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Small fluorescent nanoparticles at the nano–bio interface

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Semiconductor quantum dots and metal nanoclusters are fluorescent nanoparticles with diameters in the few-nanometer range. In recent years, they have captured a great deal of attention as experimental tools in the life sciences for diverse applications including imaging, bioassays and therapy. It is crucially important to understand how these small nanoparticles behave in complex biological environments, especially in view of their potential for biomedical applications. In this review, we shall highlight recent advances in exploring the behavior of fluorescent nanoparticles at the nano–bio interface, including their interactions with proteins and cells, their intracellular stability as well as their *in vivo* behavior.

Introduction

Fluorescence-based spectroscopy and imaging techniques have long been valued as reliable and quantitative tools in the biosciences, owing to the availability of a wide array of fluorogenic probes and straightforward bioconjugation techniques, as well as their high sensitivity and multiplexed detection capabilities [1–3]. It is important to realize that the photophysical and photochemical properties of the fluorescence markers are often key and limiting factors in these measurements. Thus, researchers keep on pursuing novel fluorescence probes with improved properties. With the rapid advancement of nanotechnology in recent years, a wide range of intrinsically fluorescent nanomaterials have become available as probes, including semiconductor quantum dots (QDs), metal nanoclusters (NCs), dye-doped silica or polymer nanoparticles (NPs), up-converting NPs and nanodiamonds [4].

Fluorescent nanomaterials frequently exhibit excellent photophysical properties, color tunability and facile synthesis. With an extremely high surface-to-volume ratio compared with their bulk materials, NPs can act as multivalent scaffolds for further supramolecular assemblies as well as controllable bioconjugation [5,6]. These properties make NP-based fluorophores promising as probes for fluorescence-related applications. Indeed, important biological and biomedical applications of NPs have been reported, such as *in vitro* and *in vivo* imaging, ultra-sensitive bioassays and photodynamic therapy [7–10].

It is crucially important to understand how fluorescent NPs behave in the complex biological environment. This is obvious when we think of biomedical applications, but we should not overlook that the widespread use of nanotechnology will inevitably lead to an unintended exposure of living systems including humans. Understandably, great concerns have recently been voiced regarding the safe use of nanotechnology [11,12], which calls for a deep understanding of the processes occurring at the interface between nanomaterials and biological systems, a.k.a. the nano–bio interface. The nano–bio interface comprises a dynamic series of interactions between nanomaterials surfaces and biomolecular surfaces, which are governed by a variety of forces including long-range forces arising from attractive van der Waals and repulsive electrostatic double-layer interactions, and short-range forces arising from charge, steric, depletion and solvent interactions. These interactions determine such processes as the formation of protein corona, cellular contact, endocytosis and intracellular transport [13].

Interactions of NPs with biological systems, including proteins, cells and living organelles, may alter the NP surface properties and thus their subsequent biological responses. Moreover, considering the large surface-to-volume ratio of fluorescent NPs, surface changes are likely to change their photophysical properties, which may in turn affect their fluorescence properties. Clearly, understanding the

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processes at the nano–bio interface of fluorescent NPs is of essential importance for their use in biological applications.

In this review, we intend to highlight several recent advances in exploring the behavior of fluorescent NPs at the nano–bio interface. Among the wide variety of fluorescent NPs, we focus here on semiconductor QDs and metal NCs. While semiconductor QDs have already become very popular as fluorescent NPs owing to their commercial availability and excellent photophysical properties [14,15], metal NCs, composed of a few to a hundred atoms, are a novel type of fluorescent NPs that recently have attracted enormous interest because of their ultrasmall size and facile synthesis [16,17].

Interactions of fluorescent nanoparticles with proteins

NPs possess a huge active surface in comparison to their bulk counterparts through which they can interact with biomatter in their environment. Once exposed to biological fluids, NP surfaces will be rapidly covered with dissolved components, in particular proteins, forming the so-called ''protein corona'' around NPs [18,19]. Consequently, the initial interactions of NPs with biological entities, such as cells, tissues and organs, will be mediated by the protein layer adsorbed on the NP surface. Based on a thorough understanding of these interactions, one can potentially predict and even control the behavior of NPs at the nano–bio interface, which will greatly assist in their safe use in biomedical applications [20].

Many experimental techniques have been employed to study NP–protein interactions, including various spectroscopy methods, size-exclusion chromatography, isothermal titration calorimetry, X-ray crystallography, surface plasmon resonance and mass spcetroscopy [21]. Intrinsically fluorescent NPs have the key advantage that their interactions with proteins can be directly investigated by fluorescence-based techniques such as fluorescence correlation spectroscopy (FCS) [22,23]. Based on the analysis of the duration of brief bursts of photons from individual diffusing emitters during their brief sojourn in the observation volume of a confocal microscope, FCS can provide quantitative information on NP–protein interactions, specifically the increasing NP size due to protein adsorption, protein binding affinity and even the protein orientation on the NP surfaces [24–26]. For instance, our group has studied the adsorption of human serum albumin (HSA) onto small carboxylic acid-functionalized CdSe/ZnS QDs with an overall diameter of 16 nm by using FCS [27]. The thickness of the HSA corona around QDs, ~3.3 nm, corresponded to a monolayer of proteins adsorbed in a specific orientation. The dissociation coefficient was in the micromolar concentration range, and an anti-cooperative binding isotherm was observed.

Protein binding to fluorescent NPs can also be monitored by simple steady-state or time-resolved fluorescence spectroscopy. Poderys and coworkers investigated interactions of thioglycolic acid-coated CdTe QDs with proteins by measuring the photoluminescence intensity of QDs upon titration with bovine serum albumin (BSA) [28]. A gradual increase in QD photoluminescence (up to 120% of the initial intensity) clearly suggested the adsorption of proteins on the QDs. The enhanced emission efficiency of QDs may result from the recovery of surface defects by the formed protein corona [29]. Recently, we investigated interactions of proteins with ultrasmall gold nanoclusters (AuNCs, diameter 3.2 nm) coated with dihydrolipoic acid (DHLA, Fig. 1) [30]. Based on the substantial increase in the fluorescence intensity of AuNCs upon protein adsorption, the apparent binding affinities of four different proteins (HSA, apotransferrin, lysozyme and apolipoprotein E4) to AuNCs were measured, yielding values in the micromolar range. Time-resolved fluorescence studies further revealed significantly enhanced long lifetime components of the AuNC luminescence decay curves upon protein association. Similar

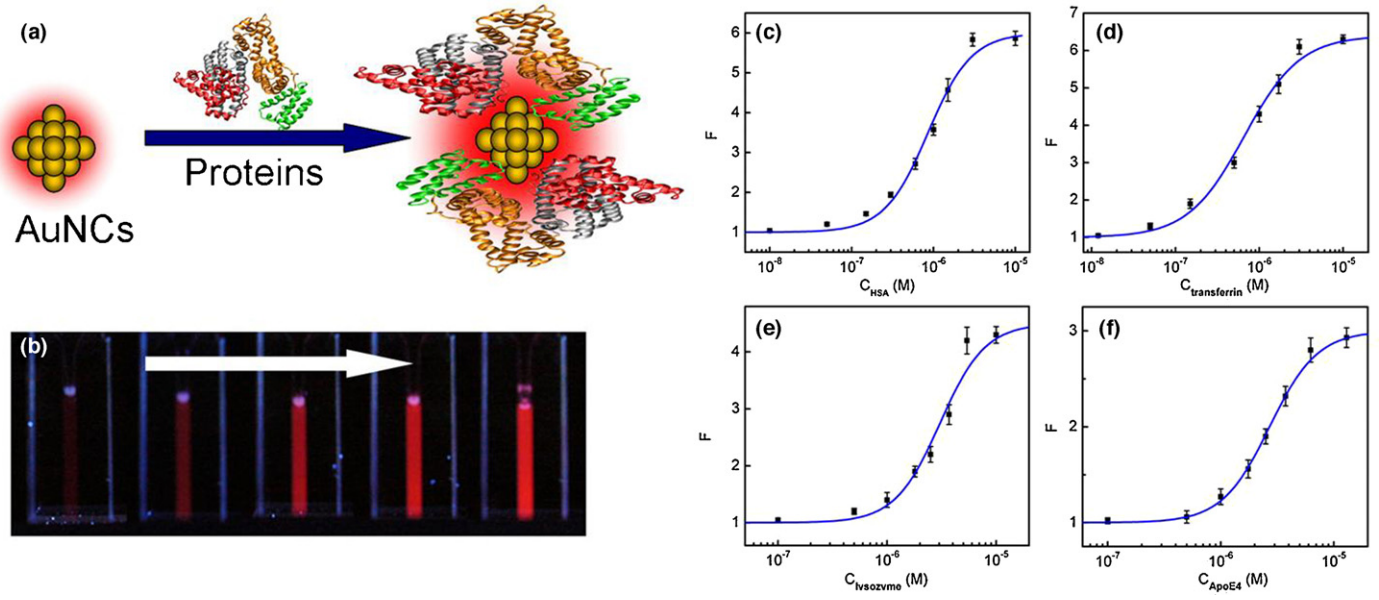

FIGURE 1

(a) Schematic illustration of protein adsorption-enhanced AuNC fluorescence. (b) Photographs of AuNC solutions in the presence of increasing protein concentration (from left to right), under a UV light source with 365 nm emission. Fluorescence intensity of AuNCs as a function of (c) HSA, (d) transferrin, (e) lysozyme and (f) apolipoprotein E4 concentration. The blue lines represent fits to the data points using the adapted Hill equation. Reproduced from Ref. [30] with permission from John Wiley and Sons.

changes in the emission decays upon protein adsorption have recently also been reported for other fluorescent NPs, such as AgNCs [31], AuAg alloy NCs [32], and nanodiamonds [33]. These results clearly show that the photophysical properties of NPs are sensitive to protein binding, and especially for their use as fluorescence markers, these results call for a thorough investigation of photophysical effects in the biological environment.

An important aspect is that proteins may change their conformation upon adsorption onto NP surfaces because they are weakly stabilized biomacromolecules that can fluctuate among a huge number of conformational substates [34,35]. Protein adsorption onto NPs may constitute a significant energetic perturbation that induces conformational changes and, concomitantly, alters its function [36–38]. Indeed, conformational changes of HSA on the surface of mercaptoacetic acid-capped CdSe/ZnS QDs (3.4 nm diameter) have been reported [39]. Based on their Fourier transform infrared (FTIR) and circular dichroism (CD) spectroscopy data, the authors suggested that HSA underwent substantial conformational changes at both secondary and tertiary structure levels upon interaction with QDs. In another study, Shen et al. [40] reported that hemoglobin adsorbed onto the surface of cationic CdS QDs (9.1 nm diameter) exhibited a loose conformation in which the α-helix content was substantially decreased. The structural alterations resulted in an orientational change of the heme vinyl groups, which was revealed by Raman spectroscopy.

Actually, ideal optical probes are expected to interfere only minimally – if at all – with the function of the conjugated biomolecules as well as the investigated biological processes. Especially fluorescent NPs with small size are favorable for biological applications [41]. Researchers have recently addressed the critical size issue by either making the NP surface coating thinner [42,43] or by producing fluorescent NPs with ultrasmall cores (e.g., few atom-composed metal NCs) [16]. In a recent paper, we reported that both tryptophan emission and CD spectra of HSA remained essentially unchanged upon binding to AgNCs with an overall diameter of 2.1 nm, suggesting that major perturbations of the protein conformation upon adsorption onto AgNC surfaces are absent [31]. In another study, Chou and coworkers observed that insulin retained its bioactivity in the protein–AuNC conjugates, evaluated by examining the regulation of the blood glucose level in mice by insulin-AuNCs [44]. These results suggest the distinct advantage of ultrasmall fluorescent NPs as promising optical labels for biological applications. However, it is very clear that further structural studies are required to advance our understanding of the protein corona.

For applications of fluorescent NPs in targeted drug delivery, protein–NP interactions need to be particularly taken into account, as protein adsorption can adversely affect the biological interactions with these NPs. In a recent study, Dawson and coworkers have shown that the formation of a biomolecular corona around the surface of NPs can alter their targeting capabilities [45]. Using transferrin-conjugated fluorescent silica NPs, they found that proteins in the media can shield transferrin from binding to its cognate receptor on cells and in solution (Fig. 2). Although NPs continue to enter cells, the targeting specificity of transferrin was lost, suggesting that the formation of a protein corona can 'screen' the targeting molecules on the surface of NPs and cause a loss of specificity in targeting. One important implication of these results is that the design of new NPs for application in biological media requires a profound understanding of the interactions between NPs and biomolecules.

Interactions of fluorescent nanoparticles with cells

Interactions of nanomaterials with cells are critical for many applications such as cellular imaging, diagnosis and therapy because applications often require NPs to enter cells [46–48]. Rising concerns over the biological safety of NPs also call for a better understanding of how NPs behave upon entering cells [49]. To this end, it is necessary to advance our knowledge of how fluorescent NPs interact with cells, including uptake and transport pathways, intracellular fate, effects on cellular function and so on.

While NPs can be delivered into cells by physical approaches such as direct microinjection and electroporation, most NPs are capable of entering cells *via* endocytosis, a fundamental process

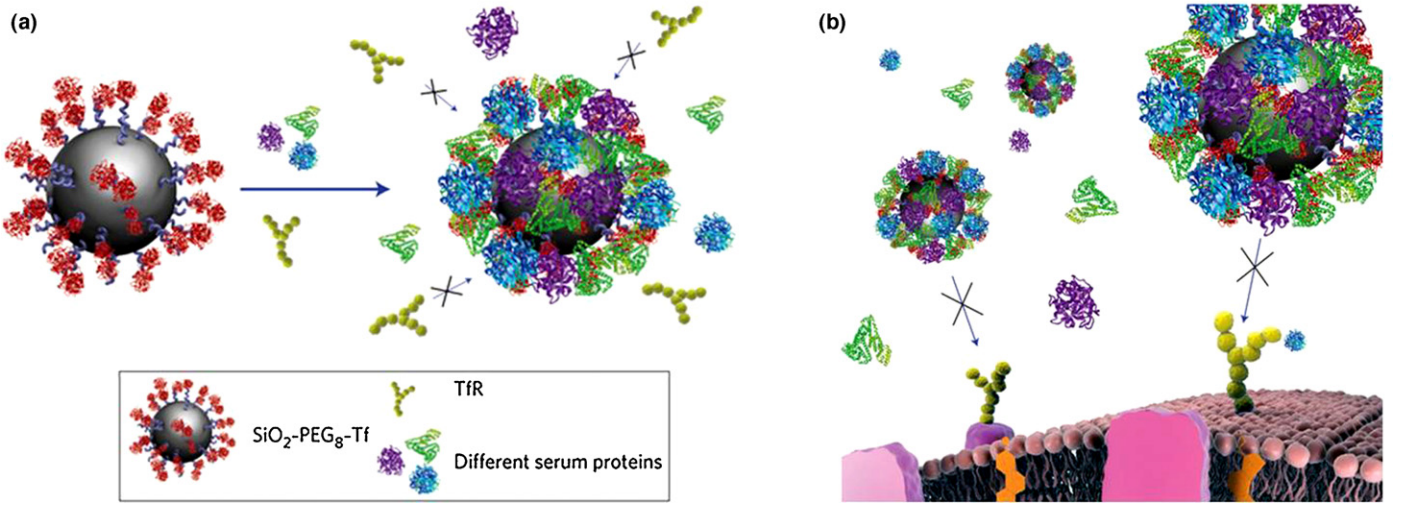

FIGURE 2

(a) Schematic depiction of blocked transferrin–transferrin receptor (Tf–TfR) interaction in solution in the presence of serum proteins. (b) Schematic representation of the loss of targeting capability of Tf-conjugated NPs toward TfR on the cell surface in the presence of serum proteins (endogenous Tf, where present, could also compete for TfR). Reproduced from Ref. [45] with permission from Nature Publishing Group.

that is used by essentially all cells to internalize (bio)molecules [50]. Indeed, many fluorescent NPs have been engineered to enter cells through endocytosis and deliver their cargo within the cell. Endocytosis encompasses several active cellular mechanisms, which are generally divided into two categories: phagocytosis and pinocytosis. Phagocytosis is efficiently carried out by specialized cells, professional phagocytes, which take up larger particles; pinocytosis of smaller NPs (a few to about a hundred nanometers) occurs in almost all eukaryotic cells [51]. Unraveling endocytosis mechanisms during NP internalization is crucial to understand the fate of NPs and the effect on the biological activity of the cargo transported inside the cells.

Fluorescent NPs allow one to directly visualize the uptake process and track NPs within live cells in real time using fluorescence microscopy [52]. Uptake and intracellular transport of D-pencillamine-coated QDs [53] (DPA-QDs) of 8 nm diameter by live HeLa cells have been investigated systematically by using spinning disk and 4Pi confocal microscopy [54]. Interestingly, these small QDs were observed to accumulate at the plasma membrane prior to internalization (Fig. 3). The same behavior has also recently been observed for the cellular uptake of AuNCs with an overall diameter of 3.3 nm [55]. Indeed, both theoretical [56] and experimental [57] studies have indicated that there exists a critical NP density/size to trigger cellular uptake, which can be achieved through clustering of small NPs so that the entire aggregate is internalized as a whole. Such a behavior is in stark contrast with the internalization of larger NPs, e.g., polystyrene NPs with 100 nm diameter, where no accumulation at the plasma membrane was observed [58,59].

By using inhibitors that interfere with particular uptake pathways, uptake mechanisms of NPs can be elucidated. For instance, our recent studies revealed that clathrin-mediated endocytosis plays a significant role in the uptake of both DPA-QDs [54] and dihydrolipoic acid-coated AuNCs [55], which were actively transported along microtubules toward the perinuclear region. Co-labeling different cell organelles such as early endosome, lysosome and nucleus further showed intracellular trafficking of the AuNCs through the endosomal pathway. These NPs were ultimately transferred to lysosomes, but did not enter the nucleus even after 24 h. The cellular uptake kinetics and mechanisms of fluorescent nanodiamonds have also been evaluated through fluorescence imaging by either taking advantage of their intrinsic emission [60] or conjugation with additional fluorophores [61]. Similarly, these nanodiamonds of size less than 50 nm enter cells mainly by endocytosis through a clathrin-mediated process, and localization studies revealed that they reside in early endosomes and lysosomes with eventual release back into the cytoplasm.

Interactions of NPs with cells depend on many parameters, including the physicochemical properties of NPs, NP surface modifications, cell types as well as the cell cycle [62–65]. Indeed, Monteiro-Riviere and coworkers [66] recently showed that the uptake mechanism of carboxylic acid-terminated CdSe/ZnS QDs is different for dendritic cells and HEK cells [67], and that uptake also depends on the state of differentiation of the dendritic cells. In another study, interactions of CdSe QDs of various sizes and shapes with live immune cells were studied by high-speed total internal reflectance fluorescence (TIRF) microscopy [68]. This work showed that both size and aspect ratio of QDs are critical characteristics, which significantly affect their interactions with the plasma membrane, cellular uptake efficiency as well as localization within intracellular vesicles. Also, QDs with the same size but varied short ligand surface functionalization were observed to enter human kidney and liver cells through lipid raft-mediated endocytosis, although to significantly different extents [69]. Polyethylene glycol (PEG) has been used frequently for coating QD surfaces to improve their biocompatibility and to prolong their blood circulation time by reducing nonspecific protein adsorption; however, cellular uptake of PEG-coated QDs was shown to be severely reduced [70,71].

To better understand the mechanisms of NP–cell interactions, several studies have examined the interactions of fluorescent NPs with cellular membranes [72,73] or synthetic lipid bilayers [74]. Jiang and coworkers have investigated the passive transport of DPA-QDs across the plasma membranes of red blood cells, which are incapable of endocytosis [72]. Fluorescence microscopy studies revealed that zwitterionic QDs penetrated cell membranes and entered the cells. Meanwhile, entrance of DPA-QDs did not cause any measurable leakage of calcein violet AM, a cell-membrane-permeable dye that becomes impermeable after hydrolysis by

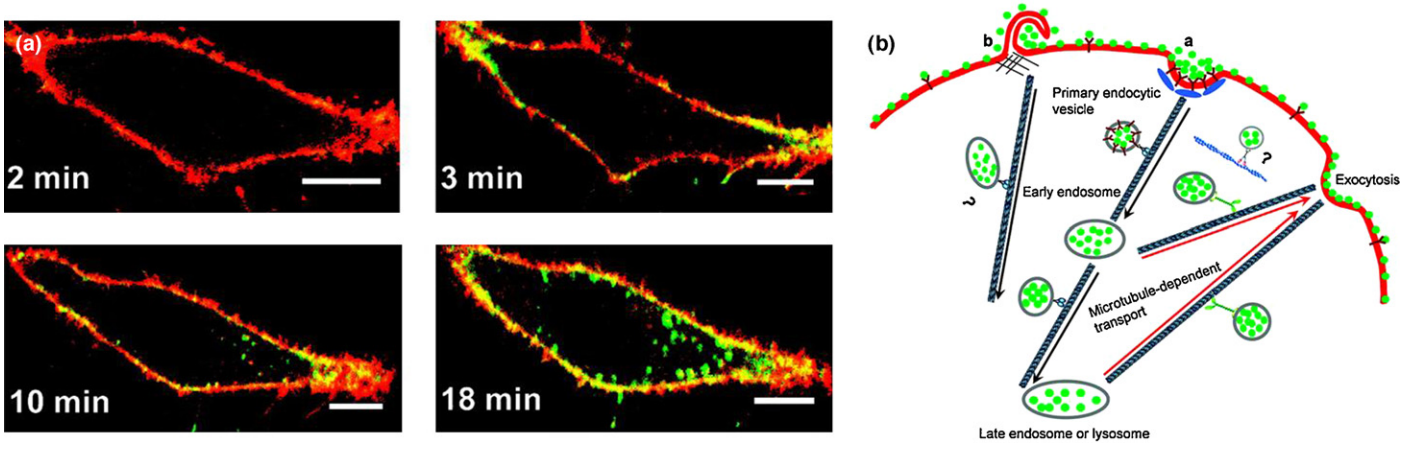

FIGURE 3

(a) Time sequence of merged two-color confocal images of a HeLa cell exposed to DPA-QDs (green) for different times. The plasma membrane was stained with CellMask Deep Red (red). Scale bar: 10 μm. (b) Schematic diagram illustrating the key steps involved in DPA-QD uptake, active transport and intracellular fate. Reproduced from Ref. [54] with permission from the American Chemical Society.

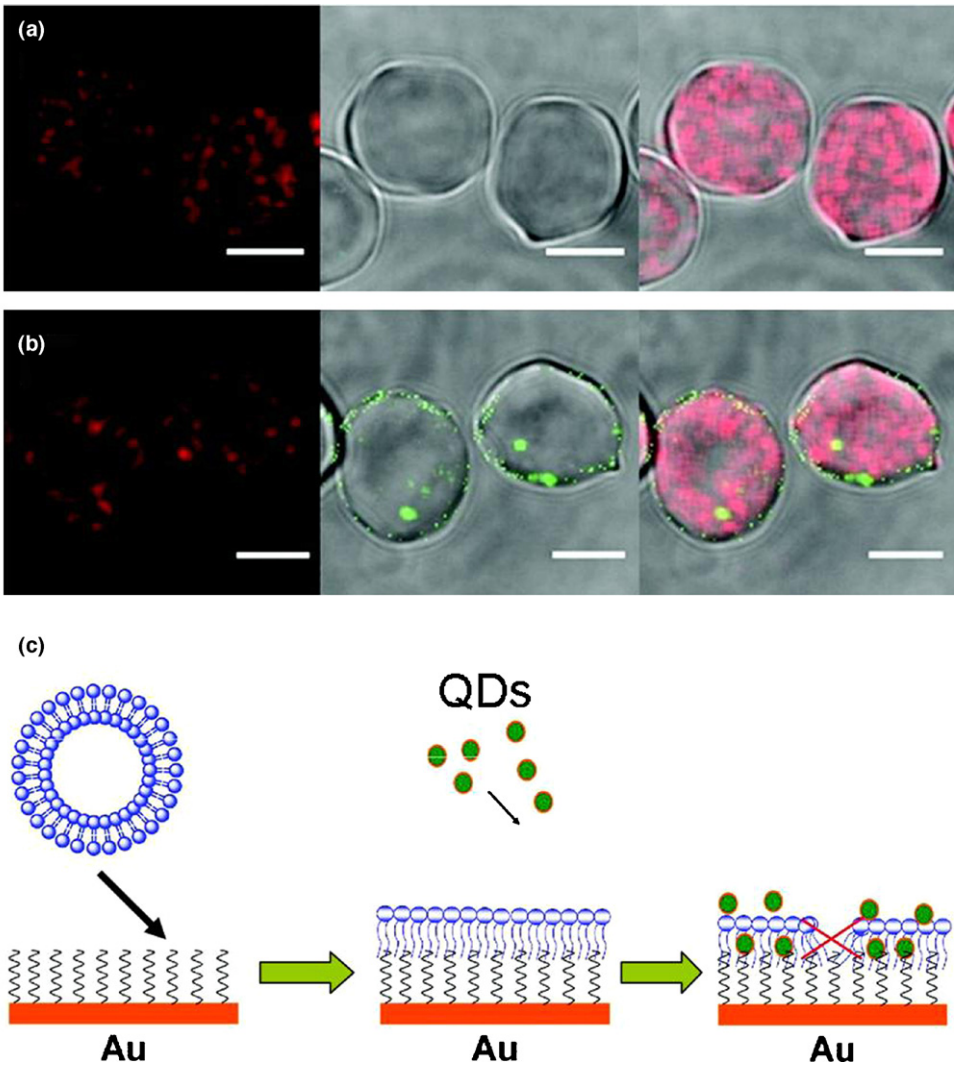

FIGURE 4

Fluorescence microscopy experiment to examine if DPA-QD uptake by red blood cells (RBCs) causes holes in the plasma membrane. RBCs were incubated with (a) calcein violet AM, and subsequently (b) with 10 nM QDs for 6 h. Scale bar: 5 μm. (c) Schematic diagram illustrating the interaction of QDs with planar membranes, formed by hydrophobic interactions between vesicles and 1-dodecanethiol monolayers self-assembled on a gold electrode. Reproduced from Ref. [72] with permission from the American Chemical Society.

intracellular esterase (Fig. 4). Moreover, surface-enhanced infrared absorption spectroscopy and electrochemistry studies revealed a markedly enhanced flexibility of the lipid bilayers in the presence of NPs and the overall membrane structure remained intact without persistent holes formation in the bilayers. Previously, NPs with very small dimensions and positive charge have been observed to pass through cell membranes by forming membrane holes, generating noticeable cytotoxic effects in the process [75,76]. Therefore, it appears feasible that carefully designed materials with optimally engineered surface properties may pass through membranes without disrupting the membrane.

Intracellular stability of fluorescent nanoparticles

Stability of NPs in live cells is an important issue that recently captured the attention of many researchers. Endocytosed NPs are passed from endosomes to lysosomes, and lysosomes have a low pH and contain proteases and other enzymes that degrade a variety of biological substances [77]. Consequently, surface coatings and even core materials of NPs face the risk of degradation in this corrosive intracellular milieu, which may not only compromise NP function but also give rise to NP toxicity. Knowledge about the intracellular stability of fluorescent NPs is important for effective utilization of these optical materials in many biological applications, and can also assist in the design of stable and biocompatible NPs [78].

The intracellular stability of fluorescent NPs has been rarely explored due to the lack of adequate experimental tools. Rotello and coworkers recently proposed a label-free method to quantify the stability of four types of cationic CdSe/ZnS QDs in live cells by a combined use of laser desorption ionization mass spectroscopy (LDI-MS) and inductively coupled plasma mass spectrometry (ICP-MS) [79]. The total molar amount of QDs taken up by cells was first quantified using ICP-MS, whereas the amount of QD surface ligands was measured by LDI-MS (Fig. 5). The difference between the amount of QDs determined by ICP-MS and the amount of surface coatings determined by LDI-MS gives the amount of

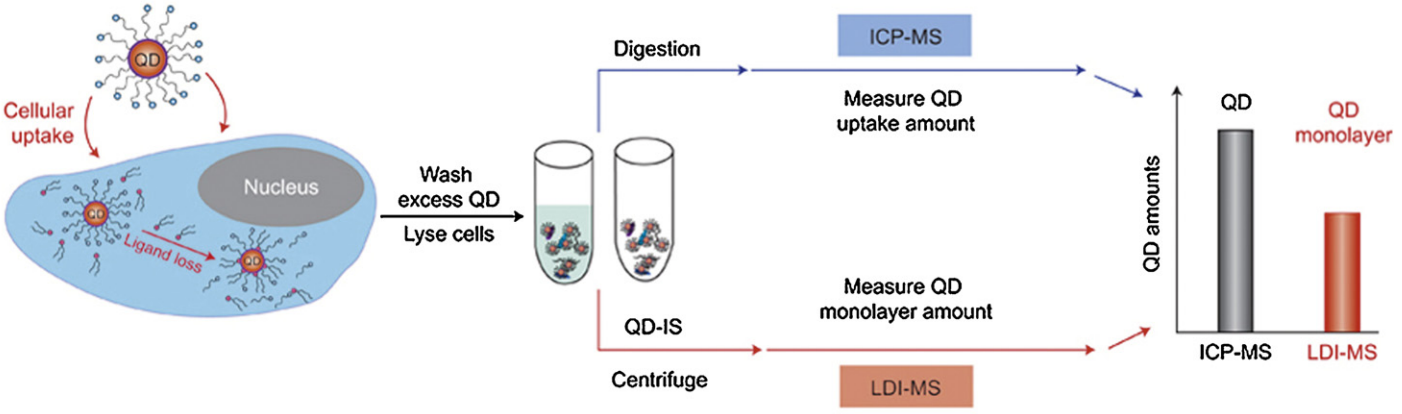

FIGURE 5

Parallel measurement of total QD uptake and monolayer amounts inside cells using ICP-MS and LDI-MS, respectively. The difference between the values obtained by ICP-MS and LDI-MS represents the amount of ligands released from QDs inside the cells. Reproduced from Ref. [79] with permission from Nature Publishing Group.

ligands released from the QDs, providing a quantitation of the stability of the coating inside the cells. With this approach, they demonstrated that QD stability is correlated with both NP size and monolayer structure: relatively small QDs with bidentate ligands such as dithiolate possess a better stability in live cell imaging applications. Indeed, previous studies by the Mattoussi lab and others have suggested an enhanced colloidal stability of QDs in biological media upon surface coating with bidentate-based ligands such as PEG-modified ones [80] or sulfobetaine-appended DHLA [81].

In another study, the intracellular aggregation kinetics of QDs with three different surface chemistries were evaluated by fluorescence microscopy observation, revealing that the QD intracellular aggregation behavior is strongly dependent on the surface chemistry [82]. QDs coated with bidentate zwitterionic ligands exhibited better stability, and diffused within the entire cell cytosol for at least 24 h. In contrast, QDs stabilized with other surface chemistries, i.e., encapsulation into phospholipid micelles or amphiphilic copolymers showed rapid aggregation in the cytoplasm. In line with these results, Chen et al. [83] recently examined the intracellular fate of polymer coatings on QDs by labeling the coatings with fluorophores. The emission of the dyes conjugated to the QD coatings remained quenched upon internalization during 4 h incubation of the cells. Fluorescence from these dyes appeared after 8 h, however, suggesting that the polymer coating dissociated from the QD surfaces in the lysosomes of the cells. These results contribute to advancing our knowledge of the fate of fluorescent NPs in biological environments. Considering the versatile coating strategies and surface chemistries of fluorescent NPs, more research will be necessary to evaluate their stability in biological systems.

In vivo behavior of fluorescent nanoparticles

Recent advances on fluorescent NPs for *in vivo* biomedical applications have prompted a close scrutiny of their behavior *in vivo*, including biodistribution, clearance, metabolism, and toxicity [84–87]. A comprehensive knowledge of the *in vivo* toxicity of these nanoscale materials is of essential importance for their safe biomedical application. While toxicology data currently are frequently derived from *in vitro* studies of cultured cells, these approaches may not capture natural *in vivo* responses. For example, several reports have demonstrated QD toxicity in cell culture studies [88,89], yet this toxicity has not been observed *in vivo* in small animals [90–92]. Evidently, the *in vivo* behavior of NPs, which is closely related to their surface chemistries, sizes, doses, and administration routes, is a rather complicated issue.

In an early study, Chan and coworkers provided the first quantitative glimpse of *in vivo* kinetics, clearance, and metabolism of semiconductor QDs following their intravenous administration to Sprague–Dawley rats [93]. QDs coated with mercaptoundecanoic acid cross-linked with lysine were observed to exhibit a lower clearance from plasma than QDs conjugated with BSA. In addition, the biodistribution of these QDs was different. The role of surface coatings on the *in vivo* behavior of QDs has also been examined by oral administration in *Drosophila melanogaster* [94]. The results indicated that cadmium-based QDs elicited a significant lifespan decrease, high levels of oxidative stress and genotoxicity, mainly due to the *in vivo* release of Cd^{2+} ions. The surface engineering of QDs may affect the uptake and bioaccumulation in the organism, thereby decreasing the overall toxicity but not entirely eliminating it. While PEG is typically considered an inert molecule, the length of the PEG chain appears to have a marked effect on the biodistribution and clearance of capped NPs, e.g., PEGylated InAs(ZnS) QDs [95]. NPs with ultrashort (i.e., dimeric) PEG chains retain a hydrophobic character and result in rapid uptake by the liver; relatively long PEG chains (i.e., 22-mers) are highly hydrophilic, so the NPS remain in the vasculature for long periods of time. NPs coated with PEGs of intermediate chain lengths exhibit specific tissue and organ distribution and clearance.

Using intravenously administered QDs in rodents as a model system, Frangioni and coworkers [96] recently reported that the *in vivo* behavior of QDs was greatly dependent on their hydrodynamic size. They suggested that QDs smaller than 5.5 nm can be rapidly and efficiently metabolized by renal clearance, while QDs larger than 15 nm escape renal excretion and accumulate in the liver and spleen. Following this study, the same group later investigated the translocation behavior of QDs from the lung airspaces

to the body [97]. NPs small than 34 nm with a noncationic surface were observed to translocate rapidly from the lung to mediastinal lymph nodes, while NPs smaller than 6 nm traffic rapidly from the lung to the lymph nodes and further on to the blood stream. Finally, they are cleared by the kidneys.

For *in vivo* biomedical applications, renal clearance is of fundamental importance to ensure that the contrast agents can be effectively and quickly cleared from the body, thereby avoiding *in vivo* toxicity due to long-term effects. Compared with other NPs, the much smaller metal NCs appear advantageous in regard to highly efficient clearing. Indeed, recent studies revealed that over 50% of 2 nm glutathione (GSH)-coated luminescent AuNPs were found in the urine of mice within 24 h after intravenous injection, and up to 65% after 72 h (Fig. 6) [98]. Only 3.7% of these luminescent AuNPs accumulated in the liver of mice. This finding is in stark contrast to the previously reported biodistribution of larger AuNPs (50–94% presence in the liver) [99,100]. The efficient renal clearance of the 2-nm AuNPs resulted not only from their small size but also from their coating with GSH, which stabilizes the luminescent AuNPs during blood circulation. In another study, Zhang et al. [101] systematically explored the *in vivo* renal clearance, biodistribution and toxicity responses of Au_{25}NCs coated with either GSH or BSA. Their results suggest that the surface of AuNCs plays a very important role in biodistribution and toxicity. These studies provide an important foundation for the design and development of fluorescent NPs for future cancer therapy, drug delivery, and bioimaging applications.

Perspective

Fluorescent NPs hold great promise as alternatives and even substitutes to conventional fluorophores for many biomedical applications. It is important to carefully characterize their behavior in the complicated biological environment prior to widespread application in biomedicine. Although impressive progress has been made in recent years in exploring the interactions of these nanoscale materials with biological systems, we are still far from the deep understanding of how they behave at the nano–bio interface, especially regarding their *in vivo* responses. Many questions remain

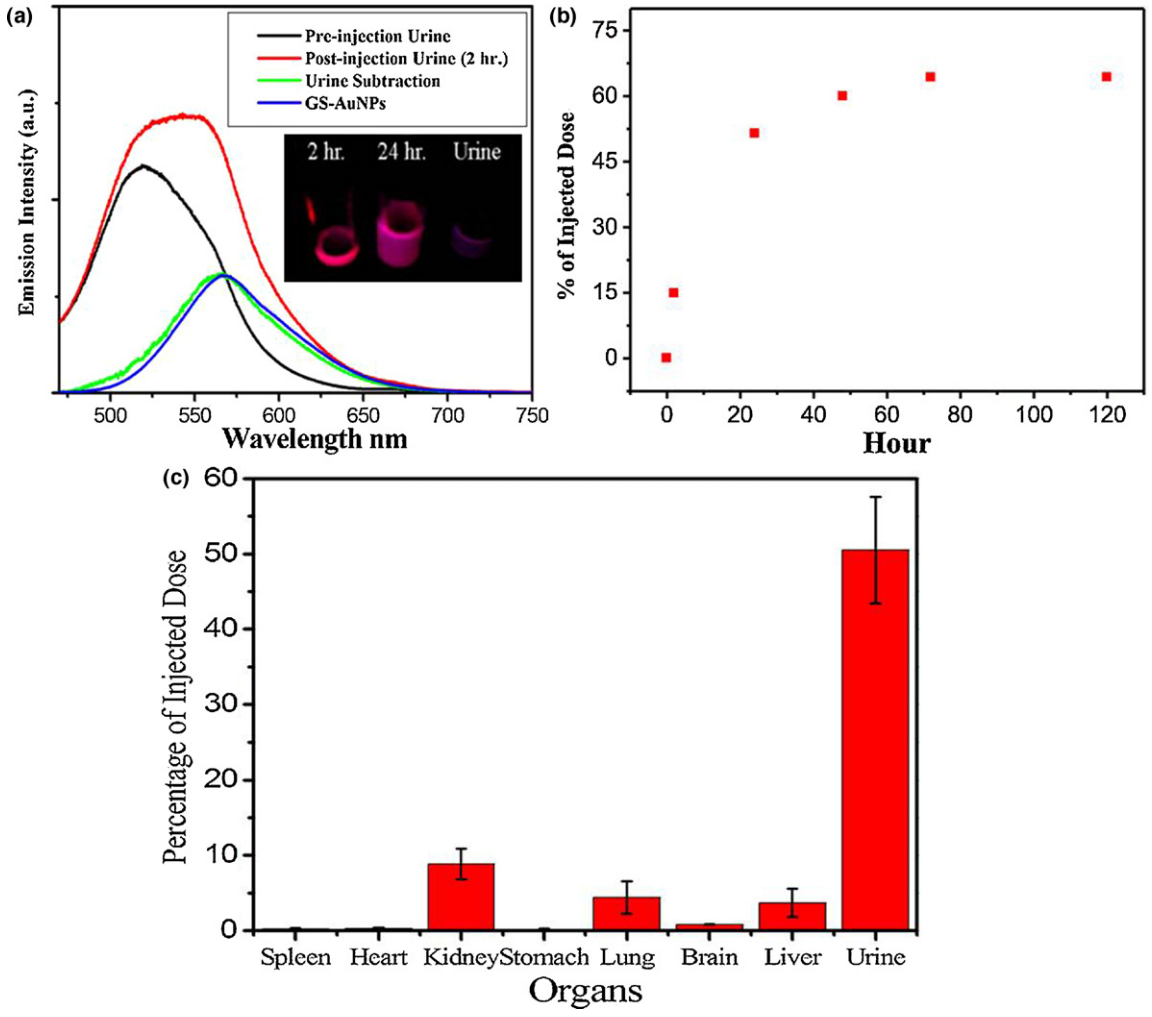

FIGURE 6

Renal clearance and biodistribution studies of GSH-AuNPs in mice. (a) Luminescence spectra of urine (black), AuNPs (blue), the urine collected 2 h post-injection (p.i.) (red), and spectrum (green) after subtracting the urine background (excitation wavelength 420 nm). (Inset) Luminescence images of GSH-AuNPs in urine 2 and 24 h p.i. and control urine upon excitation with ultraviolet (UV) light; the emitted light was collected through a 630/75-nm bandpass filter. (b) Gold concentrations in the urine at 2, 6, 24, 48, 72, and 120 h p.i. measured by ICP-MS. (c) Biodistribution of GSH-AuNPs in mice 24 h after intravenous injection. Reprinted from Ref. [98] with permission from John Wiley and Sons.

to be answered, i.e., how does protein adsorption impact the kinetics and metabolism of NPs *in vivo*? What is their long-term fate? How long can their optical properties survive in living organisms? Addressing these questions will be necessary to fully elucidate their utilization, transformation, and final fate within biological systems.

Future development of fluorescent NPs for biomedical applications will benefit from ongoing advances in the materials sciences, continually producing novel NPs with improved photophysical and chemical properties. Particularly, a more precise control over NP surface chemistry will definitely allow researchers to better understand how NP properties can affect protein adsorption, structure, and subsequent biological outcomes [102]. While conjugation of NPs with biomolecules is crucial for controlling the biological activity of the resulting NP bioconjugates, current conjugation strategies still suffer from inefficiency, cross-reactivity and reproducibility. There is an urgent need for the development of more efficient and simpler bioconjugation strategies, yet this area is presently underexplored [103]. Another point that has recently moved center stage is the establishment of reliable, standardized methods and instruments for the characterization of physical and chemical properties of NPs, and nanomaterials in general. Any serious toxicity assessment has to be based on the precise knowledge of the nanomaterials properties; therefore, characterization procedures have recently been attracting a great deal of attention in the broader nano-community [104].

Fluorescent NP-based advanced imaging techniques will offer unique tools for observing nano–bio interactions with nanoscale precision and resolution. For instance, the combination of the excellent photostability of fluorescent nanodiamonds with the sub-diffraction imaging capability of STED microscopy [105] in live cell imaging has been reported [106], which will open up many exciting new opportunities to probe intracellular interactions. Alternatively, photoswitchable fluorescent NPs [107], which can alternate their emission between two colors or between bright and dark states in response to external light stimulation, also have great potential in developing super-resolution imaging techniques [108] to visualize interactions in biological systems.

Acknowledgements

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