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DNA stabilized fluorescent metal nanoclusters for biosensor development

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Abstract

In the past decade, fluorescent silver, gold and copper nanoclusters (Ag, Au and CuNCs) have emerged as a new class of signaling moiety for biosensor development. Compared to semiconductor quantum dots, metal NCs have less toxicity concerns and can be more easily conjugated to biopolymers. Due to their extremely small size, these NCs need a stabilizing ligand. Many polymers, proteins and nucleic acids have been reported to stabilize NCs. In particular, many DNA sequences produce highly fluorescence NCs. Coupling these DNA stabilizers with other sequences such as aptamers has generated a large number of biosensors. In this review, the synthesis of DNA and nucleotide-templated NCs is first summarized; their chemical interactions are also discussed. In the second part, the properties of NCs such as fluorescence quantum yield, emission wavelength and lifetime, structure and photostability are briefly reviewed. In the last part, various sensor design strategies using these NCs are categorized into the following four classes: 1) fluorescence de-quenching; 2) generation of templating DNA sequences to produce NCs; 3) change of nearby environment; and 4) reacting with heavy metal ions or other quenchers. Finally, the future trends in this field are discussed.

***Keywords*:** nanoclusters; DNA; fluorescence; biosensors; quenching; metal

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1. Introduction

Noble metal nanoparticles have made profound impacts in biosensor development since the seminal work in 1996 by Mirkin, Alivisatos and co-workers [1, 2], where thiolated DNA strands were attached to gold nanoparticles (AuNPs). Au and AgNPs are versatile building blocks in the current analytical chemistry owning to their plasmonic [3], surface enhanced Raman scattering (SERS) [4], and fluorescence quenching properties [5]. Attaching DNA allows stimuli-responsive modulation of inter-particle distance or the distance between a particle and an organic fluorophore, generating a change in optical signals [6-10]. Each particle typically contains hundreds to thousands of metal atoms. While these noble metal NPs are best known to be fluorescence quenchers, they can be strongly fluorescent as well. For example, when the size of such NPs is reduced to below 2 nm and each particle contains only a handful of atoms, their plasmonic properties disappear. At the same time, metal atoms might organize into well-defined structures (so called nanoclusters or NCs), forming molecular energy levels to produce strong fluorescence emission [11]. Few-atom fluorescent Au and AgNCs have been known for a long time. However, they started to attract extensive interest of analytical chemists only in the past decade due to advancement in their solution phase synthesis. Since the pioneering work from the Dickson group, NCs can now be routinely prepared in aqueous solutions with a number of polymeric or thiol templates including dendrimers [12, 13], proteins [14], DNA [15], and GSH [16]. This review is focused on DNA-templated NCs.

DNA is a particularly interesting ligand for preparing fluorescent metal NCs and for developing biosensors. 1) Different emission colors ranging from blue to near IR with high quantum yield can be obtained by simply changing the DNA sequence. In this regard, DNA encodes the structure and function of AgNCs, which is more difficult to achieve with other types of polymers. 2) DNA has molecular recognition function. In addition to binding to complementary nucleic acids, DNA aptamers can selectively bind to a diverse range of analytes ranging from metal ions, small molecules, proteins and even cell surface receptors [8, 17-19]. New biosensing modalities can be achieved by rationally

designed DNA sequences to combine molecular recognition and NC templating properties. 3) DNA with arbitrary length, sequence and modification can be made via chemical synthesis. It is more difficult to achieve this level of control with any other type of polymer. With its high stability, it is convenient to use DNA for systematic mechanistic studies and sensor optimization. 4) Finally, unlike organic fluorophores or semiconductor quantum dots, NCs do not need covalent modifications on DNA, allowing a low cost for synthesis. Compared to quantum dots, NCs also have fewer toxicity concerns.

This is a rapidly growing field and the number of publications is increasing exponentially. A number of review papers have already covered the physical chemistry [20-23], synthesis [24], DNA sensing [25-27], and other applications [28, 29]. Herein, we review recent progress in using DNA-templated metal NCs for biosensor development. In particular, we focus on the general sensing strategies that can be applied to different targets by changing the aptamer sequence. Attention is also given to the mechanistic understanding and practical aspects that may impact the trend in future research and application of these new materials.

2. Synthesis of NCs.

For biosensor development, reliable methods are needed to produce stable fluorescent metal NCs in aqueous buffers. Due to their extremely small size and high surface energy, NCs tend to aggregate. Therefore, a stabilizing agent or matrix is required to cap NCs. Before the seminal work in 2002 by Zheng and Dickson who used dendrimers as a stabilizer [12], metal NCs were only prepared in cryogenic noble gases or in solid zeolites [30-32]. Following this initial discovery, the Dickson group further showed that cytosine (C)-rich DNA can also stabilize fluorescent AgNCs [15], which represents the first connection between metal NCs and DNA. There is a strong and specific coordination between the N3 position of cytosine and Ag^+ (Figure 1A), which has been utilized to develop biosensors for Ag^+ [33, 34]. Subsequent work demonstrated that all nucleotides can participate in the binding of metal NCs. Each nucleotide has at least one metal binding site (Figure 1A). Binding to thymine requires

deprotonation of the N3 nitrogen and thus is favored only at high pH. This could be the reason that poly-T DNA has not been a popular template for making NCs. It was soon demonstrated that different AgNC emission colors could be obtained by simply changing the sequence of DNA [35-38]. In 2006, Dickson and co-workers screened a diverse range of DNA sequences using a DNA microarray and identified various sequences that produce blue to near IR emitters (Figure 1B) [39]. In addition, the same AgNC might emit different colors in different local environments. For example, Werner, Martinez and co-workers showed that the sequence in Figure 1C templates a non-fluorescent AgNC. A few emission colors were generated upon hybridizing with a DNA containing different overhang sequences. However, the relationship between the DNA sequence and the optical property of the final product cannot be easily predicted. Future work including computer simulation is needed to provide atomic understandings that might facilitate rational biosensor design.

Compared to making high quality quantum dots, the synthesis of NC is quite simple. In a typical reaction, DNA and Ag^+ are mixed at a certain ratio in water or in a neutral buffer (the ratio of cytosine base to Ag^+ is usually between 1:1 to 2:1), to which freshly prepared $NaBH_4$ is added as a reducing agent. AgNCs are used as an example here since the majority of work was carried out on this material. The product is generally obtained in a few hours and the color of the solution changes to yellow, orange or red, depending on the DNA sequence. Without DNA, Ag^+ and $NaBH_4$ produces yellow or black colored AgNPs. DNA allows controlled reduction and confines the reduced Ag species within the DNA template. Most of the reactions were performed in water or at around neutral pH, where metal binding can easily take place since the pK_a value of the N3 nitrogen on cytosine is ~4.2. On the other hand, the N3 position is protonated at neutral pH on a thymine. For this reason, poly-T DNA templated synthesis was reported only at pH 11 [40]. The N7 position in A and G are high affinity metal binding sites, where other nitrogens in the purine rings can also contribute to metal coordination [41, 42]. Therefore, in a long DNA chain, it is quite difficult to rationally design and predict metal binding sites or the final NC structure within the DNA.

In addition to simple C-rich DNA, many other DNA sequences have also been tested. For example, Shao and co-workers studied the effect of abasic sites in a double-stranded DNA to understand the effect of DNA base stacking. They found that the excited state of AgNCs is stabilized by interacting with the guanine base [43]. Abasic sites can also selectively recognize DNA bases, which was detected based on AgNC formation [44]. Duplex DNA with gaps [45], and mismatches [46] was also studied for AgNC synthesis. Ren and Qu and co-workers reported the formation of AgNCs on triplex DNA [47]. Aside from the traditional scheme of one DNA one AgNC, single AgNCs can also be stabilized by multiple DNA strands [48]. In addition, covalent attachment of multiple DNA on a benzene ring allows for much stronger fluorescence than that afforded by a single-stranded DNA of the same sequence [49]. Usually, the length of DNA is around 12-mer or longer, and it has been documented that 6-mer DNA cannot support fluorescent AgNCs [50]. On the other hand, the cytosine DNA monomer (i.e. 1-mer DNA) can be used to make AgNCs in ethanol, although its synthesis in aqueous buffer has not been reported [51]. G-rich DNA or RNA and G-quadruplex DNA can also make AgNCs [35, 52, 53], but homopolymers of A or T/U cannot at neutral pH. Formation of AgNCs with different DNA sequences has also been calculated using the DFT theory [54].

Fluorescent DNA-stabilized AgNCs are quite susceptible to photobleaching and this problem might be alleviated by using AuNCs. Fluorescent AuNCs have been prepared using polymer and protein stabilizers, while DNA templated AuNCs was reported only recently [55, 56]. The choice of reducing agent has an important effect since when $NaBH_4$ was used, only large AuNPs were obtained, possibly due to its strong reducing power. We initially tested 30-mer DNA homopolymers and obtained blue emitters using citrate as the reducing agent. The C_{30} DNA produces fluorescence only at low pH with a large excess of DNA, while A_{30} worked optimal at neutral pH with the ratio between adenine and $HAuCl_4$ being ~1:1 [55]. The pH and stoichiometric requirements might be related to the coordination between the bases and Au (Figure 1A). Liu and co-workers reported red AuNC emitters from other DNA templates using dimethylamine borane (DMAB) as the reducing agent [56, 57].

Overall, it seems that the emission color of DNA-stabilized AuNCs is mainly controlled by the type of reducing agent instead of the DNA sequence.

Adenine derivatives including adenosine, AMP and ATP can also produce blue emitting AuNCs, but other nucleotides cannot [58]. Even adenine itself cannot and therefore it is important to block its N9 position (Figure 1A) [59]. Citrate was also used as a reducing agent for this reaction. Adenosine and $HAuCl_4$ form large fluorescent particles on the order of a micrometer in size while AMP and ATP form small molecular clusters due to charge repulsion. The coordination between adenine derivatives and gold has been extensively studied; each adenosine has 3 metal binding sites (N7, N1 and N3) [42]. This explains its tendency to form large network structures.

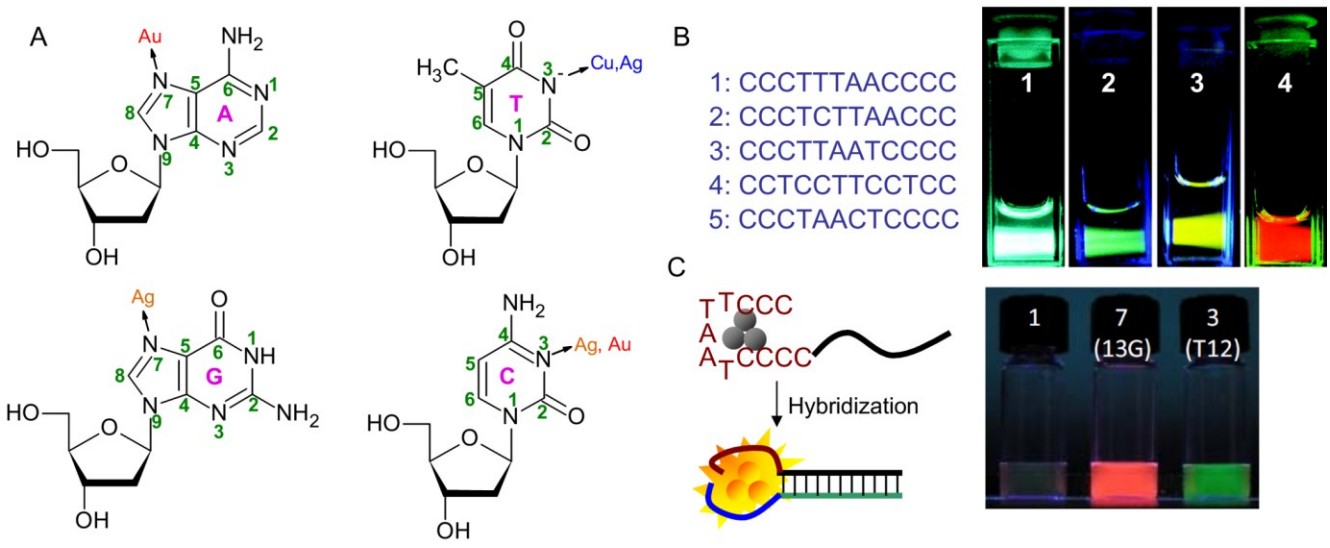

Figure 1. (A) Chemical structures of DNA nucleosides and primary metal coordination sites. The labeled metals are based on the literature reports of using related DNA or nucleotides for making NCs. (B) A few DNA sequences and the fluorescent AgNCs. DNA5 makes a near IR emitter. Reproduced with permission from ref [39]. Copyright 2008 American Chemical Society. (C) AgNCs of different emission colors produced by hybridization with a DNA containing various overhangs (the blue line in the cartoon). Reproduced with permission from ref [60]. Copyright 2010 American Chemical Society.

The amount of work on fluorescent CuNCs is relatively limited. Double-stranded DNA and thymine-rich single-stranded DNA have produced CuNCs [61-64]. Aside from using single metal elements, different emission colors can also be produced when two metals are mixed. Examples of such will be given in the following sections. In addition to making NCs in solution phase, they can also be grown on various materials such as on DNA origami [65], carbon nanotubes [66], graphene oxide [67], and in hydrogels [68, 69]. Overall, the work on synthesis in the past decade has laid a solid foundation for their analytical applications.

3. Characterization of NCs.

After synthesis, the next step is to characterize NCs. For analytical applications, fluorescence is the most important property. Since DNA absorbs strongly in the UV region around 260 nm and this absorption can be transferred to excite the associated NCs [70], a handheld UV lamp can often provide a visual perception on the emission color and intensity. Most NCs also absorb in the visible region, which can be used for directly excitation as well. To compare fluorescence intensity of different synthesis methods, quantum yield is often reported, which can range from below 0.1% to more than 50%. Fluorescence lifetime is another important characterization, reflecting the mechanism and origin of the emission. Many AgNCs have low ns lifetime, similar to organic fluorophores. On the other hand, AuNCs have much longer lifetime in the μs scale, suggesting ligand to metal charge transfer as the origin of their emission [71].

For biosensing, it is important to understand the photostability of NC emitters. Unfortunately, this information is often missing in most analytical chemistry literature. Many AgNCs have reasonable stability when stored in dark. DNA sequence also plays a very important role. For example, Sharma et al screened a large number of DNA templates and identified one particular sequence that can retain more than 30% of the fluorescence after one year [72]. For comparison, AgNCs with many other DNA

sequences are quenched by more than 50% after just one day. We found that DNA templated AgNCs were bleached very quickly when exposed to strong light. A wavelength-dependent study indicates that photobleaching takes place only by the UV light, while little effect was observed at wavelengths longer than 400 nm [73]. We attributed photobleaching to oxidation since bleached AgNCs can be recovered after adding $NaBH_4$. Such oxidation is often accompanied by the splitting of AgNCs into smaller clusters or even dispersed ions. Developing strategies to maintain long term photostability is an important task. It needs to be noted that partial oxidation is often needed for luminescence. In an experiment where oxygen was completely removed, the resulting AgNC was non-fluorescent [40]. Therefore, there is a fine requirement on the redox level of NCs to produce fluorescence. In a few papers, it is reported that some DNA can template two emitters, one with red emission containing 13 Ag atoms and the other with green emission containing 11 Ag atoms. These two are coupled with a redox reaction, where the green emitter is the oxidized form. The red and green emitters appeared to be associated with the same DNA in different conformations, as suggested by analytical gel electrophoresis [74].

Besides fluorescence, to achieve more detailed understanding of NCs, both mass spectroscopy and X-ray crystallography are important methods. Crystallography is more useful for NCs stabilized by small molecules [75-78], where the ligands are often a thiol-containing compound. Since it is more difficult to produce single crystals with DNA, mass spectrometry is a more widely used tool. In a recent report, Gwinn and co-workers screened 80 DNA sequences and used multiple stages of HPLC to purify products that are stable for the HPLC process [79]. With extensive mass spectrometry and simulation work, the authors concluded that DNA stabilized AgNCs contain roughly the same amounts of neutral silver atom and silver cation. In addition, these Ag species align in a rod-like structure instead of planar or globular structures. The reason for the presence of difference emission colors was attributed to the change of the rod length due to specific base/Ag interactions. Cartoons of this model are shown in Figure 2.

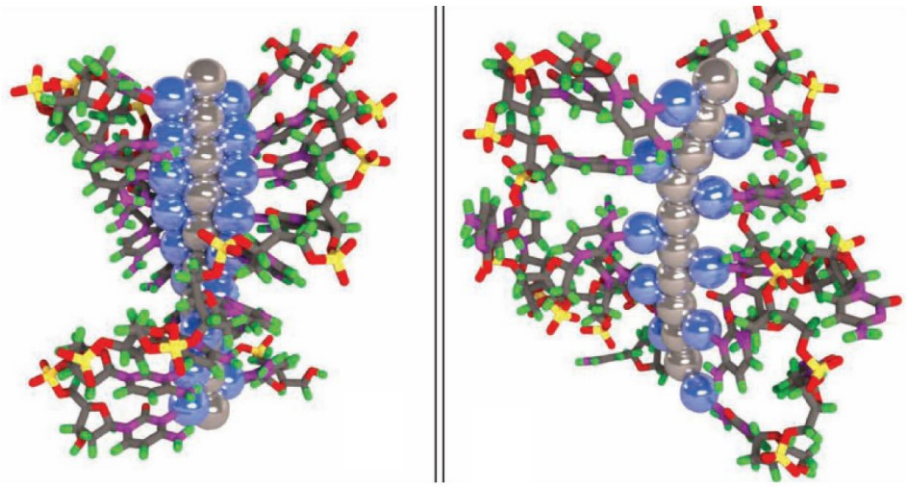

Figure 2. The rod like model of AgNCs with neutral Ag atoms (gray balls) and Ag^+ cations (blue balls) in poly-C DNA. Left panel: in repeat tetramer units. Right panel: in repeat trimer units. Reprinted with permission from ref xx. Copyright ® 2013 John Wiley and Sons.

4. Signaling methods.

An intrinsic advantage of using DNA as a stabilizer is that the programmability and molecular recognition properties of DNA can be rationally incorporated. In addition to complementary nucleic acids, DNA aptamers can selectively bind to a diverse range of analytes, including metal ions, small organic molecules, peptides, proteins and even cells [8, 9, 19, 80-83]. DNA can also be part of material assembly, linking inorganic, polymeric and biological nanoparticles in a programmable way. In other words, by designing the primary and secondary structures of DNA, it is possible to combine NC-based signaling and molecular recognition in the same molecule, which is very difficult to achieve for protein-based biosensors. These properties have offered interesting opportunities in developing NC-based biosensors. In the past few years, various signaling methods have emerged and each can detect many different analytes. Therefore, we review the progress based on the signaling mechanism instead of specific analytes.

4.1. Molecular beacons. The original concept of molecular beacon involves a DNA hairpin with a fluorophore and a quencher respectively labeled on its two ends [84]. The hairpin is initially closed to give low fluorescence. A target DNA opens up the hairpin to increase the distance between the fluorophore and quenching, leading to signal increase [85]. Molecular beacons are attractive analytical probes since they allow for simple homogeneous assays with high sensitivity. Since NCs are naturally conjugated to DNA after the synthesis, a molecular beacon setup is an obvious method of using these NCs as a fluorophore substituent. Similar applications have already been demonstrated using semiconductor quantum dots [86, 87], although it needs to be noted that it is quite difficult to conjugate DNA to an as-synthesized quantum dot. Since DNA is already a part of the NC structure, it is unnecessary to perform additional conjugation reactions. To date, no classical beacon was reported with NC fluorophores in conjunction with typical dark quenchers, but there are examples involving other type of quenchers.

Wang and co-workers discovered that the hemin/G-quadruplex complex could quench AgNC emission via photo-induced electron transfer (PET). They appended a G-rich sequence to a DNA template for AgNC formation. After making AgNCs, they observed fluorescence quenching upon adding hemin (Figure 3A). Based on this observation, they incorporated a DNA hairpin with the probe DNA sequence in the loop region (in red). Since a fraction of the hemin binding sequence is locked in the hairpin, hemin cannot bind and the fluorescence remains high. In the presence of the target, the hairpin opens, freeing the hemin binding sequence and the fluorescence drops (Figure 3B). Similar strategies have been applied to detect ATP using its aptamer [88]. Although this is not the classic molecular beacon design, modulation of the fluorophore-to-quencher distance is key for this method.

Graphene oxide (GO) is a general quencher for a diverse range of fluorophores, including semiconductor quantum dots [87, 89, 90]. DNA can be adsorbed by GO and also desorb by adding its cDNA to form a duplex [91]. Ren and Qu and co-workers used DNA-templated AgNCs as fluorophores and extended the DNA templates to include a probe sequence. They found that the AgNC

fluorescence was quenched upon adsorption of the whole complex by GO. Addition of the target DNA resulted in probe desorption and fluorescence recovery [92]. Using three different AgNCs that emit at different wavelengths, multiplexed detection was demonstrated. Recently, Willner and co-workers employed this strategy and involved aptamers [93]. The advantage of this method is that all the sensor components are pre-assembled. In addition, the fluorescence is increased upon target binding. In general, light-up sensors are more desirable than sensors that decrease fluorescence in the presence of target analytes.

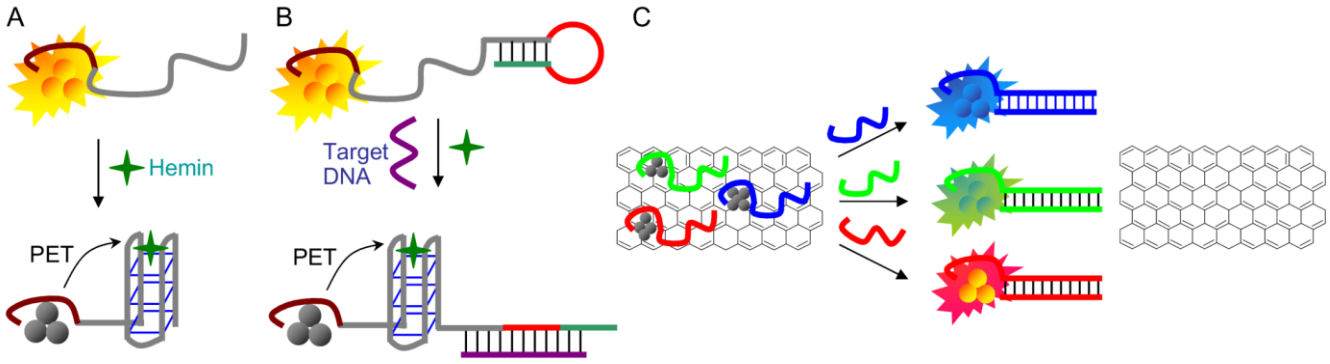

Figure 3. Molecular beacon type of signaling method involving an external quencher. (A) Hemin/DNA complex can quench AgNCs. (B) DNA detection using hemin as a quencher. (C) Multiplexed detection of DNA using AgNC probes on GO. For clarity of the figure, the oxygenated species on GO is not drawn.

4.2. Signaling based on DNA-templated NC synthesis. To prepare fluorescent AgNCs, specific DNA sequences are needed (e.g. C-rich DNA) and sometimes the sequence has to be in a special secondary structure. Therefore, it is possible to achieve detection based on the generation of such sequences or secondary structures. For example, Wang and co-workers found that a C_6 bulge on a duplex DNA can template yellow emitting AgNCs (Figure 4A). Perfect base pairing nearby the bulged region is

important and a single base mismatch results in non-fluorescent products. Based on this observation, the authors achieved sequence selective detection of DNA [94]. In a follow-up work, the authors further demonstrated that emission intensity can be modulated by hybridization or removal of the cDNA as shown in Figure 4B [95]. The cDNA was designed to have an overhang so that it can be removed with a fully complementary strand (in blue). Similar methods have also been used to detect cocaine, ATP, and Hg^{2+}, where these small molecules or ions bring two pieces of DNA together to form aptamer binding pockets, which stabilize C-rich DNA templates for producing fluorescent AgNCs [96, 97].

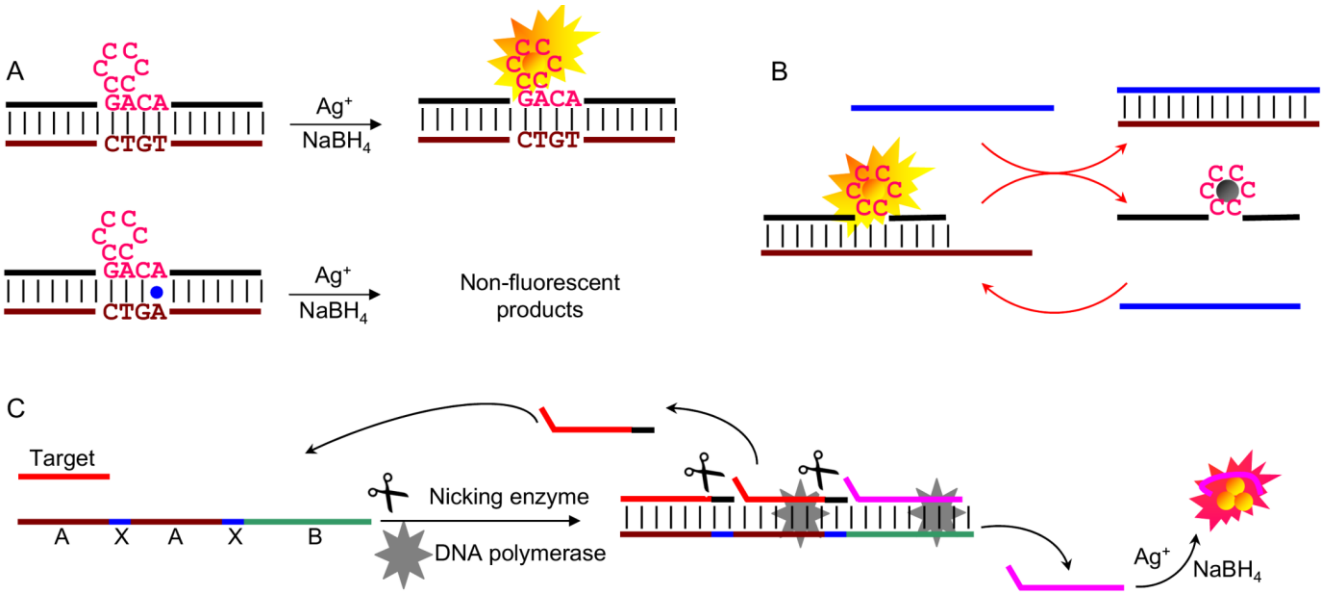

Figure 4. (A) A duplex DNA containing a C_6 bulge can template fluorescent AgNCs, while the synthesis is hindered by the presence of a single base mismatch. (B) The fluorescence of AgNC in the bulge can be modulated by hybridization and removal of the cDNA. (C) A DNA machine method to generate a DNA template for making AgNCs.

The sensitivity of a sensor might be limited if each target DNA only generates one fluorescent AgNC. To increase sensitivity, Ye and co-workers developed a target-triggered isothermal

amplification strategy, where the target microRNA (red strand in Figure 4C) was used to initiate a polymerization reaction upon hybridizing with the DNA template. The template was designed to contain two target binding sequences (brown regions marked with 'A') followed by two nick sites and then a template for making AgNCs (in green marked with 'B'). Upon target hybridization, more target sequences were generated and released with a DNA nicking enzyme. In the same process, more AgNC templating DNAs were also produced. These two amplification mechanisms allow for ultrahigh sensitivity with a detection limit of 10 aM, or ~15 copies of synthetic microRNA in 10 μL [98]. This method also represents a convenient platform for multiplexed detection of different target sequences as demonstrated by the same group [99]. They designed a few different DNA templates, each with a different target recognition region and a different template for different emission colors. In addition to nucleic acid targets, Wang and co-workers used this method for detecting Hg^{2+}, where Hg^{2+} induces a hairpin formation by paring T-T mismatches and allows the DNA polymerization reaction [100]. Similar strategies have also been applied to detect platelet-derived growth factor B-chain homodimer (PDGF-BB) [101]. While high sensitivity can be achieved, an intrinsic limitation of this type of method is that AgNC synthesis is an integrated step of the detection process and a few hours are often needed to generate fluorescence. In addition, the synthesis involves $NaBH_4$, which is an unstable chemical in aqueous solution, limiting its practical applications.

4.3. Hybridization activated fluorescence. Our understanding on the fundamental properties of these NCs is still quite limited. Many discoveries were made accidently and detailed physical explanations are still lacking. On the other hand, such observations have already found many analytical applications. An interesting class of sensor is based on NC fluorescence change upon DNA hybridization. In 2009, Martinez and Werner and co-workers reported such an example [60]. The No. 3 DNA sequence in Figure 1B was previously shown to produce yellow Ag emitters. When this DNA is appended to an A/T rich sequence intended for hybridization, the resulting AgNCs were essentially non-fluorescent. However, upon hybridization with DNA containing a poly-G overhang (Figure 1C), strong red

fluorescence was achieved and the enhancement factor reached over 500-fold. By using different overhang sequences, different emission colors were obtained (Figure 1C) [102]. The authors demonstrated that it is the proximity between the AgNC and the G-rich sequence that enhanced the fluorescence. Based on this understanding, a sensor was designed as in Figure 5A for DNA detection. The target DNA brings non-fluorescent AgNCs and the G-rich sequence close to each other, generating a three-way junction structure and fluorescence. In a follow-up work, the authors further optimized the DNA sequences and demonstrated that a single mutation in the target sequence can result in a large emission wavelength shift [103].

Other types of targets beyond DNA were detected by incorporating aptamers. For example, two aptamers are available to bind different sites on thrombin. Le and Zhu and co-workers extended one aptamer to prepare the non-fluorescent AgNC, while the other aptamer contained the poly-G overhang (Figure 5B). Flexible spacers were also used to separate the aptamers and the signaling DNA fragments. In the absence of the target protein, the affinity between these two DNA was too weak to bring the poly-G DNA close to the AgNCs. The fluorescence was only activated in the presence of thrombin, where binding of both DNA to the same protein has generated a high local effective DNA concentration, facilitating DNA hybridization. The detection limit was reported to be 1 nM thrombin [104]. Ye and co-workers inserted an adenosine aptamer between a AgNC and a piece of G-rich sequence. In the presence of adenosine, the aptamer folds into a binding structure, bringing the poly-G DNA and the AgNC together to increase fluorescence (Figure 5C) [105].

Dong and Wang and co-workers developed a dynamic strand displacement method, where the DNA probe containing AgNCs was first hybridized to a blocking DNA. The G-rich DNA cannot directly displace the blocking DNA and therefore the background fluorescence is low. The target DNA is fully complementary to the blocking DNA and can remove it from the AgNC, allowing the hybridization of the G-rich DNA. This method was applied to detect both nucleic acids and thrombin [106]. Park et al also employed a blocking strand (called competitor in their paper) to form a short

duplex with the AgNC bearing strand (Figure 5D). The sequence for making AgNCs was the same as that used by Werner and co-workers. Since this blocking strand was short, the incoming DNA was able to displace it. Instead of using a G-rich sequence, the authors used a C_{12} sequence to be close to the original AgNC. In this case, an increase of fluorescence was also observed, and the authors attributed this increase to the transfer of the AgNCs from the original template to the C_{12} DNA. In one of their systems, up to 48-fold fluorescence enhancement was observed [107].

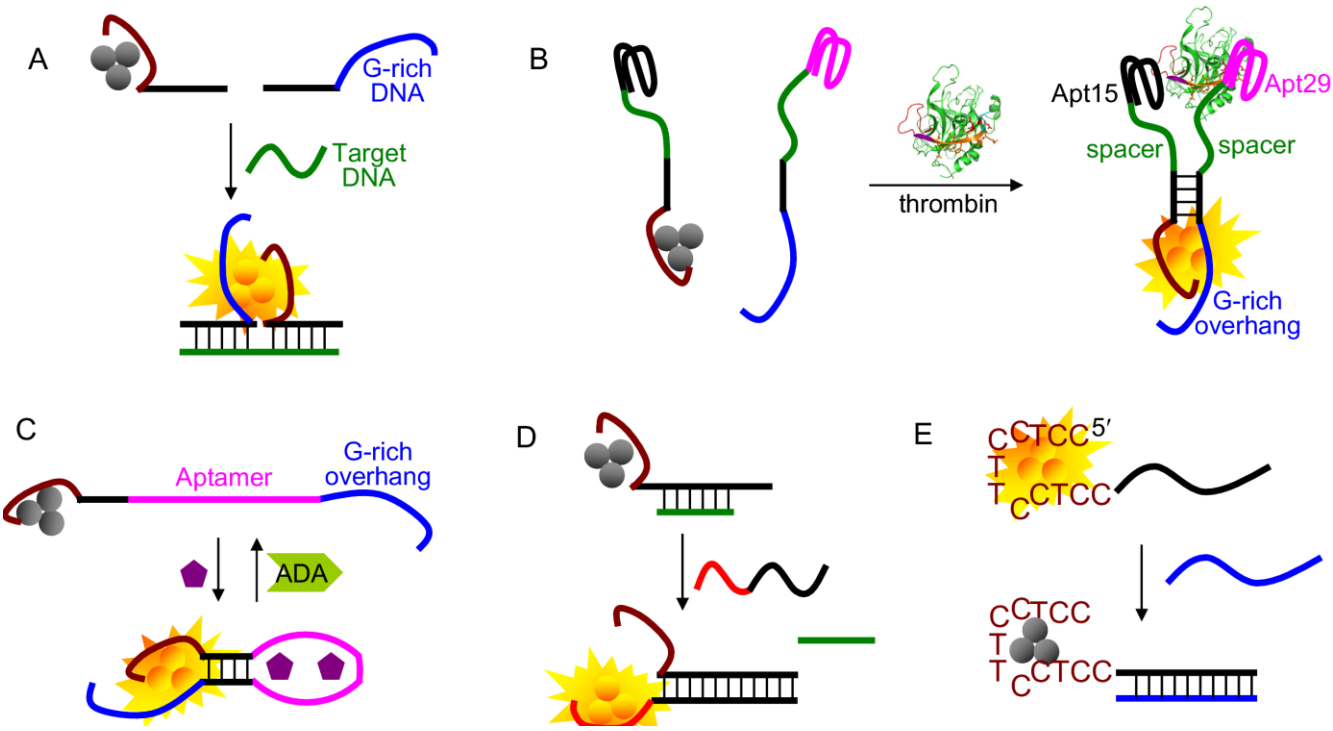

Figure 5. Sensors based on nucleic acid hybridization related to changing of the AgNC local environment. (A) Hybridization brings G-rich DNA to AgNCs to increase its fluorescence. (B) Aptamer binding assisted DNA hybridization and signaling. (C) Aptamer folding induced signaling. (D) Switching of AgNC from one DNA template to another. (E) Hybridization induced fluorescence quenching.

Yang and Vosch reported that hybridization of microRNA to a probe resulted in fluorescence quenching (Figure 5E) [108]. This DNA contained a 12-mer C-rich sequence to stabilize AgNCs while the rest of the sequence served as a probe for the microRNA. Highly fluorescent AgNCs were produced with this DNA. The authors suggested that mismatched self-dimer formation might be a reason for its strong fluorescence [109]. When hybridized with a complementary microRNA sequence, significant fluorescence quenching was observed, allowing detection of such nucleic acid targets. The reason for this hybridization induced quenching, however, is not fully clear at the moment. On the other hand, Chang and co-workers observed fluorescence enhancement upon hybridization with another set of DNA sequence (5′-C_{12}-CCAGATACTCACCGG), where the C_{12} part is for making AgNCs and the rest is a probe for the fumarylacetoacetate hydrolase gene [110].

Bringing a protein close to a AgNC could also modulate its fluorescence, which can be easily achieved by attaching an aptamer to a metal NC. For example, binding a thrombin protein led to ~80% fluorescence quenching [111]. On the other hand, when using a prion protein binding aptamer, fluorescence decreased only ~ 40% in the presence of prion. Since a lot of fluorescence still remained, this system was used for imaging cells over-expressing prion [112]. Since DNA is used as a stabilizer for AgNCs, removal of such DNA NCs might quench fluorescence as well. For example, Chang and co-workers reported that the fluorescence of a DNA-stabilized Cu/AgNC was decreased upon adding single-stranded DNA binding protein (SSB). This was attributed to the competition from SSB which weakened the interaction between DNA and the NC [113].

4.4. Reactive quenchers. The fluorescence of metal NCs can be modulated not only by changing its local environment via DNA hybridization, but also by interacting with other chemicals such as heavy metal ions and thiolated compounds. Since almost all the metal atoms in a NC are exposed, they are prone to chemical reactions, which usually lead to a change in fluorescence signal. An early work on this was reported by Ying and co-workers using BSA stabilized AuNCs [114], and then by Lu and co-workers using lysozyme stabilized AuNCs [115]. In both cases, Hg^{2+} was found to strongly quench the

fluorescence of AuNCs. Similar observations were also made with DNA-templated metal NCs. For example, Zhang and Ye reported Cu^{2+} induced fluorescence quenching of AgNCs[116]. Chang and co-workers reported quenching of AgNCs by Hg^{2+} [37], and also Au/AgNC by sulfide ions [117]. The scheme of this simple metal ion induced fluorescence quenching is shown in Figure 6A (step 1). If this reaction is reversible, adding metal ion chelators re-regenerates the fluorescence. For example, guanosine 3′-diphosphate-5′-di(tri)phosphate (ppGpp) can rescue quenching induced by Cu^{2+}, thus allowing the detection of ppGpp (Figure 6A, step 2) [118]. While heavy metal ions often quench fluorescence, they may induce enhancement of the light scattering signal at the same time due to aggregation of NCs to larger NPs [119]. For analytical applications, however, light scattering is far less sensitive compared to fluorescence.

Efforts have been made to explain metal ion induced NC quenching. Ying and co-workers proposed that d^{10}-d^{10} metallophilic interaction was the reason for Hg^{2+} binding to AuNCs [114, 120]. This explanation has been widely adopted by many other researchers. We carried out a systematic study using a set of DNA hairpins that templated red emitting AgNCs with different sensitivity to Hg^{2+}. The same trend of sensitivity was observed with light exposure, which is related to oxidation of the AgNCs [73]. It has been concluded that metal ions (usually Hg^{2+}, Cu^{2+}, Ag^{+} and Au^{3+}) can quench metal NCs due to their high redox potential. Depending on the specific reaction system, other fluorescence quenching mechanisms may also come into play. For example, Shang and Dong reported quenching of AgNCs stabilized by poly(methacrylic acid) and attributed it to the binding of Cu^{2+} to the polymer [121]. This hypothesis was supported by the lack of electronic absorption spectrum change upon Cu^{2+} addition and the inhibition of quenching by adding free monomers. Another possibility is energy transfer from AgNC to the nearby Cu^{2+}. Chang and co-workers synthesized carboxyl-capped AuNCs and showed fluorescence quenching induced by Pb^{2+}, Hg^{2+} or Cd^{2+} [122]. In this case, quenching was likely related to AuNC aggregation since these metal ions could be chelated by the surface carboxyl ligands. In another sensing system, Wang and co-workers showed the lack of

fluorescence lifetime change by Hg^{2+} induced AgNC quenching, suggesting disruption of the ground state of AgNCs [123].

In addition to heavy metals, thiol containing compounds were also potent quenchers. In 2010, Chang and co-workers reported mercaptopropionic acid quenching DNA-templated AgNCs; the fluorescence was recovered upon the addition of Cu^{2+} due to the stronger affinity between Cu^{2+} and thiol. This allowed them to detect Cu^{2+} down to 2.7 nM [124]. Acetylcholinesterase (AChE) can generate a free thiol using acetylthiocholine (ATCh) as a substrate, where the generated thiol can quench the fluorescence of Cu/AgNCs, allowing detection of AChE activity and its inhibitor [125]. Similar thiol quenching strategies have also been used to detect N-acetylcysteine [126], cysteine [127], glutathione reductase [128], and other biothiols [129]. While most studies reported thiols as quenchers, Ren and Qu and co-workers tested a number of different sequences and found that AgNC templated by C_{12} DNA could be enhanced by thiols. They attributed this increase to either new charge transfer enabled by the Ag-S bond or by the change of the local environment brought by the thiols [130]. This enhancement was utilized to detect the acetylcholinesterase activity, where a free thiol was generated via the enzymatic reaction [131].

Willner and co-workers found that quinones can also quench DNA-templated AgNCs. Based on this observation, they detected enzymes such as tyrosinase that generate quinones [132]. In the same paper, they also reported quenching of AgNCs by H_2O_2. With glucose oxidase they were able to detect glucose since H_2O_2 is a by-product of this reaction. Jiang and Yu coupled the H_2O_2 quenching reaction to an enzymatic reaction using cholesterol as the substrate, where H_2O_2 was consumed. They were able to detect as little as 200 nM cholesterol [133]. Recently, Chu and co-workers detected highly oxidative species inside cells using AuNCs decorated on silica nanoparticles [134].

Wang and co-workers reported that AgNCs can be quenched by DNA intercalators such as daunorubicin and quinacrine, and a non-intercalating binder (bisBenzimide H 33258), allowing detection of these drugs quite sensitively (detection limit = ~10 nM). In addition, fluorescence was

recovered by adding double-stranded DNA to remove the drugs from the AgNCs [135]. Aside from fluorescence quenching, some reactions also resulted in fluorescence enhancement. For example, melamine was found to enhance AgNC fluorescence [136]. Chang and co-workers reported that adding Cu^{2+} to a DNA-templated AgNC enhanced its fluorescence by ~9-fold, which was attributed to the formation of Ag/CuNCs that have stronger fluorescence [137].

As described above, metal NCs showed various types of responses. For example, both thiols and heavy metals can quench fluorescence. This is likely to be related to the sequence of DNA (or other templating molecules). We have not explicitly listed the DNA sequences in most cases, since we believe that these are specific examples instead of general observation. An immediate problem coming out of this summary is that these NCs are sensitive to many types of analytes. For each application, usually only one type of analyte is tested (metal ions, thiol compounds, metabolites that can generate H_2O_2 or other series). Therefore, we can expect specificity to be a problem if only fluorescence intensity is monitored.

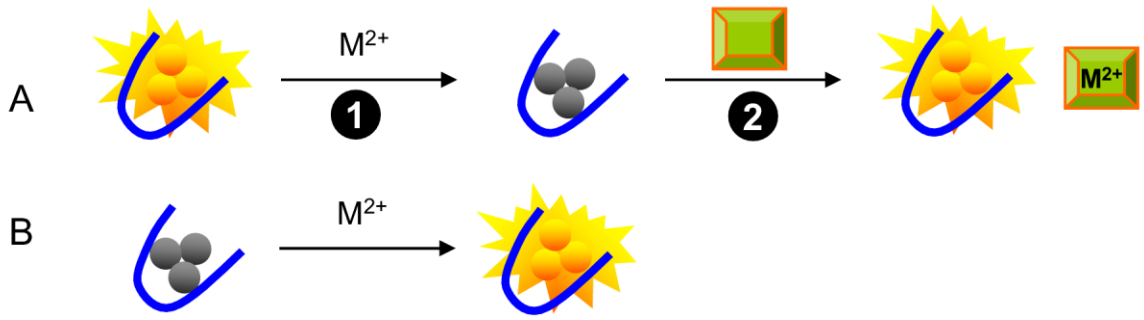

Figure 6. (A) Detection of heavy metal ions that can quench the fluorescence of DNA-templated AgNCs (step 1). If this reaction is reversible, addition of a metal chelator might recover the quenched fluorescence (step 2), allowing the detection of the chelator. (B) In some cases, non-fluorescent NCs show fluorescence enhancement upon addition of heavy metal ions.

5. Conclusions. It has been just a decade since the first report of using DNA to template fluorescent AgNCs [15]. Since then, many DNA sequences have been identified to produce different emission colors and the type of metal has expanded to Au and Cu and their mixtures. With high fluorescence yield, ease of synthesis and conjugation, and the programmable property of DNA, numerous analytical applications have already been reported, detecting analytes ranging from metal ions, small molecules, nucleic acids, proteins to cell imaging. In this review, we summarized the current state of the field based on the type of signal generation method. In general, NCs can be treated as a traditional fluorophore that can be quenched by typical quenchers. NCs can be readily generated in situ as long as the required DNA template is present. On the other hand, its fluorescence is sensitive to a diverse range of chemicals due to their susceptibility to chemical reactions. Although this might be useful for detecting those chemicals, such reactions also lack specificity since there are too many potential quenchers.

Given the progress, this field is still in its infancy and a few future directions are discussed here. On the fundamental aspect, it is important to systematically establish the relationship between the DNA sequence and the color of the resulting NCs. This will require screening a large number of DNA sequences and length. In addition, knowledge of the bonding and structure of the DNA-NC complex is needed so that the templating DNA sequences can be rationally incorporated with a high rate of success. Efforts are also to be made to understand the redox property of NCs within the DNA template. Finally, to obtain mono-dispersed NCs is a challenge; many DNA molecules produce a distribution of NC sizes and structures.

From the application point of view, one important aspect is to prepare NCs with excellent photostability. This is a particularly problem for AgNCs. More stable NCs may be achieved by optimization of the DNA sequence [72], or changing buffer/storage conditions. AuNCs might suffer less from photobleaching since they are more resistant to oxidation. However, little has been done on using DNA stabilized AuNCs for making biosensors, which is a feasible direction for future work.

Another way to extend the application of such NCs is to interface them with other types of materials. There are already reports of making NCs on DNA origami, graphene, carbon nanotubes and hydrogels. The use of hydrogels for immobilizing NCs is particularly attractive since they are transparent with a large loading capacity, allowing direct visual detection [68, 69]. A few methods for preparing such hydrogels have already been reported and more efforts are needed to optimize sensitivity and demonstrate other properties such as sensor re-generation. Additional work is needed to understand the properties of NCs in cellular conditions, where they may be used for imaging applications [138, 139]. Incorporation of other DNA functions beyond molecular recognition is also interesting. For example, Ren and Qu and co-workers reported CpG DNA templated AgNCs, combining both immune stimulation and fluorescence imaging functions [140]. Taken together, DNA-templated NCs have emerged as a useful tool for analytical chemistry and more developments in this field are expected in the near future.

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