New views on cellular uptake and trafficking of manufactured nanoparticles

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Nanoparticles (NPs) are of similar size to typical cellular components and can efficiently intrude living cells. A detailed understanding of the involved processes at the molecular level is important for developing NPs designed for selective uptake by specific cells, for example, for targeted drug delivery. In addition, this knowledge can greatly assist in the engineering of NPs that should not penetrate cells so as to avoid adverse health effects. In recent years, a wide variety of experiments have been performed to elucidate the mechanisms underlying cellular NP uptake. Here, we review some select recent studies, which are often based on fluorescence microscopy and sophisticated strategies for specific labelling of key cellular components. We address the role of the protein corona forming around NPs in biological environments, and describe recent work revealing active endocytosis mechanisms and pathways involved in their cellular uptake. Passive uptake is also discussed. The current state of knowledge is summarized, and we point to issues that still need to be addressed to further advance our understanding of cellular NP uptake.

1. Introduction

The widespread and steadily growing use of nanoparticles (NPs) and other nanomaterials in scientific [1] and commercial applications [2–5] entails their increasing proliferation and accumulation in the environment [6–9]. Thus, although a profound assessment of the risks to human health is not yet available, the chance of unintended human exposure is further increasing [10–16]. The fundamental interactions of nanomaterials with biomatter remain incompletely understood [11–16], largely due to a lack of mechanistic knowledge at the molecular level [11,17]. Even for intended therapeutic applications of NPs, possible health concerns need addressing [18–20]. This situation has spawned a large number of studies in recent years aimed at shedding light on the molecular interactions involved in the biological actions of NPs.

NPs are of similar size to typical cellular components and can efficiently intrude living cells by exploiting the cellular endocytosis machinery, resulting in permanent cell damage [20,21]. Only specialized cells such as macrophages are capable of phagocytosis, a form of endocytosis in which the cell engulfs larger particles. Almost all cells, however, can internalize NPs by pinocytosis. Four different basic pinocytic mechanisms are currently known, macropinocytosis, clathrin-mediated endocytosis, caveola-mediated endocytosis and mechanisms independent of clathrin and caveolin [22,23]. Physico-chemical properties of NPs including size [24,25], shape [24], surface charge [26–28] and surface chemistry [26,29,30] have been identified as strongly modulating the cellular uptake efficiency.

Upon incorporation by an organism, NPs interact with extracellular biomolecules dissolved in body fluids, including proteins, sugars and lipids.
prior to their encounter with the plasma membranes of cells. A layer of proteins forms on the NP surfaces, the so-called protein corona [10,31–43]. Consequently, cell surface receptors, which activate the endocytosis machinery, actually encounter NPs enshrouded in biomolecules rather than bare particles. This is an important issue for designing and engineering NPs with intentionally enhanced or suppressed cellular uptake.

In addition to intruding cells by active processes, NPs may also enter cells by passive penetration of the plasma membrane [44,45]. The ability of NPs to adhere to and penetrate cell membranes was shown to depend on their physical properties, including size, surface composition and surface charge [46–51]. Small, positively charged NPs were observed to pass through cell membranes, leading to membrane rupture and noticeable cytotoxic effects [52–55]. Even particles greater than 500 nm in diameter were reported to penetrate cell membranes by inducing strong local membrane deformations [56]. Membrane disruption can be reduced or even entirely avoided by a suitable design of the surface structure and charge density [46,57]. We note, however, that the surface properties of NPs within the organism may be significantly altered by the formation of a biomolecular adsorption layer.

Besides mere NP internalization, there is still another issue that is crucial for the assessment of biological consequences of NP exposure, namely their degradation in the biological environment. This may involve removal of the protein corona and, subsequently, a surface functionalization layer that often encloses an inorganic core particle. Exposure of the NP core to the corrosive intracellular milieu may result in its degradation and, eventually, its complete dissolution. The release of molecular debris and/or metal ions (e.g. Ag, Cd or In) will give rise to cytotoxic effects [58–60]. Adverse effects of NP incorporation will generally be a combination of ionic/molecular toxicity and toxicity aspects related to the particulate nature of the material. Recent studies of silver NPs with and without polymer surface coatings showed that formation of the protein corona strongly depended on the surface coatings around these NPs [40], whereas their severe cytotoxicity arose from the release of silver ions [61–63].

In recent years, there have been substantial activities to better understand the detailed molecular mechanisms involved in cellular NP uptake, often using fluorescence microscopy and other sophisticated biophysical techniques. In this review, we have selected a few recent studies that illustrate the approaches chosen to elucidate the molecular interactions promoting NP uptake. A thorough understanding of the involved processes at the molecular level will, on the one hand, greatly aid in the engineering of NPs that do not penetrate cells, which is relevant, for example, for NP-based contrast agents widely used in medical diagnosis. On the other hand, this knowledge is also important for developing NPs designed for selective uptake by specific cells, for example, for targeted drug delivery.

2. Effects of the protein corona on uptake efficiency

A variety of studies have reported effects of protein corona formation on the cellular response to NP exposure. For example, uptake of carboxyl-functionalized NPs by HeLa cells with adsorbed blood plasma proteins was shown to be strongly suppressed in comparison with bare NPs [64]. Immunoglobulin binding caused NP opsonization, thereby promoting receptor-mediated phagocytosis by macrophages [65]. Suppression of protein absorption onto NPs by coating them with polyethylene glycol (PEG) resulted in decreased uptake by macrophages [66] and longer circulation times as well as altered biodistribution upon injection in mice [65]. The adsorbed proteins are internalized by the cells together with the NPs and, therefore, may enter cellular compartments that they would not normally reach [67]. Understanding the formation and persistence of the protein corona on NPs is, therefore, critically important for the elucidation of cellular NP uptake.

To elucidate how the presence of a protein adsorption layer around NPs affects their cellular uptake, Jiang et al. [64] compared NP uptake by live HeLa cells in the presence or absence of human transferrin (TF) and human serum albumin (HSA) in buffer (phosphate-buffered saline, PBS) medium. Small (diameter approx. 10 nm), carboxyl-functionalized polymer-coated FePt NPs, fluorescently labelled by DY-636 dye molecules in the polymer shell, were used as model NPs. Protein concentrations of approximately 100 μM in the cell medium ensured complete coating of the NPs with these proteins, as was shown by binding studies using fluorescence correlation spectroscopy (FCS). The uptake of FePt NPs by live HeLa cells in PBS buffer was studied by quantitative confocal fluorescence microscopy.

Within minutes after exposing the cells to FePt NPs dissolved in PBS, the NPs accumulated on the cell membranes, and internalization took approximately 1 h to saturate. In the presence of 100 μM TF or HSA in the buffer, cellular uptake was significantly reduced. Control experiments were performed on fluorescently labelled (approx. 1:1 ratio) TF and HSA proteins to test the ability of HeLa cells to internalize these proteins without NPs. Uptake of TF, which is well known to be internalized via its cognate receptor, was significant, whereas HSA was barely endocytosed by HeLa cells under otherwise identical conditions.

The pronounced but comparable uptake suppression of NPs coated with TF and HSA was in marked contrast to the uptake behaviour of the individual proteins. Apparently, the TF layer on the NPs did not assist in their endocytosis, possibly because the cellular endocytosis machinery was occupied with internalization of the freely dissolved protein, which was present in 105-fold excess over the 1 nM NPs. Alternatively, binding of TF to the NPs may occur in such an orientation that its TF receptor binding site is concealed, so that triggering the receptor and subsequent uptake is precluded. For HSA, the protein corona may act as a protective layer, shielding the carboxyl-functionalized NP surface from direct interactions with receptors on the cell membrane. This work presents a vivid example how protein adsorption onto NPs markedly affects their uptake behaviour and underscores the need to understand NP-protein interactions at the molecular level.

Presently, our knowledge about conformational changes of the proteins upon adsorption onto NPs is still limited. For a number of serum proteins, Nienhaus and co-workers [36,64,68] have carefully measured the thickness of the protein corona on carboxyl-functionalized, polymer-coated NPs by using FCS. They found in all cases that the protein corona consisted of a protein monolayer that had a thickness...
that coincided with the dimensions of the adsorbed proteins binding in a specific orientation. These results suggest that the overall structure of the bound proteins was not markedly changed upon binding to the NPs. However, depending on the chemical nature of the protein–NP interaction, adsorption forces exerted on proteins may be sufficiently strong to distort or even completely disrupt the delicate architecture of these weakly stabilized, flexible macromolecules that are known to fluctuate among a vast number of conformational substrates at physiological temperatures [69,70]. In fact, protein structural changes, upon binding to NP surfaces, have been reported [10,39,40]. Conformational effects of adsorbed proteins were recently also addressed by Prapainop et al. [71], who observed that cellular uptake of NPs can be changed by a surface modification inducing protein misfolding in a component of the protein corona. For their study, they used fluorescent, hydrophobic CdSe/ZnS quantum dots (QDs) coated with amino-functionalized PEG and, for comparison, the same QDs modified by chemically attaching the inflammatory metabolite cholesterol 5,6-secosterol atheronal-B [72]. Antheronals are a class of oxysterol inflammatory metabolites [73] known to affect folding and aggregation state of several proteins, including apo-B100 [73], β-amyloid [74], α-synuclein [75] antibody light chains [76] and a murine prion protein [77]. Both QD types had the apolipoprotein apo-B100 in their hard protein corona after incubation in foetal calf serum [71,72].

Confocal fluorescence microscopy and flow cytometry studies revealed a concentration- and time-dependent QD uptake of the antheronal functionalized QDs by murine macrophage-like cells (RAW 264.7), with a measureable QD uptake at particle concentrations down to 10 nM. By contrast, the antheronal-free QDs were not internalized by the cells even at higher concentrations (100 nM). From a comparison of antheronal-coated QD (100 nM) uptake after incubation for 2 h at 37°C in Roswell Park Memorial Institute medium, supplemented with either foetal bovine serum (FBS, 1%, containing lipoproteins), lipoprotein-deficient serum (LPDS, 1%) or delipidated LPDS (1%), they concluded that low-density lipoprotein is required for cellular uptake.

With this study, Prapainop et al. [71] demonstrated that attaching small molecules to the NP surface led to misfolding of corona proteins, which then triggered NP uptake by specific cells that otherwise would not have done so. This study presents a vivid example that adsorption-induced structural changes of the proteins present in the corona may give rise to specific biological responses and underscores the importance of understanding NP–protein interactions in depth.

### 3. Cellular mechanisms involved in nanoparticle uptake

The effect of physico-chemical properties of NPs on their cellular uptake by different endocytosis mechanisms was thoroughly assessed by a variety of studies [24,30,78–81]. Recently, Kim et al. [82] studied the dependence of NP internalization on the cell cycle phase. The cell cycle consists of four phases during which the cell grows and divides. The G1 (or post-mitotic) phase is normally the major period of cell growth. The high demand of structural proteins and enzymes during this phase results in a high intracellular protein synthesis activity and a high rate of cell metabolism. The G1 phase is followed by the S (or synthesis) phase in which DNA is replicated. The subsequent G2 phase is again a period of cell growth and protein synthesis in preparation of mitosis. During the M (or mitotic) phase, the cell splits into two daughter cells. Both daughter cells will then have their own cell cycles starting with the G1 phase. Cells may temporarily stop their reproductive cycle and enter a resting phase, G0.

To test the effect of the cell cycle phase on NP uptake, Kim et al. [82] studied accumulation of NPs in human lung carcinoma cells (A549) that were incubated for up to 72 h with carboxyl-functionalized polystyrene NPs (PS-COOH, 40 nm diameter) with an overall negative ζ-potential (–34 ± 1 mV in PBS/–2 ± 1 mV in complete minimal essential medium, cMEM). These NPs were fluorescently labelled with a yellow-green dye similar to fluorescein isothiocyanate. By using confocal fluorescence microscopy, the NPs were observed to enter the A549 cells and to accumulate in lysosomes, as inferred from colocalization of the NP fluorescence, and the red fluorescence of lysosomes marked with the lysosomal-associated membrane protein 1 (LAMP1) antibody.

The mean intracellular fluorescence intensities were measured by using flow cytometry during the exponential growth phase of the cells to determine the uptake kinetics. Figure 1a shows confocal images of A549 cells that were continuously exposed to the PS-COOH NPs (25 μg ml⁻¹) in cMEM for 5, 24 and 72 h, respectively. The time-dependent intracellular NP fluorescence intensity is shown in figure 1b. Because these data are averaged over all cells, they refer to a mixture of cells in different cell cycle phases.

In a subsequent step, cells in the G0/G1, S or G2/M phases, respectively, were identified by DNA staining with 7-amino-actinomycin D, and DNA synthesis was monitored with the nucleoside analogue ethynyl deoxyuridine (5-ethynyl-2-deoxyuridine) so that contributions to the average of all cells (figure 1c–e) could be assigned. The results indicated that the NP uptake yield did not depend on the cell cycle phase during the first 10 h of uptake.

Differences started to appear once the cells had divided during NP exposure. These cells in G0/G1 showed a reduced NP concentration compared with cells in the other phases (figure 1c–e). When cells that had divided under NP exposure began to populate the S phase, their intracellular NP concentration was again reduced when compared with cells in G2/M that had not yet divided at this point. This behaviour was also observed for other cell types (SH-SY5Y neuroblastoma cells, 1321N1 astrocytoma cells and hCMEC/D3 cells) and different NPs in different media (40 nm yellow-green PS-COOH in serum-free medium, 40 nm yellow-green PS-COOH in complete medium, 100 nm yellow-green PS-COOH in complete medium and 50 nm green silica in complete medium).

These results can be understood by assuming that exocytosis is negligible in these experiments, and that the observed reduction is a mere consequence of cell division, during which the intracellular NP load is split between the daughter cells. Numerical simulations were found to agree with these interpretations. An intriguing aspect, as the authors noted [82], is that the observed NP dilution effect during cell division will likely be enhanced in tumour cells, owing to their generally enhanced cell division rate compared with non-cancerous cells. This effect can be relevant for NP-based cancer therapeutics and deserves further attention.
Although the in vivo consequences of these findings remain unclear, in vitro studies could be significantly affected. Environmental and even medical exposure scenarios normally involve markedly lower NP concentrations per cell than those used here and in most other in vitro studies of cellular NP uptake. Therefore, utmost caution needs to be exercised when arriving at conclusions from in vitro studies. For example, even a small exocytosis rate may become significant at lower NP concentrations and may efficiently counteract endocytosis. For a profound assessment of biological responses to NP exposure, in vivo conditions need to be approached as closely as possible.

The specific endocytosis pathways involved in NP uptake can be revealed by means of inhibitory drugs that specifically interfere with one or the other pathway. By using this strategy, Jiang et al. [23] investigated the uptake of polystyrene NPs by mesenchymal stem cells using spinning-disc confocal optical microscopy combined with quantitative image analysis. These experiments were carried out in PBS buffer to avoid problems arising from protein adsorption onto the NPs. Two types of anionic polystyrene (PS) NPs with essentially identical sizes (approx. 100 nm) and z-potentials were compared: carboxyl-functionalized PS NPs (CPS) and plain PS NPs; both were coated in addition with anionic detergent for colloidal stabilization.

In contrast to smaller NPs (approx. 10 nm diameter), which accumulated on the cell membrane prior to internalization [64], the 10-fold larger polymeric NPs were not observed to adsorb onto the cell membrane (figure 2a), indicating that they were internalized as soon as they touched the membrane. CPS NPs internalized more rapidly and accumulated to a much greater extent inside the cells than plain PS NPs.

The presence of dynasore, a drug that suppresses endocytic processes involving dynamin, a large multi-domain protein involved in clathrin- and caveolin-mediated endocytosis [83], led to a significant uptake by approximately 70 per cent for CPS NPs in comparison with a control experiment without the inhibitor. However, no effect was observed for PS NPs under the same conditions (figure 2a). These observations were corroborated by quantitative image analysis (figure 2b), showing clearly that both the intracellular fractions and those close to the plasma membrane were affected to a similar degree. These results suggest that the carboxylic acid groups on the CPS NPs caused a strong...
preference for a dynamin-dependent endocytosis pathway. A comparable effect was observed with another inhibitory drug, chlorpromazine, known to interfere with clathrin-mediated endocytosis by disrupting the assembly of the clathrin lattice forming the endocytic pit at the plasma membrane [84]. The analysis (figure 2) showed that the uptake of CPS NPs was also suppressed by approximately 70 per cent, whereas again little effect was seen for PS NPs. Similar results were obtained with potassium-depleted cells as an alternative strategy for the inhibition of clathrin-mediated endocytosis [85]. Uptake of CPS NPs was reduced by approximately 60 per cent, whereas the uptake of PS NPs was again unaffected (figure 2).

Taken together, the inhibition studies revealed that uptake of CPS NPs proceeded predominantly via dynamin- and clathrin-dependent pathways; the PS NPs apparently had a preference for dynamin- and clathrin-independent mechanisms. The observation that markedly different mechanisms were involved in the endocytosis of two types of NPs with identical properties, except for the surface functionalities, exemplifies that cellular uptake pathways and NP properties crucially depend on specific interactions with cell surface receptors, which subsequently activate different pathways.

To elucidate intracellular trafficking of NPs, Sandin et al. [86] investigated collocalization of internalized NPs with Rab family GTPases. The Rab family of small GTPases is a major class of proteins regulating intracellular traffic [87]. Rab5A is typically the first Rab family GTPase encountered during endocytosis, mediating early endosome fusion [87,88]. Rab7 is essential for maturation of late endosomes and their subsequent fusion with lysosomes [89,90]; Rab9 is involved in the recycling of acid hydrolase receptors from late endosomes to the trans-Golgi network [91]; endocytic recycling processes involve Rab11A [92].

Sandin et al. [86] used 40 nm carboxylated polystyrene NPs in their study, carrying a negative $\zeta$-potential of...
–12.3 mV in cDMEM (Dulbecco's modified Eagle medium plus 10% FBS), by live HeLa cells using spinning disc confocal microscopy. To visualize the Rab proteins, the cells were transfected with DNA encoding for fusion proteins of the earlier-mentioned Rab GTPases with the fluorescent protein mCherry to the specific Rab proteins [93].

The cells were subjected to a 1 min pulse of NPs followed by a chase in NP-free medium. Images were taken every 15 min for up to 4 h. The time-dependent collocalization profiles (figure 3) showed an increasing collocalization of NPs and Rab7 until a constant level was reached after approximately 90 min. For collocalization of NPs with Rab5, an initial increase peaking approximately 45 min after NP exposure was followed by a subsequent decline. The suggested interpretation for this behaviour was a transient occupancy of the NPs in Rab5-labelled membranes before their release into other endocytic intermediates.

The collocalization profile with Rab9, however, was markedly different from the one acquired with Rab5. The increase in
collocalization of NPs with this marker was coincident with the decline of collocalization with Rab5, indicating that the NPs become trapped in Rab9-positive structures. Finally, a low degree of collocalization of NPs and Rab11A was measured, which suggests that the NPs used in this study are not quantitatively exocytosed. However, our earlier-mentioned caveat regarding the relevance of NP concentrations also applies here.

The experiments described here begin to shed light on the intracellular fate of NPs following their internalization. We conclude this section by pointing out that the chemical composition and, in consequence, other parameters such as pH of the immediate NP surroundings change dramatically during their transport through the endosomal pathway. The influence of such changes on the degradation of the protein corona or the NP itself as well as the influence of protein or ligand release into the cell interior as a consequence of possible exchange reactions on the NP surfaces are far from being thoroughly understood.

4. Dose dependence of nanoparticle uptake

Intriguingly, small NPs (diameter approx. 10 nm) coat the plasma membrane before incorporation [64], whereas large NPs (100 nm) are directly internalized without any prior accumulation on the cell membrane [23]. A small NP may not be able to trigger endocytosis by itself because it interacts with an insufficient number of receptors. Thus, several NPs may be necessary to activate pit formation, and a nonlinear dependence of the uptake yield on the overall NP concentration should result. Jiang et al. [94] investigated this effect by monitoring the incorporation of 8 nm diameter β-penicillamine-coated quantum dots (DPA-QDs) by live human cancer (HeLa) cells. NP uptake was assessed quantitatively using spinning disc and 4Pi confocal microscopies. Following exposure of HeLa cells with PBS solution containing 10 nM DPA-QDs, cells were imaged for typically 1 h. Within 1 min, QDs were observed at the cell membrane and subsequently accumulated there. Over time, more and more QDs were internalized by the cells, forming large clusters in the perinuclear region within 1 h. A significant fraction of endocytosed QDs was localized in lysosomes, and some of these QD clusters were observed to actively being transported to the cell periphery for exocytosis. This report of a relevant exocytosis activity contrasts the claims of several other studies where exocytosis was found to be negligible [82,95–97]. In addition to the possible impact of NP concentration discussed earlier, NP size could be a decisive parameter for this behaviour as the DPA-QDs used in this study were considerably smaller than the NPs investigated in previous studies of NP exocytosis [82,95–97].

Confocal images taken after 1 h incubation at different QD concentrations (10, 3 and 1 nM; figure 4a–c) revealed the NP concentration dependence of the amount of DPA-QDs both at the plasma membranes and inside the cells. About equal...
amounts of QDs were associated with the cell membrane and found inside the cell after 1 h at 10 nM QD concentration.

With decreasing QD concentration, the membrane-associated fraction decreased in a linear fashion with the NP concentration, whereas the intracellular fraction decreased much more strongly. Kinetic analysis of these processes (figure 4d–f) lent further support to these findings [94]. With 1 nM NP concentration, only very few bright spots were visible inside the cells. These results support the notion that, for very small NPs, a critical threshold density of QDs on the cell membrane has to be exceeded to trigger the internalization process. This study again underscores the importance of understanding dosage effects on NP uptake and intracellular fate. This issue is challenging to address because only a few highly sensitive and sophisticated methods are available for studies at particle concentrations comparable to those of relevant exposure scenarios.

Further issues of particle dosage during in vitro studies arise from the consequences of NP degradation, which can significantly affect the results of such experiments [10,39,61,98,99]. The problem of NP stability in biological environments, containing high concentrations of proteins and ions, was also investigated by Treuel and co-workers [39], who studied the influence of a protein corona formation around initially citrate stabilized silver NPs on their colloidal stability. Their NPs were further stabilized by the formation of a protein corona, and this effect could be used to measure protein binding affinities to the NP surfaces [39].

Most in vitro experiments studying cellular uptake of NPs expose cells at the bottom of a culture plate. Xia and co-workers [99] studied the effect of NP sedimentation and diffusion on the cellular uptake of gold NPs (nanospheres, nanocages and nanorods) by human breast cancer cells (SK-BR-3, ATCC HTB-30). They compared uptake efficiencies of their particles in a classical upright set-up to data acquired in an inverted set-up with cells being positioned at the ceiling of the culture chamber and observed a higher uptake of NPs in the upright configuration compared with the inverted set-up. Not surprisingly, this difference increased with the sedimentation velocity of their NPs.

Controlling the NP dosage is important for obtaining meaningful results in both in vivo and in vitro experiments. Besides NP degradation during the experiment, NP transport in the extracellular medium, notably sedimentation, may result in an erroneous result for NP uptake. This issue presents additional complications when comparing results obtained with other NPs with dissimilar sedimentation characteristics.

5. Modelling forces acting on membrane-bound nanoparticles

The interpretation of experimental results from cellular uptake studies can be facilitated by quantitative modelling of the observed behaviour. Lunov et al. [19] used carboxyldextran-coated superparamagnetic iron oxide NPs of 60 nm (SPIO) and 20 nm (USPIO) diameters that are widely used as contrast agents in magnetic resonance imaging. They analysed the uptake of these NPs by human macrophages and found that endocytosis occurred via a clathrin-mediated, scavenger receptor A-dependent mechanism. Intriguingly, they also measured NP uptake as a function of time and presented a mathematical model that allows several mechanistic parameters to be estimated. This model describes internalization by initial binding of NPs to receptors and subsequent wrapping a membrane patch around them for internalization. The overall uptake was assumed to be governed by the wrapping time, $\tau_w$, whereas it is known that relaxation mode at earlier models [100] had suggested that NP diffusion through the membrane controls the wrapping time.

By using quantitative confocal fluorescence microscopy, the time dependence of NP uptake, $N(t)$, was observed to be exponential,

$$N(t) = N_s \left(1 - \exp \left(\frac{-t}{T}\right)\right),$$

where $N_s$ is the number of NPs at saturation (approx. $10^7$) and $T$ is the characteristic time (approx. 1 h). At short times after exposing the cells to the NPs, the uptake rates, $dn(0)/dt$, for SPIO and USPIO were approximately 25 000 and approximately 2500 s$^{-1}$, respectively. The overall rate uptake per cell, $dn/dt$, can be recast into the rate per individual clathrin-coated pit-forming event, $dn/dt$,

$$\frac{dn}{dt} = \frac{a^2 dN}{L^2 dt},$$

by introducing two parameters, the lateral dimension of the macropath, $L$ (approx. 20 μm), and the characteristic footprint of an individual endocytosis pit, $a$. However, neither $dn/dt$ nor $a$ are directly accessible from kinetic experiments on entire cells. The rate $dn/dt$, however, is the inverse of the uptake time, which may be approximated by the wrapping time, $\tau_w$. A simple force model was introduced to calculate the wrapping time (figure 5a),

$$F(x) = F_0(x) + 6\pi\eta R \frac{dx}{dt}. $$

Here, the total force that the cytoskeletal actin structure has to exert along the direction perpendicular to the membrane, $F(x)$, consists of the elastic membrane deformation force, $F_0(x)$, and the viscous drag force, which depends on the cytoplasmic viscosity, $\eta$, the particle radius, $R$ and the velocity, $dx/dt$. The attachment of the NP during incorporation has to be ensured by receptor interactions ($f_{rec}$ in figure 5a). From these calculations, $\tau_w$ was obtained as a function of $F(x)$, as shown in figure 5b. For the same actin force, the model reveals that SPIO and USPIO wrapping times differ by a factor of approximately 10. The rate of individual uptake events, $dn/dt$, can be estimated as 10–100 s$^{-1}$, yielding reasonable values of 0.3–4 μm for the characteristic length scale $a$ associated with clathrin-coated pits. Given that the overall number of receptors per cell is $(2–4) \times 10^4$ [101], one concludes that approximately 2–20 receptors are involved in NP binding during an individual endocytosis event.

Overall, the analysis of the experimental data obtained by confocal microscopy using the model presented by Lunov et al. [19] produced reasonable parameters and emphasized the value of even rather simplistic physical models for furthering our understanding of complex biological processes.

6. Passive mechanisms of nanoparticle uptake by cells

In addition to active cellular uptake, NPs may also translocate passively through cellular membranes. These processes may
remain unnoticed because active endocytosis often predominates. To focus on passive transport of NPs across cellular membranes, red blood cells (RBCs) have frequently been used as model systems [44,45,102,103] because these cells are highly specialized and lack a cell nucleus, most organelles and the endocytic machinery [104].

Recently, Wang et al. [44] studied interactions between DPA-QDs and RBCs. DPA is a small, zwitterionic acid ligand and the charges on the amino and carboxylic acid groups are balanced at neutral pH. RBCs were incubated with 10 nM DPA-QDs in PBS solution for different time periods and subsequently centrifuged the solution to separate free DPA-QDs from the RBCs. The sedimented cells were then transferred into a microscope sample cell and imaged using confocal fluorescence microscopy. In figure 6a, overlaid bright-field and fluorescence confocal images are shown for a control sample (without DPA-QD exposure) and for samples incubated with DPA-QDs for 1, 4 and 8 h. They clearly reveal that DPA-QDs adhere to RBC membranes, and the number of fluorescence spots, either close to the cell membranes or inside the cells, increases with exposure time.

The internalization kinetics was calculated from the integrated fluorescence intensity of the internal spots (figure 6a, red circles), and a normalization to the cellular area in the observation plane was applied to account for the different dimensions of the cells (figure 6b). For the internalized fraction, a half-life of 1.7 h was determined. The images showed that the adsorbed DPA-QDs did not induce a strong local membrane deformation and that penetration of DPA-QDs into RBCs apparently did not disturb the integrity of the membrane. Naturally, these statements only hold for spatial scales that can be resolved by optical microscopy.

The integrity of the RBC membrane during DPA-QD internalization was examined by observing possible escape of a tracer dye from the cytosol. RBCs were preincubated with calcein violet AM, a cell-membrane-permeant dye that becomes impermanent after hydrolysis by intracellular esterases. Subsequently, the cells were incubated with DPA-QDs for 6 h (figure 6c,d). The data clearly showed that internalization of DPA-QDs did not cause any detectable loss of cellular fluorescence from the dye, suggesting that the RBC membranes remained largely intact during NP penetration of the bilayer.

These data were further complemented by electrochemical studies of the interaction between DPA-QDs and a planar model membrane. Briefly, vesicles with the lipid content of the outer or inner leaflets of the RBC lipid bilayer were fused onto a gold electrode by the interaction between the vesicles and the hydrophobic surface of a self-assembled monolayer of 1-dodecanethiol (DT) pre-adsorbed onto the electrode. With these bilayers prepared on a gold electrode, cyclic voltammograms of 5 mM [Fe(CN)6]3− in a solution of 0.1 M KCl were acquired before and after treating the membrane with DPA-QD solution for several hours. A leakage current owing to the presence of NPs was not detectable, supporting the notion that the presence of DPA-QDs does not cause pores to form in the outer and inner lipid layers of the model membrane, which would allow ions to penetrate the bilayer and diffuse to the gold electrode.

Moreover, surface-enhanced infrared absorption spectroscopy (SEIRAS) was carried out on the same model membrane preparation. SEIRAS allows infrared spectra to be acquired from extremely thin molecular layers by using the strong enhancement of the infrared absorption of molecules in near-field distance to nano-structured metal films [105,106]. The analyses of the frequencies of the stretching modes of the CH2 groups in the lipid tails revealed that the bilayer structure is softened in the presence of DPA-QDs interacting with either side of the model membrane. Its higher dynamics may facilitate penetration of DPA-QDs into the lipid bilayer without pore formation.

As was shown earlier [46,57], NP-induced hole formation in a membrane can be avoided by suitably modulating the NP surface charge density or structure. Cationic Au NPs with 50 per cent charge density (relative to hydrophobic ligands) effectively penetrated the lipid membrane without forming holes, whereas significant membrane disruption occurred at higher charge densities [46,57]. Certain cell-penetrating peptides are also known to translocate across membranes without lipid bilayer disruption [107–109]. The zwitterionic functionalization of the DPA-QDs used by Wang et al. [44] may be responsible for the ability of these NPs to penetrate lipid membranes without compromising bilayer integrity.

7. Conclusions and outlook

The results discussed in this review have a number of implications for the field of NP exposure to cells and entire
organisms, including humans. The first consequence of such exposure is usually a protein adsorption layer that forms around NPs upon their exposure to biofluids that can markedly modify cellular uptake [10,64]. The current state of knowledge is, however, still incomplete, especially with respect to the detailed physico-chemical processes occurring at the molecular level. The kinetics of corona formation, stability and ageing effects need further attention, especially under complex biological conditions, with proteins exchanging with a multitude of competing proteins, is still only poorly understood [10,110–113]. We stress the need to bridge the gap between the \textit{in vitro} results, acquired under highly controlled conditions, and the \textit{in vivo} consequences.

Little is known yet about the change of protein structure upon adsorption. It depends both on the nature of the adsorbing protein and on the NP surface [10,39–41]. On some NP surfaces, a particular protein may stay native-like, whereas other surfaces may cause severe denaturation. To coat NP surfaces with small molecules that give rise to denaturation of particular corona proteins [71] can be extremely useful for understanding the \textit{in vitro} behaviour of NPs. For application \textit{in vivo}, however, we also need to know how ligands or denatured corona proteins are exchanged on relevant timescales.

It is by now well established that NP uptake occurs mainly via the endocytic machinery of the cell [22,23,45]. Simple physical models have been developed and tested that can reveal key parameters of endocytosis. Quantitative modelling, however, is still at an early stage and awaits further development. For smaller NPs, a critical threshold density on the cell membrane has to be exceeded to trigger the internalization process, as inferred from the nonlinear dependence of the uptake on NP concentration [94]. Quantitative details of such threshold densities and their dependence on NP characteristics are still missing. Frequently, \textit{in vitro} uptake experiments are carried out with higher NP concentrations than those expected for environmental exposure of cells. However, such conditions may bear relevance in biomedical applications. Especially for targeted drug delivery, it is of utmost importance to know the uptake yields at particular NP concentrations as well as the specific pathways that are used by the NPs.

Intracellular pathways have been revealed for a variety of NPs as, for instance, by the study reviewed here using colocalization of NPs with Rab-family GTPases [86]. How the intracellular fate of NPs can be controlled by their properties is a further important issue for successful drug delivery strategies and, likewise, for a reduction of NP toxicity.

The ability of NPs to adhere to and penetrate cell membranes by processes that do not involve any active uptake machinery is well documented. Overall, the way in which NP surface properties govern their behaviour in passive uptake has not yet been thoroughly characterized. Passive uptake routes may well play an important role during long-term exposure to low NP concentrations. Whenever threshold densities of NPs on cellular membranes are

\begin{figure}
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\includegraphics[width=\textwidth]{figure6.png}
\caption{Cellular uptake of NPs by red blood cells (RBCs) via passive membrane penetration. (a) Overlay of bright-field and confocal fluorescence images (scale bar: 5 \mu m) of DPA-QD internalization by RBCs after incubating with PBS (control) and 10 nM DPA-QDs for 1, 4 and 8 h. (b) Normalized fluorescence intensity of intracellular bright spots plotted as a function of time. (c,d) Images from fluorescence microscopy experiments (scale bar: 5 \mu m) probing the integrity of the RBCs plasma membrane during DPA-QD uptake. RBCs were incubated with (c) calcein violet AM, and subsequently (d) with 10 nM DPA-QDs for 6 h. (e) Mean fluorescence intensities of calcein violet AM labelled cells. Adapted from Wang et al. [44]. Copyright 2011 American Chemical Society.}
\end{figure}
not reached, passive uptake of NPs may become a significant contribution to their overall internalization.

The impact of cell division on the NP load within cells is another issue that needs attention. Especially, for targeted delivery of NPs to cancer cells, the effect of cell division on intracellular particle concentrations was pointed out to be potentially crucial, as cancer cells often divide faster and, thus, reduce their NP load faster, than the surrounding non-cancerous cells [82]. However, the relative timescales of NP uptake and cell division have to be considered, in addition to the acute NP toxicity, to assess the relevance of this effect in different exposure scenarios.

We have also discussed the importance of a detailed characterization of colloidal stability in any NP studies and its consequences for NP uptake yields in biological studies. This is very relevant for in vitro studies and can also be relevant in biomimetic exposure with high NP concentrations. In most environmental exposure situations, the NP concentration will likely be so low that extracellular agglomeration of NPs would occur on a much longer timescale than cellular uptake, reducing the relevance of this effect.

To conclude, thanks to substantial efforts by many laboratories, general mechanisms of NP uptake by cells have been identified, and correlations between uptake behaviour and NP properties have been observed, although many details remain to be explored. The importance of specific interactions between NPs and cell surface receptors has been shown, but the effects of physical and chemical properties of the NP surfaces deserve further attention. The key relevance of the protein corona in modulating cellular interactions has been recognized, but still very little is known about the dynamics of protein adsorption onto NPs in complex biological fluids, protein conformational changes associated with corona formation and the ensuing effects on cellular responses. Considering the wide variety of existing NPs and relevant cell types, substantial variations in their mutual interactions can be expected, and much work remains to be done.

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References


