formaline solution for fixation for 20 minutes. After fixation, cells were pelleted again by centrifugation for 3 minutes at 1500rpm and re-suspended in 1ml PBS. Samples were stored at 4 °C for approximately one hour before fluorescence levels were detected using the Flow Cytometer.

The results are reported as the mean of the distribution of cell fluorescence intensity, obtained on at least 15000 events (cells), averaged between 3 independent replica. Error bars are the standard deviation between the replica. The full time curves were performed at least 3 times.
Normalisation of the flow cytometry data. As shown in Figure S4B, without normalisation of the data, the fluorescence of the cells due to SiO$_2$ nanoparticle uptake is higher for larger particles. Given that cells are treated with the same mass of nanoparticles, which means that very different particle numbers are presented to the cells, and that the intensity of the starting dispersions are slightly different for the different SiO$_2$ nanoparticle sizes (as shown in Figure S2B), the raw data for each particle size have been divided by the intensity of fluorescence of a single nanoparticle of that particle size. This normalisation factor is obtained by dividing the intensity of emission of the starting dispersion (100 µg/ml in PBS as obtained in Figure S2B) by the number of particles in 100 µg for each particle size calculated assuming monodisperse spherical particles. After this normalisation, the fluorescence profiles are inverted (as shown in Figure 1B) and this indicates that uptake is larger for the smaller SiO$_2$ nanoparticle sizes, as one would expect for non-phagocytic cells.

Confocal Imaging. Cells were plated on 35mm plates with 15mm diameter glass coverslips at densities ranging from 1.25x10$^5$ to 1.8x10$^5$ cells and treated as described above for flow cytometry sample preparation. For actin visualization, cells were washed with 3 x 1ml PBS, permeabilized for 5 minutes with 0.1% saponin from Quillaja bark (Sigma, St. Louis, MO), washed again with 3 x 1ml PBS then incubated at room temperature for 20 minutes with a 2% Texas Red-X Phalloidin (Invitrogen) and 0.5% BSA in PBS. Slides were then washed with 3 x 1ml PBS, treated for 3 minutes with DAPI to stain the nuclei, washed with 1ml PBS, and then mounted onto slides for imaging. For lysosome and early endosomes staining, samples were washed with 3 x 1ml PBS, fixed for 20 minutes with 1ml 4% Formaline, permeabilized for 5 mins of 1ml 1% saponin from Quillaja bark (Sigma), and incubated for 30 minutes at room temperature with a blocking solution of 1% Albumin Bovine Serum Fraction V (Sigma) in PBS-T to prevent non specific binding. Samples were incubated for 1 hour at room temperature with a primary antibody of 1:330 mouse mAb to EEA1 (Abcam, Cambridge, UK) or 1:200 mouse mAb to LAMP [H4A3] (Abcam, Cambridge, UK), washed with 3 x 1ml PBS, and then incubated at room temperature for 1hr with 1:400 dilution of AlexaFluor 647 Goat Anti-mouse IgG (H+L) as a secondary antibody. Samples were washed 3 x 1ml PBS and incubated for 3 minutes with DAPI before
mounting with MOWIOL on slides for imaging. The cells were observed using a Carl Zeiss LSM 510 Meta laser scanning confocal microscope (Zeiss, Munchen, Germany) with lasers at 364nm (DAPI), 488nm (FITC labelled SiO$_2$ nanoparticles), 543nm (Phalloidin), and 633nm (EEA1 and LAMP antibodies).

Electron Microscopy. A549 cells treated as described above were fixed at room temperature in 2.5% glutaraldehyde in 0.1 M Sorensen phosphate buffer (pH 7.3) for 1h, rinsed with Sorensen phosphate buffer (pH 7.3), and then post-fixed for 1h in 1% osmium tetroxide in deionised water. After dehydrating the samples in increasing concentrations of ethanol (from 70% up to 100%), they were then immersed in an ethanol/Epon (1:1 vol/vol) mixture for 1h before being transferred to pure Epon and embedded at 37 °C for 2 hours. The final polymerization was carried out at 60 °C for 24 hours. Ultrathin Sections of 80nm, obtained with a diamond knife using an ultramicrotome Leica U6, were mounted on copper grids, and stained with uranyl acetate and lead citrate before being examined with an FEI TECNAI Transmission Electron Microscope. The Electron Microscope images were acquired, for each condition, on (technical) replicate samples and three independent biological replicas (that is, different experiments) to confirm the validity of the observed results. An average of about 50 images was taken for each sample. Representative images have been selected for publication. Whilst the data and imaging seem compelling, it will in future be useful to strive toward more statistical analysis in imaging.

SDS PAGE. SDS Polyacrylamide Gel Electrophoresis (PAGE) gels (4% stacking gel and 10% resolving gel) were used to estimate the ratio of fluorescence due to the SiO$_2$ nanoparticles and that due to residual labile dye that elutes from the particles. Particles (25mg/ml) were diluted 1:1 with loading buffer (10% mercaptoethanol, and 20% glycerol in Tris/HCl 25 nM ph 6.9, with 10% SDS), and loaded into the stacking gel. Gels were run at 120mV for 45-60 minutes. Pictures were acquired using a Fujifilm Intelligent dark box LAS-3000, exciting the gels with a blue light at 460nm and emission was recorded with a 515nm filter. Images were taken at 2 second exposure times.
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Figure 1. **1A**: Three replicas of a time profile for endocytosis of 50 nm SiO$_2$ nanoparticles exposed to A549 cells at 100 µg/ml. **1B**: Normalised time profile of uptake of 50, 100 and 300 nm SiO$_2$ nanoparticles exposed to A549 cells at 100 µg/ml. The cell fluorescence intensity is normalised by the fluorescence intensity and the number of nanoparticles in the starting dispersion, as described in the Methods section.
**Figure 2**: Co-localisation of green SiO\textsubscript{2} nanoparticles with lysosomes in A549 cells. Confocal and EM images of A549 cells after 24h of exposure to 100 µg/ml 50 nm (A and C) and 100 nm (B and D) green SiO\textsubscript{2} particles. Red: immunostaining of lysosomes with LAMP1 antibody (secondary Alexa-647 antibody). Blue: DAPI staining of nuclei. Arrows indicate the localization of nanoparticles in the cells.
Figure 3: EM images of A549 cells exposed for 4h (A and B) and 24h (C and D) to 100 µg/ml 50 and 100 nm green SiO$_2$ particles (A, C and B, D respectively), showing the later stages of uptake and nanoparticles sub-cellular localisation. Arrows indicate the localization of nanoparticles in the cells.
**Figure 4**: EM images of the early events of uptake of (A) 50 nm and (B) 100 nm green SiO$_2$ nanoparticles. (C-E): 50 nm green SiO$_2$ nanoparticles in early endosomes, multilamellar bodies and multivesicular bodies, respectively. Arrows indicate the localization of nanoparticles in the cells.
Figure 5: A: Energy dependence of endocytosis of 50 and 100 nm SiO$_2$ nanoparticles exposed to A549 cells at 100 µg/ml at 4°C or with 5 mg/ml NaN$_3$. B: Export of 50nm and 100nm SiO$_2$ nanoparticles: after 17h exposure to 100 µg/ml nanoparticles in complete media, the particle source is removed to study if there is export or degradation of the internalized nanoparticle load, which would be observed as a decrease in the average cellular fluorescence. The data are normalized for the starting cell fluorescence intensity immediately prior to particle removal (Export time 0h).
Figure 6: EM images of A549 cells exposed for 4h to 100 µg/ml 50 nm green SiO$_2$ nanoparticles, followed by replacement of the nanoparticle-containing media with fresh (particle-free) media and imaging after an additional 2 (A), 4 (B) and 24h (C) of incubation. Arrows indicate the localization of nanoparticles in the cells.
Uptake of silica nanoparticles is resolved from the early events of entry to the final localization inside the cell.

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