Overview

Ultrasmall metal nanoclusters for bio-related applications

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The study of ultrasmall metal nanoclusters (NCs, ranging from subnanometer to ca 2 nm) is evidently a quickly evolving field in current nanoscience and nanotechnology research. Metal NCs, typically composed of several to hundreds of metal atoms, have attracted great interest in recent years owing to their unique properties including ultrasmall size and enhanced photoluminescence, together with other properties such as excellent photostability, low toxicity, and good biocompatibility desired for biological applications. This review summarizes recent advances in the field of bio-related applications of metal NCs materials. We highlight the applications of metal NCs for biosensor development, fluorescent biological imaging, and biomedical research, and finally discuss briefly some current challenges and future work. © 2013 Wiley Periodicals, Inc.

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INTRODUCTION

Metal nanoparticles (NPs, typically 1–100 nm) of Au and Ag have received significant attention in biological applications. The majority of bioapplications are based upon the optical properties of NPs, including absorption, scattering, and surface-enhancing capability. Spherical gold NPs of ~5–20 nm display a strong resonant absorption band in green ($\lambda_{peak} \sim 520$ nm), which is not seen in the optical spectrum of bulk gold, hence, it is a unique excitation mode to NPs, called surface plasmon resonance (SPR). The SPR wavelength redshifts with increasing particle size (e.g., 520–570 nm for 5–100 nm particles). The nature of SPR is due to collective excitation of free electrons in the particle upon excitation by light. This collective excitation mode of conduction electrons also accounts for the optical scattering of larger Au NPs (typically >30 nm).

For plasmonic metal NPs, they are *metallic*, i.e., the free electron picture in bulk metals is essentially applicable to plasmonic NPs, even down to $D \sim 5$ nm. The distinct SPR is a size effect of conduction electrons confined in the particle and can be well modeled by classical electrodynamics, hence, the SPR mode is a *classical* size effect, meaning that the underlying physics is essentially handled by *classical physics* while no quantum physics is needed for particles in this size regime.

On the other hand, as the size of metal NPs shrinks below ca 2 nm (diameter), significant changes occur to the properties of particles; for instance, the SPR band disappears and step-like multibands are emerged in the optical spectrum. These ultrasmall NPs are often called nanoclusters (NCs)[1] to distinguish them from the plasmonic NPs. Quantum confinement effects make metal NCs no longer *metallic*, manifested in quantized electron energy levels with a sizable energy gap. Such NCs also exhibit greatly enhanced fluorescence[2,3] compared to plasmonic NPs[4] and bulk gold. For a review of the fluorescent NCs, the reader is referred to Ref 2. Both characters—ultrasmall size and photoluminescence—of metal NCs can be utilized in bio-related applications, which constitute the focus of this review.

In regard to the photoluminescence of metal NCs, there are several distinct features such as large Stokes shift and good photostability.[2,3] The exact origin of photoluminescence from metal NCs has not been fully understood. Nevertheless, some factors have been found to influence the photoluminescence of

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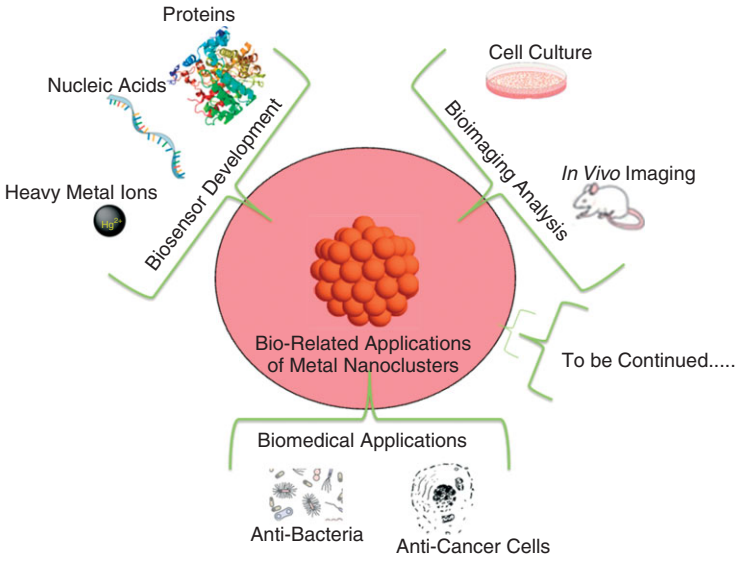

SCHEME 1 | Ultra-small metal nanoclusters for bio-related applications.

NCs, including the electron energy quantization effect, metal–ligand interaction, and metal core charge state, as well as the surface effect. With decreasing size from NP to NC, the resultant discrete electronic structure in NCs is partially responsible for the fluorescence properties.[1–4] Compared with the well-explored field of synthesis of large metal NPs,[5–7] synthesis of metal NCs is a new field.[8–11] Moreover, for purification of the crude metal NCs, more complicated processes such as polyacrylamide gel electrophoresis (PAGE) and high-performance liquid chromatography (HPLC) are sometimes required.[12,13] Among the various reported metal NCs such as Au, Ag, Cu, Pt, Pd NCs,[1,10,11,14–17] Au and Ag NCs are most popular, mainly because of their extraordinary stability, strong fluorescence, good biocompatibility, and tunable emission in the visible to near-infrared spectral region.[18] A variety of Au and Ag NCs capped with various ligands, such as thiolates, proteins, and DNA, can generate fluorescence at different wavelengths with good quantum yield.[10] In addition, compared with plasmonic metal NPs which are usually larger than the biomolecules of interest such as proteins (typically 2–5 nm), metal NCs are very small (typically < 2 nm) and therefore the normal biological functions of these biomolecules may not be disturbed or at least minimally disturbed.[19,20] Due to all these advantages, bio-related applications of metal NCs have attracted great interest in recent years.

Herein, we highlight some recent advances of using metal NCs, especially Au and Ag NCs, in biosensor development (Metal NCs for Biosensor Development), bioimaging analysis (Metal NCs for Bioimaging Applications), and biomedical studies (Metal NCs for Biomedical Applications), Scheme 1. In the last, we provide our perspectives on some challenges and future efforts. Overall, we hope to convey that NCs, especially those with atomic precision,[8] may offer some unique opportunities that are not attainable with conventional plasmonic NPs (5–100 nm). Many potential applications of ultrasmall metal NPs remain to be further developed in the fields of nanomedicine and nanobiotechnology.

METAL NCs FOR BIOSENSOR DEVELOPMENT

In this section, we summarize recent applications of metal NCs for the detection of various biomolecules such as biohazard chemicals (e.g., toxic ions), proteins, nucleic acids.

Detection of Biohazard Chemicals

Heavy-metal ions such as mercury and lead can pollute soil and water and are highly toxic to our environment. They are dangerous to human health because of toxicity caused by bioaccumulation. Therefore, it is necessary to develop analytical methods to detect heavy-metal ions. It was shown that heavy-metal ions, such as Hg^{2+}, Cd^{2+}, and Pb^{2+}, can cause the aggregation of plasmonic Au NPs which in turn changes the UV–vis absorption or color of NPs.[21–24] Compared with those methods based on metal NPs, different detection mechanisms have been applied in the case of metal NCs for the detection of heavy metal ions (vide infra).

One common strategy is to monitor the fluorescence quenching after the interaction between

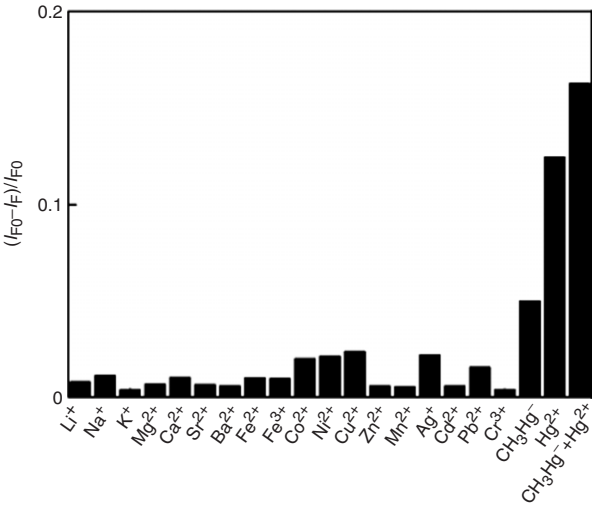

FIGURE 1 | Lysozyme-protected, red fluorescent Au nanoclusters (NCs) for metal ion detection. Relative fluorescence intensities $[(I_{F0} - I_F)/I_{F0}]$ at 631 nm emission of solutions of lysozyme-Au NCs after addition of Hg^{2+} (100 nM), CH_3Hg^+ (100 nM), and other metal ions (50 μM). (Reprinted with permission from Ref 28. Copyright 2010 American Chemical Society)

heavy metal ions and metal NCs. The heavy metal ions such as Hg^{2+} can interact with the NC surface, resulting in quenching of the fuorescence of Au or Ag NCs. In some cases (e.g., Ag^+ detection), fluorescence enhancement may also be observed. The detailed mechanism of metal ion detection by NCs is quite complicated; many effects could occur, such as the change in the metal core charge state, replacement of Au or Ag atoms in the cluster, and so on.

Guo et al.[25] synthesized fluorescent Ag NCs using denatured bovine serum albumin (dBSA) as the stabilizing agent. The fluorescence of Ag NCs was quenched in the presence of Hg^{2+}. The limit of detection (LOD) of this method was 10 nM.[25] Similarly, the Wang group synthesized fluorescent oligonucleotide-stabilized Ag NCs for Hg^{2+} detection and obtained a LOD of 5 nM.[26] In another study, Adhikari et al.[27] also found that the Ag NCs can be used as fluorescence probes to detect Hg^{2+} with ultrasensitivity (LOD = 100 pM). In addition, by using lysozyme-stabilized Au NCs, Lin et al.[28] reported an ultrasensitive method to detect Hg^{2+} with a LOD as low as 3 pM, and the selectivity of this probe is more than 500-fold for Hg^{2+} over any other metal ions (Figure 1). Lin et al. also found that lysozyme-Au NCs provided an approximately 330-fold improvement in the detection of Hg^{2+} than BSA-Au NCs.[28] In addition, lysozyme-Au NCs also provided the first example for detecting CH_3Hg^+ (LOD: 4 nM).

Besides the detection of Hg^{2+}, other heavy metal ions are also detectable by using metal NCs. For example, Goswami et al.[29] demonstrated that water-soluble Cu NCs capped with BSA can selectively and sensitively detect Pb^{2+} ions at ppm concentrations. For the detection of Cu^{2+} ions, Xavier et al.[30] used lactoferrin-stabilized Au NCs and reached the sensitivity of ppm concentrations.

In addition, metal NCs has been proved feasible to detect other biohazard chemicals such as cyanide in the solution. Cyanide is very toxic to human health and environments.[31,32] Although gold is chemically inert and few anions can react with it, cyanide can form very stable $Au(CN)_2^-$ complex in the presence of O_2 in aqueous solution. This complex formation will lead to fluorescence quenching of Au NCs.[33,34] Using this strategy, as shown in Figure 2, Liu et al.[34] demonstrated selective and sensitive detection of cyanide by Au NCs with a detect limit around 200 nM, which is 10-fold less than the maximum level of cyanide in drinking water permitted by the World Health Organization.

In addition to the strategies using fluorescence quenching of metal NCs to report signals, metal NCs have also been demonstrated to detect some chemicals with enhanced fluorescence or luminescence. Habeeb-Muhammed et al.[35] showed that when BSA-Au NCs was incubated with Ag NPs, a ninefold maximum luminescence enhancement was observed. The enhanced fluorescence might be caused by altering the radiative decay rate or increasing the absorption of the fluorescing BSA-Au NCs.[35] In another example, as shown in Figure 3, Kawasaki et al.[36] found that the pepsin-mediated Au NCs (e.g., 25-atom Au_{25}) were able to generate enhanced fluorescence in the presence of Pb^{2+} ions, but Hg^{2+} ions resulted in dramatic fluorescence quenching when interacting with this pepsin-Au NCs.

On the other hand, Pu et al.[37] utilized fluorescence resonance energy transfer (FRET) between blue-fluorescent conjugated oligomer-substituted polyhedral oligomeric silsesquioxane (POSSFF) and red-fluorescent Au NCs for the development of a detection scheme for Hg^{2+} ion sensing both in solution and in cell. They observed that efficient FRET from POSSFF to Au NC led to pink fluorescence upon excitation of donor (i.e., POSSFF). In the presence of Hg^{2+} ions, the pink fluorescence specifically turned to blue fluorescence. This is a visual Hg^{2+} ion detection method with a LOD of ~0.1 nM. Moreover, they also applied this probe for multicolor intracellular sensing of mercury ion.[37]

Detection of Important Biomolecules

Proteins and nucleic acids play the most important roles in the life. Development of methods for

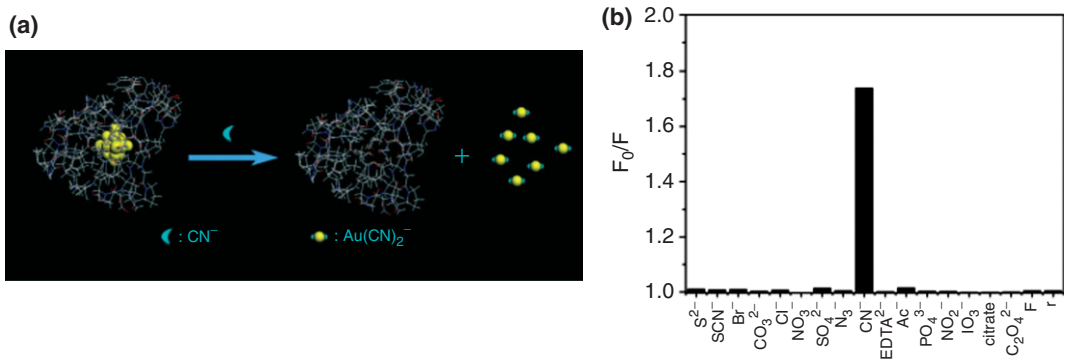

FIGURE 2 | The Au nanocluster (NC)-based sensor for CN^- detection. (a) Schematic representation, (b) selectivity of the Au-NC-based sensor for CN^- over other anions at pH 12: the concentration of each anion was 5×10^{-6} M. (Reprinted with permission from Ref 34. Copyright 2010 Wiley)

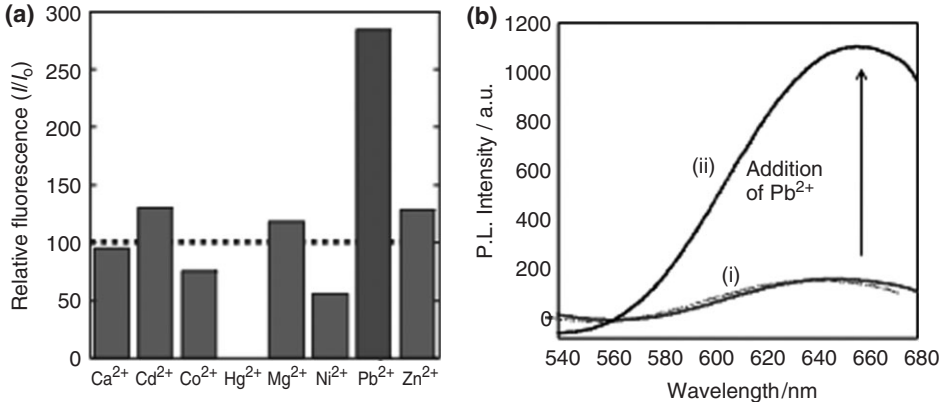

FIGURE 3 | (a) Relative fluorescence (I/I_0) of pepsin-mediated Au_{25} NCs at excitation $\lambda_{ex} = 360$ nm in the presence of various metal ions (Ca^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Mg^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}) at pH 8. I and I_0: the fluorescence intensities of pepsin-mediated Au_{25} NCs in the presence and the absence of the metal ion (10 μM), respectively. (b) Fluorescence spectra of aqueous solution of pepsin-mediated Au_{25} NCs (i) in the presence and (ii) in the absence of Pb^{2+} ions (50 μM). (Reprinted with permission from Ref 36. Copyright 2011 Wiley)

detection of these important biomolecules is fundamentally required for biomedical and biotechnological research. In this part, we will summarize recent advances of using metal NCs for detection of important biomolecules such as proteins, small molecules (e.g., amino acids and glucose), nucleic acids.

Triulzi et al.[38] prepared an antihuman IgG/PAMAM-Au NCs complex and used it to detect human IgG antigen; of note, PAMAM refers to poly(amido amine) dendrimers. The mechanism is that, through interactions between IgG and the antibody (i.e. anti-human IgG in the complex), aggregation of the Au NCs occurred, leading to decreased fluorescence intensity. This method can detect as low as nanomolar range of IgG. Similarly, another group, Shiang et al.[39] employed protein A/Au NC complex as a luminescence sensor for the detection of human IgG, in which protein A can specifically recognize IgG. They reached a LOD of 10 nM for IgG detection and demonstrated that this probe can be directly used in plasma samples without any need for sample pretreatment.

The interaction between aptamer and its antigen was also used for protein detection by Au NCs. Huang et al.[40] conjugated anti-PDGF AA (platelet-derived growth factor AA) aptamer on 13 nm Au NPs and PDGF AA on Au NCs, respectively. Through the interaction between anti-PDGF AA aptamer and PDGF AA, the fluorescence of Au NCs was quenched by Au NPs. This system can also be used to detect the PDGF receptor as a result of competitive interactions of the aptamer-Au NPs and the PDGF receptor with the PDGF AA-Au NCs. The LODs for PDGF AA and PDGF receptor were 80 pM and 0.25 nM, respectively. The same group also conjugated mannose onto the surface of Au NCs. Through the interaction between mannose and Concanavalin A (Con A) or *Escherichia coli* (*E. coli*), they can sensitively sense Con A (LOD = 75 pM) and *E. coli* (LOD = 7.20×10^5 cells/mL) in aqueous solution.[41]

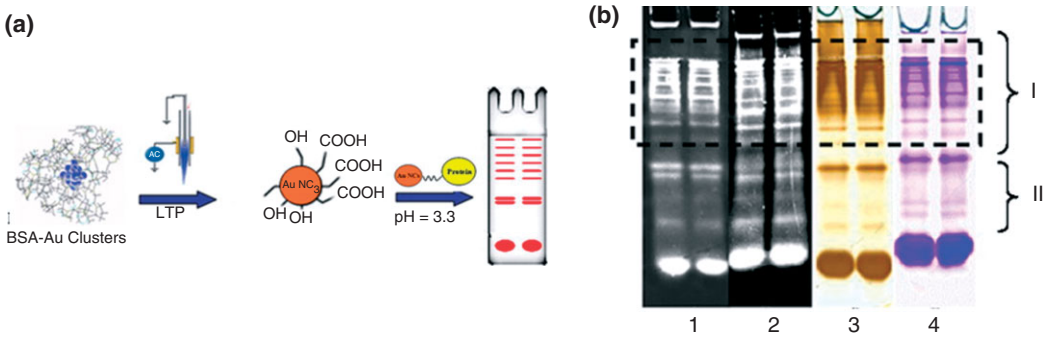

FIGURE 4 | (a) The schematic illustration of the low-temperature plasma treatment of bovine serum albumin (BSA)-Au nanoclusters (NCs) and their interaction with proteins. (b) The detection of proteins in human serum after 1D polyacrylamide gel electrophoresis (PAGE). (1) BSA-stabilized Au NC-based fluorescence imaging (NaAc solution, BSA-stabilized Au NCs 0.59 mM, pH 3.3). (2) Fluorescence image with oxygen low-temperature plasma (LTP)-treated Au NCs. (3) Silver stain. (4) CBB-R250 stain. The serum samples were diluted to a 1/10 ratio in the sample buffer (6.67% glycerin and 0.05% bromophenol blue). (Reprinted with permission from Ref 44. Copyright 2012 Elsevier)

In another example, antithrombin aptamer/Ag NC complex was applied for the specific and sensitive detection of thrombin. Jaswinder et al.[42] conjugated a cytosine-rich DNA sequence on Ag NCs for generating fluorescence and an antithrombin aptamer sequence for specifically recognizing thrombin. Thrombin binding induced a structural change in the chimera of the Ag NC complex, resulting in fluorescence quenching. The LOD is 1 nM for thrombin.

Lan et al.[43] prepared a DNA-Cu/Ag NC complex for the detection of single-stranded DNA-binding protein (SSBP). When SSBP interacted with the complex, the conformation of the DNA template was changed and thus induced a decrease of the fluorescence intensity. This DNA-Cu/Ag NC complex can sensitively and selectively detect the SSBP with a LOD of 0.2 nM.

Another interesting application is to apply Au NCs in the fluorescence imaging of human serum proteins after native PAGE. As shown in Figure 4, Zhang et al.[44] used the fluorescent BSA-stabilized Au NCs to improve the sensitivity of the detection of human serum proteins after native PAGE. They presented a novel method which has a sensitivity of 7–14 times higher than that of the traditional staining detection methods. Some low abundance proteins were easily detected. In addition, they used this Au NCs-based fluorescence imaging method to distinguish between the serum samples of patients with liver diseases and those of healthy people, indicating that this novel method has potential capabilities for clinical diagnosis.

Besides proteins, metal NCs were also applied for detection of other important biomolecules. For example, Tran et al.[45] reported the first method to use the ultrasmall Au NCs in isolation/enrichment of *N*-glycosylated peptides. Hydrazide-functionalized Au NCs exhibited a large capacity for glycoproteins capturing. They demonstrated that more than 90% of captured product was glycopeptides through this method. These results demonstrate that the Au NCs can be used for a high-throughput analysis platform of glycoproteins.

In regard to the detection of biologically important small molecules, Wang et al.[46] reported a novel sensory method for the identification of amino acids by BSA-Au NCs' dual fluorescence emissions: the blue at 425 nm from the oxides of BSA, and the red at 635 nm from Au NCs. They observed that when BSA-Au NC probes modulated by four different metal ions were used together, different amino acids were clearly discriminated by the distinctive patterns of four ratiometric fluorescence responses. They provided a new strategy for the determination of amino acids. Au NCs stabilized with the amino acid L-cysteine showed blue fluorescence emission. Hussain et al.[47] used this functional Au NC complex as a detection probe for glucose sensing with a LOD of 2.5 mM of β-D-glucose. This sensor was also applied for detection of glucose levels in serum samples.

On the other hand, development of highly sensitive, selective, and inexpensive approaches to nucleic acid detection is of great importance in basic research and many applied areas such as clinical diagnoses of infectious and genetic diseases, forensic and food analysis, and environmental monitoring. Below we illustrate with a few typical examples.

Lan et al.[48] conjugated Ag NCs with a fluorescent base DNA motif and a specific sequence that recognizes a gene for fumarylacetoacetate hydrolase. Through the interaction between DNA-Ag NCs and target DNA, the conformation of the DNA template was changed and thus induced a decrease in the fluorescence intensity. The LOD of this method is 14 nM. Similarly, Yang et al.[49] designed a

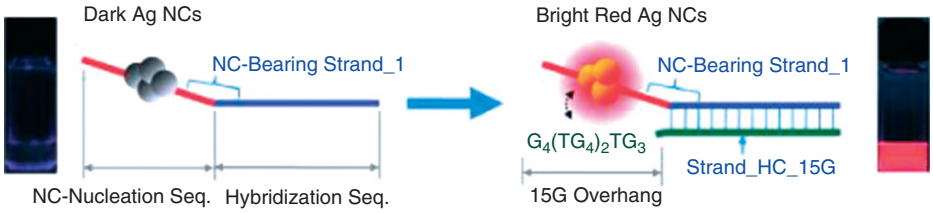

FIGURE 5 | Schematic showing red fluorescence enhancement of DNA/Ag nanoclusters (NCs) through proximity with a G-rich overhang, 3′-$G_4(TG_4)_2TG_3$, caused by DNA hybridization and photographs of the resulting emission under UV (366 nm) irradiation. (Reprinted with permission from Ref 50. Copyright 2010 American Chemical Society)

DNA-Ag NC probe for detection of miRNA. The red fluorescence of the DNA-Ag NC complex is diminished upon the presence of target miRNA. This turnoff of DNA-Ag NCs has a LOD of 20 nM for the target miRNA.[49]

Another approach to detect DNA by metal NCs is based on a 'light-up' mechanism (as opposed to the 'turnoff' one). As shown in Figure 5, Yet et al.[50] found that red fluorescence of the DNA-Ag NCs can be enhanced by 500-fold in the presence of a proximity guanine-rich DNA. Accordingly, they designed a NC beacon which 'lights up' upon target binding. They demonstrated NC beacon detection of an influenza target with a signal to background (S/B) ratio of 175, a factor of 5 better than a conventional molecular beacon probe.[50]

METAL NCs FOR BIOIMAGING APPLICATIONS

Although organic dyes with bright fluorescence have been widely employed as fluorescence markers for many years,[51] their shortcomings such as limited photostability and small Stokes shift may restrict their use in long-term tracking or multicolor imaging applications.[52] Semiconductor quantum dots have excellent brightness and photostability, however, diameters of many quantum dots are often above 5 nm, which may cause major kinetic or steric hindrance problems for studying biomolecule interactions or tracking biological processes.[53] Fluorescent metal NCs have been demonstrated as useful probes for bioimaging. Their advantages such as ultrasmall size (typically < 2 nm metal core), good photostability, low cytotoxicity, good biocompatibility, large Stokes shift, high emission rates, and near-infrared (NIR) emission make metal NCs as attractive fluorescent probes for bioimaging.[54–59] Among various metal NCs, fluorescent Au and Ag NCs have been mostly pursued for bioimaging application because of their high stability and well-developed synthesis techniques.[60,61]

Cellular Imaging

Yu et al.[62] presented an early work of using Ag NCs for bioimaging study. They synthesized the polycytosine-Ag NCs which have high fluorescence intensity. Then they conjugated avidin or antibodies onto the Ag NCs. Because the Ag NCs is significantly less than the labeled protein molecular weight, the biological functions of proteins are not significantly interfered. Employing either avidin–biotin or antibody–antigen interactions, these Ag NCs were demonstrated to be able to stain the cell surface and be internalized.

Liu et al.[63] synthesized the insulin-conjugated Au NCs, in which insulin retained the same bioactivity as commercial insulin in reducing blood glucose. They also observed that the uptake efficiency of insulin-Au NCs for C2C12 myoblasts was able to distinguish the differentiated versus undifferentiated C2C12 (Figure 6). Interestingly, the fluorescent insulin-Au NCs were successfully applied in X-ray computed tomography imaging.[63]

On the other hand, the characteristic NIR emission from metal NCs made it possible to image the clusters under 700–800 nm emission which are highly favorable for biomedical imaging; the 700–800 nm regime is much less interfered by the self-fluorescence of biological samples and light in this region also has a higher penetration capability compared to the often used blue and green fluorescence. Retnakumari et al.[64] conjugated folic acid (FA) to BSA-Au NCs. This FA-conjugated Au NCs can specifically recognize the FA receptors on cancer cells. They demonstrated NIR imaging applications using these Au NCs in FR^{+ve} oral squamous cell carcinoma and breast adenocarcinoma cell MCF-7, with significantly higher internalization to the negative control cell lines. Similarly, fluorescent Au NCs capped with multidentate polymer were used as NIR probes to label hematopoietic cancer cells,[65] which incorporated more Au NCs than normal cells. In addition, Le Guevel et al.[66] produced transferrin-stabilized Au NCs with strong NIR emission for cell imaging and targeting. They showed NIR imaging of A549 cells by targeting transferrin receptors.

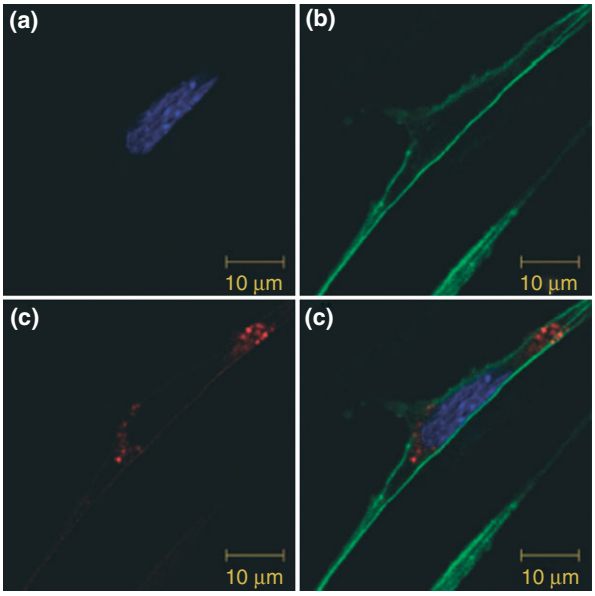

FIGURE 6 | Microscopic observation of internalization of the insulin-Au nanoclusters (NCs). Differentiated C2C12 myoblasts were treated with insulin-Au NCs for 2 h. (a) Cell nucleus stained with 4′,6-diamidino-2-phenylindole (DAPI, blue). (b) Actin fiber stained with Alexa Fluor 488 phalloidin to confirm the cell boundary (green). (c) Insulin-Au NCs exhibit red luminescence. (d) Fluorescence image overlay of the three images. (Reprinted with permission from Ref 63. Copyright 2011 Wiley)

On the other hand, two-photon excitation with near-infrared light can reduce phototoxicity to biosamples.[67] The outstanding two-photon absorption cross-sections of Au NCs make them excellent probes for two-photon cellular imaging. Chou et al.[68] produced dextran encapsulated Au NCs and used them for two-photon imaging of human mesenchymal stem cells. Upon two-photon excitation with 800 nm, bright luminescence from Au NCs was observed in the cells. Similarly, glutathione (GSH)-Au NCs[54] and D-penicillamine-Au NCs[69] were also applied in two-photon cellular imaging.

Of particular interest is that the ultrasmall size of metal NCs renders them a much higher chance to enter nuclei of cells. Lin et al.[70] presented that upon functionalization of Au NCs with a nuclear-localization peptide, SV40, these peptide-coated Au NCs were distributed within both the cytoplasm and the nucleus, as revealed by colocalization experiments. In another work, as shown in Figure 7, herceptin was conjugated with fluorescent BSA-protected Au NCs (referred to as Her-Au NCs) and Wang et al.[71] demonstrated using these Au NCs for imaging SK-BR3 cells, which overexpress human epidermal growth factor receptor 2 (HER2) on the cell membrane. The Her-Au NCs were localized to the nucleus.

In addition, Ag NCs have shown stronger staining of nucleoli, which is reminiscent of a relevant result that Ag ions preferentially bind argyrophilic proteins located in nucleoli.[62,72] Penetratin-C 12-Ag NCs were demonstrated to be capable of crossing the cell membrane and entering nuclei within a few minutes, either in NIH 3T3 or Bovine Pulmonary Artery Endothelial Cells (BPAEC) cells.[73] Similarly, aptamer-Ag NCs (Sgc8c-Ag NCs) were applied for specific targeting of the nuclei of tumor cells.[74]

Sousa et al.[75] reported an imaging approach which enables unbiased detection and quantification of individual ultrasmall metal NCs and aggregates in cells. They synthesized the Au NCs coated with GSH or both GSH and cell-penetrating peptide Trans-acting Activator of Transcription (TAT). HeLa cells were then incubated with both Au(GSH) and Au(GSH)-TAT complexes, and imaged without silver enhancement of the Au NCs in unstained thin sections by quantitative scanning transmission electron microscopy. This approach can be used to quantify the Au NCs in the cytoplasm and nucleus of the cell.

The mechanisms of how metal NCs enter cells have been studied. It was reported that Au NCs may enter cells via endocytosis.[54,69] More recently, Yang et al.[76] revealed that cellular uptake of Au NCs is energy dependent and involves multiple mechanisms: clathrin-mediated endocytosis and macropinocytosis appear to play a significant role, whereas the caveolin-mediated pathway only contributes to a lesser extent.

In Vivo Imaging

Compared with cellular imaging, *in vivo* imaging suffers difficulties such as the poor transmission of visible light through biological tissues.[77,78] Because of the NIR emission properties, ultrasmall metal NCs would be an ideal type of probes for live animal imaging. We highlight some recent works which are important for future development of metal NCs as contrast agents for *in vivo* imaging applications. It is worth noting that fluorescent quantum dots (such as CdSe) are less desirable due to their high toxicity to living animals.

The first application of Au NCs-based *in vivo* fluorescence imaging was presented by Wu et al.[55] They used NIR-emitting BSA-stabilized Au NCs as imaging agents for tumor fluorescence imaging *in vivo*. Immediately after tail vein injection of Au NCs, bright fluorescence from the superficial vasculature of the whole bodies of mice could easily be visualized. These Au NCs accumulated predominantly in tumor areas of mice and the fluorescence decreased noticeably within

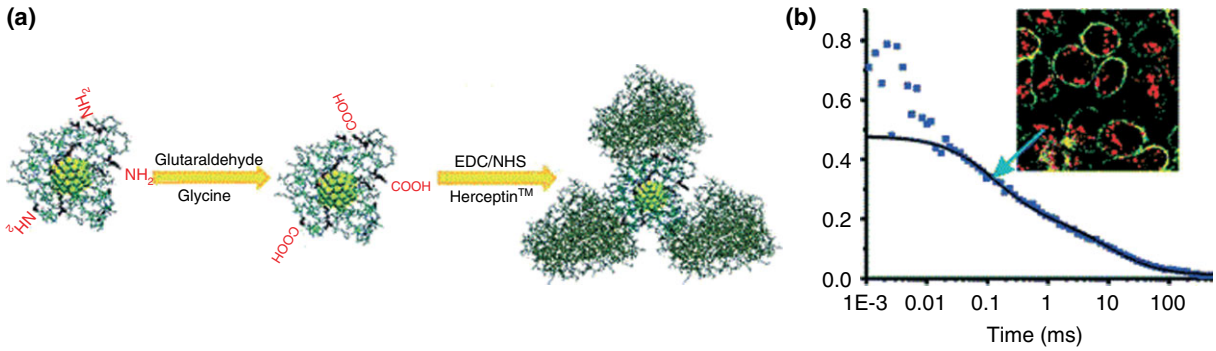

FIGURE 7 | (a) Schematic of Au nanoclusters (NCs)-Her conjugation. (b) Typical autocorrelation curve (solid squares) of Au NCs-Her diffusing inside the nucleus fitted with two components (black solid line). Inset is the image of SK-BR3 cells incubated with Au NCs-Her-Alexa647 for 4 h. (Reprinted with permission from Ref 71. Copyright 2011 American Chemical Society)

a day. Interestingly, they also found that, compared with other NP-based contrast imaging agents, uptake of Au NCs by the reticule-endothelial system (e.g., liver and spleen) is small due to their ultrasmall hydrodynamic size.[55]

Sun et al.[79] prepared red-emitting ferritin-coated Au NCs as fluorescent probes for *in vivo* imaging. They injected these Au NCs via the lateral tail vein into mice. Fluorescent regions with a kidney-like shape were observed on either side of the spine 30 min postinjection and remained visible for at least 7 h, probably because of the specific uptake mechanism related to the existence of certain ferritin receptors in kidney. Inductively coupled plasma mass spectrometry revealed that liver, kidney, and spleen were the major target tissues of ferritin-Au NCs.

In another study, Zhou et al.[80] studied renal clearance of GSH-coated luminescent Au NCs and observed that only 3.7% of the clusters accumulated in the liver; over 50% of the clusters were found in urine within 24 h after intravenous injection, which is comparable to the case of quantum dots.[59] In addition, they also revealed that the renal clearance of GSH-Au NCs was more than 10–100 times better than that of the similar sized nonluminescent Au NPs coated by bis(*p*-sulfonatophenyl)phenylphosphine and cysteine. Urinary excretion of the particles was imaged with X-ray computed tomography and luminescence techniques in real time.

More recently, Huang et al.[81] found that 2 nm Au@tiopronin NCs show high levels of accumulation in tumor tissue in mice after a single intravenous injection. Furthermore, the ultrasmall Au NCs were distributed throughout the cytoplasm and nucleus of cancer cells *in vitro* and *in vivo*, whereas 15 nm Au NPs were found only in the cytoplasm.

Another recent interesting study is to construct a multimodal *in vivo* imaging technique using Au NCs. As shown in Figure 8, Zhang et al.[82] used Au NCs as dual-modality imaging contrast agents for fluorescent and X-ray dual-modality imaging. Because of advantages of Au NCs such as ultrasmall sizes, reliable fluorescent emission, high computed tomography value, and fine biocompatibility, authors successfully demonstrated that Au NCs have potential for fluorescent/X-ray enhanced dual-modality imaging *in vivo*. Moreover, the abundant chemical groups on the surface of Au NCs and their feasibility for further surface labeling with various biological molecules.

METAL NCs FOR BIOMEDICAL APPLICATIONS

Metal NCs also show great potential for biomedical applications. For example, Chen et al.[20] demonstrated that lysozyme-directed generation of Au NCs are potential antimicrobial agents for antibiotic-resistant bacteria and broad labeling agents for pathogenic bacteria. They used lysozyme as the sequester and reducing agent for the functional Au NCs generation under microwave irradiation. Because the ultrasmall size of the Au NCs, the bioactivity of the lysozyme on the Au NCs was retained. Therefore, the lysozyme-Au NCs can be used to effectively inhibit the cell growth of notorious antibiotic-resistant bacteria. In another study, Zhang et al.[83] presented that ultrasmall Au NCs conjugated to doxorubicin (Au-Dox) are 20-fold more cytotoxic to B16 melanoma cells than the equivalent concentration of Dox alone, and act up to six times more quickly. Because of their ultrasmall size, Au-Dox can enter the nucleus as seen by confocal and electron microscopy, in distinct contrast to larger Au NPs conjugated to Dox, which show no nuclear entry.[84] In addition, they also revealed that the Dox-resistant Hela cells are not resistant to Au-Dox, suggesting the high potential in killing of apoptosis-resistant cancer

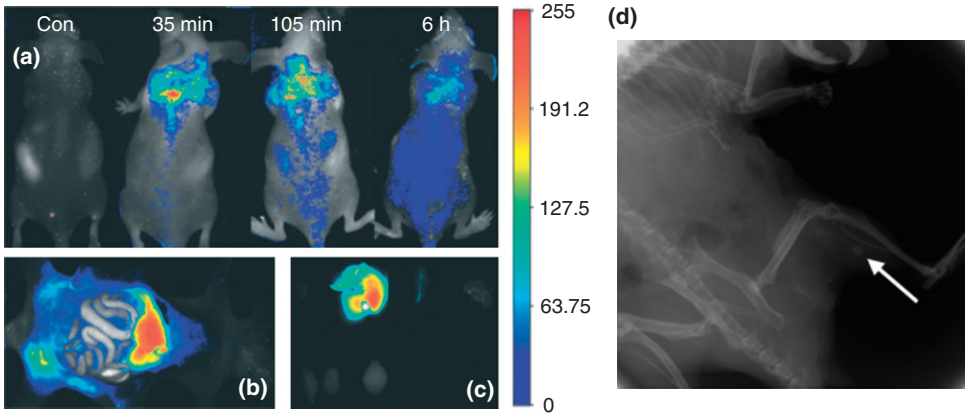

FIGURE 8 | (a) Real-time *in vivo* imaging of 250 μL of Au nanoclusters (NCs) intravenously injected at different time points, postinjection. (b) *Ex vivo* optical imaging of anatomized mice with injection of 200 μL of Au NCs and some dissected organs during necropsy at 6 h postinjection. (c) The organs are heart, liver, spleen, and lung from left to right at up-level, and left kidney, right kidney, and head from left to right at down-level. (d) *In vivo* X-ray images of 0.1 mL of Au NCs CAs solution intramuscularly injected in the right leg of the mouse. The white arrow points to the Au NCs injection region. (Reprinted with permission from Ref 82. Copyright 2012 Elsevier)

cells. Later, the same group[85] found that although the Au NCs alone are non-toxic and Indium phosphide (InP) is moderately toxic, Au-Dox is more effective than InP quantum dots-Dox against the Dox-resistant cancer cell line. Electron and confocal microscopy and atomic absorption spectroscopy revealed that over 60% of the Au-Dox conjugates reach the cell nucleus, in distinct contrast to InP-Dox which enters cell nuclei to a very limited extent.[85]

More recently, Chen et al.[86] presented that Au NCs-poly(L-lactide) (PLA)-sulfated Gynostemma Pentaphyllum Polysaccharide (GPPS)-folate (FA) copolymers (Figure 9) gain specificity to target some cancer cells because of the enhanced cell uptake mediated by FA moiety. Furthermore, the Au NC complex has high anticancer activity against Hela cells. They demonstrated that the Au NCs copolymers not only have great potential as tumor-targeted drug delivery carrier, but also have an assistant role in the treatment of cancer.

CONCLUSIONS AND OUTLOOK

In this review, we have summarized the recent research progress on bio-related applications of ultrasmall metal NCs (with size ca 2 nm or smaller). When bulk metals are reduced to metal NCs containing only 10^1–10^2 atoms, unique chemical and physical properties are manifested in such ultrasmall metal NCs, which offer extraordinary advantages compared with larger metal NPs, including ultrasmall size, significantly higher photoluminescence and NIR emission, large Stokes shift, good photostability, low cytotoxicity, good biocompatibility. With these advantages, metal NCs have been demonstrated in many applications including biosensor development, bioimaging, and biomedical research as discussed in this review.

Despite substantial progress in applying metal NCs for bio-related applications, some challenges still remain and should be resolved in future work. First of all, when applying metal NCs for *in vivo* study, knowledge of their interactions within the complex biological environment is still quite limited. The effects of metal NCs on cell growth and living organisms over extended periods of time need to be extensively studied. Secondly, most of current studies focus on Au, Ag, and Cu metal NCs, it would also be interesting to explore the synthesis and application of *bimetallic* metal NCs using doping or alloying strategies to further enhance their photoluminescence so that metal NCs can compete with quantum dots. Thirdly, it is known that these metal NCs exhibit good catalytic properties and have been demonstrated their catalytic efficiency in some chemical reactions.[87–89] However, how to apply their good catalytic properties for bioreactions involving biofuels still remains to be explored.

Overall, ultrasmall metal NCs possess some unique and attractive features for many bio-related applications. With respect to particle size, large NPs (e.g., 13 nm Au NPs as well as 5–10 nm quantum dots) may cause major kinetic or steric hindrance problems for studying biomolecular functions and interactions, while for metal NCs, their disturbance to biological functions of proteins is the least since their size is significantly less than the labeled protein. Another unique advantage of ultrasmall sized NCs lies in that they have a high chance to enter the nucleus. Future

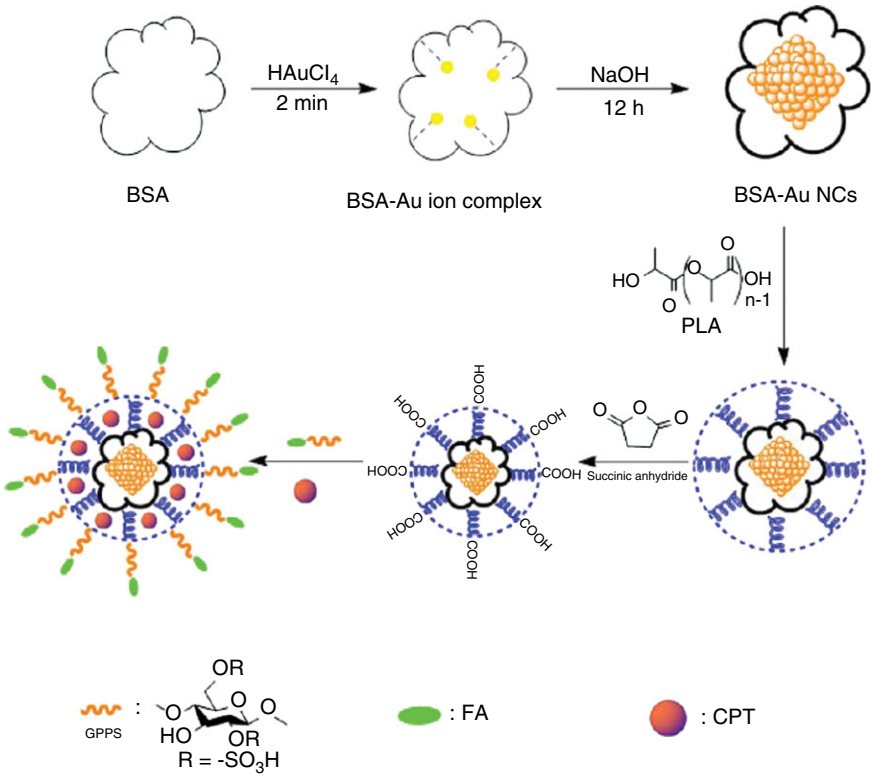

FIGURE 9 | Schematic illustration of the Au nanoclusters (NCs)-PLA-GPPS-FA copolymers. (Reprinted with permission from Ref 86. Copyright 2012 American Chemical Society)

work should focus on the mechanism of NC entering nucleus and how the NCs will affect the activities of the nucleus. In addition, metal NCs as contrast agents for *in vivo* imaging holds great potential, especially for dual mode imaging and related scenarios. Taken together, more opportunities are to be opened up in future work to explore their potential applications in fields of nanomedicine and nanobiotechnology such as biofuels, drug delivery, antimicrobial agents, and clinical imaging.

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