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Research Update: Interfacing ultrasmall metal nanoclusters with biological systems

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Metal nanoclusters (NCs), a new type of nanomaterial with unique physicochemical properties, show great potential in many biomedical applications. Understanding their behavior in the complex biological environment is critical not only for designing highly efficient NC-based nanomedicines but also for elucidating the biological impact (e.g., toxicity) of these emerging nanomaterials. In this review, we give an overview of recent progress in exploring interactions of metal NCs with biological systems, including protein adsorption onto NCs, NC interactions with cells, and also the *in vivo* behavior of NCs. We also discuss the biological responses to the interactions, key parameters defining the interactions, and current challenges in the exploration of NCs in the complex biological environment. [http://dx.doi.org/10.1063/1.4974514]

INTRODUCTION

Metal nanoclusters (NCs), composed of several to a few hundred metal atoms, have received enormous attention over the past decade.[1,2] With attractive physicochemical features including distinct molecular-like photoluminescence, high stability, excellent biocompatibility, and ultrasmall size, metal NCs have emerged as a new type of luminescent nanomaterial with promising applications in many areas including the biological and environmental sciences.[3–5] In particular, there is an increasing interest in exploring NC applications in the biomedical field.[6–9] For example, Irudayaraj and coworkers reported the specific targeting of ErbB2 over-expressing breast cancer cells and tumor tissues by using biofunctionalized gold nanoclusters (AuNCs).[10] They observed that the therapeutic efficacy of Herceptin was markedly enhanced upon conjugation with AuNCs. Recently, metal NC-based nanocomposites have also been successfully applied in gene therapy, as demonstrated by a 2–3 times higher transfection efficiency of polyethyleneimine (PEI)-templated AuNCs than PEI only.[11] More recently, Tao *et al.*[12] reported the use of ovalbumin-CpG oligodeoxynucleotide conjugates-templated AuNCs as smart self-vaccines for enhanced immune response. Both *in vitro* and *in vivo* experiments demonstrated that these engineered AuNC-based vaccines possess high immunogenicity.

Whenever NCs are internalized by an organism, they will inevitably interact with molecules in the fluidic biological environment. For example, if NCs are being injected into the circulatory system, a large variety of biomolecules, notably proteins, will immediately adsorb onto the nanoparticle

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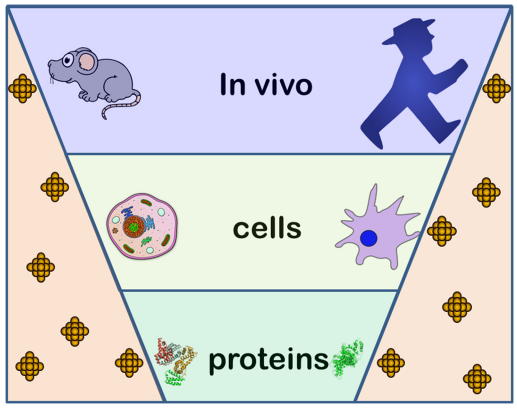

FIG. 1. Schematic illustration of metal NCs interacting with biological systems at different levels: proteins, cells and *in vivo*.

surface, forming a so-called protein corona that governs the subsequent biological interactions with the coated nanoparticles.[13] Depending on their physicochemical properties, nanoparticles can be taken up by cells and delivered to different organelles, while nanoparticle degradation may occur and lead to toxicity.[14] In higher organisms, the biomedical efficacy of NCs greatly depends on whether they accumulate in certain organs such as liver or kidneys, or are swiftly cleared from the body.[15] In general, all these biological processes have important implications for the safe and efficient use of nanoparticles in biomedical applications. In light of the large surface-to-volume ratio of ultrasmall metal NCs, their interactions with biosystems are expected to have more significant consequences, and understanding the interactions at the nano-bio interfaces of metal NCs is highly relevant for their biomedical application.[16] In this brief review, we intend to highlight recent advances in exploring interactions of metal NCs with biological systems at different levels: proteins, cells, and living organisms (Figure 1). Important progress will be overviewed to present an up-to-date perspective. Among the different types of metal NCs, we mainly focus on the biological behavior of AuNCs, which are the most widely investigated NCs owing to their good stability and biocompatibility.

PROTEIN CORONA FORMATION ON METAL NC SURFACES

It has been well recognized by the community that a protein adsorption layer ("corona") forms whenever nanoparticles enter a biological fluid, which critically defines the biological identity of the nanoparticles.[17,18] Although surface modification such as PEGylation and zwitterionic ligand coating may reduce nonspecific protein binding, it remains challenging to completely suppress it.[19–21] On the one hand, the protein layer can modify the physicochemical and biofunctional properties of coated nanoparticles, which in turn may affect their performance in nanomedicine applications.[22,23] On the other hand, protein corona formation on nanoparticles can also trigger the unfolding, denaturation, and even aggregation of serum proteins, giving rise to unexpected biological consequences.[24,25] Thus, a thorough understanding of NC-protein interactions is a fundamental prerequisite for advancing the biomedical utilization of metal NCs.

Thanks to their intrinsic luminescence, metal NC interactions with proteins can be directly studied by optical spectroscopy and microscopy techniques in real time without disturbing the biological environment. Although the detailed luminescence mechanism of metal NCs is still in dispute, researchers all agree on the important role of surface metal-ligand interactions in modulating their luminescence.[26,27] For example, Wu and Jin reported that electron-rich atoms or functional groups of the surface ligands can enhance the luminescence of AuNCs via surface interactions.[28] Consequently, one should expect that the photophysical properties of metal NCs also change due to protein adsorption. Indeed, the near-infrared photoluminescence of AuNCs protected by dihydrolipoic acid (DHLA) was found to be significantly altered upon adsorption of different serum proteins, including human serum albumin (HSA), transferrin, and apolipoproteins (Figure 2).[29] Besides the emission intensity, the luminescence decay of AuNCs was also markedly modified upon protein association. Apparently,

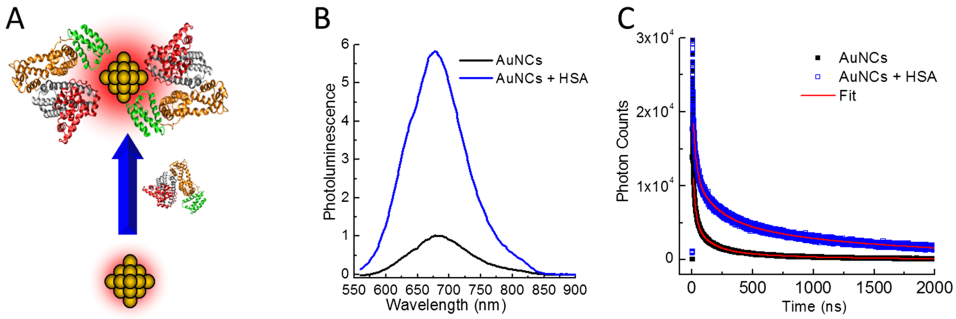

FIG. 2. Protein adsorption affects the luminescence of AuNCs (a), enhancing the emission intensity (b) and increasing the luminescence lifetime (c). Images adapted from Ref. 29.

adsorption of proteins on the AuNC surfaces alters the local environment in a way that affects the photophysical processes of luminescence generation. Specifically, the local dielectric environment of AuNCs becomes less polar upon coating with a protein adsorption layer in comparison to direct exposure to aqueous solvent.

Similar observations have been reported for other metal NCs such as silver NCs (AgNCs)[30] and Au–Ag alloy NCs.[31] These findings have important implications for the utilization of metal NCs as optical markers, especially in quantitative bio-applications. For example, when using AuNCs for temperature sensing within cells, not only the absolute luminescence intensity and lifetime were modified from those in phosphate buffered saline but also their temperature sensitivity turned out to be very different.[32] These findings thus exemplify the importance of a thorough evaluation of the photophysical effects of metal NCs in the exact biological environment intended for their quantitative application. Interestingly, the protein adsorption-sensitive luminescence response of metal NCs has been harnessed for developing a new platform for protein discrimination, in which metal NCs function as both sensing and reading units.[33,34] As demonstrated by several recent studies, robust metal NC-based sensor systems can be established to detect multiple proteins with high specificity and sensitivity.[35,36]

Interactions between nanoparticles and proteins depend on many parameters, including size, shape, and surface ligands of the nanoparticles, composition of the surrounding medium, temperature, and the physicochemical properties of the adsorbing proteins.[37,38] Among these, charge interactions play an essential role in mediating protein corona formation because most proteins and nanoparticles possess abundant surface charges. This also holds true for protein adsorption onto metal NC surfaces. This was elegantly shown by using HSA as a model protein, for which the strength of the AuNC–HSA interactions changed substantially upon variation of its surface charge by controlled succinylation or amination.[39] Importantly, such modifications of the protein layer can further modulate cellular interactions of AuNCs, vividly illustrating the important role of charge interactions in defining the biological behavior of nanoparticles.

CELLULAR INTERACTIONS WITH METAL NCs

In many promising biomedical applications such as cellular imaging and medical therapy, metal NCs have to intrude cells by penetrating the cell membrane. Knowledge about the uptake mechanism and the intracellular fate after NC internalization is needed to ensure successful utilization.[40,41] Moreover, rising concerns over the biocompatibility of engineered nanomaterials call for a thorough evaluation of how metal NCs interact with cells.[15] Our recent work revealed that metal NCs typically intrude cells via endocytosis.[42] Specifically, clathrin-mediated endocytosis and micropinocytosis dominate internalization of DHLA-coated AuNCs. Interestingly, ultrasmall-sized NCs (hydrodynamic diameter 3.3 nm) accumulate on the cell membrane prior to internalization, as was observed by using spinning disk confocal fluorescence microscopy of living cells. This is distinctly different from internalization of 100 nm-sized polystyrene nanoparticles,[43,44] where no such membrane accumulation was observed during their cellular uptake. These observations clearly show that nanoparticle

internalization is strongly size dependent.[45] Small nanoparticles such as metal NCs have to work cooperatively and cluster together in order to trigger the internalization machinery, as supported by both experimental and theoretical studies.[46,47] Actually, similar behavior has also been reported for other small nanoparticles, i.e., semiconductor quantum dots (QDs).[48]

After entering cells, nanoparticles will be delivered to different cellular compartments. In most cases, metal NCs internalized via endocytosis will end up in lysosomes. However, they may also be intentionally designed to bypass endocytosis or escape lysosomal trapping.[49] One of the most distinct features of the lysosomal compartment is its acidic milieu, with pH in the range of 4-5, which can cause destabilization or even degradation of nanomaterials.[50] By using fluorescence lifetime imaging microscopy, we recently evaluated the stability of AuNCs inside HeLa cells for up to 72 h.[51] We observed that intracellular DHLA-AuNCs undergo a slow degradation, as indicated by their luminescence lifetime decreasing by ca. 19% over 72 h (Figure 3). By using model solvents, NP corrosion within the acidic lysosomal environment was identified as the main effect responsible for intracellular degradation of DHLA-AuNCs. Thus, there is a need to further improve the stability of metal NCs to resist the reactive biological environment inside the cells. A potential strategy towards this goal is to cross-link the surface ligands of metal NCs so that they can better protect the NCs from degradation. Zhou *et al.*[52] deposited polymer-like layers around AuNCs by covalently linking the scaffold (protein) and ligands, rendering the AuNCs highly stable against reactive oxygen species (ROS) and protease degradation inside living cells. By contrast, AgNCs are more likely to be dissolved than AuNCs in the cell milieu. Recent studies reported that metal-ligand bond stability plays an important role in AgNC stability: Ag^0-rich NCs were observed to be less stable in the acidic environment of lysosomes than Ag^+-rich NCs.[53] Consequently, more Ag^0 species are released and oxidized to Ag^+, leading to an increase of intracellular ROS and associated cellular responses.

With their exceptionally high surface-to-volume ratio, the chemical nature of the NC surface is expected to have a profound effect on their cellular interactions. For instance, the presence of corona proteins on the surface of AgNCs affects their internalization efficiency as well as their cytotoxicity.[30] Le Guével and co-workers studied the effect of AuNCs stabilized by different ligands on intracellular accumulation and the immune response of human monocyte-derived

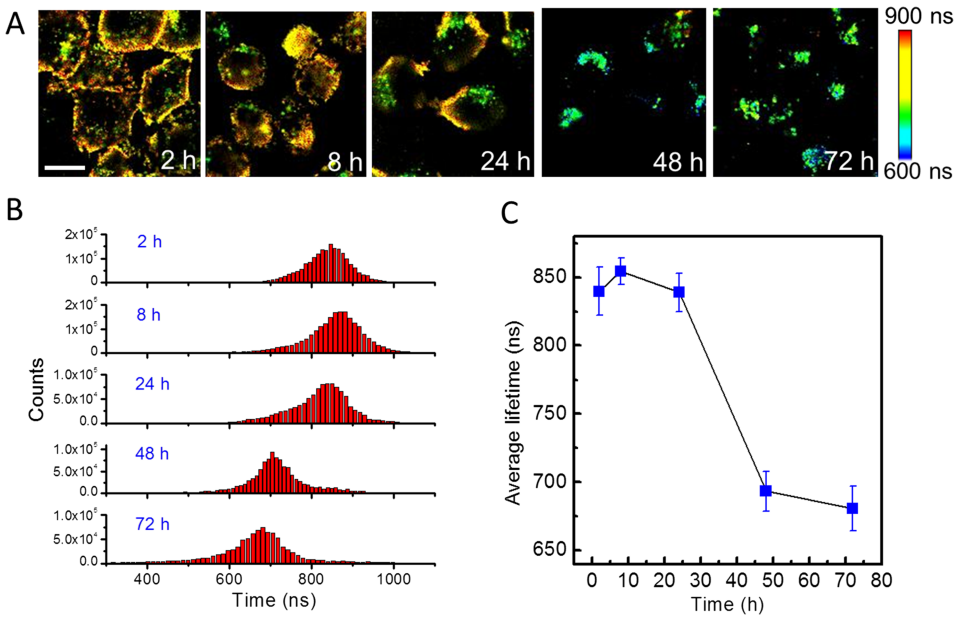

FIG. 3. (a) Typical fluorescence lifetime images of DHLA-AuNCs at different times after internalization by HeLa cells. Scale bar: 20 μm. (b) Histograms of luminescence lifetimes of DHLA-AuNCs in HeLa cells after incubation for specific times. (c) Plot of the average luminescence lifetime of versus time. Images adapted from Ref. 51.

dendritic cells.[54] Their results indicate that the uptake efficiency of AuNCs is strongly determined by the nature of the surface ligand, with zwitterionic ligands being more effective than PEGylated ones. It has also been shown that mercaptopropionic acid-capped AuNCs are biologically more reactive than glutathione-capped AuNCs, as judged by the enhanced production of mitochondrial superoxide anion and cytoplasmic ROS inside the cells.[55] These results provide concrete perspectives for the rational engineering of functionalized metal NCs for biological applications.

IN VIVO BEHAVIOR OF METAL NCs

Although many different types of engineered nanomaterials show potential for cancer diagnosis and therapy, translating them into clinical practice has been severely hampered by toxicity induced by unintended accumulation in healthy tissues/organs. The *in vivo* toxicity of nanoparticles depends on many parameters, of which the size plays a dominant role. Key physiological thresholds exist for the nanoparticle diameter: ~6 nm for glomerular wall filtration in the kidneys, ~5 nm for rapid extravascular equilibration, and ~20 nm for enhanced permeability and retention (EPR).[20,56,57] Therefore, metal NCs, with typical core sizes of ~1 nm are expected to overcome these size barriers and minimize nonspecific accumulation. Indeed, after intravenous injection, more than 50% of 2 nm glutathione-coated AuNCs were found in the urine of mice within 24 h, and less than 4% of these NCs were retained in the liver.[58] This result is in stark contrast to the biodistribution profile of larger Au nanoparticles, where a 50%-94% accumulation in the liver was reported.[16,59] It is also worthwhile mentioning that ultrasmall metal NCs, i.e., AuNCs, typically possess exceptional colloidal stability in biological media, even in the blood circulation system. This is important in this context because aggregation can otherwise give rise to much larger agglomerated particles.[60]

Apart from the widely recognized parameters, net charge and hydrodynamic diameter, biological adsorption, distribution, metabolism, excretion, and pharmacokinetics of AuNCs upon organismal exposure are also greatly influenced by the presence of exposed hydrophobic surfaces and the surface charge density.[61] For instance, enhanced coverage of oligoethylene glycol moieties on the NC surface increases their hydrophobicity and results in higher retention of NCs in the reticuloendothelial system. In a similar study, Zhang *et al.*[62] found that bovine serum albumin (BSA)-protected Au_{25}NCs exhibit higher toxicity and lower renal clearance than GSH-coated Au_{25}NCs (Figure 4). Particularly, small GSH-AuNCs can be metabolized by renal clearance and, thus, their toxicity is low. In contrast, BSA-AuNCs form large compounds *in vivo* and further accumulate in the liver and spleen, causing irreparable damage. These studies are important for advancing clinical applications of metal NCs, as they can provide valuable guidance to the design and development of NC-based nanomedicines with minimized toxicity and maximized efficiency.

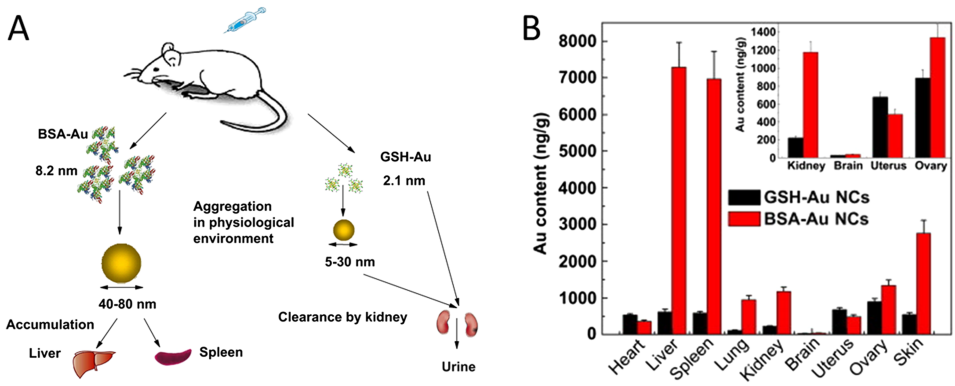

FIG. 4. (a) Schematic illustration of the biodistribution and renal clearance of GSH- and BSA-protected AuNCs. (b) Quantitative analysis of the gold content distribution of GSH- and BSA-protected AuNC-treated mice at 48 h. Images adapted from Ref. 62.

SUMMARY AND PERSPECTIVES

Considerable amounts of effort have been devoted to unveiling the complicated interactions between engineered nanomaterials and biological systems. Metal NCs, a novel type of nanoprobes, have shown many promising biomedical applications. Knowledge about their behavior in the biological environment will be an important prerequisite for their future use in practical applications. Although the field is still at an early stage, several key findings have been revealed: (1) protein adsorption significantly affects the physicochemical properties of metal NCs; (2) cellular interactions of metal NCs are mediated by many parameters including protein adsorption, charge interactions and surface ligands; (3) the ultrasmall size of metal NCs is generally favorable for their clearance *in vivo*, causing less toxicity.

To obtain a profound picture of the behavior of metal NCs in the biological environment, further systematic, in-depth studies are required. For this research, the NCs should be structurally very well defined to enable a robust comparison of the effects of particular physicochemical parameters. Of note, there is presently still a high demand for simple synthesis routes for creating highly monodisperse, atomically precise metal NCs with well-defined properties.[63] The use of sophisticated analysis tools such as computer simulations[64] and super-resolution microscopy will yield new insights.[65] Moreover, further *in vivo* data on their biodistribution, trafficking, degradation in tissues/organs, and their influence on the immune system will be greatly appreciated.[66] We hope that this brief overview on the biological interactions of metal NCs will be helpful for researchers working in this field, so that NCs will eventually find wide-spread use as powerful tools in biomedical applications.

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