Enzymatic Drug Release

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Physicochemical Properties of Protein-Coated Gold Nanoparticles in Biological Fluids and Cells before and after Proteolytic Digestion**

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Owing to their size-related physical and chemical properties, nanoparticles (NPs) are not only considered promising tools for various fundamental in vitro and in vivo studies, but also for many biomedical applications, particularly as imaging contrast agents and drug-delivery vehicles.[1] However, little is known about the fate of NPs in biological systems, especially in terms of their physicochemical properties after their internalization by cells and organisms. Whereas nearly all biorelevant physicochemical properties of NPs can be characterized well before their uptake by cells,[2] they are hard to assess intracellularly. Important parameters, such as NP uptake, their intracellular localization, and toxicity, can be readily determined by means of various microscopy techniques (e.g. by fluorescence microscopy or transmission electron microscopy)[3] or by a variety of viability assays.[4] Although the assessment of these parameters has been the subject of extensive studies during the last decade,[1a,4a,5] it is still necessary to increase our understanding of what happens to NPs once they are taken up by cells. In particular, the physicochemical characterization of internalized NPs has scarcely been reported so far.

Upon internalization, NPs typically reside in endocytic vesicles (e.g. endosomes, lysosomes),[5a] in which the conditions are completely different to those outside the cell; that is, NPs are exposed to a lower pH, different salts, and also different kinds of proteins and enzymes. For example, in the presence of salts or under extreme pH conditions, NPs (which are often stabilized by charge) may lose their colloidal stability as a result of charge-screening effects. Additionally, in the presence of proteins, NPs tend to bind such proteins, which form a protein corona on the NP surface.[2a–c,e] The bound proteins cause NP agglomeration[2a,b] and also influence the interaction of NPs with cells in an ambiguous manner.[1a] As it is known that the extracellular protein corona around NPs evolves with time,[2b] indications also exist that proteins associated with internalized particles in general are degraded intracellularly.[6] Consequently, the properties of internalized NPs are expected to be significantly different from those in extracellular fluids[7] (in which they are typically determined[2b]), and thus, correlation of the physicochemical properties of NPs outside cells with their behavior inside the cells is not straightforward[2a] and may lead to ambiguous and vague conclusions. Moreover, the experimental characterization of the fundamental physicochemical parameters of internalized NPs, such as their colloidal stability (hydrodynamic diameter, state of agglomeration) and surface properties (surface charge, nature of the coating layer or protein corona), is an intricate problem, since common techniques for assessing these parameters cannot be applied within cells. Therefore, the characterization of the physicochemical properties of NPs located in intracellular space has been a formidable challenge.

Herein, we present gold NPs capped with various proteins (Au@protein) and labeled with different fluorescent dyes (Au@protein-F) as a model system for the investigation of the physicochemical properties of the NPs before and after internalization by cells, especially after enzymatic degradation of the protein coating under different intracellular conditions. We demonstrate that such Au@protein NPs are colloidally stable in physiological media in the presence of salts and free proteins, but more strikingly, also in the presence of proteolytic enzymes, which can degrade the stabilizing protein coating.

Au@protein NPs were synthesized by coating presynthesized highly monodisperse citrate-coated gold NPs (Au@citrate, $d_{core} = 15$ nm) with various proteins of different molecular weights (Figure 1; see also Figure S1 in the Supporting Information), namely, insulin (5808 Da, Ins), β-lactoglobulin (18.3 kDa, monomeric, β-LG), and bovine serum albumin (64 kDa, BSA), through ligand exchange, as previously reported.[8] For the synthesis of the fluorescently labeled NPs (Au@protein-F), two different synthetic routes comprising either one or two synthetic steps were used (Figure 1a). In the one-step synthesis (route a), a commercially available fluorescently labeled protein, ovalbumin labeled with BODIPY FL (45 kDa, Ova-DQ; BODIPY FL = 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pro-

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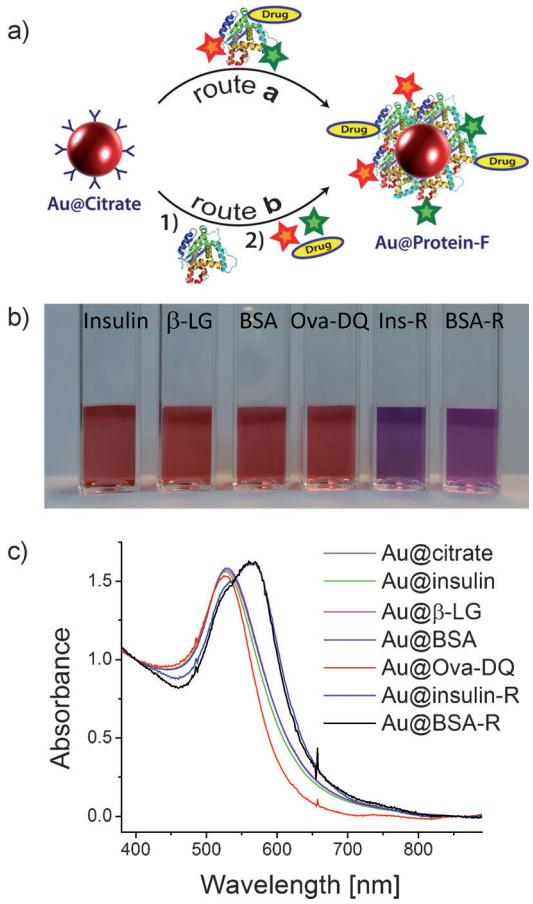

Figure 1. a) Schematic illustration of the one-step (route a) and two-step (route b) synthesis of the drug/fluorescent-dye-functionalized protein-coated gold NPs (Au@protein-F). The red and green stars represent the fluorophores; the yellow ellipses represent drug molecules. b) Photographs and c) UV/Vis/NIR absorption spectra of dispersions of gold NPs coated with different labeled and unlabeled proteins. The spectra are normalized at $\lambda = 400$ nm.

pionic acid), was used to coat the Au NPs, whereas in the two-step synthetic method (route b), the fluorescent dye tetramethylrhodamine isothiocyanate (TRITC) was covalently bonded to the presynthesized Au@protein NPs (Au@insulin and Au@BSA) through standard amine-coupling reactions (see the Supporting Information for more details). In general, synthetic route a offers the clear advantage of precise control over the degree of conjugation during protein labeling, but also involves arduous purification steps. On the other hand, route b enables fast and facile purification, which can be readily accomplished by centrifugation.

The resulting Au@protein NPs (Au@insulin, Au@β-LG, and Au@BSA) were red in color (Figure 1b) and exhibited a localized surface plasmon resonance (LSPR) band centered at 525 nm (Figure 1c). The rhodamine-labeled NPs (Au@insulin-R and Au@BSA-R) were purple (Figure 1b) and exhibited a second absorption peak due to TRITC (λ_{max}(excitation) $\approx$ 550 nm, λ_{max}(emission) $\approx$ 570 nm, quantum yield: 0.15[9]), unlike Au@Ova-DQ, for which the absorption band of the BODIPY dye (λ_{max}(excitation) $\approx$ 505 nm, λ_{max}(emission) $\approx$ 515 nm, quantum yield: $>$ 0.8[10]) overlapped with the LSPR band of the gold NPs (Figure 1c). Furthermore, all Au@protein NPs exhibited remarkably high colloidal stability in physiological media, including various culture media containing salts and proteins. This stability stems from the interplay of three major effects that nature employs to make proteins stable under physiological conditions: 1) electrostatic stabilization (negative charge at pH > pI, positive charge at pH < pI; pI = isoelectric point) due to the numerous charged groups on the protein, 2) steric stabilization as a result of the macromolecular character of the protein, and 3) the ability of the protein to form hydrogen bonds with water molecules to provide the protein with a hydration shell.[11] Therefore, the NPs could be readily purified and concentrated to NP concentrations in the micromolar range (expressed in terms of number of NPs per volume, which corresponds to a concentration of Au atoms in the millimolar range) in pure water, but also in culture media, without affecting their colloidal stability.

Indeed, high NP concentrations are utterly important for any serious biomedical applications, since the administration volumes should preferentially be kept as small as possible.[12] Although several research groups have reported low cellular toxicity of gold NPs upon cellular uptake (mostly at [Au^0] < 100 μM),[4c–e] it is still necessary to study the dose-dependent biocompatibility/cytotoxicity of these NPs. Such studies are particularly important for very high NP concentrations ([NP] $\geq$ 1 μM; that is, [Au^0] $\geq$ 100 mM), since NPs could evoke toxicity or other side effects at a high enough dose ("*dosis venenum facit*"—Philippus Aureolus Paracelsus, 1538). Thus, we studied the cell viability of 3T3 embryonic mouse fibroblasts after incubation with three different Au@protein NPs at different NP concentrations for 24 h. The viability of the cells was quantified by a standard resazurin-based cell-viability assay,[13] and the data were normalized to the control value (without NPs) of each experiment (see Figure S2). Although these data do not enable a detailed assessment of NP biocompatibility and possible toxicity mechanisms, they indicate whether the conjugation of proteins affects toxicity. Under culture conditions, NPs are usually associated with protein coronas,[14] and serum albumin is a major constituent of these coronas,[15] which under isolated conditions form a tight monolayer around each NP.[16] Thus, our Au@BSA NPs are comparable to the NPs typically assayed in previous studies on the cytotoxicity of Au NPs. Our data indicate that the attachment of proteins other than BSA, in particular, β-LG and insulin, can in fact affect biocompatibility. Whereas Au@β-LG has a comparable effect on cell viability to that of Au@BSA, Au@insulin is clearly less biocompatible than Au@BSA (reduced cell viability was observed for Au@insulin at an NP concentration one order of magnitude lower than that of Au@BSA; see Figure S2). Still, Au@protein NPs at concentrations above 1 μM ([Au^0] > 100 mM) are so highly concentrated that the NP dispersions are extremely viscous and even exhibit a golden shimmer (see Figure S3).

In comparison to many other coating materials, such as poly(ethyleneglycol) (PEG), polyelectrolytes, and other ionic and nonionic polymers, protein coatings not only provide high colloidal stability in physiological media,[8a] but they are also

biologically degradable upon enzymatic digestion, which might be harnessed for drug-delivery applications.[17] Drug or marker molecules can be covalently attached to the NPs and subsequently released in a sustained manner exclusively upon enzymatic digestion of the protein coating by proteolytic enzymes, known as proteinases or proteases (Figure 2a).

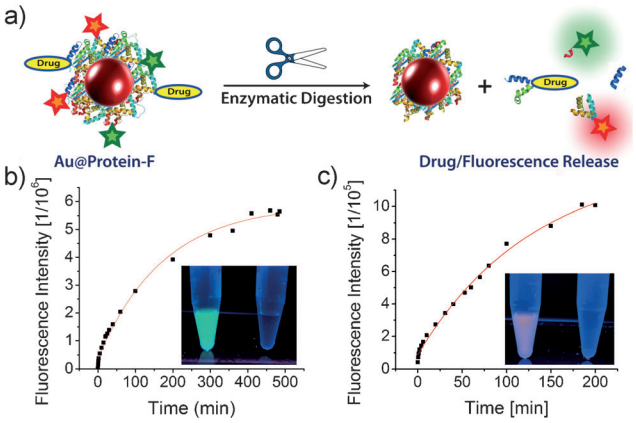

Figure 2. a) Schematic illustration of enzymatic fluorescence/drug release from dye/drug-functionalized Au@protein NPs upon protein digestion. The NPs are nonfluorescent owing to the quenching effects caused by the gold NPs and the high loading of the fluorescent dyes (autoquenching). b,c) Time-dependent fluorescence increase of Au@Ova-DQ NPs (green emission, b) and Au@insulin-R (red emission, c) as a result of the release of the fluorescent dye upon enzymatic digestion of the protein coating by proteolytic enzymes (pronase). The photographs show the supernatant of the respective NP dispersions with (left vials) and without (right vials) proteolytic enzymes (incubation time: 24 h).

In particular, the fluorescently labeled Au@protein-F NPs are very interesting, as they feature a smart on/off system that depends on the presence of proteolytic enzymes (Figure 2b,c). When attached to the plasmonic Au NPs,[18] but also due to the autoquenching effects mediated by the high fluorophore concentration on the coating protein,[6] the fluorescence emission of the fluorescent dyes is completely quenched,[6,18] resulting into non-fluorescent NPs (off state). Upon proteolytic digestion, however, the fluorophores are gradually released from the NPs, so that the quenching effects are cancelled out, and the fluorescent molecule can emit again when excited (on state).[6] This process was also observed in living cells by incubating 3T3 mouse embryonic fibroblasts with Au@protein-F NPs and recording fluorescence microscopy images at different times (Figure 3; see also Figure S4). In contrast to the untreated cells (without NPs), the fluorescence inside the cells treated with Au@Ova-DQ increased with time as a result of the enzymatic release of the (previously quenched) fluorescent dye (Figure 3).

There is general agreement that NPs are typically incorporated by endocytic pathways[5a,19] when no special surface modifications, such as cell-penetrating peptides[20] or hydrophobic/hydrophilic patterns,[21] are used. This process involves transport of the NPs to endocytic vesicles,[5a,19a] in which proteolytic enzymes are present that digest the protein

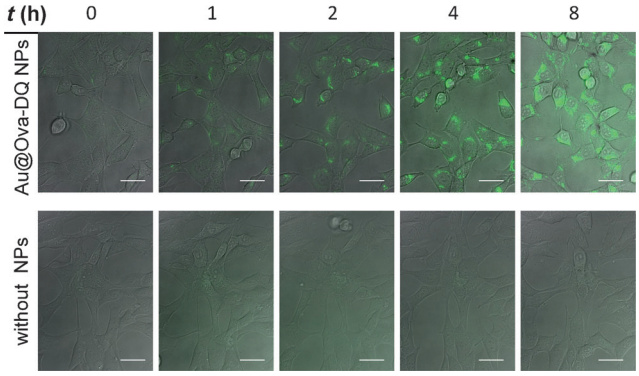

Figure 3. Time-dependent fluorescence release of Au@Ova-DQ in 3T3 fibroblasts. Confocal laser scanning microscopy (CLSM) images of 3T3 fibroblasts treated with (top) and without (bottom) the Au@Ova-DQ NPs. The images were recorded at the same position at different times, as indicated. The images are an overlay of the fluorescence (green) and transmission channel. Scale bars: 20 μm.

(Figure 2 and Figure 3). The dotted patterns of the intracellular NPs in confocal microscopy images (see Figures S4 and S5) also indicate that the Au@protein NPs are most likely endocytosed. The distribution of the released protein fragments was similar for Ova-DQ and insulin, which suggests that Au@Ova and Au@insulin were incorporated through a similar pathway. Overall, our data clearly indicate that proteins bound to the surface of NPs can be cleaved inside digestive vacuoles (e.g. lysosomes). Clearly, changes in the protein shell of the NPs may have a significant impact on the physicochemical properties of the NPs, in particular on their colloidal stability.

As the colloidal stability of internalized NPs is experimentally hard to assess, we set out to define the colloidal stability of the Au@protein NPs under the different environmental conditions in the relevant intracellular compartments. The extracellular matrix (ECM) is a complex fluid, but the overall pH value is the physiological pH value of 7.4. High colloidal stability of the Au@protein NPs under physiological conditions was already demonstrated; thus, the NPs were individually dispersed and non-agglomerated in the ECM. On the other hand, the interior of lysosomes is known to be acidic, usually around pH 4–5. Since the Au@protein NPs are highly pH-sensitive and agglomerate exactly in the pH range of 4–5 (owing to the pI value of the coating proteins),[8] we assumed that the NPs would agglomerate inside lysosomes. Besides their low pH value, lysosomes contain proteolytic enzymes, which, as already shown, can degrade the protein coating. For this study, we first dispersed different Au@protein NPs in phosphate-buffered saline (PBS buffer; pH 7.4) in the presence and absence of proteolytic enzymes (proteases) and incubated the dispersions at 37 °C for 3 days (Figure 4a).

Surprisingly, the NPs in the protease–PBS solution, in contrast to dispersions in pure PBS (see Figure S6), remained completely stable at pH 7.4 (even longer than 3 days, data not shown), and even upon the enzymatic degradation of the stabilizing protein coating (Figure 2b,c and Figure 4a). However, this remarkably high colloidal stability of Au@protein NPs in the presence of proteolytic enzymes is difficult to

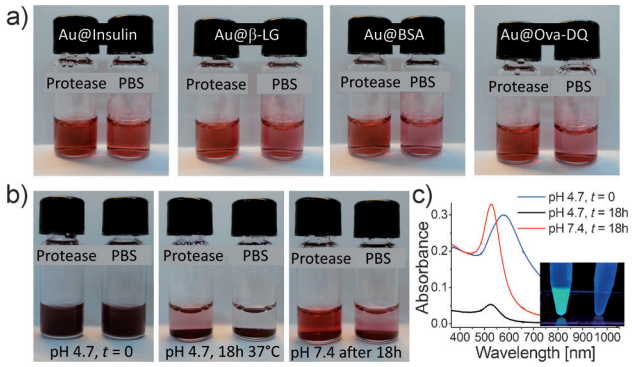

Figure 4. a) Long-term stability of various Au@protein NPs under physiological conditions (PBS) in the presence (left vials) and absence (right vials) of proteases (0.2 mg mL^{-1}). The NPs were incubated for 3 days at 37 °C. The particles in PBS climbed the glass wall (indication of hydrophobicity; see the Supporting Information). The NPs in the PBS-protease solution remained stable with a ζ potential of around −(30±12) mV. The ζ potential of the NPs in pure PBS was in the range of −20 to −25 mV. b) Stability of Au@protein NPs (Au@Ova-DQ) under lysosomal conditions, that is, under acidic conditions (pH 4.7) in the presence of proteases. The NPs aggregate owing to their pH-sensitive character. After incubation for 18 h at 37 °C (middle photograph), the pH of the dispersions was raised to the physiological pH of 7.4 (right photograph). c) UV/Vis spectra of the protease-containing mixtures in (b). The inset shows the supernatant of the centrifuged dispersions from (b) over a UV lamp.

explain. Dynamic light scattering and ζ-potential measurements did not reveal any satisfactory explanation owing to the small changes that occur on the NP surface upon digestion of the proteins; these small changes could not be detected by these techniques. However, even if we assume complete degradation of the protein coating by the enzymes, the cysteine-containing peptides should still remain bound to the NP surface through strong gold–sulfur bonds and provide the NPs with sufficient colloidal stability. Moreover, the cleaved peptides in the solution, and most probably also the enzymes themselves, which are also proteins, can be adsorbed onto the NPs or even replace the bound peptides. We believe that Au@protein NPs are extremely stable in protein/enzyme solutions for these reasons and therefore that they should also be highly stable in neutral biological fluids, such as the ECM.

However, as mentioned above, the Au@protein NPs agglomerate at low pH values, at which the proteolytic enzymes in lysosomes exhibit their highest enzymatic activity. To evaluate the effect of agglomeration on the enzymatic digestion of the protein coating, we dispersed Au@Ova-DQ NPs in pure PBS and in a PBS–protease solution and adjusted the pH value of the dispersions to pH 4.7. The NP dispersion in pure PBS served as a control. Both with and without the proteases, the dispersions turned purple, which indicated strong particle agglomeration (Figure 4b, left and Figure 4c, blue curve). The dispersions were incubated at 37 °C for 18 h. After this time, the NPs in pure PBS had completely agglomerated and sedimented, whereas in the presence of the proteases, a small fraction of the NPs were dispersed in the solution (Figure 4b, center). The UV/Vis spectrum of the supernatant revealed an LSPR maximum at 525 nm, which indicates that the NPs are individually dispersed and non-agglomerated, even at this low pH value (Figure 4c, black curve). This result suggests that in the presence of proteolytic enzymes, the pI of the NPs shifts toward basic pH values; the NPs are therefore stable at low pH as a result of either protein digestion or the self-adsorption of enzymes that exhibit a basic pI (e.g. trypsin: pI = 10.1-10.5). Nevertheless, a large fraction of the NPs was in an agglomerated state at low pH, which however redispersed completely when the pH of the solution was increased to 7.4.

In contrast to the dispersion in the presence of the proteases, the NPs in pure PBS did not redisperse completely. This result shows the remarkable stability of digested and nondigested Au@protein-F NPs in the presence of proteins or enzymes (Figure 4b, right and Figure 4c, red curve). Moreover, the enzymatic digestion of the protein coating of the NPs in their agglomerated state was not really affected. After 18 h, the supernatant of digested NP dispersions showed strong fluorescence, which indicated the release of the fluorophore from the NP coating (Figure 4c, inset). Hence, the release of attached (drug) molecules from the Au@protein NPs would not really be affected by their pH-sensitive agglomeration, for example, in the lysosome, and the NPs would redisperse completely after leaving the acidic milieu upon secretion.

In conclusion, gold NPs capped with various proteins exhibit remarkably high colloidal stability not only in physiological buffer solutions, but also in protein-containing cell-culture media, and thus mimic the intrinsic stability of proteins. As a result of the multifunctional molecular structure of proteins, the Au@protein NPs exhibit pH-responsive reversible agglomeration/disagglomeration behavior and therefore disagglomerate after a return to physiological conditions. This behavior of the Au@protein NPs indicates that the physicochemical properties of such NPs can be very different in biological and nonbiological fluids, but also in various intracellular compartments, and gives us grounds to believe that the Au@Protein NPs would also be very stable in biological fluids, such as the cytosol, the ECM, or even blood. Like proteins, the Au@protein NPs offer possibilities for straightforward further functionalization. Thus, the NPs can be loaded with model drug molecules, or as in this case with fluorescent dyes, which were exclusively released in a proteolytic milieu upon enzymatic digestion of the capping protein layer of the NPs. Furthermore, we could show that the colloidal stability of the NPs is not affected by the enzymatic degradation of the protein corona at pH 4.7, and that the NPs redisperse when the pH of the medium returns to a physiological value. Hence, Au@protein NPs represent a smart in vitro drug-delivery system based on plasmonic particles and biomolecules. They also serve as a unique and convenient tool for the investigation of the stability of such NPs in biological fluids. In this way, novel information can be obtained on the physicochemical properties of NPs in biological systems.

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