Luminescent metal nanoclusters: Biosensing strategies and bioimaging applications

Yan Xiao1,2  |  Zhennan Wu1  |  Qiaofeng Yao1  |  Jianping Xie1,3

1 Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore, Singapore
2 College of Chemistry and Chemical Engineering, Hubei University, Wuhan, Hubei, China
3 Joint School of National University of Singapore and Tianjin University, International Campus of Tianjin University Binhai New City, Fuzhou, China

Correspondence
Jianping Xie, Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, 117585 Singapore, Singapore.
Email: cheyaoq@nus.edu.sg (Q.Y.); chexiej@nus.edu.sg (J.X.)

Funding information
Ministry of Education, Singapore, Grant/Award Numbers: R-279-000-580-112, R-279-000-538-114; China Scholarship Council, Grant/Award Number: 201908420311; Ministry of Education, Singapore, Grant/Award Numbers: R-279-000-580-112, R-279-000-538-114; China Scholarship Council, Grant/Award Number: 201908420311

1 INTRODUCTION

Metal nanoclusters (MNCs) are a unique class of ultra-small particles consisting of few to hundreds of metal atoms.[1] They feature a metal core of <2 nm, which is formed by the controlled aggregation of metal atoms (M), and is further passivated by a monolayer of M-L motifs (L denotes organic ligand).[2] The M-L motifs anchored on the cluster core can well protect the MNCs from agglomerating to large nanoparticles (NPs) or being destroyed by the etchants to form M-L complexes, thereby giving MNCs excellent solution stability. The continuous development of cluster chemistry shows that in this ultra-small size regime, the unique aggregation or packing patterns of metal atoms and organic ligands will largely determine the physicochemical properties of MNCs, which are distinctly different from that of their larger counterparts, i.e., plasmonic metal NPs.[3] It is well known that plasmonic metal NPs exhibit characteristic surface plasmon resonance (SPR), which is responsible for their applications in different fields.[4] As the size of MNCs is reduced to less than 2 nm and close to the Fermi wavelength of electrons, the motion of electrons becomes extremely restricted. This feature leads to a breakup of the continuous energy levels of metal NPs into discrete energy levels.[5] As a result, MNCs no longer support SPR, but feature a variety of molecule-like properties, including highest occupied molecular orbital-lowest occupied molecular orbital transition, luminescence, intrinsic chirality, etc.[6,7]

Among these molecule-like properties, luminescence has attracted tremendous research interest because it has

Abstract
Metal nanoclusters (MNCs) are ultrasmall metal–organic aggregates, composed of a metal core less than 2 nm and a protecting shell of metal–organic ligand motifs. The controlled aggregation of metal atoms (in the cluster core) and metal–organic ligand motifs (around the cluster core) renders MNCs with numerous molecule-like properties, among which strong and bright luminescence has attracted extensive basic and applied interests. It has now known that aggregation-induced emission is a feasible mechanism for controlling luminescence of MNCs, which makes it particularly useful in biosensing and bioimaging applications. Although the luminescence fundamentals and design principles largely determine the practicality and effectiveness of MNCs in biosensing and bioimaging applications, a systematic summary of this topic is lacking in the current literature. In this review, we aim to provide a concise discussion of the latest developments in biosensing and bioimaging applications of luminescent MNCs, highlighting their luminescence mechanisms, biosensing principles, and bioimaging strategies. Specifically, we first introduce the recent advances in the synthetic chemistry of MNCs, and then briefly discuss the luminescence fundamentals of MNCs. Then the design strategy and practicality of luminescent MNCs in biosensing and bioimaging applications are exemplified. We conclude the review with our perspectives on the further development of MNC-based optical probes in biosensing and bioimaging applications. Our review is expected to provide guidance for the future practice of designing and synthesizing luminescent MNCs for biomedical and other applications.

KEYWORDS
aggregation-induced emission, bioimaging, biosensor, luminescence, metal nanoclusters
evoked cluster applications in various fields, such as environmental monitoring, energy conversion, and biomedicine.[8]

However, compared with conventional luminescent materials (e.g., organic dyes and quantum dots), the quantum yield (QY) of MNCs is relatively low. Fortunately, the aggregation-induced emission (AIE) originally discovered by Tang and co-workers has paved an effective way to enhance the emission of MNCs, thereby promoting the application of MNCs as luminescent probes.[9] The remarkable advantages of using luminescence of MNCs in biosensing and bioimaging mainly include the following aspects: 1) tunable emission from ultra-violet (UV) to near-infrared-II (NIR-II) region; 2) considerable QYs, up to 60%; 3) large Stokes shift, effectively avoiding the interference by excitation light; 4) excellent photostability for long-time and reliable illuminating. Due to these excellent optical properties of MNCs, as well as their facile synthesis and good biocompatibility, MNCs have broad application prospects in the fields of biosensing and bioimaging. Although great achievements have been made in the past decade, there is a lack of systematic summary of the design principles and fundamental chemistry governing the biosensing and bioimaging applications of luminescent MNCs in the current literature.

In this manuscript, we summarize the latest developments in biosensing and bioimaging applications of luminescent MNCs, focusing on the luminescence fundamentals of MNCs and the design principles of optical probes for the aforementioned applications. Notably, considering their excellent stability and high QYs are beneficial for biological applications, gold and silver NCs will be used as paradigm clusters throughout the discussion in this review. In detail, we first introduce the synthesis strategies of luminescent MNCs, in which atomic precision has been persistently pursued. Following the synthetic discussion is an anatomy of the luminescence mechanism of MNCs, highlighting the dictating roles of the aggregation states of M atoms and M-L motifs on the luminescence of MNCs. In the subsequent sections, we elaborate various biosensing and bioimaging strategies based on luminescent MNCs (Figure 1). This review is then concluded with our perspectives on the further development of luminescent MNCs for biosensing and bioimaging applications. In this vein, we hope that the basic and applied principles summarized in this review can increase the acceptance of luminescent MNCs in biological and many other applications.

2 | SYNTHESIS STRATEGIES

The successful synthesis of ligand or matrix-protected MNCs can be dated back to the 1970s.[10] In the following decades, MNCs were mainly synthesized and stabilized in solid matrices, such as glass and zeolite, for chemical catalysis and data storage.[11,12] With the continuous development of nanoscience and nanotechnology, luminescent MNCs in solution have been gradually prepared with high yields through facile methods.[13,14] In the past two decades, the synthesis of MNCs has experienced rapid development, in which the pursuit of high QYs and atomically precise cluster structures has continued.

2.1 | Classic synthetic methods

Generally, the synthetic methods of MNCs can be categorized into two classic strategies, namely the “bottom-up” and “top-down” synthetic routes (Figure 2). Both routes require protection of the templates or organic ligands during the synthesis process, because MNCs without stabilization have high surface energy, which will cause irreversible agglomeration of MNCs in solution. In a typical “bottom-up” approach, the synthesis of MNCs is achieved by reducing a metal precursor (usually a metal salt) under the protection of an appropriate template or organic ligand. In the presence of the templates (or organic ligands) and reducing agents, the metal precursors will be reduced to zero-valence metal atoms, and then these newly generated M(0) atoms will aggregate to form the M(0) core of MNCs. At the same time, the growing M(0) core will be passivated by templates or organic ligands, which may be diverse, including small thiol molecules, DNA, peptides, proteins, and polymers. Therefore, the feeding ratios of metal precursors, templates/organic ligands and reducing agents have a significant effect on the nucleation and/or growth of MNCs. Generally, in order to effectively protect the M(0) core during the synthesis process, the amount of ligands is larger than that of the metal precursors. For example, in the synthesis of thiolate-protected MNCs, the feeding ratio of ligand-to-metal is about 3:1.[15-17] Regarding reducing agents, their dosage depends on their reducing power. For example, NaBH₄ is one of the most used reducing agents in the synthesis of MNCs, and has a strong reducing power. In a recent contribution, Chen et al. demonstrated that only a stoichiometric amount of NaBH₄, based on the equation 32/9 [Au(SR)]₂⁺ + 8 e⁻ → [Au₂₅(SR)₁₈]⁻ + 7 [Au(SR)]₂⁻ (where SR denotes thiolate ligand), is required to produce...
atomically precise [Au\textsubscript{25}(SR)\textsubscript{18}]\textsuperscript{−} with high yield.[18] However, for most of other clusters, the stoichiometry synthesis by NaBH\textsubscript{4} remains as a major challenge because of lacking molecule-level knowledge on their formation mechanism. Using excessive or insufficient amount of NaBH\textsubscript{4} in synthesis of MNCs is likely to cause the impurity and polydispersity in the as-synthesized MNCs. In a typical “top-down” approach, MNCs are prepared by etching pre-formed large metal NPs using strongly interacting ligands. When small thiol molecules are used as etching ligands, this route is usually used in the synthesis of Au or Ag NCs. Based on these two synthetic strategies, remarkable progress has been made in the synthesis of MNCs in solution in the past two decades, and a wide diversity of MNCs have been successfully synthesized with atomic precision. However, the delicate formation process and mechanism are still not very clear for the majority of MNCs. More efforts are still required to understand the formation kinetics and dynamics of MNCs.

2.2 Toward atomic precision

In the early days of cluster research, MNCs are usually prepared in the form of mixtures of different sizes and compositions. The polydispersity of MNCs is one of main obstacles to their practical applications, because polydispersity not only makes it difficult to understand the correlation of structures and properties, but also leads to many uncertainties or deviations in their materials performance. Therefore, the synthesis of monodisperse MNCs with their size and composition controllable at the atomic level is of core interest. “One batch for one size” has become a long-term dream in the synthesis of MNCs, and various methods have been developed to achieve this goal. Good examples of such methods include CO reduction,[19] stoichiometric NaBH\textsubscript{4} reduction,[18] size-focusing,[20] ligand-exchange induced size/structure transformation,[21] and many others.[3] Based on these synthetic methods, a series of atomically precise MNCs with molecular purity, such as Au\textsubscript{15}(SR)\textsubscript{13}, Au\textsubscript{18}(SR)\textsubscript{14}, and Au\textsubscript{25}(SR)\textsubscript{18}, have been successfully prepared. For example, a “size-focusing” method was developed to synthesize Au\textsubscript{25} with different thiolate ligands, which subsequently became a common method for preparing atomically precise MNCs.[20] The size-focusing method is based on the conversion of polydisperse crude products into thermodynamically favorable monodisperse MNCs by ligand etching under prolonged reaction time. Other MNCs with different atom numbers, such as Au\textsubscript{28}, Au\textsubscript{36}, and Au\textsubscript{44}, can also be obtained by this method.[22]

Building upon the prosperity of synthetic chemistry is the determination of MNC structure with atomic precision. In 2007, Jadzinsky et al. reported for the first time the successful crystallization and X-ray crystal structure determination of a thiolate-protected MNC, namely Au\textsubscript{102}(SR)\textsubscript{44}.[23] In 2008, Zhu et al. and Heavens et al. independently determined the crystal structure of Au\textsubscript{25}(SR)\textsubscript{18} by X-ray crystallography.[24,25] Inspired by these pioneering work, many MNCs, such as [Ag\textsubscript{44}(SR)\textsubscript{30}]\textsuperscript{4−}, [Ag\textsubscript{25}(SR)\textsubscript{18}]\textsuperscript{−}, [Ag\textsubscript{32}Au\textsubscript{12}(SR)\textsubscript{30}]\textsuperscript{4−}, and [Au\textsubscript{52}Cu\textsubscript{72}(SR)\textsubscript{55}]\textsuperscript{+}, have been probed by X-ray crystallography, and their atomic packing structures have been resolved accordingly.[26–29] These works have opened up new horizons for the basic research of MNCs, because not only they advanced the structural anatomy of MNCs into atomic precision, but also they proved that the packing or aggregation mode of metal atoms in this ultra-small size regime can be distinctly different from the crystalline packing patterns observed in plasmonic metal NPs.

Almost all of the abovementioned synthesis and structure determination successes are achieved in the organic phase. The relatively low QYs and poor water solubility of these organic-soluble MNCs largely hinder their biomedical application.
applications, such as biosensing and bioimaging. In order to tackle these challenges, our group has developed a series of synthetic methods for water-soluble MNCs with atomic precision.[30] For example, by using carbon monoxide (CO) as a mild reducing agent, we developed a simple large-scale synthesis of water-soluble [Au$_{25}$(SR)$_{18}$]$_{n}^+$ NCS.[31] A salient feature of this method is that it is easy to control the size and protecting ligands of MNCs, among which Au$_{10,12}$(SR)$_{10,12}$, Au$_{15}$(SR)$_{13}$, Au$_{16}$(SR)$_{14}$ and Au$_{25}$(SR)$_{18}$ were produced by using varied thiolate ligands (e.g., glutathione (GSH) and 3-mercaptopropionic acid (MPA)).[19] Compared with commonly used reducing agent NaBH$_4$, gaseous CO possesses a milder reducing ability, which is conducive to establishing slow and controllable reaction kinetics for cluster growth. Furthermore, the mild reduction kinetics also allows the cluster growth pumped by CO readily quenchable at desired stage, providing a good platform not only to trap the cluster growth into designed size, but also to reveal the size growth mechanism of MNCs. Therefore, using CO as the reducing agent is a promising synthesis route for molecularly pure MNCs with size and composition tunable in a wide spectrum. A minor concern of this strategy is the toxicity of CO, which requires extra care during its usage. It is worth mentioning that based on the interesting AIE feature of Au(I)-SR complexes, we designed a simple and effective method to synthesize highly luminescent Au(0)@Au(I)-SR NCs (QY up to ~15%), where the Au(I)-SR complexes were incubated at an elevated temperature (e.g., 70 °C) to produce a mixture of Au$_{25}$(SR)$_{27}$, Au$_{30}$(SR)$_{32}$, Au$_{35}$(SR)$_{32}$, Au$_{39}$(SR)$_{35}$, and Au$_{45}$(SR)$_{37}$ NCS.[32] In a follow-up contribution, the AIE properties of Au(I)-SR complexes have also been used to synthesize Au$_{25}$(SR)$_{18}$, which is the first highly luminescent Au NC produced with atomically precise formula.[33] The production of atomically precise Au$_{25}$(SR)$_{18}$ should be predominantly attributed to the controlled anchoring and aggregation of Au(I)-SR complexes on Au$_{14}$(SR)$_{14}$ NCS.[34] In addition to developing synthesis routes, elucidating the detailed formation mechanism of atomically precise MNCs is another center of current cluster research, which can provide the molecular- and atomic-level guidance for the rational synthesis of MNCs.[35–37] Regarding the future development of synthesis research, more atomically precise MNCs with pre-designed size, composition, and structure should be synthesized by facile and versatile approaches, conducive to numerous basic and applied research involving atomically precise MNCs.

3 | LUMINESCENCE PROPERTIES

The understanding of the luminescence properties of MNCs is not only essential for the rational design of highly luminescent MNCs, but also for their applications in a wide range of fields such as biomedicine, energy conversion, and light-emitting devices. Since the early 2000s, the luminescence properties of MNCs have attracted extensive research interest from the cluster research community.[38] However, largely due to the unsatisfactory size monodispersity of MNCs in the early stage, it is difficult to establish a reliable structure-property relationship at the atomic precision for luminescent MNCs. With the development of synthesis chemistry and structure determination technology (for more information, see section 2 above), in the past two decades, important hints have been revealed at the atomic level to understand the luminescence fundamentals of MNCs.

3.1 | Luminescence mechanisms

In the early stage, the luminescence of MNCs was mainly attributed to the strong quantum confinement effect in the size range of less than 2 nm.[39] Since there is no bandgap and electron-hole separation or recombination process in plasmonic metal NPs, they hardly emit light.[40] Nevertheless, as the size of metal NPs decreases, their metallic properties gradually disappear and molecule-like characteristics become dominant. When the size of MNCs is close to the Fermi wavelength of electrons, the motion of electrons in MNCs is severely restricted, which converts the energy bands of the MNCs into discrete energy levels. This inherently different electronic structure produces completely different physicochemical properties between MNCs and plasmonic metal NPs.[1,39] After absorbing light, electrons will be excited, and the excited electrons are prone to radiative relaxation between discrete energy levels, resulting in bright luminescence. The emission of MNCs can span a wide spectrum from UV to the NIR.[41] In the quantum size effect scheme, the excitation and emission wavelengths of MNCs are highly dependent on size. As the size of MNCs decreases, the relaxation energy gap of electrons increases, therefore the emission wavelength of MNCs shifts to the hypochromic end.[41]

With the deepening of the optical research of MNCs, more and more exceptions have been documented against the abovementioned size-dependence of cluster luminescence. For example, Au$_{25}$ NCs with the same core size but different ligands emit different wavelengths.[42,43] This readily indicates that in addition to the size of MNCs, the protecting ligand also contribute to their luminescence. The observations of Wu et al. further support this conclusion. The authors found that the ligand-to-metal charge transfer (LMCT) through the metal-sulfur bond has a significant impact on the luminescence properties of Au$_{25}$(SR)$_{18}$.[44] Keeping the core size of Au NCS unchanged, the luminescence of Au$_{25}$(SR)$_{18}$ can be largely promoted by using ligands with electron-rich atoms and groups. In another study, Chen et al. separately studied the contribution of ligand shell and metal core of poly(methacrylic acid) (PMAA)-Ag NCS to their luminescence. The authors proposed that the luminescence origin of PMAA-Ag NCS is the ligand-to-metal-metal charge transfer (LMMCT) between Ag(0) atoms and Ag(I)-carboxylate complexes.[45] Indeed, as the exploration of MNC luminescence continues to develop, more and more observations have shown that not only the metal core but also the ligands play a vital role in the luminescence of MNCs. In most cases, LMMCT/LMCT and quantum size effect have been widely recognized as the luminescence source of MNCs.[46] Recently, through the use of a series of “monocuboctahedral kernel” Au NCs, including [Au$_{25}$(SR)$_{16}$]$^+$, [Au$_{25}$(SR)$_{12}$(PCP)]$^+$, [Au$_{19}$Cd$_{2}$(SR)$_{16}$]$^+$, Au$_{21}$(SR)$_{15}$, and Au$_{25}$(SR)$_{15}$ (PCP = PPh$_2$-CH$_2$-PPh$_2$) as models, Jin and co-workers further improved their understanding of cluster luminescence. The authors suggest that although the surface is essential for the room-temperature luminescence of this series of MNCs, the luminescence origin is indeed the kernel.
emission rather than the surface emission through the LMCT mechanism.[47]

In addition to the luminescence mechanisms discussed above, the original discovery of AIE by Tang and co-workers also provides another mechanism for understanding the strong emission of MNCs. For example, Luo et al. discovered the AIE phenomenon of Au(I)-thiolate complexes and synthesized a type of ultra-bright Au(0)@Au(I)-thiolate core-shell NCs.[32] These Au NCs with a core-shell structure are formed by the controlled aggregation of Au(I)-thiolate complexes on the surface of the Au(0) core. Due to the AIE of the surface Au(I)-thiolate complexes, this type of Au NCs has a strong emission and QY is about 15%. In addition, Wu et al. further studied the influence of Au(I)-thiolate motifs on the AIE of Au NCs.[48] They systematically studied the AIE behavior of a series of Au(I)-SR complexes and Au NCs. They found that the emission mechanism of Au NCs is intrinsically affected by the length of Au(I)-SR motifs. The AIE of the long Au(I)-SR motifs on the Au(0) core can sustain strong luminescence. In this case, AIE is the dominant emission mechanism for Au NCs. In sharp contrast, when the Au(I)-SR motifs have a relatively short length on the cluster surface, the cluster emission mainly originates from the Au(0) core. Bain et al. also revealed the important role of surface Au(I)-thiolate motifs on the AIE of bi-metallic AuAg NCs.[49] They reported that doping of Ag into Au NCs could change the structure of surface Au(I)-thiolate motifs and further regulate AIE of AuAg NCs. Specifically, the Ag(I) first acted as linker between Au(I)-thiolate motifs, followed by reaction with Au(0) core via an anti-galvanic reduction mechanism. Such anti-galvanic reaction ultimately led to the formation of M(0)@Au(I)-thiolate (M = Au or Ag) alloy NCs with bright luminescence. By using this strategy, the emission intensity of MNCs could be significantly enhanced and the emission maxima could be tuned from 735 nm via 674 nm and finally to 574 nm. These studies clearly show that the emission of MNCs can be derived from the AIE of Au(I)-thiolate complexes on the surface of MNCs, and AIE can provide a good means to enhance the luminescence of MNCs. Although these recent studies have made some breakthroughs, a comprehensive and systematic understanding of the luminescence mechanism of MNCs has not yet been established. It is essential to synthesize highly luminescent MNCs with known atomically precise structure, to further reveal its luminescence mechanism at the atomic level.

3.2 Factors affecting luminescence

Although the exact luminescence origin and mechanism of MNCs are not yet fully understood, some factors that influence the luminescence behavior of MNCs have been identified through the joint efforts of theoretical and experimental chemists. The metal core, surface ligands, and surrounding environment of MNCs are the key factors affecting their luminescence.

Tuning the size and composition of the metal core can regulate the emission of MNCs. Since the electronic transition in the M(0) core is an important source of cluster emission, changing the aggregation size and pattern of metal atoms in individual MNCs is an effective method to adjust the luminescence of MNCs. In this scheme, the dependence of emission energy on the number of metal atoms in individual cluster can be estimated through the free electron (Jellium) model.[41] For example, a series of Au NCs of different sizes encapsulated by poly(amidoamine) dendrimers (PAMAM), such as Au5, Au8, Au13, Au23, and Au31, have been prepared with emission peaks at 385, 456, 510, 751, and 879 nm, respectively.[41] Consistent with the prediction of the Jellium model, the emission energy of these Au NCs can be estimated by the relationship of $E_{\text{Fermi}} = N/3E_F$, where $E_{\text{Fermi}}$ and $N$ represent the Fermi energy of bulk gold and the number of Au atoms in individual cluster, respectively. Recently, Yang et al. reported that the addition of an Ag atom into the metal interstice of a radar-like $[\text{Ag}_{27}(\text{StBu})_{14}(\text{S})_2(\text{CF}_3\text{COO})_9(\text{DMAc})_4]\text{DMAC NC}$ (DMAC = dimethylacetamide) can light-up its luminescence (Figure 3A).[50] In addition, doping MNCs with heterogeneous metal atoms can also be used to tailor their luminescence properties. The electronic synergy between different metal atoms will change the electronic structure of MNCs. Wang et al. reported that doping $[\text{Au}_{25}(\text{PPPh}_3)_{10}(\text{SC}_{2}\text{H}_4\text{PH}_3)\text{Cl}_2]^{2+}$ with 13 Ag atoms can greatly increase its emission by 200 times (Figure 3B).[51] In addition, the emission of Au25 NCs was enhanced by 26 times by doping with Au heteroatoms.[52] Moreover, it is reported that the oxidation state of the metal core has an effect on the optical properties of MNCs. After being reduced by NaBH4, the luminescence of Au NCs stabilized by polyethyleneimine (PEI) can change from green to blue.[53]

Surface ligands can not only protect MNCs from agglomeration, but also affect the electronic transitions of MNCs. As mentioned above, in Au25 NCs stabilized by ligands with different electron-donating abilities, the emission properties are very different. In a recent study, by simply incorporating a short bidentate dithiol ligand (as a co-ligand) into the surface of the mercapthoxenoic acid (MHA)-Au25 NCs, their luminescence can be shifted to the shortwave IR region (900-1700 nm) with enhanced emission intensity.[54] In addition, the vibration and rotation of the ligands will also affect the emission intensity of MNCs. The vibration and rotation of the ligands will induce the non-radiative decay, which is one of the main relaxation pathways for the excited electrons. Therefore, the luminescence of MNCs can be enhanced by restricting the thermal motion of ligand molecules. This can be easily achieved by the controlled immobilization and aggregation of M(I)-L complexes (L denotes protecting ligand) on the M(0) core of MNCs. As mentioned above, Luo et al. demonstrated that the vibration and rotation of the SR ligands can be significantly inhibited by inducing the aggregation of the Au(I)-SR complexes on the Au(0) core, yielding a new AIE-type Au(0)@Au(I)-SR NCs emitting strong orange light (Figure 3C).[32] Similar strategies have been widely used to synthesize various Au, Ag, and alloy NCs.[33, 55, 56] In addition, the QY of AIE-type Au25(SG)13 can be further increased to 60% by coating with tetra-octyl-ammonium (TOA) cations to further rigidify their protecting shell (Figure 3D).[57] Besides, impregnating MNCs within biopolymers (e.g., chitosan, polypeptide etc.) is another effective route to improve the luminescence of AIE-type MNCs.[58, 59] For example, chitosan, a natural polymer with abundant positively charged amine groups in the backbone, can electrostatically interact with negatively charged thiolate-stabilized MNCs, leading to the formation of self-assembled MNCs/chitosan.
aggregates. The “spatial confinement” effect in these aggregates can largely restrict the intramolecular motion of the surface ligands of MNCs and promote the luminescence of MNCs. A marked advantage of using these biopolymers to regulate the luminescence of MNCs is their excellent stability and biocompatibility, which could vastly add to the biomedical applications of MNCs.

In addition to changing the metal core and surface ligands, the luminescence properties of MNCs can also be adjusted by changing external environment factors, such as temperature, pH, and solvent viscosity. An increase in temperature will cause more intense molecular vibration or rotation, thereby promoting the non-radiative relaxation process and reducing the emission intensity of MNCs. On the contrary, limiting the non-radiative energy dissipation of the excited state by suppressing the vibration or rotation upon cooling will enhance the luminescence of MNCs. Almost all MNCs show temperature-dependent emissions, but their detailed response to temperature may vary. The change of pH usually changes the charge state of the water-soluble ligands of MNCs, thereby affecting the solubility, aggregation state and binding behavior of MNCs in the solution. Since all these attributes can affect the electron transfer process between the ligands and the metal core, it is not surprising that the pH value has an effect on the luminescence of MNCs. The rigidity of the surrounding environment of MNCs also plays a critical role in regulating the luminescence of MNCs. This is because that the motion of surface ligands of MNCs is largely affected by the rigidity of the surrounding solvent. A rigid solvation environment would limit the intramolecular rotations and vibrations of the protecting motifs/ligands of MNCs, and subsequently suppress their non-radiative decay pathways. Hence, improving the environmental rigidity can provide an alternative strategy for enhancing the luminescence of MNCs. Several groups have demonstrated that the luminescence of MNCs is highly dependent on solvent viscosity, as the viscosity is one of decisive factors affecting the rigidity of the surrounding environment of MNCs.

4 BIOSENSING STRATEGIES

Due to its ease of synthesis and functionalization, good luminescence properties, water solubility and biocompatibility, MNCs have become an attractive class of luminescent materials suitable for biosensing applications. For this purpose, several strategies have been developed to effectively and accurately modulate the luminescence signal of MNCs, which helps to selectively and sensitively detect various biological analytes, including ions, small biomolecules, DNA/RNA, proteins and cells. Here, we summarize the main strategies for constructing biosensing systems based on MNCs in recent years. Representative examples of these strategies and the performances of MNC-based luminescent probes in these examples can be found in Table 1.

4.1 Composition change

Since the luminescence properties of MNCs largely depend on their composition (e.g., number, valence and aggregation patterns of metal atoms or protecting ligands), changing the composition of MNCs by the analytes will affect their luminescence, which can be used as a convenient mechanism for


| Strategies | Nanoclusters | Analytes | Detection range | Limit of Detection | Ref. |
|------------|--------------|----------|-----------------|--------------------|------|
| Composition Change | PMAA-AgNCs | Cys | 0.025-6.0 μM | 20 nM | [68] |
| DNA-Ag NCs | Acetylcholinesterase | 0.1-1.25 U/L | 0.05 U/L | [69] |
| HRP-Au NCs | H₂O₂ | 0.1-100 μM | 30 nM | [70] |
| GSH-Au NCs | hROS | 0.2-100 μM for •OH | 0.1 μM | [71] |
| BSA-Au NCs | Glucose | 5.0 μM-2.5 mM | 0.30 μM | [72] |
| His-Au NCs | GSH | 150-1200 μM | 0.2 μM | [73] |
| Peptides-Au NCs | Protein Kinase A Casein Kinase II | 0.4-3 U/ml | 0.1 U/ml | [74] |
| DNA-Ag NCs | S1 nucleases | 2-70 U/ml | 0.14 U/ml | [75] |
| Interaction with the core/ligands of MNCs | BSA-Au NCs | Hg²⁺ | 1-20 nM | 0.5 nM | [76] |
| ATT-Au NCs | Arginase | 0.0625-1.15 U/ml | 0.056 U/ml | [77] |
| ATT-Au NCs | AflatoxinB1 | 0.01-30 ng/ml | 0.34 pg/ml | [78] |
| Energy/electron transfer | DNA-Ag NCs | Pathogenic DNAs/ATP/Thrombin | – | 0.5/2.5/2.5 nM | [79] |
| L-proline-Au NCs | AflatoxinB1 Zearealenone | 0.005-100 ng/ml | 0.34 pg/ml | [80] |
| BSA-Au NCs | | 0.005-100 ng/ml | 0.53 pg/ml | |
| FA-Au NCs | Butyrylcholinesterase | 5-100 ng/ml | 4 ng/ml | [81] |
| DNA-Ag NCs | DNA | 1.0-100 mM | 0.6 mM | [82] |
| DNA-Ag NCs | ATP | 0.02-1.0 μM | 8.0 nM | [83] |
| Cys/NAC-Au NCs | Acid phosphatase | 0.1-5 U/L | 0.05 U/L | [84] |
| Internal Filter Effect | BSA-Au NCs | H₂O₂ | 1-100 μM | 0.8 μM | [85] |
| MUA-Au NCs | Acid phosphatase | 1-100 μM | 1.4 μM | [86] |
| β-CD-Au NCs | Nitrophenol isomers | 1-50 μM | 0.21 μM | [87] |
| Aggregation-Induced Emission/Quenching | GSH-Ag NCs | Pyrophosphatase | 2.1-35.0 μL | 0.7 U/L | [88] |
| DNA-Ag NCs | ATP Cytochrome c | 1-200 nM | 0.42 nM | [89] |
| Microenvironmental Impact | GSH-Au NCs | Temperature | 25-45°C | – | [61] |
| NAC-Au NCs | pH | 6.05-6.40 | – | [90] |
| Nanoclusters Formation | DNA-Ag NCs | MicroRNA | 1 aM-1 nM | 2 aM | [91] |
| DNA-Ag NCs | Adenosine deaminase | 0-100 U/L | 0.05 U/L | [92] |
| PMAA-AgNCs | Sarcosine oxidase | 2.5-15 units | 0.04 unit | [93] |

Sensor development. Since S has a strong affinity for noble metals (e.g., Au and Ag) and can induce etching or decomposition reaction of the corresponding MNCs, thiol-containing biomolecules are one of the most common analytes that can be detected by this method. For example, Shang et al. synthesized Ag NCs protected by PMAA and found that their emission can be quenched by cysteine (Cys), which should be attributed to the binding of the thiol group of Cys to Ag NCs.[68] This luminescence quenching mechanism allows sensitive detection of Cys with a limit of detection (LOD) of 20 nM. Besides PMAA-protected Ag NCs, many other MNCs also use strong Ag/Au-S bonds to induce fluorescence turn-on or turn-off to detect thiols.[94–96] However, since the Ag/Au-S interaction is not specific to a particular thiol ligand, most of these sensing systems are lacking of selectivity. Therefore, Yuan et al. proposed that the steric hindrance of biothiols may provide a good means for its selective detection. The authors developed a luminescence probe based on GSH-protected Ag NCs (Figure 4A), which can selectively detect less-bulky Cys against GSH.[97] In addition to directly sensing thiol compounds, thiol-induced etching or decomposition reactions can also be used to detect the activity of enzymes that catalyze the biological reactions producing thiols. For example, because acetylcholinesterase (AChE) can catalyze the hydrolysis of acetylthiocholine to produce thiocholine, Zhang et al. developed an assay based on DNA-protected Ag NCs to evaluate the activity of AChE, where the hydrolysate thiocholine can react quickly with Ag NCs and change their luminescence.[69] Similarly, based on the catalytic conversion of oxidized glutathione (GSSG) to GSH,
the activity of GSH reductase can be evaluated by a similar method.\[98\]

Some small biomolecules and species with redox activity, such as hydrogen peroxide (H$_2$O$_2$), ascorbic acid (AA), dopamine (DA), and reactive oxygen species (ROS), can react with MNCs to change the valence of metals in MNCs, which provides another mechanism for changing their luminescence. Based on this redox reaction of MNCs, many analytes can already be detected by MNC-based luminescent probes. For example, Wen et al. used horseradish peroxidase (HRP) as a functional template to directly synthesize luminescent Au NCs. HRP can also catalyze the reaction between Au NCs and H$_2$O$_2$ to detect H$_2$O$_2$ sensitively.\[70\] Chen et al. developed a dual-emission nanocomposite by decorating dye-encapsulated silica particles with GSH-Au NCs, which can be used for in situ ratiometric detection of highly reactive oxygen species (hROS).\[71\] Jiang and co-workers functionalized GSH-Au NCs with positively charged ligands, such as thiolated quaternary ammonium salt (QA), Tat and oligoarginine peptides, to improve the cellular uptake of Au NCs for intracellular hROS detection (Figure 4B).\[99\] Similarly, biomolecules related to the production or elimination of reactive redox substances can also be detected based on luminescent MNCs. For example, the emission change of bimetallic Au/Ag NCs induced by AA can be used to detect acid phosphatase that can catalyze the hydrolysis of 2-phospho-L-AA to AA.\[100\] In addition, based on redox-induced changes in the composition of MNCs, glucose can be detected (e.g., glucose can be hydrolyzed to produce H$_2$O$_2$).\[72\]

Since the ligands on the surface of MNCs play a vital role in the luminescence of MNCs, the emission change caused by the change or removal of the ligands can be used for biosensing applications. For example, the ligand exchange between histidine (His)-Au NCs and GSH can enhance the emission intensity of Au NCs, providing a simple mechanism for detecting GSH.\[73\] In addition, the hydrolysis of DNA or peptides in MNCs protected by peptides has become a versatile method for quenching cluster luminescence. The hydrolysis-induced luminescence changes can be used to selectively detect the corresponding peptidases and nucleases. For example, Qiu and co-workers developed a simple sensor to monitor the activity of protein kinase A and carboxypeptidase Y, based on the quenching of MNC emission induced by the digestion of their peptide ligands.\[74,101\] Similarly, the S1 nuclease that can hydrolyze the phosphodiester bond in the nucleic acid backbone, can be detected by using DNA-Ag NCs.\[75\]

Changing the composition of MNCs to modulate their luminescence is a simple and effective strategy to design optical biosensors with high sensitivity. This strategy is direct, convenient, easy to implement, and has achieved great success. However, the practical application of MNC-based biosensors requires some attention. A noteworthy issue is their selectivity, especially in complex biological and physiological environments. It is not easy to improve their selectivity and anti-interference ability in those complex environments, which requires intense research efforts from various research communities.

### 4.2 Interaction with the core/ligands of MNCs

In addition to changing the core or ligands of MNCs, certain ions or molecules can also interact weakly with MNCs to form complexes. In this way, the luminescence of MNCs can also be adjusted since the complexation process may change the electronic structure or ligand vibration and rotation of MNCs. Therefore, based on the weak interaction between the analytes and the MNCs, several biosensors have been...
developed. For example, Xie et al. reported for the first time that Hg$^{2+}$ can react with the Au(I) species of bovine serum albumin (BSA)-Au NCs through 5d$^{10}$-5d$^{10}$ interactions, thereby changing the electronic structure of Au NCs to quench their strong red emission (Figure 4C).[76] Therefore, the authors were able to create a sensitive Au NC-based sensor for detecting toxic Hg$^{2+}$. Since then, by using similar metallophilic interactions, a large number of MNCs, including poly(N-isopropylacrylamide)-Au NCs, GSH-Ag NCs, and DNA-Ag NCs, have been used for sensitive detection of Hg$^{2+}$.[102–104] In addition, it has been documented that host-guest recognition interaction can enhance the luminescence of Au NCs, which may limit the vibration and rotation of surface ligands.[105] After introducing L-arginine (Arg) into 6-aza-2-thiethymine (ATT)-Au NCs, Arg can form a supramolecular host-guest assembly with ATT through hydrogen bonding. This host-guest interaction can inhibit the vibration and rotation of the ligands on the surface of the Au NCs, resulting in a greatly enhanced cluster emission (Figure 4D). On this basis, some sensing systems have been constructed. For example, Deng et al. reported a simple fluorescent (FL) method for continuous detection of arginase and its inhibitors based on the aforementioned host-guest interaction.[77] Arginase can catalyze the hydrolysis of Arg and therefore reduce the emission intensity of Arg/ATT-Au NCs hybrids. Similarly, by using this hybrid and competitive immunoreaction, Wang et al. designed an enzyme immunoassay for the detection of aflatoxin B1 (AFB$_1$).[78] The immunoreaction was carried out on AFB$_1$-BSA-coated microplate, and arginase-labeled anti-AFB$_1$ antibody was used as a competitor. AFB$_1$ will compete with the arginase-labeled anti-AFB$_1$ antibody, thereby reducing the binding of the antibody on the microplate. The free arginase-labeled anti-AFB$_1$ antibody in the solution can catalyze the hydrolysis of Arg and reduce the emission of Arg/ATT-Au NCs hybrids.

The non-destructive interaction with MNC core or ligands provides another effective mechanism for the development of biosensors. However, there are relatively few biosensors developed based on this detection principle, because a limited number of analytes can exhibit specific and non-destructive interactions with MNCs. The range of analytes detectable by this sensing strategy needs to be expanded in the near future.

### 4.3 Energy/Electron transfer

Generally, energy transfer (ET) has two models: 1) Förster resonance energy transfer (FRET); and 2) nano-surface energy transfer (NSET).[106] They are both non-radiative processes through dipole-dipole or dipole-surface interaction between the energy donor and the nearby acceptor. The efficiency of ET depends on the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, as well as the distance between the donor and the acceptor. ET is very attractive for biological analysis because it is inherently sensitive to the nanoscale variation of the donor/acceptor separation distance and the interference of matching energy (spectrum).[107,108] With their adjustable emission and absorption features, MNCs can be used as a donor or acceptor in an ET-based sensing system. The sensing mechanism is usually based on the analyte-induced separation of the donor and acceptor or changes in the absorption of the acceptor. As energy donors, MNCs can be integrated with multiple types of acceptors to construct ET-based biosensors. Commonly used acceptors include organic dyes, graphene oxide (GO), MnO$_2$, WS$_2$, CoOHH/FcOOH, metal NPs, polydopamine, etc.[79–81,109–113] For example, Liu et al. designed a hybrid system consisting of DNA-Ag NCs as the donor and GO as the acceptor for the selective detection of DNA, adenosine triphosphate (ATP) and thrombin (Figure 5A). GO can absorb single-stranded DNA-stabilized Ag NCs onto its surface through π–π interactions. The ET generated between GO and Ag NCs can effectively quench the NC luminescence.[79] After introducing the analytes (e.g., complementary DNA strands), DNA-Ag NCs can be desorbed from the GO matrix through the complementary DNA pairing reaction. Since ET is highly dependent on distance, the desorption of Ag NCs from GO will inhibit the ET process and regenerate the cluster emission as a readout signal for detection. It should be noted that single-stranded DNA can be used as an aptamer to specifically bind to the substrate molecules (e.g., ATP and thrombin). Based on this specific interaction, GO decorated with DNA-Ag NCs has also been developed as an aptamer sensor. In a separate contribution, Zhang et al. combined FeOOH nanorods with folic acid (FA)-Au NCs to establish a sensing platform for butyrylcholinesterase (BChE) (Figure 5B).[81] FeOOH quenched the luminescence of FA-Au NCs by ET. However, in the co-existence of BChE and acetylthiocholine, such an ET pathway may be ineffective, in which FeOOH was effectively decomposed by the enzymatic hydrolysis product of acetylthiocholine (i.e., thiocholine). The emission of Au NCs can then be recovered, thus constituting a luminescence “turn-on” mechanism for detecting BChE. In addition to energy donors, MNCs can also act as energy acceptors in ET-based sensing systems. For example, Pu et al. developed an ET-based probe for intracellular Hg$^{2+}$ detection, where polyhedral oligomeric silsesquioxane (POSSFF) was used as the donor and BSA-Au NCs was used as the acceptor.[114] Xiao et al. studied ET from the organic dyes to DNA-Ag NCs, and constructed an ET-based biosensing platform for analyzing nuclease activity.[75]

Photo-induced electron transfer (PET) is another widely used method of modulating the luminescence of MNCs. Unlike energy transfer based on dipole-dipole or dipole-surface interactions, PET usually occurs in the strong coupling range, involving electronic interactions between electron donors and acceptors.[115] Rather than the spectral overlap requirement in energy transfer, the occurrence of electron transfer requires a match between the excited state energy and the redox state of the electron transfer pair.[116] Therefore, PET-based sensors are constructed based on changing the distance between the electron donor and acceptor or their redox state. For example, PET from DNA-Ag NCs to G-quadruplex/hemin complexes has been documented (Figure 5C).[82] Based on the formation of hemin complex near Ag NCs or the distance change between Ag NCs and hemin complex that are induced by the recognition interaction between the sensor and the analyte, the PET system enables the specific and versatile detection of biomolecules, such as DNA, ATP, and Argonaute-2.[82,83] In addition, Deng et al. successfully designed a PET sensor for evaluating acid phosphatase (ACP) activity, where Cys/N-acetyl-l-cysteine (NAC)-Au NCs and pyridoxal phosphate...
(PLP) were used as electron acceptor and donor, respectively (Figure 5D).[84] Since PLP can be converted to pyridoxal (PL) by ACP, the electron transfer process established between Au NCs and PLP will be disturbed in the presence of ACP. The luminescence change of Au NCs can then reflect the activity of ACP.

MNCs-based analysis systems that use energy/electron transfer as the sensing mechanism are relatively more complicated than the systems discussed in the previous sections. Although delicate efforts may be required to investigate the appropriate conditions for energy/electron transfer to occur, in biosensing applications, the strict electronic and distance requirements in energy/electron transfer events can indeed be easily transmitted into high selectivity and sensitivity. Therefore, the rational design of energy/electron transfer in the MNCs-based analysis systems is very attractive and has potential applications in various situations.

### 4.4 Internal filter effect

Inner filter effect (IFE) refers to the absorption of the excitation or emission spectrum of fluorophores by an absorbing species in the same detection system.[117] The overlap between the excitation or emission spectrum of the fluorophores and the absorption spectrum of the absorbers makes this possible. Unlike the energy/electron transfer processes discussed above, IFE does not require a fluorophore-absorber distance. Therefore, distance control cannot be used to design IFE-based sensing methods. Instead, this sensing mechanism only depends on the construction or destruction of spectra overlap. For example, by using BSA-Au NCs and PVP-Au NPs as the fluorophore and absorber, a simple IFE-based assay was developed for the detection of H$_2$O$_2$ and cholesterol (Figure 6A).[85] In this assay, the catalytic growth of PVP-Au NPs by H$_2$O$_2$ caused the SPR band of Au NPs to overlap with the excitation band of Au NCs, thereby quenching the cluster emission by IFE to detect H$_2$O$_2$. This method can also be used to detect cholesterol that produces H$_2$O$_2$ in the presence of O$_2$ and cholesterol oxidase. In another study, Liu et al. developed a new method for measuring alkaline phosphatase (ALP) by inhibiting the IFE between MUA-Au NCs and p-nitrophenylphosphate (pNPP).[86] The absorption spectrum of pNPP overlaps well with the excitation spectrum of MUA-Au NCs. Therefore, in the presence of pNPP, the luminescence of Au NCs can be significantly quenched by IFE. ALP can catalyze the hydrolysis of pNPP to p-nitrophenol, which shows a different absorption spectrum from that of pNPP. The resultant absorption spectrum overlaps less with the excitation spectrum of Au NCs, which leads to the recovery of the emission of Au NCs (Figure 6B).

Yang et al. reported a rapid identification method of nitrophenol isomers by using various IFEs with Au NCs that emit different colors.[87] Because the IFE in the detection system lacks distance dependence, compared with the energy transfer discussed in the previous section, IFE is usually more likely to occur between MNCs and other substances in complex biological systems. However, relatively loose distance requirements also make them vulnerable to environmental interference.
4.5 AIE/quenching

AIE is a photo-physical phenomenon in which materials exhibiting non-luminescence or weak luminescence emit intensely when aggregated, where aggregation suppresses the non-radiative relaxation pathway of these materials.[118] Since Tang and co-workers discovered the AIE effect in 2001, it has evoked great interest in the field of biosensing.[119] By noting that Au(I)-SR complexes are a good family of AIE luminogens (or AIEgens for short), where the non-luminescent Au(I)-SR complexes show strong emission after solvent- or ion-induced aggregation, our group introduced the AIE mechanism into the MNC research to rationalize and customize the emissions of MNCs.[32] Based on this concept, a series of AIE-type MNCs can be obtained by modulating the M(I)-SR motifs on the surface of MNCs, which can be further used to construct luminescent probes for biosensing applications. For example, we reported the aggregation of short Au(I)-SR motifs on the surface of GSH-protected Au NCs via Ag⁺ bridging, significantly enhancing the luminescence of the pristine Au NCs.[120] Based on this AIE method, an effective optical probe can be developed to detect trace amounts of Ag⁺ with a linear detection range of 0.5-20 μM.[121] Moreover, by triggering the aggregation of GSH-Ag NCs by Al³⁺, an AIE-based pyrophosphatase (PPase) assay was developed (Figure 6C).[88] It was found that pyrophosphate ions (PPi) have the ability to coordinate to Al³⁺ competitively, which can prevent the formation of Ag NCs-Al³⁺ aggregates. PPase catalyzes the hydrolysis of PPi to release Al³⁺, which triggers the aggregation of Ag NCs, thereby enhancing the luminescence of Ag NCs as a “turn-on” signal to detect PPase. Besides thiolate-protected MNCs, DNA-Ag NCs have recently been shown to have AIE activity.[89] It was found that DNA-Ag NCs have enhanced emission after being immobilized on the surface of GO. Based on this observation, DNA-Ag NCs/GO hybrid was fabricated for label-free detection of ATP and cytochrome c (Figure 6D).

AIE is an interesting strategy to enhance the luminescence of MNCs. This strategy is particularly effective for constructing luminescent “turn-on” probes that can deliver relatively high sensitivity in biosensing applications. However, due to its short development history, the development of AIE-based biosensors is still at an early stage. In future research, it is still crucial to develop more AIE-type metal NCs and more effective strategies to trigger AIE of MNCs. Moreover, because the aggregation of MNCs is mostly sustained by weak supramolecular interactions between MNCs, the stability of aggregates in complex biological matrices should be carefully evaluated to ensure their reliable performance in the in vivo applications.

The aggregation-induced quenching (AIQ) phenomenon, which is contrary to AIE, has also been demonstrated in MNCs, and has also been used to design sensing systems. For example, Li et al. prepared H⁺-triggered self-assembled Au NCs (AuEH) based on egg white-functionalized Au NCs.[122] These AuEH can be used as probes for the detection of multianalytes. One of the analytes is Fe³⁺ that can trigger the aggregation of AuEH by the complexation of Fe³⁺ with amino acids on the surface of AuEH. The aggregation induced by Fe³⁺ can quench the luminescence of AuEH, and this AIQ mechanism can be used to quantitatively determine the concentrations of Fe³⁺. The AIQ strategy has also been used for the detection of other biological substances including AA,[123] melanin,[124] Acinetobacter baumannii,[125]
etc. However, the AIQ-based assays usually adopt the luminescence turn-off mechanism, which is prone to the interference by other quenchers in the environment. False-positive signal and low accuracy of this analytical strategy are critical problems that need to be addressed.

4.6 Microenvironmental impact

The microenvironment (e.g., temperature and pH) surrounding the MNCs also has a significant impact on the luminescence of MNCs. Basically, the assay based on the impact of the microenvironment of MNCs is to use the MNCs to directly detect environmental parameters or analytes that can change environmental parameters. For example, based on the high temperature sensitivity of lipoic acid-protected Au NCs, intracellular thermometry can be developed to detect temperature differences in living cells.[60] A temperature resolution of about 0.3-0.5 °C within a detecting range of 14–43 °C can be achieved by the as-designed thermometer in HeLa cells, which is even more sensitive than many molecule-based optical thermometers. In order to improve the accuracy and reliability of MNC-based temperature sensing, a dual-emission carbon dots/Au NCs nanohybrid was developed to construct a ratiometric FL thermometer in living cells.[61] Such a thermometry would eliminate possible interference caused by probe concentration, excitation efficiency and other local environmental parameters, thereby achieving high sensitivity and reliability.

It is important to accurately estimate the pH value in the biological environment in many biological or disease progressions. It is widely found that the luminescence of MNCs is sensitive to pH. For example, it is proved that the luminescence of NAC-Au NCs is extremely sensitive to pH.[90] When the pH changed from 6.05 to 6.40, a significant decrease in luminescence was recorded on NAC-Au NCs. This ultra-sensitive pH responsive characteristic can be used to develop an effective sensing platform for urea, urease, and urease inhibitors. The hydrolysis of urea catalyzed by urease produces ammonia, which is a basic molecule that can change the pH of the surrounding environment of Au NCs. In a recent study, Au NCs stabilized by nicotinamide adenosine dinucleotide (NAD) with two acidic phosphoric groups were used as ratiometric FL pH sensors with a working pH range of 3 to 11.[126] Specifically, the change of pH value will change the chelation degree of bidentate ligands with Au atoms on the surface, resulting in different emission of Au NCs for ratiometric pH sensing (Figures 7A and B).

In addition to small molecules that are sensitive to the environment, the sensitivity of large biomolecules to the environment can also be used in biosensing applications of MNCs. A large number of studies have shown that the luminescence of DNA-Ag NCs is sensitive to DNA sequences and other nanomaterials around them.[127,128] Due to the limited understanding of the structure and luminescence mechanism of DNA-Ag NCs, the underlying principles governing this environmental sensitivity are still unclear. However, this strategy has been used to develop analytical methods for various biomolecules. For example, Yeh et al. reported that the luminescence of DNA-Ag NCs can be increased by 500 times when they are placed in proximity to the guanine-rich DNA sequences.[129] Based on this interesting finding, some sensing platforms have been developed to detect various analytes.[130–132] The sensing mechanism is mainly based on the analyte-induced approaching/separation of the guanine-rich DNA to/from the Ag NCs. For example, a FL “turn-on” method for thrombin detection was developed according to the above-mentioned sensing mechanism.[130] The two aptamer sequences of thrombin are respectively connected to the nucleation sequence of Ag NCs and a G-rich sequence, respectively. Recognizing thrombin with two aptamer sequences will bring Ag NCs close to the G-rich sequence, thereby enhancing the emission of Ag NCs. In
addition, Liu et al. found that the luminescence enhancement of Ag NCs can be achieved by placing a weakly FL DNA-Ag NC pair in proximity. The hybridization of the two DNA sequences of the Ag NC pair with the target DNA would bring the Ag NC pair together. Considering that base mismatches will affect the hybridization and distance of two Ag NCs, this finding can be used to pinpoint the location of single nucleotide polymorphisms.

4.7 | Nanoclusters formation

As discussed above, MNCs can be conveniently prepared by reducing metal ions in various templates. Generally, the synthesis of MNCs is a mild process, easy to operate and sensitive to the scaffold condition. This motivates researchers to control the formation of MNCs to develop sensing methods. In the current strategies for the formation of MNCs, in situ generation of MNCs in DNA templates is the most commonly used approach for this purpose. The growth of MNCs shows a sensitive dependence on the sequence and structure of the template DNA, where only the selected DNA templates can adapt to the growth of MNCs. Therefore, the controlled release of a specific DNA sequence can be used to modulate the formation process of MNCs, thereby regulating the luminescence of the resulting MNCs. For example, based on the release of template DNA sequences induced by the analyte, DNA-protected Ag NCs were synthesized and utilized as label-free molecular beacons for the quantitative detection of multiplexed DNA (Figures 7C and D). DNA-protected Ag NCs can be synthesized through the formation of a six-base cytosine loop induced by the analyte. This mechanism has been used to detect adenosine deaminase and its inhibitors.

In addition to DNA templates, polymer templates can also be utilized to accommodate the in situ formation of MNCs, providing another strategy for biosensor development. For example, by using PMAA as a stabilizer, Zhao et al. designed a chemical information processing system based on Ag NCs to detect various metabolites and enzymes. The authors used oxidases to promote the production of H2O2. Through the reaction with Fe2+, H2O2 can generate hydroxyl radicals and further initiate the polymerization of MAA into PMAA as a protecting template for Ag NCs. The production rate of PMAA and Ag NCs is closely related to the activity of the oxidases, and can be used to analyze a series of oxidases.

Using the formation mechanism of MNCs as the detection principle is a special strategy for establishing MNC-based sensing systems, because it is usually difficult to implement with other luminescent materials. The realization of this detection principle benefits from the simple and mild synthesis conditions of MNCs. This detection principle based on cluster formation enriches the sensing strategies of MNCs. However, since the formation of MNCs requires a certain concentration of reactants, the developed analytical methods may be less sensitive. Only when a sufficient amount of template molecules and metal ions are present can the formation of MNCs be triggered. Therefore, this sensing principle can be combined with some signal amplification strategies to improve sensitivity.

5 | BIOIMAGING

An effective way to study biological components in living organisms or to understand biological processes occurring in vivo is to make them visible. Optical imaging technique is a useful tool to achieve this goal. By using FL imaging agents, visualization of various biological components and real-time monitoring of dynamic biological processes can be achieved. Compared with other traditional imaging methods, FL imaging has the advantages of non-invasiveness, high signal-to-noise ratio, and excellent temporal and spatial resolution. So far, several FL imaging materials have been developed, and these materials can be mainly divided into two categories: organic molecules and inorganic nanomaterials. Generally, most organic dyes suffer from photo-bleaching or phototoxicity. In recent years, some luminescent inorganic nanomaterials, such as quantum dots, rare-earth doped NPs, and carbon materials, have also found potential applications in bioimaging. However, they often face some inherent challenges that limit their bioimaging applications. These challenges include complicated synthesis procedures, high in vivo toxicity, difficulty in biodegradation, and short emission wavelengths. Luminescent MNCs can bypass some inherent problems of organic dyes and conventional inorganic nanoparticles. Luminescent MNCs possess a series of attractive features such as easy synthesis and conjugation, excellent photostability, efficient renal clearance, large Stoke shift, long emission lifetime, and tunable emission wavelength, which make them a promising class imaging agents for biomedical applications.

5.1 | Single modal imaging

Among the various types of MNCs, biomolecule-protected MNCs with biological activity are particularly suitable for bioimaging. For example, GSH is a thiol-containing tripeptide, which exists in almost every cell of the human body and maintains the reducing environment in the cells by scavenging ROS or reactive nitrogen species (RNS). Due to the overproduction of ROS and RNS in cancer cells, the over-expression of GSH in cancer cells has been largely demonstrated. At the same time, GSH is a common ligand for synthesizing MNCs, leveraging on its high affinity to noble metals. Taking these aspects together, Wang et al. reported the in situ synthesis of GSH-protected Au NCs in cancer cells, which can selectively image cancer cells through the strong luminescence of Au NCs. The higher concentration of GSH in cancer cells can immobilize more Au3+, and the in situ reduction of these Au3+ by GSH can produce higher concentration of luminescent Au NCs in tumor tissues. Similarly, the biosynthesis of GSH-Ag NCs can also be performed in situ and used for selective self-imaging of cancer cells and tumors.

Some biomolecules can maintain their biological functions after being incorporated into the protecting shell of MNCs, endowing these biomolecule-stabilized MNCs with specific biological activity. For example, transferrin (Tf), which has a specific ability to recognize Tf receptors in the cell membrane, can be used as a template for Au NC synthesis. The resultant Tf-Au NCs, without any post-synthesis
modification, have specific targeting ability to cells overexpressing Tf receptors. Based on the targeting ability of Tf-Au NCs, a turn-on FL probe was constructed by integrating Tf-Au NCs with GO, which can effectively image HeLa cells (and HeLa tumor-bearing mice) overexpressing Tf receptors. Due to the good fluorescence quenching properties of GO, the fluorescence of Tf-Au NCs disappeared after adding GO. In the presence of Tf receptors, the specific interaction between Tf and its receptors can competitively remove GO from the Tf-Au NC/GO nanocomposite, resulting in the restoration of luminescence of Tf-Au NCs, which can be used to stain cells overexpressing Tf receptors.

In addition to proteins, peptides and DNAs can also serve as both stabilizers and functional surface moieties of MNCs. For example, the cyclic arginine-glycine-aspartic acid (RGD) peptide was used to synthesize Au NCs for targeting and FL imaging of melanoma A375 cells.[148] Zhu et al. used a simple NIR-light assisted method to synthesize Au NCs protected by antimicrobial peptides (AMP), which can simultaneously diagnose and treat cancer and bacterial infections.[149] Both G-quadruplex AS1411 and MUC1 aptamer have been used as ligands and targeting moieties in the synthesis of Ag NCs. In this way, specific recognition and imaging of cancer cells overexpressing nucleolin and mucin can be achieved, respectively.[150, 151] In order to improve the stability of DNA-Ag NCs in the physiological environment, Lyu et al. used cationic polyelectrolytes to modify DNA-Ag NCs.[152] The modified Ag NCs not only have enhanced stability against the nuclease digestion in biological environments, but also have rapid cell imaging capabilities.

Although some achievements have been made in bioimaging applications of MNCs, the wavelength of light used in these systems is mostly in the range of visible to NIR-I (400-900 nm), which has a weak penetration depth in biological tissues. Hemoglobin absorbs light below 650 nm, and some biological tissues have auto-fluorescence in the visible light range (400-700 nm).[153, 154] These interferences will inevitably compromise the performance of MNC-based bioimaging agents and lead to low signal-to-noise ratios in bioimaging events. In order to overcome these obstacles, NIR-II (1000-1700 nm) FL imaging has been proposed in recent years.[155-158] By using the long-wavelength emission in the NIR-II region, the corresponding FL imaging technique shows a series of advantages, such as deep tissue penetration, reduced photo-damage to biological samples, and low background interference (from auto-fluorescence or light scattering), providing broad prospects in high-resolution visualization of biological components. Recently, several interesting contributions have been reported in MNC-based bioimaging in NIR-II window. For example, Chen et al. reported that Au NCs capped by lipoic acid-based sulfobetaine showed an emission peak at ~1000 nm, which subsequently proved their potential for in vivo imaging of blood vessels in mice.[159] More recently, by noting that the atomically precise Au25(SG)18 can emit strongly in the NIR-II region of 1100–1350 nm, Liu et al. used Au25(SG)18 to perform in vivo imaging of brain vessels and monitor the process of tumor metastasis in a mouse model (Figure 8A).[160] In order to improve the NIR-II QY of Au NCs, Yu et al. induced heterogeneity in the protecting shell of Au25 NCs by using MHA and tetra(ethylene glycol) dithiol as mixed ligands.[161] These dual-ligand-protected Au NCs showed strong emission in the NIR-II region with a QY as high as 6%, which allowed the visualization of mouse vascular networks with enhanced spatial resolution and contrast. Recently, Wang et al. found that incorporation of protein corona structure on the surface of Au NCs can shift their emission to the NIR-II region and increase their QY to 1.9% at the same time.[162] The constructed Au NCs showed good photostability under acid conditions and can be used for gastrointestinal imaging.

In addition to NIR-II imaging, another effective strategy is two-photon (TP) FL imaging (a type of anti-Stokes emission technique that is excited by simultaneously absorbing two NIR photons), which can avoid the interference of auto-fluorescence and improve the penetration depth and spatial resolution.[163, 164] The excellent TP absorption cross-section of MNCs makes them suitable for TP bioimaging applications. DNA-Ag NCs were reported to exhibit an ultra-high TP excitation cross-section, up to 35000–50000 Goppert-Mayer (GM) units.[165] In recent years, MNCs with good TP emission properties have been synthesized and used in bioimaging of living cells and biological processes. For example, Oh et al. synthesized Au NCs by using PEG-dithiolane as a protecting ligand to track cellular uptake events by TP emission.[166] Khandelia et al. used BSA-Au NCs loaded with the anticancer drug doxorubicin (DOX) for TP imaging and treatment of HeLa cells (Figure 8B).[167] Jiang et al. prepared Au NCs co-stabilized with zwitterionic ligands and 11-mercaptoundecanoic acid. The synthesized Au NCs have a TP absorption cross-section of 2.27 × 104 GM at ~770 nm, which can be used for one-photon, TP and fluorescence lifetime imaging.[168]

5.2 | Multimodal imaging

The demand for precise medical diagnosis and treatment has motivated the development of various imaging techniques. In addition to FL imaging, there are other imaging methods, such as X-ray computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), photoacoustic (PA) imaging, and ultrasound (US) imaging, which are widely used in the field of bioimaging.[171-174] CT shows high-resolution 3D structural details of hard tissues, but it is difficult to distinguish soft tissues. MRI can provide high spatial resolution, but its sensitivity is relatively low. PET has the highest detection sensitivity, but the spatial resolution is poor. As discussed above, the application of traditional FL imaging technology is limited by the penetration depth of light. Since each imaging technique has its inherent advantages and disadvantages, the integrated multimodal imaging based on two or more imaging techniques has attracted great interest in order to circumvent the limitations of single-modal imaging, as well as to combine the advantages of different imaging techniques into one imaging entity. Therefore, it is promising to integrate FL imaging technique based on luminescent MNCs with other imaging techniques (e.g., CT, PET, MR and PA) to achieve high-resolution and sensitive bioimaging.

Due to its high atomic number and electron density, gold has a high X-ray attenuation coefficient, making it a promising probe for CT and PA imaging. Therefore, Au NCs themselves can be used for FL/CT/PA multimodal imaging
without the need to integrate external functional moieties. For example, GSH-Au NCs and lysozyme-Au NCs were both used as FL/CT dual-modal imaging agents. After being combined with a targeting moiety (e.g., folic acid), they were used to targeted imaging of MGC-803 and HeLa tumors, respectively.[175,176] Pea protein isolates-protected Au NCs loaded with indocyanine green (ICG) were used for FL/CT dual-modal imaging and synergistic treatment of A549 cells.[177] FL and PA channels have also been used in tandem to track the cellular uptake of zwitterion-functionalized Au NCs.[178] These in vitro and in vivo studies prove the usefulness of Au NCs in FL/CT/PA multimodal imaging.

Considering the advantages of MRI, MNCs have also been combined with magnetic agents, such as Gd-based compounds and Fe₃O₄ NPs, for FL/MRI dual-modal imaging. Gd³⁺ is a good MRI agent because it has seven unpaired electrons and exhibits paramagnetism. For example, Liang et al. used cyclodecapeptides as protecting ligands to synthesize Au NCs, and then incorporated with Gd³⁺ for FL/MRI dual-modal imaging.[179] Diethylene triaminepentaacetic acid and diethylene triaminepentaacetic acid dianhydride have also been conjugated to the surface of Au NCs to achieve Gd³⁺ coordination.[180,181] In addition, gadolinium oxide (Gd₂O₃), with a large proton relaxivity than Gd³⁺ chelates, was integrated with BSA-Au NCs and aptamer-Ag NCs for FL/MRI dual-modal imaging.[182,183] In a similar way, iron oxide NPs with excellent magnetic properties can be easily hybridized with MNCs for multimodal imaging.[184]

PET is another non-invasive technique that can be used for multimodal imaging. ⁶⁴Cu is a well-known β⁺ radioisotope and is often used for PET imaging. For example, Hu et al. reported the design and preparation of self-illuminating ⁶⁴Cu-doped Au NCs for FL/PET dual-modal imaging based on Cerenkov resonance energy transfer (Figure 8C).[169] The ⁶⁴Cu dopant is used not only as an energy donor for Au NCs excitation, but also as a positron-emitting radionuclide for PET imaging. At the same time, Au NCs excited by ⁶⁴Cu can self-illuminate in the NIR region for FL imaging.
Compared with dual-modal imaging, triple-modal imaging that combines three functional moieties into one hybrid is usually more accurate in clinical diagnosis. In recent years, we have witnessed some developments of triple-modal imaging techniques based on MNCs. For example, the Gd₂O₃-Au NCs-ICG composites can perform FL/CT/MRI triple-modal imaging and cancer therapy (Figure SD).[170] Ga³⁺-aggregated Au NCs also successfully achieved FL/CT/MRI triple-modal cancer imaging.[185] It has been proved that the integration of Au NCs in the mesoporous silica shell has FL/PA/MRI triple-modal imaging capability.[186]

MNCs with unique fluorescence properties and good biocompatibility have shown great potential in NIR-II, TP and multimodal bioimaging. In order to better use MNCs in bioimaging research, some important issues, such as their affinity for specific biomolecules, the stability in the physiological environment, and cell penetration efficiency, require further systematic and comprehensive research efforts, because these factors directly affect the pharmacokinetics, biodistribution, and clearance of MNCs. In addition, the customizability of the QYs and emission wavelengths of the luminescent MNCs is another area that needs further development. Generally, the NIR-II QY of MNCs is less than 1%, and their emission peaks are shorter than 1100 nm. Therefore, it is desirable to develop MNCs with high QYs and long emission wavelengths in the NIR-II region in biological and clinical applications.

6 | CONCLUSIONS AND OUTLOOKS

In summary, the past decades have witnessed rapid development in the field of luminescent MNCs. The breakthrough in the synthesis of MNCs provides a good foundation for obtaining high-purity MNCs at the unprecedented molecular and atomic level, which is conducive to establishing a reliable structure-property relationship for metal-based nanomaterials. Studies have shown that in-depth research on the mechanism and key influencing factors of cluster luminescence will help to improve the luminescence properties and materials performance of MNCs. Based on the above two developments, MNCs have achieved encouraging applications in the field of biosensing and bioimaging in the past years. Various sensing strategies utilizing MNCs as luminescent probes have been developed to analyze many important biomarkers. The sensing strategies mainly depend on composition changes, interaction with core/ligands, energy/electron transfer, internal filter effects, AIE/quenching, microenvironmental impact, and controlled formation of MNCs. In addition, the applications of MNCs as bioimaging agents have been extensively explored. The imaging window of MNCs can now be extended to the NIR-II regime with deep tissue penetration, reduced photo damage and background interference. In order to integrate the advantages of different imaging techniques, multimodal imaging based on MNCs has attracted increasing interest.

Although significant progress has been made in the biosensing and bioimaging applications of MNCs, there are still some important issues and challenges that need to be addressed. First, only a limited number of atomically precise luminescent MNCs are available in aqueous solution, which largely hinders the biological applications of MNCs. The development of facile and large-scale methods for the synthesis of water-soluble luminescent MNCs with atomic precision is still at the forefront of cluster research. Second, compared with other luminescent materials, such as quantum dots and organic dyes, most luminescent MNCs have relatively low QYs. Efforts should be made to reveal the luminescence mechanism of MNCs and improve their luminescence properties. Third, although many strategies have been developed for the construction of MNC-based biosensors, the existing strategies still have some common problems, such as weak anti-interference capability, limited analysis targets, complex design, and unsatisfactory sensitivity. For the practical applications of MNCs in biological systems, it is equally important to develop new sensing strategies and improving existing sensing strategies. In addition, the in vivo pharmacokinetics, biodistribution, and clearance of MNCs also play a crucial role in their biosensing and bioimaging applications, and therefore these research topics deserve more in-depth investigation.

In summary, luminescent MNCs with numerous unique advantages have shown great potential in biosensing and bioimaging applications. With the collective efforts of experimental and theoretical scientists, we expect that the existing problems and challenges of MNCs will be satisfactorily addressed in the near future, and luminescent MNCs may find broad applications in biomedicine and many other fields.

ACKNOWLEDGMENTS

The authors acknowledge the financial support from Minstry of Education, Singapore (under Research Grant R-279-000-580-112 and R-279-000-538-114) and China Scholarship Council (under Grant No. 201908420311).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ORCID

Jianping Xie https://orcid.org/0000-0002-3254-5799

REFERENCES

1. L. Shang, S. Dong, G.U. Nienhaus, Nano Today 2011, 6, 401.
2. X. Yuan, Z. Luo, Y. Yu, Q. Yao, J. Xie, Chem. Asian J. 2013, 8, 858.
3. I. Chakraborty, T. Pradeep, Chem. Rev. 2017, 117, 8208.
4. S. Gao, E. Wang, Nano Today 2017, 6, 240.
5. S. Maity, D. Bain, A. Patra, Nanoscale 2019, 11, 22685.
6. P. Yu, X. Wen, Y.-R. Toh, X. Ma, J. Tang, Part. Part. Syst. Charact. 2015, 32, 142.
7. Y. Li, T. Higaki, X. Du, R. Jin, Adv. Mater. 2020, 32, 1905488.
8. A. Mathew, T. Pradeep, Part. Part. Syst. Charact. 2014, 31, 1017.
9. H. Zhang, Z. Zhao, P.R. McGonigal, R. Ye, S. Liu, J.W.Y. Lam, R.T.K. Kwok, W.Z. Yuan, J. Xie, A.L. Rogach, B.Z. Tang, Mater. Today 2020, 32, 275.
10. G.A. Ozin, H. Huber, Inorg. Chem. 1978, 17, 155.
11. G. De Cremer, B.F. Sels, J. Hotta, M.B. Roeffaers, E. Bartholomeeusen, Coutinho-Gonzalez, V. Valtchev, D.E. De Vos, T. Vosch, J. Hofkens, Adv. Mater. 2010, 22, 957.
12. T. Sun, K. Seff, Chem. Rev. 1994, 94, 857.
13. Y. Lu, W. Chen, Chem. Soc. Rev. 2012, 41, 3594.
14. Z. Luo, K. Zheng, J. Xie, Chem. Commun. 2014, 50, 5143.
15. M. Zhu, E. Lanni, N. Garg, M.E. Bier, R. Jin, J. Am. Chem. Soc. 2010, 132, 1138.
16. M. Zhu, H. Qian, R. Jin, J. Phys. Chem. Lett. 2010, 1, 1003.
17. Q. Yao, Y. Yu, X. Yuan, Y. Yu, J. Xie, J.V. Lee, Small 2013, 9, 2696.
18. T. Chen, V. Fung, Q. Yao, Z. Luo, D.E. Jiang, J. Xie, J. Am. Chem. Soc. 2018, 140, 11370.
AUTHOR BIOGRAPHIES

**Yan Xiao** received her B.S. (2010) and Ph.D. (2015) degree from the College of Chemistry and Molecular Sciences, Wuhan University. She is currently an associate professor of College of Chemistry and Chemical Engineering, Hubei University. Her current research interest is the study of the luminescence properties of metal nanoclusters and their bioanalysis applications.

**Qiaofeng Yao** received his B.S. (2010) from University of Science and Technology of China (USTC). He then earned his Ph.D. (2015) from National University of Singapore (NUS), under the co-supervision of Prof. Jim Yang Lee and Prof. Jianping Xie. He is currently working as a research fellow with Prof. Jianping Xie at NUS. His current research interests focus on the development of total synthesis and self-assembly chemistry of atomically precise metal nanoclusters.

**Jianping Xie** is currently a Dean’s Chair Associate Professor at the Department of Chemical & Biomolecular Engineering, NUS. He received his B.S. and M.S. in Chemical Engineering from Tsinghua University, China, and his Ph.D. from the Singapore MIT Alliance (SMA) program. He joined NUS in 2010 and established the BioNanoMetals research group. His group is known for their work on engineering ultrasmall metal nanoclusters for biomedical and catalytic applications. His research interests include noble metal nanoclusters, nanomedicine, and cluster catalysis.

**How to cite this article:** Xiao Y, Wu Z, Yao Q, Xie J. Luminescent metal nanoclusters: Biosensing strategies and bioimaging applications. *Aggregate*. 2021;2:114–132. [https://doi.org/10.1002/agt2.11](https://doi.org/10.1002/agt2.11)