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Uptake and Intracellular Fate of Surface-Modified Gold Nanoparticles

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ABSTRACT Understanding and controlling the interactions between nanoscale objects and living cells is of great importance for arising diagnostic and therapeutic applications of nanoparticles and for nanotoxicology studies. Here we report a detailed transmission electron microscopy (TEM) study of the uptake of *ca.* 16 nm surface-modified gold nanoparticles by human fibroblast cells (HeLa cells). It is demonstrated that the well-established endosomal route of cellular uptake can be bypassed to a significant extent by controlling the uptake mechanism either *via* the delivery of the nanoparticles by liposomes or by surface modification of the nanoparticles with so-called cell penetrating peptides (CPPs). Successful nuclear targeting is demonstrated using surface modification with a cocktail of CPPs and a peptide acting as a nuclear localization signal (NLS).

KEYWORDS: cellular uptake · gold nanoparticles · liposomes · cell penetrating peptides · nuclear targeting · transmission electron microscopy

A number of recent developments in the biomedical sciences involve the interaction of synthetically produced nanoparticles with living organisms, often on the subcellular level.[1–3] These include hyperthermia cancer therapy,[4–6] targeted gene and drug delivery,[7,8] optical[9–13] and electron microscopy,[14] and magnetic resonance imaging.[15,16] In parallel with such promising applications, there is a growing interest in the toxicology of nanoparticles, which is still in its infancy.

Given the breadth of currently arising opportunities and concerns associated with nanoparticles in living systems, it is of great importance to develop an understanding of the complex processes that govern their cellular uptake and intracellular fate. Here we present a first systematic approximation to this multidimensional problem and demonstrate a perhaps unexpected wealth of scenarios even for relatively simple systems. This study is limited to investigating the interactions between HeLa cells, a well-known human fibroblast epithelial cell line, and *ca.* 16 nm colloidal gold particles with a range of chemical surface modifications. For simplicity, effects of particle size and shape and differences that would arise from the use of different cell lines have not been taken into account here. Gold particles have been chosen as a model system for their stability, ease of preparation and detection by electron microscopy, and our ability to precisely control their surface chemistry by a range of well-established methods.

Our main objective has been to control cellular uptake and to determine what happens to the particles once they are inside the cell, based on the assumption that these processes predominantly depend on the specific surface chemistry of the particles. It is generally believed that nanoparticles will remain in the endosome created by the endocytosis process[17] unless they are microinjected or brought into the cell by any other mechanically disruptive means such as a gene gun, sonication, or the application of an osmotic shock. This appears to be in contradiction with a number of reports on successful nuclear targeting and transfection.[18–21] While many of our results confirm that cells indeed tend to deal with intruding nanoparticles by confining them to the endosome, there are measures that can be taken to avoid this endosomal pathway. As shown below, this involves manipulating the uptake mechanism, either *via* the use of liposomes as delivery agents or by appropriate modification of the particles with so-called cell penetrating peptides (CPPs). We also investigated nuclear targeting by additional modification of the particles with a nuclear localization signal peptide.

RESULTS AND DISCUSSION

All gold particles used were based on colloidal preparations by the classical

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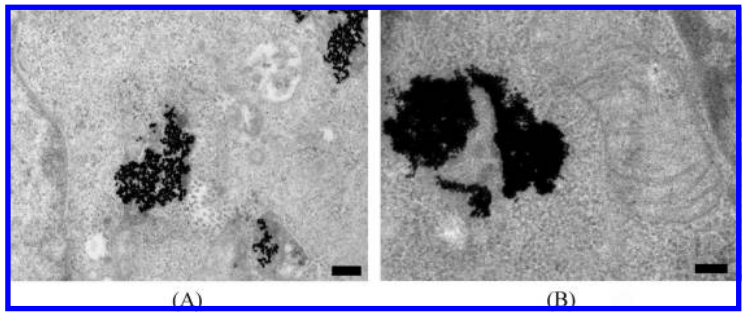

Figure 1. Typical TEM images of (A) as-prepared citrate-stabilized and (B) CALNN-modified gold nanoparticles confined to the endosome after cellular uptake. Scale bars are 200 nm.

Turkevich−Frens[22,23] method using trisodium citrate as a reducing agent in aqueous solution. The simplest experiment involved the direct exposure of the as-prepared particles to the cell culture. Surprisingly, the charge-stabilized colloid remains stable after addition to the cell culture, which is clearly indicated by the permanence of its characteristic red color. This is also confirmed by UV−vis spectra that do not show any significant red shift or broadening after expose of the particles to the cell serum. Destabilization would lead to aggregation and to a concomitant color change from red to blue. Usually, citrate-stabilized particles aggregate rapidly upon slight changes in the ionic strength of the medium unless there is an additional capping agent in the solution that can impart higher stability. In the case of the cell serum, it is assumed that the cocktail of proteins secreted by the cells and the presence of fetal bovine serum (FBS) are responsible for the observed stability.[24] Generic proteins such as BSA are well-known to adsorb to gold particles and provide additional stability.

More importantly for this study, it was found that these standard particles were readily taken up by the cells and ended up in the endosome. A typical example of this type of cellular uptake is shown in Figure 1A. The particles are quite densely packed, show clear signs of aggregation, and are strictly confined to the endosomes. Individual particles freely dispersed in the cytosol are not even observed as a minority species. To obtain an estimate of the total number of particles taken up by the cells, the gold content of the entire cell culture (*ca.* 26 000 000 cells) was determined by atomic emission spectroscopy (AES). Cells incubated for 2 h with a 5 nM solution of 16 nm citrate-stabilized particles contained 3.59 ppm of gold in a final volume of 10 mL. Assuming that each particle contains 126 500 gold atoms, this corresponds to an average of *ca.* 33 400 particles per cell. On the basis of these findings, and assuming a cell diameter of 16 μm and a section thickness of 50−75 nm, we would expect to find between 100 and 160 nanoparticles per cell section. This number is in reasonable agreement with the electron microscopic images, although the particles were found not to be evenly distributed across the sections, and counting them within the densely loaded endosomes is difficult.

Particles similar to those used in this experiment are commercially available and all have in common that aggregation is prevented by adsorbed ionic charge and not by ligands. As mentioned before, they are thus amenable to fortuitous attachment of serum proteins or other molecules present in the cell culture resulting in an unknown chemical composition of the particle surface at the moment of cellular uptake.

More controlled conditions are achieved if the particles are coated with a well-defined ligand shell prior to incubation. This can be obtained by standard procedures.[25,26] Cellular uptake can be altered dramatically if the adsorption of proteins from the serum is blocked by the presence of such a ligand shell. For example, gold particles originally prepared by the same citrate reduction method but subsequently modified with a ligand shell of monohydroxy 1-mercaptoundec-11-yl tetraethylene glycol (PEG)[25] are not taken up at all by HeLa cells. Even after prolonged incubation for 24 h or after 10-fold increase in concentration, no particles were found inside the cells by TEM, and no gold could be detected by AES. If the ligand shell instead is composed of a pentapeptide with the amino acid sequence CALNN, chosen for its well-established ability to stabilize gold nanoparticles,[26] the uptake becomes again comparable to that of particles that have been added to the cell culture without prior coating (Figure 1B). Notably, the CALNN-coated particles within the endosomes show even less stability against aggregation than the nominally uncoated particles. This destabilization could be either due to ligand exchange reactions within the cell as suggested by Rotello and coworkers[27] or may be the result of the digestion of the ligand shell by proteases inside the endosome. Both CALNN- and PEG-modified particles are completely stable against aggregation in the cell serum as confirmed by UV−vis.

The quantitative gold analysis by AES resulted in an estimate of 11 600 particles per cell or 35 to 65 particles per cell section, again broadly consistent with the TEM observations when averaged over all sections inspected. Although in the case of CALNN-stabilized particles the endosomes appear to be more densely packed, fewer of them are found in each cell section when compared to cells incubated with citrate-stabilized particles.

For most practical purposes, it is necessary that at least a fraction of the particles taken up by the cells are freely dispersed in the cytosol. In fact, a lot of scepticism regarding the use of nanoparticles as intracellular probes and agents is based on the notion that, with the exception of microinjection, there are no reliable and well-understood methods to achieve this. To address this problem, we have investigated different

routes to circumvent the endosomal pathway and to specifically target designated sites within the cell (*e.g.*, the nucleus). An unexpectedly broad spectrum of scenarios was found that raises new questions regarding uptake mechanisms and the possibility of endosomal escape.

In order to change the uptake mechanism, we have investigated the effect of the presence of commercially available neutral liposomes that can contain the gold particles and should be able to deliver them to the interior of the cell by fusion with the cell membrane. To avoid competition with uptake mechanisms that are independent of the presence of the liposomes, we used the PEG-modified particles for which we had already established that, by themselves, they are not taken up by the cells. Interestingly, in the presence of liposomes, PEG-modified particles are taken up very efficiently by a number of different routes, not all of them directly mediated by the liposomes. For example, a clear association of particles with caveolae is observed (Figure 2A).

Caveolae represent the major non-clathrin-dependent route from the cell surface to endosomes.[28] Internalization *via* clathrin-coated vesicles is also seen (Figure 2B), indicating that both major routes of endocytosis are utilized in the presence of liposomes. Direct delivery of particles to the cell membrane by liposomes is shown in Figure 2C,D. The images shown have been selected from a large number of observations that show liposomes of a range of sizes between 40 and 300 nm, many of them empty, and others only containing a small number (<10) of particles in accordance with the estimated nanoparticle to liposome ratio of 1:1. Interestingly, in some liposomes, the PEG-modified particles appear to be aggregated, albeit without noticeable coalescence. Such, probably reversible, aggregates are not found inside the cells and may occur due to the specific conditions inside the liposome.

Inside the cell, the majority of particles are found in the endosomes, but particles are also observed freely dispersed in the cytosol. Closer inspection of the endosomes reveals that there appear to be two types of particles present inside, as illustrated in Figure 2E.

The particles marked by arrows are surrounded by a membrane which indicates that they have been taken up into the endosome from the cytosol, while the other particles in the endosome will have been delivered *via* a vesicular intermediate directly from the plasma membrane. Importantly, the particles do not aggregate, which represents a major difference in the behavior of citrate- or CALNN-protected particles. Such findings are common to all samples in this series of experiments and support the existence of delivery directly across the plasma membrane (fusion of liposomes with plasma membrane) in addition to the caveolar−clathrin-dependent pathways. Under the same experimental conditions, both the particles stabilized with the pentapeptide CALNN and the as-prepared citrate-stabilized particles are also taken up in the same way, and some particles are found freely dispersed in the cytosol, al-

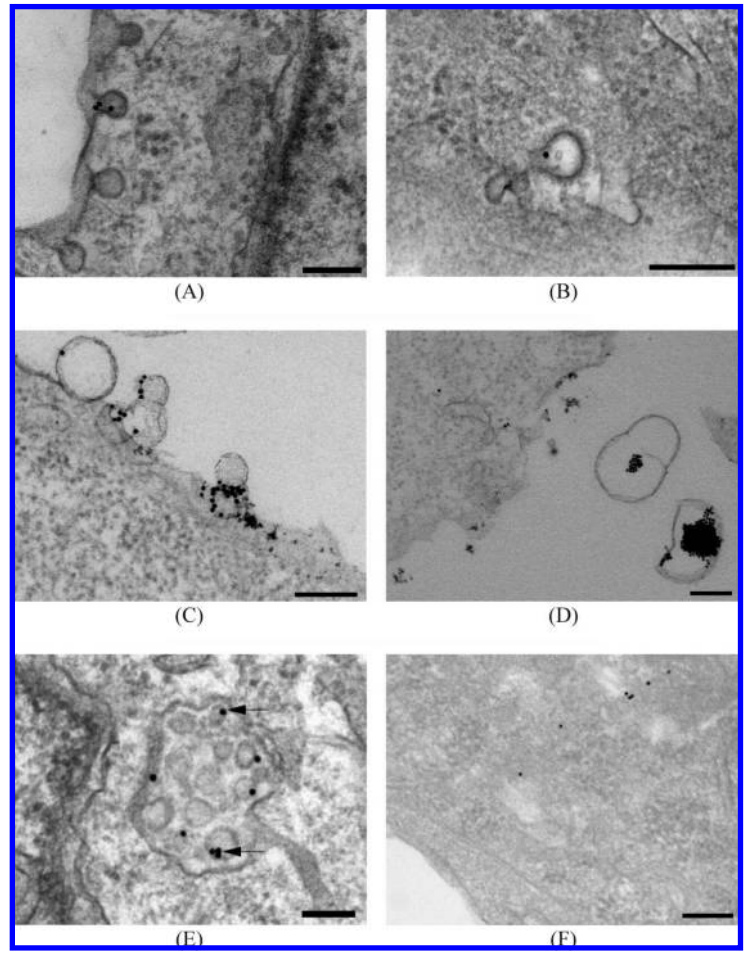

Figure 2. Uptake of PEG-modified gold nanoparticles in the presence of liposomes. Observed uptake mechanisms without apparent direct involvement of the liposomes include (A) caveolae and (B) clathrin-mediated endocytosis. Particle delivery by liposomes and subsequent uptake are shown in (C) and (D). Perhaps reflecting the wealth of different uptake mechanisms available under these conditions, nanoparticles are found in the endosome (E) or free in the cytosol (F). Note that some nanoparticles in the endosome are surrounded by an additional membrane (arrows), which indicates that they have been taken up by the endosome from the cytosol. Scale bars are 200 nm.

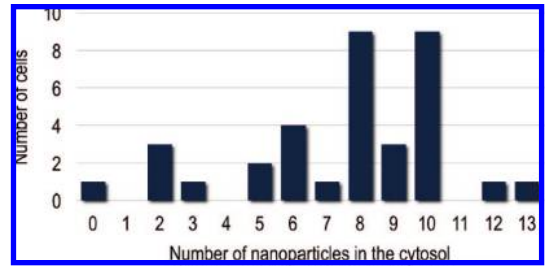

Figure 3. Uptake of PEG-modified nanoparticles in the presence of liposomes. The graph shows the number of cells in which any given number of nanoparticles was found freely dispersed in the cytosol. For example, in nine cells of the section, eight nanoparticles were found, while in three cells of the same section, two nanoparticles were found.

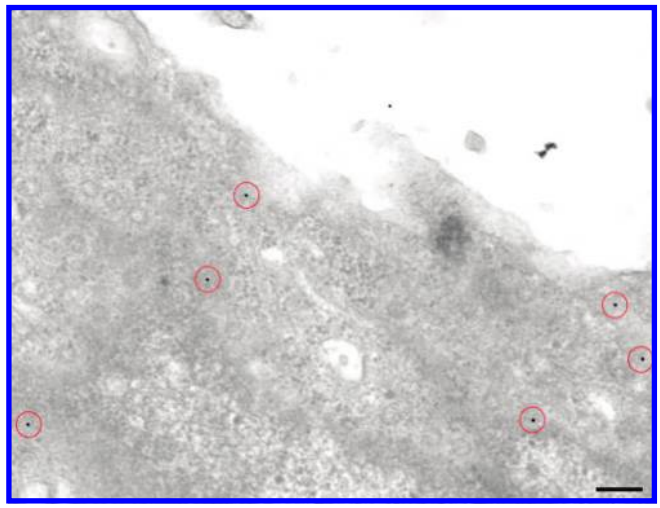

Figure 4. Functionalization of PEG-modified nanoparticles with CPPs (2% TAT and 2% Pntn) circumvents the endosomal path and leads to a significant number of nanoparticles freely dispersed in the cytosol (red circles). Scale bar is 500 nm.

though the endosomal uptake mechanism observed before in the absence of liposomes remains clearly dominant. These findings indicate that liposomes provide access to a further uptake mechanism that will deliver particles to the cytosol. As quantitative chemical analysis cannot distinguish between the different locations of particles inside the cell, the number of particles present in the cytosol (*i.e.*, not in the endosome) was obtained for each individual cell by counting the particles present in one representative section (Figure 3).

A potentially more elegant albeit less well understood approach to avoiding the accumulation of particles in the endosomes involves modifying the particle surface with so-called cell penetration peptides (CPPs), for example, the oligopeptides TAT (AGRKKRRQRRR[18]) from HIV and Pntn (GRQIKIWFQNRRMKWKK[29]) from Antennapedia protein from *Drosophila*. This may facilitate the direct transfer of particles across the membrane by a mechanism that is not yet understood.[30] For this purpose, we first prepared particles with a ligand shell containing approximately 99% of our usual PEG stabilizer and 1% of CALNN elongated with TAT. This ratio was arbitrarily chosen but keeping in mind that a high percentage of PEG in the ligand shell will prevent the particles from aggregation inside the cells. Experiments to optimize the proportion of the TAT ligand are subject to an ongoing study. The particles were taken up by the cells; however, none were encountered in the cytosol. Instead, stable particles were found in the endosomes, supporting the assumption that the predominant uptake mechanism enabled by TAT is endocytosis.[31] Contrasting the findings with citrate- or CALNN-stabilized particles, no signs of aggregation were observed in the endosomes. This is not surprising since the ligand shell contains predominantly PEG ligands, which are known to infer superior stability to the particles, are less prone to ligand exchange, and do not undergo enzymatic digestion. Replacing TAT with the other available penetration factor Pntn gave the same results, while, surprisingly, a combination of both TAT and Pntn present in the ligand shell resulted in finding a significant number of particles in the cytosol in addition to endosomal uptake, which remains the predominant path (Figure 4).

Although the efficiency of this route appears to be comparatively poor, it demonstrates the possibility to deliver nanoparticles directly to the cytosol without physical rupture or fusion of the cell membrane, merely by modifying the ligand shell of the particles with the appropriate combination of membrane penetrating peptides.

The next higher level of control is targeting specific sites inside the cell, such as the nucleus or other organelles. To achieve this, we prepared PEG-stabilized particles with 5% of the nuclear localization sequence NLS (GGFSTSLRARKA[19]) appended to CALNN in the ligand shell. Again, optimization of the proportion of the functional ligand in view of maximizing both stability and functionality is subject to ongoing work. Even in the absence of membrane penetration factors, particles were taken up and were found in the endosomes and in the nucleus but not in the cytosol (Figure 5).

Even more interesting results have been obtained with PEG-stabilized particles modified with a combination of NLS (2%), TAT (2%), and Pntn (2%), each appended to CALNN. These proportions are, as in all previous experiments, still subject to optimization. As may be expected from our previous findings, particles were found in the cytosol, in the endosomes, and in the nucleus (Figure 6A), but in addition to this, a large proportion of the particles seem to group in the vicinity of the nucleus and are associated to hitherto unobserved extended membrane structures, which appear to be damaged or aberrant endosomes (Figure 6B,C).

This finding suggests that the combination of peptides used here to modify the particles is capable of disrupting endosomal morphology and dissolving the endosomal membrane, leading to the release of its content. While the organelles ob-

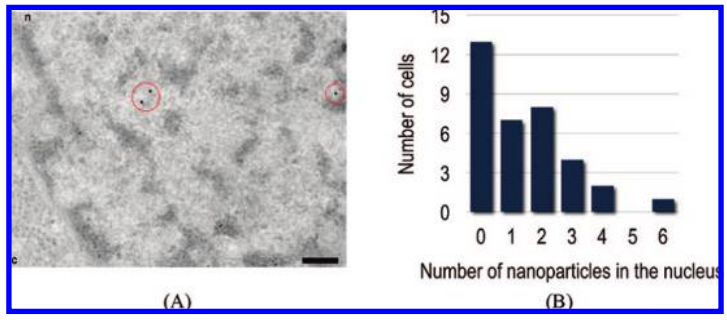

Figure 5. TEM image (A) demonstrating successful nuclear targeting with PEG-modified nanoparticles functionalized with 5% NLS. The nucleus is denoted n, and the cytosol c. No CCPs were used in this experiment. Correspondingly, the occurrence of nanoparticles in the nucleus is relatively low (B). Note that in 13 cells no particles were found in the nucleus. Scale bar is 200 nm.

served in Figure 6B,C are unlike anything normally found in a cell, we are confident that they represent disrupted endosomes based on residual morphology, location near to the nucleus, and the high density of gold nanoparticles that is only ever found within endosomes in all other conditions.

In conclusion, we have presented a detailed electron microscopic study of cellular uptake and intracellular fate of chemically modified gold nanoparticles. This investigation has necessarily been limited to a relatively small number of standard conditions. Like many initial studies aiming to gain simplified insight into a complex phenomenon, this report raises more questions than it answers. There is no doubt that particles can enter cells by a number of different routes, which may also be available in parallel. Not all of them are well understood, and it is not yet clear which factors favor any particular uptake mechanism. We have confirmed that endocytosed nanoparticles are often confined to the endosomes. Nevertheless, nanoparticles can avoid this fate if they are delivered to the cells *via* liposomes or if they are chemically modified with membrane penetrating peptide sequences. In the latter case, the particles appear to enter the cytosol either directly through the cell membrane or by endosomal escape. Further studies are needed to explain their appearance in the cytosol in association with membrane-like structures that appear to be destroyed endosomes. Besides uptake, our second focus has been the intracellular fate of nanoparticles. For gene delivery, in particular, but also to label designated sites, the ability to direct nanoparticles toward targets inside the cell is of great interest. In agreement with the findings of others,[19,21] we have presented here some evidence that nuclear targeting, albeit with moderate yield, can be achieved by modifying the particles with a nuclear localization sequence in addition to membrane penetration peptides. Further studies are needed to optimize particle delivery. These will focus on achieving the best combination of uptake strategy and intracellular targeting by finding the optimal proportion of membrane penetrating and localization factors in the ligand shell. In addition, the role of particle charge will have to be studied to complete the picture.

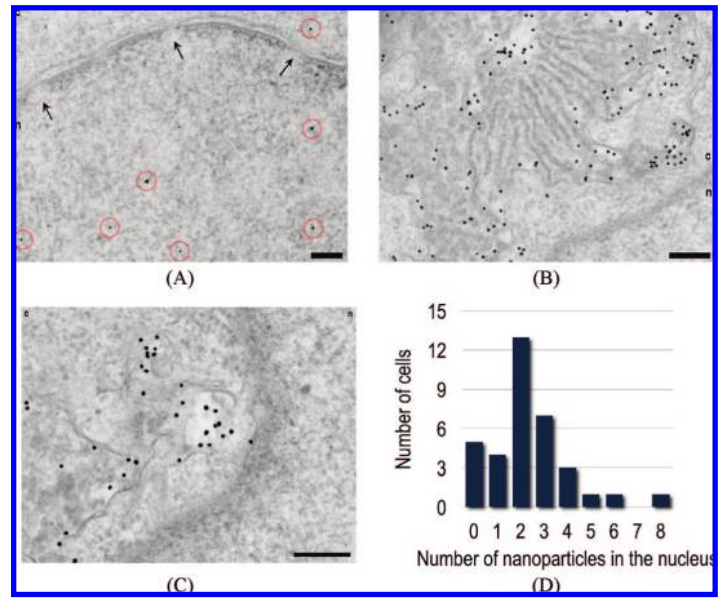

Figure 6. Nuclear targeting (A) by PEG-modified nanoparticles functionalized with a combination of CPPs (2% TAT and 2% Pntn) and 2% NLS. For clarity, nanoparticles are highlighted by red circles. The nuclear envelope with nuclear pores (arrows) is clearly shown in this image. The nucleus is denoted n, and the cytosol c. Unusual perinuclear membranous structures (B and C) that are highly loaded with nanoparticles are typically also observed under these conditions. It is assumed that these are endosomes disrupted by the presence of the CPP-functionalized nanoparticles. Nuclear targeting is enhanced in comparison with experiments in the absence of CPPs (D). Note that now only five of the analyzed cells do not show any nanoparticles in the nucleus. Scale bars are 200 nm.

EXPERIMENTAL METHODS

Cell Culture and Nanoparticle Uptake. HeLa cells (American type Culture Collection) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and nonessential amino acids without addition of antibiotics at 37 °C in a humidified atmosphere of 5% CO_2. Cells were then incubated with 5 nM gold nanoparticle solution (1 mL of 10 nM solution of gold nanoparticles added to 1 mL of medium in a 3 cm culture dish) for 2 h under the same conditions. Six, 24, and 48 h chase experiments have been performed for citrate-stabilized and CALNN-modified particles. It was found that the particles were largely cleared from the cells after 48 h. This process appeared to be slower for the citrate-stabilized particles.

Transmission Electron Microscopy. The medium containing the gold nanoparticles not taken up by the cells was discarded, and the cells were thoroughly washed with PBS buffer. Cells were then scraped from the culture dish and centrifuged at 5000*g* for 5 min, and the supernatant was removed. The cell pellets were fixed in a 0.1 M PBS solution containing 2.5% gluteraldehyde and 4% paraformaldehyde for 1 h. They were then rinsed with 0.1 M PBS, embedded in 2% agarose gel, postfixed in 4% osmium tetroxide (caution! Extremely toxic) solution for 1 h, rinsed with distilled water, stained with 0.5% uranyl acetate for 1 h, dehydrated in a graded series of ethanol (30, 60, 70, 90, and 100%), and embedded in epoxy resin. The resin was polymerized at 60 °C for 48 h. Ultrathin sections (50−70 nm) obtained with a LKB ultramicrotome were stained with 5% aqueous uranyl acetate and 2% aqueous lead citrate and imaged under a 120 kV FEI Tecnai Spirit TEM.

Image Analysis. Cells imaged by TEM were screened for association of nanoparticles with cellular organelles, which were identified morphologically. AnalySIS software (Soft Imaging Systems) was used to outline cell peripheries and calculate cross sectional areas enabling the determination of the nanoparticle concentration per unit area. Extrapolations of total number of nanoparticles per cell are based on average HeLa cell volumes.

Determination of Gold Content. Cells were incubated for 2 h with the nanoparticles as described above, washed five times with PBS buffer, fixed with glutaraldehyde and paraformaldehyde, scraped from the dish and collected in PBS (4 mL final volume). The sample was digested with *aqua regia* (highly corrosive and damaging to skin and eyes! Extreme caution is required when handling!), and the gold content was determined by atomic emission spectroscopy (AES).

Gold Nanoparticles and Liposomes. Gold hydrosols of approximately 16 nm particle diameter with a narrow size distribution were prepared following the standard Turkevich−Frens procedure.[22,23]

Briefly, 0.03 g (0.8 μmol) of hydrogen tetrachloroaurate (trihydrate) was dissolved in 300 mL of water and heated to near boiling temperature. To this was added a hot (*ca.* 60 °C) 1% aqueous trisodium citrate solution (9 mL), and the mixture was refluxed for 40 min. The ruby red solution was characterized by UV−vis spectroscopy, giving the typical plasmon band at 520 nm. A particle size of 16 ± 1.2 nm was determined by TEM. The particles were then either added to the cell medium as prepared or modified with subsequently added protecting ligands of either the pentapeptide CALNN, monohydroxy 1-mercaptoundec-11-yl tetraethylene glycol (PEG), or mixtures of the latter with a small proportion of CALNN elongated by the respective CPPs or NLS sequences. Addition of the ligand shell was carried out as described by Levy et al.[26] Typically, a total of 4×10^{-7} mols of ligand were added to 1 mL of a 2 nM dispersion of citrate nanoparticles. Prior to addition to the cell culture, surface-modified nanoparticles were purified by repeated centrifugation and redispersion. Nonionic liposomes COATSOME EL11N (stated mean diameter: 137 nm with a standard deviation of 36%) were purchased as a kit from NOF Europe and applied following the instructions. They were applied together with gold nanoparticles at an estimated ratio of one liposome per gold nanoparticle.

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