Experimental and theoretical approach to comparative nanoparticle and small molecule intracellular import, trafficking, and export

Anna Salvati,[12] PhD, Christoffer Åberg,[1] PhD, Tiago dos Santos,[1,2] Juan Varela,[1] Paulo Pinto,[1] Iseult Lynch,[1] PhD, Kenneth A. Dawson[1*], Prof.*

[1]Centre for BioNano Interactions, School of Chemistry and Chemical Biology & [2] UCD Conway Institute for Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland.

Short title: Nanoparticle import, trafficking and export

*Corresponding authors: Kenneth A. Dawson (Kenneth.A.Dawson@cbni.ucd.ie); Anna Salvati (Anna.Salvati@cbni.ucd.ie)

Centre for BioNano Interactions, School of Chemistry and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland. Phone: +353 (0)1 716 6928; Fax: +353 (0)1 716 2415.

Funding for the project has been generously provided by EU FP6 NanoInteract (NMP4-CT-2006-033231), SFI RFP (06/RFP/CHP031 and 09/RFP/MTR2425), EU MC RTN Arrested Matter (MRTN-

CT-2003-504712), EU FP6 SIGHT (IST-2005-033700), the Irish Research Council for Science, Engineering and Technology (A.S. and C.Å.), and is additionally based upon works supported by Science Foundation Ireland under Grant No. SFI/SRC/B1155. No conflict of interests is reported for any of the authors of this manuscript currently and within the past five years.

Manuscript word count: 4883. Number of figures: 6

Central to understanding how nanoscale objects interact with living matter is the need for reproducible and verifiable data that can be interpreted with confidence. Likely this will be the basis of durable advances in nanomedicine and nanosafety. To develop these fields, there is also considerable interest in advancing the first generation of theoretical models of nanoparticle uptake into cells, and nanoparticle biodistribution in general. Here we present an uptake study comparing the outcomes for free molecular dye and nanoparticles labeled with the same dye. A simple flux-based approach is presented to model nanoparticle uptake. We find that the intracellular nanoparticle concentration grows linearly in time, and that the uptake is essentially irreversible, with the particles accumulating in lysosomes. A wide range of practical challenges, from labile dye release, to nanoparticle aggregation and the need to account for cell division, are addressed to ensure these studies yield meaningful kinetic information.

Keywords: nanoparticle uptake, kinetics, flow cytometry, flux-based phenomenological model

Background

Radically new considerations emerge in the interactions between nanomaterials and living matter for sufficiently small nanoparticles.[1-3] Smaller than about 200 nm particles may enter unspecialized cells with great ease,[1] less than 35 nm particles sometimes enter the nucleus,[4, 5] and less than 30 nm particles are capable of olfactory neuronal transport into the Central Nervous System.[6] Small apolar molecules typically partition across organs and cellular compartments according to equilibrium principles, but nanoscale objects are processed by the cellular machinery, and are thereby trafficked by active processes in much the same manner as biomolecules.[7-15] This provides new opportunities in nanomedicine, and necessitates careful consideration of nanosafety issues. Whilst there is a legitimate concern about the safety of nanomaterials, there is as yet very limited evidence of hazards, although

institutions across the world have exercised caution nevertheless.[16, 17]

The present paper seeks to clarify a systematic and ultimately quantitative approach to determine nanoparticle uptake and sub-cellular distribution, and to describe the theoretical models required in framing the broader scientific issues underlying nanoparticle and small molecule internalization by cells. However, in providing the examples cited here, we seek to clearly acknowledge the challenges, many not yet commonly known, in achieving these objectives. In particular we will also seek to highlight some technical issues (such as labile fluorescent labels, nanoparticle dispersion quality, imaging and biological preparation) that, currently lacking simple solutions and standardized approaches, need to be considered carefully in future studies.

Some of the challenges, ranging from labile fluorescent labels, to tissue culture reproducibility, and nanoparticle dispersion control and characterization, are somewhat known, but not effectively translated across to bionanoscience.[18, 19] A widespread, but poorly appreciated problem is the presence of residual 'labile' dye that is released from nanoparticles in a biological milieu, and in particular within the cell. Such nanoparticle impurities are found not to be easily removed by classical dialysis (or indeed other) methods,[20] and the reasons are sufficiently general to require careful note.

This basic background means that overall molecule and nanoparticle uptake kinetics can be determined, interpreted and phenomenologically modeled in characteristic manners, based on competing kinetic terms that differ for small molecules and nanoparticles.

Intracellular fluorescence from solutions of the molecular dye increases rapidly via conventional physical transport, and is barely cellular-energy dependent. The resulting intracellular fluorescence is then distributed between compartments in the cell, mostly in the endoplasmic reticulum, as for many other hydrophobic dyes. Fluorescence accumulation is reversible, and one can apply conventional physiochemical kinetics ideas in multi-compartment models. The key idea is that competing entry and exit kinetics of the dye leads to a steady state (in fact equilibrium) intracellular concentration, with equilibrium partitioning between the compartments.

The growth of intracellular fluorescence carried by nanoparticles is, on the other hand, strongly dependent on the cellular energy used to drive active cellular transport. Following exposure of cells to the nanoparticles dispersion, nanoparticles are observed to populate early endosomal structures within ten minutes, later reaching lysosomes. Similar results are reported in literature for nanoparticles of different sizes and composition.[21-24] When the particle source is removed, competing export processes are negligible, at least for the cases studied here, but also for most systems that terminate in lysosomal population.[21, 22] However, in the presence of a continuous source of nanoparticles, a new steady state emerges at longer times, when cell division competes against nanoparticle uptake. It is important to note the origin of this saturation phenomenon so that it is not confused with explicit extracellular export.

From a more technical point of view, where there is a comparable amount of labile and nanoparticle associated dye, the residual (possibly irremovable) labile dye leads to a mixture of molecule and nanoparticle uptake behaviors. The exceptional capacity of the intracellular machinery to mobilize previously immobile label (and likely other small molecule species) leads to the risk of confusing the labile portion of the dye label for nanoparticles. This easily leads to mistakenly associated co-localization of nanoparticles to other organelles. For these cases, comparison of import and export kinetics, along with energy depletion, can be used to isolate (and quantify) the contributions from the pure molecular or nanoparticle behaviours.

Methods

Commercially available fluorescently labeled polystyrene nanoparticles of 40-50 nm diameter have been used to quantify nanoparticle uptake into cells.

All studies were performed using A549 cells (lung carcinoma), seeded and grown for 24 hours in complete MEM (cMEM), with the medium then being replaced by nanoparticle containing dispersions or free dye (also in cMEM) for differing times.

Individual intracellular fluorescence intensity is measured via flow cytometry and averages are taken

over large numbers (typically 15,000-50,000) of fixed cells to produce a time-resolved average intracellular fluorescence curve. Results are correlated with confocal and epifluorescence microscopy images of replicate samples treated in the same way.

The detailed description of the materials used and the experimental methods is given in the Supplementary Material.

Results

Materials and Their Quality

Different kinds and batches of fluorescently labeled polystyrene of 40-50 nm diameter, commonly available for reference purposes, have been used as a model to study nanoparticle uptake. These nanoparticles have no effect on cell viability.[24] Their measured size, size distribution and zeta potential under the relevant dispersion conditions are reported in Table S1 in the Supplementary Material, including in cell medium containing 10% FCS (cMEM) at the initial time (i.e. at the time of mixing). Electron Microscopy characterization of their dry size is shown in Figure S1.[21] To fully describe the real complexes formed *in situ* upon contact with serum / cell culture medium, a detailed analysis of the biomolecule corona composition is needed, as reported in ref.[25] for similar systems. Here, the results in Table S1 constitute a control of the size and polydispersity of the applied batches of particles, and are shown to imply an informal biocolloidal reference state for the dispersions as presented to cells.

Nanoparticles labeled by embedding a fluorescent marker in the polymeric matrix often suffer leakage of free dye once in contact with cells, and this can create artifacts in uptake studies. Under common laboratory dialysis conditions, although some labile dye is removed, another fraction remains unavailable to the usual cleaning procedures. Though little known, this phenomenon is present in many labeled nanoparticle systems, besides those discussed here. Even dialysis against SDS can leave a residue that becomes mobilized once the particles come into contact with the cells, and this could lead to highly undesirable artifacts in the literature. Several methods have been developed to measure the

release of hydrophobic drugs from nanoparticles[26] and to measure drug transfer between lipid particles.[27] Here, gel electrophoresis under appropriate conditions has been used to estimate the ratio of fluorescence due to nanoparticles and labile dye, as illustrated by the results shown in Figure S2. The presence of significant amounts of labile dye in the gels is quite well correlated with the dye released into cells. However, as shown in Figures 1 and 4, the analysis of the kinetic profiles and their energy dependence is a more certain strategy to isolate the pure nanoparticle (uptake) behavior in cells.

Method to Determine Kinetics of Uptake Using Average Intracellular Fluorescence

Representative flow cytometry distributions of cell fluorescence intensity in a cell population exposed to labeled nanoparticles is given in Figure S3, where we also show typical forward and side scattering results.

In order to be able to reproduce and quantify nanoparticle uptake in cells, care has been taken to develop protocols for the various steps in the experiments, ranging from preparation of nanoparticle dispersions in the cell culture medium containing serum, to exposure of the cells to nanoparticles and preparation of the samples for fluorescence assessment by flow cytometry. It should be noted that to control these experiments considerable efforts are required, and in their absence, particle uptake between nominally identical systems can differ strongly. On the other hand, Figure S4 shows a comparison of different time resolved average intracellular fluorescence curves, obtained for the same nanoparticles by different operators, using different flow cytometry instruments, illustrating the reproducibility that can be achieved by applying appropriate protocols. The overlap is promising and constitutes a basis to allow a quantitative analysis and modelling of nanoparticle uptake by cells.

An overall summary of the early stage kinetics of uptake of the different nanoparticles studied is given in Figure 1A and the same uptake curves over significantly longer times are recorded in Figure S5. Figure 1B shows the decay of intracellular fluorescence after 4 hours of uptake, followed by rapid washing, and addition of fresh medium (without nanoparticles or dye).

During uptake (Figure 1A), for some samples (e.g. samples P_1 and P_2) the fluorescence rises rapidly, followed by an apparent saturation phenomenon at a fixed concentration on the timescale of 30 minutes. For other samples (e.g. sample I_1) the fluorescence rises quite quickly, then, after a change of slope, continues to grow linearly, without any accessible saturation concentration at shorter time-scales. Curves that do and do not saturate correspond respectively to cells treated with samples P_1 and P_2 with extensive labile dye and with sample I_1, with little labile dye (as shown in Figure S2), so saturation is consistent with the presence of larger amounts of labile dye.

Illustrative confocal fluorescence images of the qualitatively different systems are given in Figure 2. In the sample with a large amount of labile dye (P_1) the fluorescence is spread across the intracellular space, although co-staining (Figure S6A-C) indicates significant association of the dye with the endoplasmic reticulum, and the lipidic compartments of the cell. This is consistent with images of cells exposed to hydrophobic molecules such as Nile red (Figure S6D) and the pure YG dye (Figure S7). In contrast, for the nanoparticle-dominated case (I_1) the fluorescence distribution is localized. Figure 3 shows the co-localisation of the nanoparticles with early endosomes and lysosomes as a function of time, after exposure to nanoparticles and washing ('pulse and chase'), together with representative confocal images. Some co-localisation of nanoparticles occurs with both early endosomes and lysosomes during the period of the pulse. Thereafter, the cells are washed, and the system is chased with medium. Both from the images and the co-localization correlation coefficients we see that particle localization with the early endosomes decreases and particle localization with lysosomes increases with the same characteristic time-scale of around one hour. It is also interesting that co-localization with lysosomes increases, plateaus, and then decreases slowly over much larger time-scales.

In terms of the export kinetics, for cells treated with pure dye, or labile dye rich nanoparticles (P_1 and P_2) (Figure 1B), there is a rapid decay of fluorescence over several minutes, followed by a slightly slower decay process. For nanoparticle samples in which there is little labile dye (I_1), after a small rapid

decay, fluorescence decrease is very slow, and falls to half of its original value only after 24 hours.

Energy-dependence of uptake and export processes

The impact of energy depletion (pre-treatment of the cells with sodium azide or performing the experiment at 4 °C) on cellular uptake of the nanoparticles is used to determine whether uptake is active (requiring cellular chemical-mechanical energy) or passive (a purely physiochemical diffusive process).

For samples respectively poor and rich in labile dye we see significant differences in the uptake behavior under the different energy conditions. In the first case, (Figure 4A), for nanoparticles I_1 there is a linearly growing uptake of fluorescence which is essentially stopped by energy depletion. In samples where there is mostly labile dye (P_1), neither the value of the fluorescence as it reaches saturation, nor the steady state values are much affected by energy depletion (see Figure S8A). The same occurs for the free YG dye, as shown in Figure S8B, together with the uptake under the different energy conditions of FITC dextran as a control (Figure S8C). The results for energy depleted cells are confirmed by the corresponding confocal images, also shown in Figure S7.

Strikingly in Figure 4B we show that, in the case of particle export, what little decay of fluorescence there is in the nanoparticle sample with limited labile dye (I_1) is effectively stopped when cellular energy is depleted, whereas both the labile dye rich sample (P_1), and the pure YG dye have a similar rapid decay of fluorescence, even when energy is depleted. The presence, even under energy depleted conditions, of a starting rapid decay of fluorescence in the nanoparticle dominated case is again consistent with the presence of a small fraction of labile dye also in this sample.

Discussion

The uptake of fluorescence for a number of the nanoparticle samples studied here (for example P_1 and P_2) is dominated by the presence of labile dye, rather than by the particles themselves, which constitute a relatively small part of the intracellular fluorescence. Fluorescence saturation at a level that depends

on the extracellular concentration is suggestive of a simple equilibration across a semi-permeable membrane, consistent with the behavior of the pure YG dye. Dead and energy depleted cells give similar results for those samples, again consistent with the usual physiochemical process of diffusion across the cell membrane, which occurs without the cells expending energy.

'Standard' model of reversible kinetics for small molecules

The uptake process of small molecules can be -described well by a simple reversible first order kinetics model with equilibrium rate constants, although there are several timescales involved in the kinetics. A simple sketch of such a model is illustrated in Figure 5A, together with a description of the kinetic model for nanoparticles in Figure 5B. This model could also be used to fit the uptake of nanoparticles containing a high amount of labile dye (P_1 and P_2).

Phenomenological model for Nanoparticle Import and Intracellular Trafficking

The samples in which the fluorescence is nanoparticle borne (I_1) behave quite differently. The uptake has a fast small rise within the first few tens of minutes, followed by essentially linear uptake kinetics over significant time periods (Figure 1A). It is likely that the initial fast rise, at least in part, is due to a remnant of the labile dye, as shown in Figure S2, although a contribution from nanoparticles associated to the cell membrane and not fully washed away, cannot be excluded. The linear uptake in energy depleted cells vanishes, suggesting energy dependent uptake processes (Figure 4A).

The decay of intracellular fluorescence (after a pulse of nanoparticles, followed by a chase period in medium without nanoparticles) is also studied in order to provide information on nanoparticle export (Figure 1B). The small initial rapid decay is likely due to a remnant of the labile dye (or residual nanoparticles dissociating from the cell membrane), and there is then a relatively long period over which the intracellular fluorescence does not change much. Still, the decay on long time scales, from an

intensity of around 0.8 and onwards, represents a genuine decrease in the fluorescence due to nanoparticles. Extensive study of many examples of time resolved images of cells where the fluorescence decreases suggests no evidence of nanoparticles exiting from lysosomes once they have arrived there. This conclusion is confirmed by the corresponding confocal live-cell imaging of cells (see movie S1 for example), which also allows exclusion of artifacts due to the fixation procedure. Moreover, similar studies ensure the stability of the labeled nanoparticles over time, even in the acidic conditions of the lysosomal compartment. Similar conclusions have been reported for other materials also accumulating in the lysosomes, where no strong evidence of particle export nor degradation could be observed for several hours after cellular uptake.[22, 23] In essence, all evidence points to the simple conclusion that once nanoparticles arrive in lysosomes, they remain there. In fact, over time, the only changes observed (by optical imaging techniques) suggest that the intracellular load of nanoparticles is divided between daughter cells quite equally upon cell division (Figure S9, also observed for silica nanoparticles accumulated in the lysosomes[22]). It is also worth noting that, based on a cell population doubling time of 22 hours (as indicated in the ATCC bank characterisation for this cell line), one could attribute all of the fluorescence decay observed to cell division (see Figure 5C, and the discussion below). Our interpretation is supported by the fact that the energy depleted case (Figure 4B) shows essentially no reduction, after the initial transient, in the apparent intracellular nanoparticle load during this period, and independent studies also show that under these conditions cell division is arrested.

Thus, the whole context of energy dependent cellular processes gives no reason to suppose that there are reverse (exit) processes for nanoparticles. For nanoparticles to exit the cell a specific nanoparticle surface signal would be required to harness an export pathway. There is no evidence that such a signal exists for these particles (or most others not specifically engineered to express such a signal), and the chances of the appropriate specific intracellular corona arising from non-specific protein binding during uptake are slim.[28]

Results presented in Figure 3 allow us to interpret what is happening inside the cell after the

nanoparticle pulse, when total intracellular fluorescence is relatively constant (Figure 1B). The co-localization probability kinetics may be interpreted as giving the characteristic relaxation (exit) time from the early endosomal compartment and the lysosomal uptake time. As expected, these are similar (τ~1 hour), consistent with the idea that there is a single onward type of pathway from endosomes to lysosomes, with a single mass transport coefficient. Intriguingly, on much longer time-scales (23 hours) the endosomal co-localization, having already fallen to its minimum, is unchanged, whereas lysosomal-particle co-localization has a long slow decay. As no significant degradation of the fluorescent dye, nor any loss of lysosomal association could be found (movie S1), the slow decay is likely predominantly due to the formation of new lysosomes, which do not contain particles, in the daughter cells after cell division.

Collectively, these results suggest the minimalist phenomenological model laid out in Figure 5B. , There nanoparticle import is determined by time-independent fluxes (J_{01} = flux across the membrane, J_{12} = flux towards the endosomes and J_{23} = flux between endosomes and lysosomes) and nanoparticle exit processes are absent. All fluxes are functions of cellular energy, and are potentially dependent on, respectively, extracellular and other organelle concentrations. One of these fluxes will limit the overall uptake kinetics, otherwise nanoparticles would 'pile up' at certain cellular locations, and we label this limiting flux J.

In the scheme shown in Figure 5B, we have not taken into account nanoparticle transport towards the plasma membrane in the extracellular medium. Though previous studies have reported that this step is rate-limiting for a different system and under different conditions,[29] we deem that this is not the case for the system in the present study. Transport in the extracellular medium is determined by a combination of hydrodynamics, diffusion and sedimentation. While sedimentation is likely completely negligible for these particular low-density (1.05 g/ml) nanoparticles, it is not clear whether hydrodynamics or diffusion is most important. Certainly there will be strong hydrodynamical flows when the nanoparticle-containing solution is added to the cells, while diffusion might be more important later on (though weak

hydrodynamical flows are likely always present). However, to assess if transport within the extracellular medium is rate-limiting, a lower bound of transport in the extracellular solution can be found by considering only diffusion in the full volume for all times, as detailed in the Supplementary Material. For an extracellular concentration of 100μg/ml, this gives around 20,000 internalised nanoparticles after exposure of nanoparticles to an average cell for 5 min (Figure S10A), and to essentially depletion of nanoparticles from the extracellular medium after 24h. Taking into account also hydrodynamical flows and sedimentation would increase the uptake. In contrast, identification of particles in a confocal 'z stack' image indicates only around 500 particles under the same conditions (Figure S10B), and independent studies show no decrease in the nanoparticle concentration of the extracellular medium. Therefore, we conclude that transport in the extracellular medium is not the rate-limiting step for the system and conditions used in this study.

With time-independent fluxes, the intracellular nanoparticle concentration will rise linearly, as is indeed observed after the initial transient (Figure 1A) for intermediate time-scales. At longer time scales (of the order of 22 hours) however (see Figure 5B) intracellular nanoparticle dilution occurs due to cell division. Assuming that the intracellular load of nanoparticles is split evenly between mother and daughter, cell division alone would amount to an exponential decay of the intracellular nanoparticle concentration. A combination of constant flux uptake, competing with cell division leads to the equation $dC/dt = J - \lambda C(t)$, where $\ln(2)/\lambda$ is the cell population doubling time. The relevant solution to this equation is

$$C(t) = \frac{J}{\lambda}\left(1 - e^{-\lambda t}\right) \quad (1)$$

Note that Equation (1) reduces to a linear uptake process (as observed) for $t < 1/\lambda$. In Figure 5C we show the data (J is obtained by a fit) without (dotted line) and with (solid curve) the assumption of cell division. It is interesting that, at much longer time scales ($t \gg 1/\lambda$), the concentration tends to a new

limit, J/λ, this (in contrast to equilibrium constants) now representing the fundamental constant characterizing the steady state. This constant is a function of cellular energy, in contrast to the chemical equilibrium constant which is a function of the thermodynamic energy (temperature).

Providing that the volume of extracellular nanoparticle 'source' is large, and its concentration, C_0 does not effectively vary over the time of the experiment, we may expect (after some transient perhaps) the time independent flux, J, to depend on the extracellular concentration of nanoparticles. Certainly if the flux-limiting step in the consecutive uptake processes is membrane crossing, the dependence of this flux is expected to be linear in extracellular nanoparticle concentration, providing it does not represent a flux limiting bottleneck for increasing numbers of nanoparticles. As a simple example, if nanoparticles entered via a single set of receptor-mediated processes (an unlikely outcome for typical particles) or some other limited set of entry portals, then as C_0 increases, these would become saturated, leading to a deviation from linearity. One should exercise caution in these arguments as, for example, aggregation at higher particle concentration can also lead to a lowering of the effective concentration of available nanoparticles, and thereby similar effects.

Using the linear portions of the uptake curves we may determine the dependence of J on extracellular concentration, as illustrated in Figure 6A and B. Though broadly linear, there is a hint of saturation at the highest nanoparticle concentration investigated (Figure 6B) at which aggregation appears to play no role. Whilst concentrations and time periods much exceeding those reported here could lead to complications of aggregation, the range reported in this study seems not to be affected, and the deviation from linearity seems genuine.

In summary, with several rather simple assumptions we can make a simple phenomenological model of uptake of nanoparticles into cells, which is valid over time periods from tens of minutes to 24 hours. Naturally, a more detailed model that includes also intracellular detail will require more effort, though one may expect it to reduce to some simple picture, similar to that we outline above. It is also of interest to interpret in some more detail the variations of the flux, $J(E,C_0)$ in terms of cellular energy E,

extracellular concentration C_0, and other variables.

These remarks provide an overall framework, both experimental and theoretical, for future studies to fully clarify the kinetic processes of uptake of nanoparticles by different cell types. If fundamental questions, such as the role of the protein corona in directing uptake, sub-cellular localization and potential nanoparticle export from cells,[25, 30] are to be addressed effectively, then such a reliable platform of data will be required. Similarly, the foundations for modeling nanoparticle uptake should be addressed relatively rapidly, before large amounts of experimental information begin to accumulate.

The task of modeling, and ultimately predicting, the distribution and fate of nanoparticles represents an interesting, and quite new challenge that will have profound implications for both safety and nanomedical applications. The fact that the import processes are irreversible and energy dependent, and that export processes are absent, casts doubt on the validity of the simplistic application of ADME (absorption, distribution, metabolism, and excretion) pharmacokinetics approaches, that are used for small molecules, to the more complex case of nanoparticle uptake and biodistribution. This is obvious at the single cell level, but one may equally expect it to be true at biological barriers that themselves are the key factors to determine *in vivo* distributions. Clearly new theoretical directions will be required for future work on nanoparticle biological modeling.

Finally, the need to address more thoroughly the quality, provenance and control of nanomaterials in order to answer questions of science is clear, and similar issues also pertain for regulatory purposes. Significant implications also arise from the observation that nanoparticle exit processes from cells are very slow or non-existent, for this suggests that nanomaterials, whether due to accidental exposure or used for delivery purposes, will bioaccumulate, and this should be considered in the future development of nano-applications.

ACKNOWLEDGMENTS. YG dye was a kind gift from Polysciences. Use of the UCD Conway Institute Flow Cytometry and Confocal Microscopy Core Facilities is kindly acknowledged. Prof. Jeremy Simpson is acknowledged for assistance in image acquisition and co-localisation analysis. Fruitful discussions with Francesca Terraneo-De Lorenzi are acknowledged.

REFERENCES

1. Royal Society of Chemistry and Royal Academy of Engineering. Nanoscience and nanotechnologies: opportunities and uncertainties. Two-year review of progress on Government actions: Joint academies' response to the Council for Science and Technology's call for evidence. 2006.

2. Prina-Mello A, Whelan AM, Atzberger A, McCarthy JE, Byrne F, Davies G-L, Coey JMD, Volkov Y, Gun'ko YK. Comparative Flow Cytometric Analysis of Immunofunctionalized Nanowire and Nanoparticle Signatures. Small 2009**;** 6: 247-255.

3. Gu Y-J, Cheng J, Lin C-C, Lam YW, Cheng SH, Wong W-T. Nuclear penetration of surface functionalized gold nanoparticles. Toxicol Appl Pharmacol 2009**;** 237: 196-204.

4. Colvin VL. The potential environmental impact of engineered nanomaterials. Nat Biotech 2003**;** 21: 1166-1170.

5. Donaldson K, Stone V, Tran CL, Kreyling W, Borm PJA. Nanotoxicol Occup Environ Med 2004**;** 61: 727-728.

6. Oberdörster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, Cox C. Translocation of Inhaled Ultrafine Particles to the Brain. Inhalation Toxicology: International Forum for Respiratory Research 2004**;** 16: 437 - 445.

7. Watson P, Jones AT, Stephens DJ. Intracellular trafficking pathways and drug delivery: fluorescence imaging of living and fixed cells. Adv Drug Del Rev 2005**;** 57: 43-61.

8. Conner SD, Schmid SL. Regulated portals of entry into the cell. Nature 2003**;** 422: 37-44.

9. Mayor S, Pagano RE. Pathways of clathrin-independent endocytosis. Nat Rev Mol Cell Biol 2007**;** 8: 603-612.

10. Panyam J, Labhasetwar V. Dynamics of Endocytosis and Exocytosis of PolyD,L-Lactide-co-Glycolide Nanoparticles in Vascular Smooth Muscle Cells. Pharmaceut Res 2003**;** 20: 212-220.

11. Jiang W, KimBetty YS, Rutka JT, ChanWarren CW. Nanoparticle-mediated cellular response is size-dependent. Nat Nanotech 2008**;** 3: 145-150.

12. Kanno S, Furuyama A, Hirano S. A Murine Scavenger Receptor MARCO Recognizes Polystyrene Nanoparticles. Toxicol Sci 2007; 97: 398-406.

13. Rejman J, Oberle V, Zuhorn IS, Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. Biochem J 2004; 377: 159-169.

14. Gratton SEA, Ropp PA, Pohlhaus PD, Luft JC, Madden VJ, Napier ME, DeSimone JM. The effect of particle design on cellular internalization pathways. Proc Natl Acad Sci USA 2008; 105: 11613-11618.

15. Jin H, Heller DA, Strano MS. Single-Particle Tracking of Endocytosis and Exocytosis of Single-Walled Carbon Nanotubes in NIH-3T3 Cells. Nano Lett 2008; 8: 1577-1585.

16. Allianz and OECD. Small sizes that matter: Opportunities and risks of Nanotechnologies. 2005.

17. European Commission. Nanotechnologies: A Preliminary Risk Analysis on the Basis of a Workshop Organized by the Health and Consumer Protection Directorate General of the European Commission. 2005.

18. Jiang J, Oberdörster G, Biswas P. Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies. J Nanopart Res 2009; 11: 77-89.

19. Pietzonka P, Rothen-Rutishauser B, Langguth P, Wunderli-Allenspach H, Walter E, Merkle HP. Transfer of Lipophilic Markers from PLGA and Polystyrene Nanoparticles to Caco-2 Monolayers Mimics Particle Uptake. Pharmaceut Res 2002; 19: 595-601.

20. Preston RA, Murphy RF, Jones EW. Apparent endocytosis of fluorescein isothiocyanate-conjugated dextran by Saccharomyces cerevisiae reflects uptake of low molecular weight impurities, not dextran. J Cell Biol 1987; 105: 1981-1987.

21. Lesniak A, Campbell A, Monopoli M, Lynch I, Salvati A, Dawson KA. Serum Heat Inactivation Affects Protein Corona Composition And Nanoparticle Uptake. Biomater 2010; 31: 9511-9518.

22. Shapero K, Fenaroli F, Lynch I, Cottell DC, Salvati A, Dawson KA. Time and space resolved uptake study of silica nanoparticles by human cells. Mol BioSyst 2011; 7: 371-378.

23. Shi H, He X, Yuan Y, Wang K, Liu D. Nanoparticle-Based Biocompatible and Long-Life Marker for Lysosome Labeling and Tracking. Analyt Chem 2010; 82: 2213-2220.

24. Bexiga MG, Varela JA, Wang F, Fenaroli F, Salvati A, Lynch I, Simpson JC, Dawson KA. Cationic nanoparticles induce caspase 3-, 7- and 9-mediated cytotoxicity in a human astrocytoma cell line. Nanotoxicol 2010; Epub ahead of print.

25. Lundqvist M, Stigler J, Elia G, Lynch I, Cedervall T, Dawson KA. Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. Proc Natl Acad Sci USA 2008; 105: 14265-14270.

26. Shabbits JA, Chiu GNC, Mayer LD. Development of an in vitro drug release assay that accurately predicts in vivo drug retention for liposome-based delivery systems. J Controll Rel 2002; 84: 161-170.

27. Petersen S, Fahr A, Bunjes H. Flow Cytometry as a New Approach To Investigate Drug Transfer between Lipid Particles. Molecul Pharmaceut 2010; 7: 350-363.

28. Agarraberes FA, Dice JF. Protein translocation across membranes. Biochim Biophys Acta 2001; 1513: 1-24.

29. Limbach LK, Li Y, Grass RN, Brunner TJ, Hintermann MA, Muller M, Gunther D, Stark WJ. Oxide Nanoparticle Uptake in Human Lung Fibroblasts: Effects of Particle Size, Agglomeration, and Diffusion at Low Concentrations. Environmen Sci Technol 2005; 39: 9370-9376.

30. Cedervall T, Lynch I, Lindman S, Berggard T, Thulin E, Nilsson H, Dawson KA, Linse S. Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. Proc Natl Acad Sci USA 2007; 104: 2050-2055.

31. Chinga G, Syverud K. Quantification of paper mass distributions within local picking areas. Nordic Pulp Paper Res J 2007; 22: 441-446.

Figure 1 Comparison of the kinetics of uptake and export by A549 cells treated with 25 µg/ml of the different polystyrene nanoparticles (P_1, P_2 and I_1, shown in Figure 1), as determined by flow cytometry. A) Uptake curves. B) Kinetics of export after 4 hours of uptake followed by removal of the nanoparticle-containing medium and replacement with fresh medium (no nanoparticles). Cell fluorescence has been normalized for the fluorescence at time zero. The point at 22 hours (X symbol) is a reference for the expected fluorescence intensity decrease due to cell division, omitting the initial transient.

Figure 2 Confocal images of A549 cells treated with 25 µg/ml green fluorescent polystyrene nanoparticles for 1h. Blue: DAPI stained nuclei (Magnification 63X and enlarged detail in the lower right corners). A) Sample P_1. B) Sample I_1.

Figure 3 Co-localisation of nanoparticles with early endosomes (EEA1 staining) and lysosomes (LAMP1 staining) as a function of time, after 1 hour exposure of A549 cells to 25 µg/ml of red fluorescent polystyrene nanoparticles (sample I_1). Values lower than 0.1 and higher than 0.5 indicate insignificant and large co-localization, respectively. Inserts: confocal images of A549 cells exposed for 1h to the same nanoparticles (25 µg/ml) and treated, after fixation, with LAMP1 antibody for lysosomes. Green: LAMP1 staining with Alexa488-secondary antibody. Red: nanoparticles. Blue: DAPI stained nuclei.

Figure 4 Energy dependence of uptake and export of polystyrene nanoparticles. A) Cells were treated with polystyrene nanoparticles I_1 at 25 µg/ml under normal cell culture conditions (37°C, cMEM), and in medium containing 5mg/ml NaN_3, or at 4°C. B) Cells were treated with the YG dye

(unknown concentration) or 25µg/ml of nanoparticles (samples P_1 and I_1) for 4 hours, after which the medium was replaced with fresh cMEM containing 5mg/ml NaN_3. Cell fluorescence has been normalized for the fluorescence at time zero. Error bars are the standard deviation among the 3 replicates. The point at 22 hours (X symbol) is a reference for the expected fluorescence intensity decrease due to cell division, omitting the initial transient.

Figure 5 Simple model representation of the cellular uptake of A) a fluorescent dye and B) nanoparticles labeled with fluorescent dye (e.g. sample I_1). The cell is made up of two containers representing the general intracellular space (cytoplasm) and the endoplasmic reticulum in the case of the dye (A) and the endosomal and lysosomal compartments in the case of the nanoparticles (B). Note that reverse processes are absent in the case of nanoparticles. At longer timescales the competing process of cell division becomes significant. C) 30 hour nanoparticle uptake profile. The solid line shows a fit of the data (excluding the initial data points at t=0) to Equation (1), while the dashed line is the linear approximation valid for short times.

Figure 6 A) Kinetics of cellular uptake of polystyrene nanoparticles (sample I_1) by A549 cells, at different extracellular particle concentrations, as determined by flow cytometry, with their linear fits. B) Fluxes, J, (as determined by the fits of the uptake profiles in panel A) as a function of extracellular nanoparticle concentration. The solid line shows the deviation of the linear trend at the highest concentration.