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NanoCluster Beacon – A New Molecular Probe for Homogeneous Detection of Nucleic Acid Targets

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Abstract— **Oligonucleotide-templated nanoclusters consisting of a few atoms of silver (DNA/Ag NCs) have been made into a new molecular probe that "lights up" upon target DNA binding, termed a NanoCluster Beacon (NCB). We discovered that interactions between silver nanoclusters and a proximal, guanine-rich DNA strand can lead to tremendous red fluorescence enhancement [1]. Here we show that dark silver nanoclusters templated on an ssDNA can be lit up into a palette of colors (green, yellow/orange, and red) by employing different proximal sequences, potentially enabling the use of NCBs in multiplexed assays. We tested a suite of nanocluster-nucleation sequences and found the sequences that created strong red fluorescence enhancement share a common 5'-C_3NNNNNC_4 motif, where N is either a thymine (T) or an adenine (A) base. We optimized the design of NCBs by testing the effect of different lengths of an interaction stem. The highest signal-to-background (S/B) ratio, 175, was achieved when the stem length was 3 base pairs long.**

Noble metal nanoclusters; light-up probes; DNA-templated silver nanoclusters; fluorescent probes

I. INTRODUCTION

Noble metal nanoclusters are collections of small numbers of gold or silver atoms (2-30 atoms) with physical sizes close to the Fermi wavelength of an electron (~0.5 nm for gold and silver) [2]. Providing the missing link between atomic and nanoparticle behavior in noble metals, these nanoclusters have shown dramatically different optical, electronic, and chemical properties as compared to those of much larger nanoparticles or bulk materials [2-4]. Owing to discrete density of states, metal clusters behave like molecular systems and yield fluorescence emission in the UV-visible range. Gold and silver clusters were first made in rare gas matrices, where their fluorescence emission and absorption spectra were correlated to size differences, as predicted by the jellium model [2]. The first organic synthesis produced clusters with little fluorescence and limited solubility. It was not until early 2000's when highly fluorescent, water-soluble noble metal nanoclusters were reported, opening new opportunities for biological labels [5, 6]. Till now, a variety of organic materials and biomolecules have served as templates (or encapsulation agents which stabilize nanoclusters with well-defined sizes and protect them against agglomeration following reduction) for aqueous synthesis of fluorescent nanoclusters, including dendrimers [7, 8], polymers [9], small molecule ligands [3, 4], peptides [10], proteins [11], and oligonucleotides [1, 12-16].

Among those water-soluble noble metal nanoclusters newly developed, DNA-templated silver nanoclusters (DNA/Ag NCs) have attracted great interest in analytical chemistry and quantitative biology owing to a number of useful photophysical properties (see Discussion for details). Although DNA/Ag NCs have been demonstrated in cellular imaging [17] and metal ion detection [18, 19], the understanding of this new type of organic/inorganic composite fluorophores is still limited. In the relatively unexplored physical size region of nanoclusters, many unknowns, such as the transition from fluorescent cluster behavior to the non-fluorescent behavior of larger nanoparticles and the detailed physical chemistry properties of clusters, remain to be addressed. From an application perspective, making fluorescent silver clusters into Főrster energy transfer pairs and finding effective methods to turn on clusters' fluorescence (i.e. light-up probes) or shift their spectra (i.e. colorimetric probes) upon target recognition can lead to the development of new molecular sensing tools, accelerating the widespread use of nanocluster fluorophores in analytical chemistry and quantitative biology.

Recently we demonstrated controlled conversion of DNA/Ag NCs between bright and dark states by guanine proximity (Fig. 1a), with bulk fluorescence changed more than 500 fold [1]. Based on this finding, we designed a new molecular probe, termed a NanoCluster Beacon (NCB), that "lights up" upon binding with a DNA target. As illustrated in Fig. 1b, an NCB consists of two linear probes, one bearing non-fluorescent silver nanoclusters (i.e. NC probe) and the other having a guanine-rich tail (i.e. G-rich probe). The two probes are designed to bind in juxtaposition to a target DNA, allowing guanine bases on one probe to interact with non-fluorescent nanoclusters on the other probe, transforming those non-fluorescent nanoclusters into bright red-emitting clusters. Not relying on Főrster energy transfer as the fluorescence on/off switching mechanism, NCBs have the potential to reach an ultrahigh signal-to-background (S/B) ratio in molecular sensing. Since the fluorescence enhancement is caused by intrinsic nucleobases, our detection technique is simple, inexpensive, and compatible with commercial DNA synthesizers. While NCBs are clearly promising as future probes in quantitative biology, their design rules have yet been addressed. Here we show that the dark nanoclusters templated on an ssDNA can be lit up into three distinct colors (green, yellow/orange, and red) by employing different proximal sequences, potentially enabling the use of NCBs in multiplexed assays. We also tested different nanocluster-nucleation sequences and found the sequences that created strong red fluorescence enhancement share a common 5'-C_3NNNNNC_4

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14. ABSTRACT
Oligonucleotide-templated nanoclusters consisting of a few atoms of silver (DNA/Ag NCs) have been made into a new molecular probe that âlights upâ upon target DNA binding, termed a NanoCluster Beacon (NCB). We discovered that interactions between silver nanoclusters and a proximal, guaninerich DNA strand can lead to tremendous red fluorescence enhancement [1]. Here we show that dark silver nanoclusters templated on an ssDNA can be lit up into a palette of colors (green, yellow/orange, and red) by employing different proximal sequences, potentially enabling the use of NCBs in multiplexed assays. We tested a suite of nanocluster-nucleation sequences and found the sequences that created strong red fluorescence enhancement share a common 5â-C3NNNNNC4 motif, where N is either a thymine (T) or an adenine (A) base. We optimized the design of NCBs by testing the effect of different lengths of an interaction stem. The highest signal-to-background (S/B) ratio, 175, was achieved when the stem length was 3 base pairs long.

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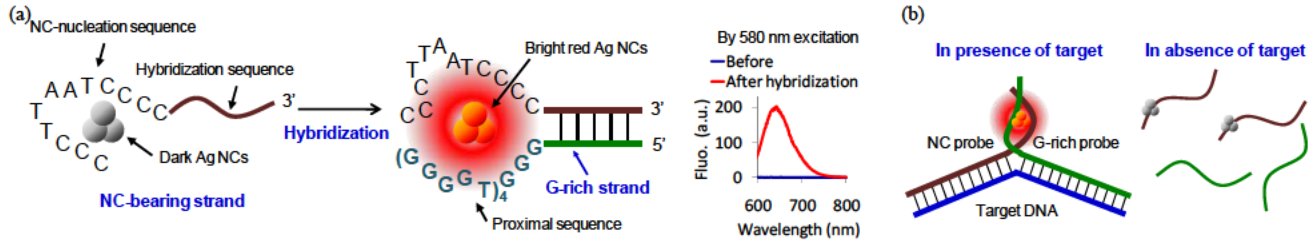


Figure 1. (a) Schematic and data showing the red fluorescence enhancement of DNA-templated silver nanoclusters (DNA/Ag NCs) caused by guanine proximity. The excitation and emission peaks for the light-up NCs are at 580 nm and 636 nm, respectively. (b) NanoCluster Beacon (NCB, consisting of a NC probe and a G-rich probe) detection scheme. NCBs light up in presence of DNA target. In absence of target, NCBs remain dark.

motif, where N is either a thymine (T) or an adenine (A) base. We optimized the design of NCBs by testing the effect of different lengths of an interaction stem. The highest signal-to-background (S/B) ratio of 175, a factor of 5 better than conventional molecular beacons, was achieved when the stem length was 3 base pairs long.

II. Methods

A. *Preparation of silver nanoclusters on DNA*

All DNA strands were purchased from Integrated DNA Technologies Incorporated and were purified by desalting. DNA/Ag NCs were made using the protocol developed by [14]. NC-bearing strand was first dissolved in ultrapure deionized water. Silver nanoclusters were formed by adding $AgNO_3$ (99.9%, Sigma-Aldrich) to the DNA solution, followed by reduction with sodium borohydride. Final concentrations were 15 µM in DNA strand, 90 µM in $AgNO_3$, and 90 µM in $NaBH_4$ in 20 mM pH 6.6 sodium phosphate buffer. The aqueous solution of $NaBH_4$ was prepared by dissolving $NaBH_4$ powder in water and adding the required volume to the DNA/Ag^+ mixture within 30 seconds, followed by vigorous shaking for 5 seconds. The reaction was kept in the dark at room temperature for 18 hours before use.

B. *Experiments*

Fluorescence was measured using a Varian Cary Eclipse Fluorescence Spectrophotometer. The images of samples were acquired by a digital camera while the samples were placed on a gel imager (InGenius, Syngene).

III. Results and Discussion

An important feature of NCBs is that dark Ag NCs on a DNA motif can be lit up into distinct colors, creating a complementary palette. This is achieved by bringing different DNA sequences (i.e. proximal sequences) into proximity of the originally dark Ag NCs (templated on a 5'-C_3TTAATC$_4$ motif). As shown in Fig. 2, three distinct light-up colors (green, yellow/orange, and red) were obtained by employing three different proximal sequences. This important characteristic, having multiple light-up colors from the same origin, is not commonly shared by organic dyes or semiconductor quantum dots, opening opportunities for NCBs in multiplexed assays.

Our initial study investigated the magnitude of fluorescence enhancement created by proximal sequences of varying guanine content [1]. Here, we further investigate the red fluorescence enhancement by testing six NC-bearing strands having an identical hybridization sequence but different NC-nucleation sequences. As shown in Fig. 3, strong red fluorescence emission after hybridization was seen from Seq_1, Seq_2 and Seq_5 samples. These three sequences share a common 5'-C_3NNNNNC$_4$ motif, where N is either a thymine (T) or an adenosine (A) base. However, only Seq_1 and Seq_2 had low background fluorescence before hybridization. Another important feature to note in the Fig. 3 experiment is that the emission spectra of all the six samples changed in some ways after hybridization – either from nearly no fluorescence to a strong emission (e.g. Seq_1 sample) or from one color to another (Seq_4 and Seq_5 samples). This characteristic leads to the possibility of creating NCBs with a variety of color-change scenarios upon target recognition, which will enrich our fluorescence detection tool box in the near future.

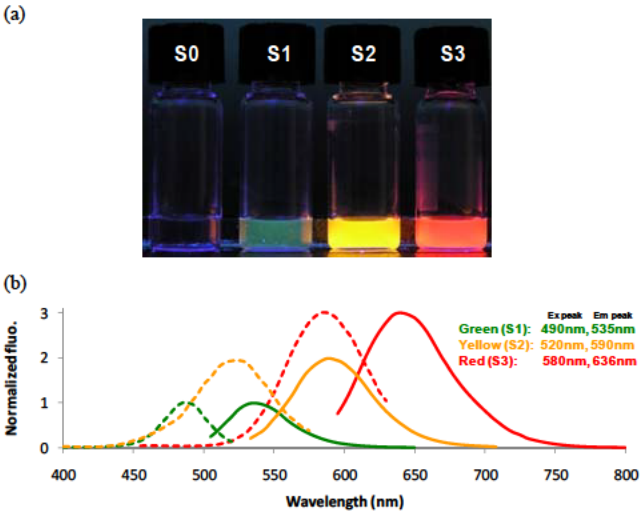
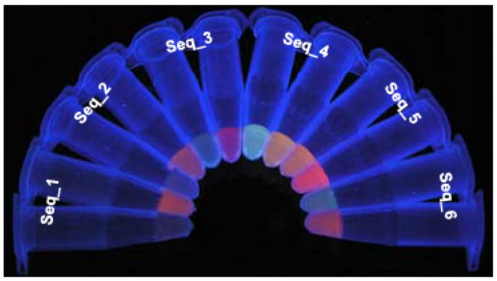


Figure 2. Dark DNA/Ag NCs templated on the NC-nucleation sequence 5'-C_3TTAATC$_4$ can be lit up into three distinct colors by bringing different DNA sequences (proximal sequences) into their proximity. (a) Photograph of the four samples under UV (365 nm) irradiation. The proximal sequences used are: none (S0), 3'-T_{12} (S1), 3'- $(G_4A)_3G_3$ (S2), and 3'-$(G_4T)_3G_3$ (S3), respectively. (b) Normalized excitation/emission spectra of S1, S2 and S3 samples. Dashlines represent the excitation spectra and solid lines represent the emission spectra. Normalization scale is set differently for easy visualization.

The design of NCB can be optimized by allowing the NC probe and the G-rich probe to form a short "stem" on the NCB interaction arm (Fig. 4a). Such a short stem helps to bring the NC-nucleation sequence closer to the proximal sequence, resulting in tighter interactions. As a consequence, the light-up

(a)



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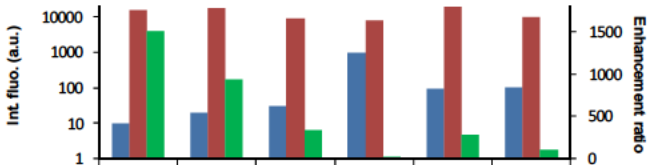


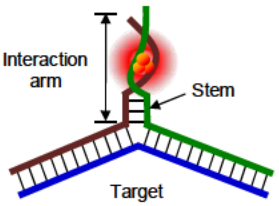
	Seq_1 (CCCTTA ATCCCC)	Seq_2 (CCCTTT AACCCC)	Seq_3 (CCTCCT TCCTCC)	Seq_4 (CCCCCC CCCCCC)	Seq_5 (CCCTAT AACCCC)	Seq_6 (CCCTAA CTCCCC)
Before hybridization	10	19	29	984	94	99
After hybridization	15225	17684	9432	7736	26283	9812
Enhancement ratio	1511	931	326	8	280	99

Figure 3. (a) Photograph of 6 pairs of samples under UV (365 nm) irradiation. In each pair of samples, the sample on the left contains one of the six NC-bearing strands (with Ag NCs on them). The sample on the right contains both the NC-bearing strand and a common G-rich strand (having proximal sequence of 3'-$(G_4T)_3G_3$). (b) Chart showing the integrated red fluorescence emission (595 nm – 800 nm, by 580 nm excitation) of the six NC-bearing strands (NC-nucleation sequence shown in parenthesis) before and after hybridization with the common G-rich strand and the associated enhancement ratio. Seq_1, Seq_2 and Seq_5 samples showed strong red fluorescence emission after hybridization. Using a Ag:DNA molar ratio of 12:1 during NC formation process, the enhancement ratio for Seq_1 sample was found greater than 1,500×. The NC-nucleation sequences Seq_1, Seq_2 and Seq_3 are from [20]. Seq_4 is from [14, 16] and Seq_5 and Seq_6 are from [21].

emission is enhanced. As shown in Fig. 4b, target-specific fluorescence increased with increasing stem length up to 4 base pairs, but background fluorescence continued to grow beyond that. The highest S/B ratio, 175, was achieved when the stem was 3 base pairs long, which is five times better than the S/B ratio obtained by the stem length of 2 base pairs.

At present, we are still investigating the underlying mechanism for the observed guanine proximity-induced fluorescence enhancement. A number of reports have studied the interactions between guanine bases and organic dyes [22-24]. In most cases [22, 23], but not all [24], guanine-dye interactions led to fluorescence quenching of excited fluorophores. Believed to be a photoinduced charge transfer phenomenon [22, 23], guanine-mediated fluorescence quenching has been studied systematically on a variety of DNA sequences and structures [25]. Guanine has the lowest oxidation potential of all nucleobases and can donate electrons to nearby fluorophores, quenching their fluorescence. Charge transfer between nucleobases and Ag NCs has been recently reported to lead to a long-lived, charge-separated trap state that causes fluorescence intermittency (i.e. blinking) of Ag NCs on microsecond time scale [26]. In our case, it is possible that guanine bases serve as electron donors, converting oxidized-NC species (in this case, non-emissive NCs) into reduced-NC species (bright red-emitting NCs). To prove this electron-transfer hypothesis, a proximal sequence rich of 7-deazaguanines, which are stronger electron donors than guanines [27], was made and tested [1]. Surprisingly, we found no light-up effect from such a deazaguanine-rich proximal sequence, weakening the electron transfer hypothesis. Another experimental result that weakens the electron-transfer hypothesis is that thymine proximity produced a green fluorescence enhancement (S1 in Fig. 2), while adenine proximity did not generate any measurable fluorescence enhancement, with thymine being a worse electron donor than adenine. The only difference between guanine and 7-deazaguanine is the nitrogen atom at the guanine N7 site (in 7-deazaguanine, it is replaced with a CH group). It was previously reported that the guanine N7 site may be the primary location for silver attachment to a DNA duplex [13]. Our 7-deazaguanine experiment (shown in [1], supporting information) indicates that the guanine N7 site plays a critical role in the observed red fluorescence enhancement.

(a)



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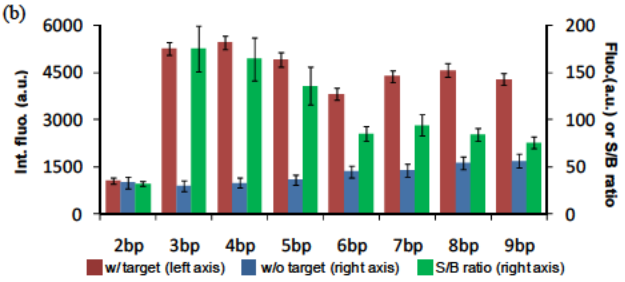

Figure 4. Optimization of NCB by changing the length of "stem" on the interaction arm. (a) Schematic of stem length optimization. (b) Integrated fluorescence with and without target, and S/B ratio at different stem length. A stem length of 3 base pairs gave the highest S/B ratio of 175.

As mentioned above, many intriguing and useful photophysical properties of DNA/Ag NCs have been discovered and reported in recent years, including high fluorescence quantum yield (> 50%) [15], good photostability [15, 16], absorption/emission features throughout the visible region [15, 20, 21], blinking only on a microsecond time scale [16, 26, 28], fluorescence recovery with low-energy secondary excitation [29], strong two-photon-induced fluorescence [21], and fluorescence recovery upon nanocluster transfer [17]. Our discovery of guanine proximity-induced red fluorescence enhancement adds to this list. As new signal transduction modes emerge rapidly, a whole new class of nanobiosensors based upon noble metal nanoclusters is expected to be realized in the years to come. Our development of NCBs served as one example and one starting point. There are many benefits of using NCBs, including design simplicity, low cost, "one-step"

preparation process, and potential to achieve an extraordinarily high S/B ratio as it does not rely on Főrster energy transfer as fluorescence on/off switching mechanism. Our method is unique not only because it requires only a single preparation step (i.e. nanocluster formation on NC probes), but because there is no need to remove excess silver ions or borohydride ions from solution after NC formation is completed, as these are essentially non-fluorescent. All these benefits were made possible by taking advantage of a poorly understood conversion dynamic process of DNA/Ag NCs, a dynamic process neither being shared by organic dyes nor by semiconductor quantum dots. While dynamic processes in fluorophores are often viewed as a drawback, we have learned how to reprogram/control such a process to create a new signal transduction mode for molecular sensing.

IV. Conclusion

Recently we demonstrated controlled conversion of DNA/Ag NCs between bright and dark states and, based upon this finding, designed a new molecular probe, NanoCluster Beacon, for homogeneous detection of nucleic acid targets [1]. Not relying on Főrster energy transfer as the fluorescence on/off switching mechanism, NCBs have the potential to reach an ultrahigh signal-to-background (S/B) ratio in molecular sensing. Since the fluorescence enhancement is caused by intrinsic nucleobases, our detection technique is simple, inexpensive, and compatible with commercial DNA synthesizers. Here we demonstrate a palette of NCB light-up colors can be produced from the same origin by employing different proximal sequences. We also show the nanocluster-nucleation sequences capable of achieving strong red fluorescence enhancement share a common 5'-$C_3NNNNNC_4$ motif.

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