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NanoCluster Beacons as Reporter Probes in Rolling Circle Enhanced Enzyme Activity Detection†

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Abstract

As a newly developed assay for the detection of endogenous enzyme activity at the single-catalytic-event level, Rolling Circle Enhanced Enzyme Activity Detection (REEAD) has been used to measure enzyme activity in both single human cells and malaria-causing parasites, *Plasmodium sp.*. Current REEAD assays rely on organic dye-tagged linear DNA probes to report the rolling circle amplification products (RCPs), the cost of which may hinder the widespread use of REEAD. Here we show that a new class of activatable probes, NanoCluster Beacons (NCBs), can simplify the REEAD assays. Easily prepared without any need for purification and capable of large fluorescence enhancement upon hybridization, NCBs are cost-effective and sensitive. Compared to conventional fluorescent probes, NCBs are also more photostable. As demonstrated in reporting the human topoisomerases I (hTopI) cleavage-ligation reaction, the proposed NCBs suggest a read-out format attractive for future REEAD-based diagnostics.

Keywords

NanoCluster Beacons; silver nanoclusters; nucleic acid detection; endogenous enzyme detection; rolling circle amplification; topoisomerase I

†Electronic Supplementary Information (ESI) available: The detailed steps of NCB preparation, REEAD assay and STEM imaging. The sequences of the sNCB and the REEAD substrate. See DOI:

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Introduction

Rolling Circle Enhanced Enzyme Activity Detection (REEAD) is a novel method to detect enzymatic activities by turning single enzyme catalytic activities into isothermally amplified nucleic acid products.^{1–4} REEAD has been demonstrated for measuring cancer-relevant enzymes in single cells^{1–3} and for detecting an enzyme activity specific to the malaria-causing *Plasmodium* parasites.⁴ While the REEAD assay is robust, specific, and capable of multiplexed detection of target enzyme activities even in crude cell extracts, the assay cost is high and sample preparation requires multiple steps of washing and separation, as well as an addition of the anti-fading agent to ascertain the detectable fluorescence signal. To push for the use of REEAD for detection of infectious diseases at point-of-care settings, it is necessary to reduce the cost and simplify the preparation procedures of this assay while maintaining its reliability. Current REEAD assays rely on organic dye-tagged linear probes to report the rolling circle amplification products (RCPs).⁵ While these organic dye-tagged reporters are easy to use, they are not cost-effective and can only provide modest target-to-background (T/B) ratio even with the intervention of anti-fading agents.

Among all the fluorescent reporters available, activatable probes,^{6,7} in particular, offer a high T/B ratio in molecular detection – they fluoresce only upon binding with specific target molecules but otherwise remain dark. While a high T/B ratio makes quantification easier and elimination of the need to remove the unbound probes simplifies the assay,⁷ activatable probes are often much more expensive than the organic dye-tagged reporter probes and difficult to prepare. For instance, molecular beacons,⁸ the most widely used activatable probes for DNA detection, need to be dually labeled (i.e. with an organic dye at one end of the hairpin and a quencher at the other end). Removal of excess dyes and singly labeled impurities during the manufacturing process is necessary, adding preparation complexity and cost to the molecular beacons. Both semiconductor quantum dots and fluorescent proteins have been converted to activatable probes for DNA detection,^{9–11} but again the material costs are high and the preparation processes are not straightforward.

Here we present a versatile strategy to design activatable probes for REEAD assays that are not only simple but cost-effective. Our probes use few-atom silver nanoclusters (Ag NCs) as fluorescent reporters that can be prepared at room temperature *via* sequential mixing of three inexpensive components in a buffer: a cytosine-rich oligonucleotide, a silver salt, and a reducing reagent (see ESI for detailed processes).^{12–14} There is no need to remove any excess reactants as they are essentially non-fluorescent, thus eliminating any purification cost. Moreover, upon interaction with a nearby DNA sequence (called an enhancer sequence¹⁵), silver clusters exhibit fluorescence activatability^{15–17} and tunability^{17,18} that cannot be obtained with organic dyes, luminescent nanocrystals and fluorescent proteins.¹² These properties have led to the development of NanoCluster Beacon (NCB, an activatable probe) for DNA detection¹⁶ and chameleon NanoCluster Beacon (cNCB, a multicolor probe) for single-nucleotide polymorphism identification.¹⁸ In particular, not relying on resonance energy transfer as activation or color-switching mechanism, NCBs have achieved a T/B ratio that is five times better than that of a conventional molecular beacon in DNA sensing.¹⁶ While being explored by researchers in the detection of proteins^{19,20} and metabolites,²¹ NCBs have not been used in surface-based DNA detection. Due to their low

cost and high performance (high T/B ratio and good photostability), NCBs are perfect reporter probes for REEAD assays.

Results and discussion

In REEAD assays (Fig. 1A),^{1–4} the enzyme (e.g. human topoisomerase I, hTopI) under detection first converts a custom-designed linear DNA substrate (the dumbbell structure) into a circularized product through cleavage and ligation. The circularized substrate is then used as the template for isothermal rolling circle amplification (RCA) on a glass surface (see ESI for detailed processes), leading to multiple ($\sim 10^3$) tandem copies of the circularized substrate called rolling circle amplification products (RCPs). Traditionally, these RCPs are visualized under a fluorescence microscope by labeling them with organic dye-tagged DNA probes.⁵ This organic fluorophore labeling allows direct quantification of single enzymatic events by simply counting the resulting fluorescent RCP dots.

By replacing organic dye-tagged reporter probes with NCBs, we take advantage of the low cost and the “fluorescing-upon-hybridization” nature of NCBs.^{12,16,17} The conventional NCB has a binary probe configuration (Fig. 1B),⁶ having two oligonucleotide strands (an NC probe and an enhancer probe) that bind in juxtaposition to a DNA target. Such a juxtaposition binding enabled the enhancer sequence to interact with the silver cluster, transforming the cluster from a non-emissive species to a highly fluorescent species. Fluorescence thus occurs only when a specific DNA target is present in the sample.¹² Extended from the conventional binary NCB design (Fig. 1B), we have developed a simplified strategy to label RCPs that further reduces the probe cost. In this new design, which we termed single NCB (sNCB, Fig. 1C), the enhancer sequence is programmed to be embedded in the RCPs, which functions both as a target and an enhancer (Fig. 1C). In this case, the sNCB consists only of the NC probe as there is no need of G-rich enhancer probe.

We examined a dumbbell DNA substrate designed for topoisomerase I activity detection. As shown in Fig. 1A, an sNCB (refer to Fig. S1 for sequence information) was designed to hybridize with the corresponding RCPs. This sNCB design was first tested on a truncated RCP-mimicking synthetic target (60nt long) in a standard homogeneous solution assay.¹⁶ Upon hybridization (i.e. fluorescence activation), sNCB lit up in solution (Fig. S1A), to produce red emission (Fig. S1B–C) and displayed an ensemble enhancement ratio greater than 1000-fold (see Fig. S1D for the enhancement ratio definition). A molecular beacon (MB)^{8,16} using FAM/Dabcyl as the FRET pair was also designed to hybridize to the same synthetic target for comparison. As shown in Fig. S2, the signal-to-background (S/B) ratio of sNCB was found ~ 30 -fold higher than that of MB on the same target, making sNCBs a better choice for our surface-based assay.

The procedure for REEAD has previously been described^{3,4} and is summarized in the Supporting Information (Materials and Methods and Fig. S3). Initially, RCPs were labeled with multiple rhodamine (Rh)-tagged DNA probes for visualization. However, these Rh-tagged probes bleached rapidly (Fig. 2A). Compared to the organic dyes, Ag NCs have previously shown a better photostability^{22–24} and stronger single-fluorophore brightness.^{22,23} Therefore we expected that when sNCBs were used as reporter probes, we

would see RCPs that are brighter and longer-lasting. Indeed, under identical illumination and imaging conditions, sNCB-labeled RCPs looked bright and their fluorescence faded away much slowly (Fig. 2A and Movies available in ESI). The average decay times are 28 s for Rh probes and 186 s for sNCBs (Fig. 2B).

Although the synthetic yield of fluorescent silver cluster can vary between 5%^{16,24} and 45%²⁵ and the sNCBs used in this study were not purified (i.e. containing non-functional sNCBs), the sNCB-labeled RCPs were about 10× brighter than the Rh-labeled RCPs in frame 1 (Fig. 2A). We believe this difference in the “appearing” brightness was mainly caused by the difference in the photostability between rhodamine and Ag NCs (in frame 1, many Rh probes had photobleached already). As sNCB-labeled RCPs still bleached after long illumination, we knew that those bright spots were not impurities (e.g. particles from chip dicing) which did not bleach at all.

One may ask why we didn't use RCPs directly as templates to synthesize fluorescent Ag NCs and the *in situ* synthesized Ag NCs as reporters for RCPs. Similar ideas have been previously discussed in solution-based isothermal amplification assays.²⁶ To test this idea, we submerged the REEAD chips with and without RCPs in a silver nitride solution and then added sodium borohydride to the solution. On both chips, many bright spots were seen after silver reduction. Those bright spots were nonspecific to RCPs and increasing in overall numbers after long illumination (Fig. 2A). These evidences led us to believe that the observed bright dots were silver oxide (Ag₂O) nanoparticles²⁷ rather than silver clusters. Photoactivation of individual Ag₂O nanoparticles has been previously reported.²⁷ We found that fluorescence of silver oxide particles can be activated more effectively with shorter wavelength excitation (e.g. blue light), which agrees well with the literature.²⁷ These silver oxide particles emit as single-quantum systems – they blink under the microscope (Fig. 3A) and bleach in a single step (Fig. 3B). On the other hand, fluorescently RCPs bleach gradually (because multiple emitters are incorporated in each RCP rather than a single emitter, see Fig. 3C) and the average dot size is larger than the diffraction limit spot size. We hypothesize that the surface coating of our REEAD chips (CodeLink® activated slides from SurModics), a hydrophilic polymer containing N-hydroxysuccinimide (NHS) ester reactive groups, supports the formation of silver islands during the reduction process, which then become silver oxide nanoparticles upon exposure to air.²⁷ As a result, *in situ* synthesis of fluorescent Ag NCs cannot be used to report the existence of RCPs here.

The labeled RCPs had a size around 1 μm when observed using standard fluorescence microscopy. We further examined the morphology of RCPs under a scanning transmission electron microscope (STEM). STEM images (Fig. 2C) show RCPs with an average size of 1 μm and an inter-RCP distance ranging from 6 to 20 μm, similar to the inter-dot distance that we observed *via* fluorescence microscopy. We noticed that RCPs did not have a wool ball-like conformation as shown in the previous report,²⁸ but rather display three-dimensional plate-like wavy structures (Fig. S4). Through STEM, we could not tell the difference in morphology for the unlabeled RCPs, RCPs labeled with Rh probes, and RCPs labeled with NCBs.

We compared the quantification results of endogenous hTopI activity (primary target for several cancer chemotherapeutics) in crude human cell extract using Rh-tagged probes and sNCB as reporters, respectfully (Fig. 4). Since rhodamine bleaches quickly under illumination (Fig. 2A), an anti-fading mounting medium, Vectashield®, was used to improve the quantification of Rh-labeled RCPs. Figure S5 shows the quantification results from three spike-in replicates of 10, 100 and 100 cells in the proposed sNCB/REEAD assay. Human topoisomerase I (hTopI) signals were analyzed using Image J software. The number of hTopI signal increases significantly with the increasing number of spike-in HEK 293 cells. Although in both cases (rhodamine probe and sNCB) the hTopI signals are correlated to the spike-in cell amount, sNCB detection result is superior to that of rhodamine probe at the low cell quantities (10 and 100 cells). These results demonstrated that sNCBs can substitute rhodamine probes in the REEAD assays. Since Ag NCs are more photostable than rhodamine, an anti-fading medium is not needed when sNCBs are used as reporters. This further reduces the cost of the sNCB/REEAD assay.

In conclusion, we have demonstrated a versatile strategy to design new activatable probes, single NanoCluster Beacons (sNCBs), for imaging and quantifying rolling circle amplification products (RCPs) in an enzyme activity assay (REEAD). Distinct from the conventional binary NCBs^{15,16} and the chameleon NCBs¹⁸ reported before, single NCBs have their guanine-rich enhancer sequence directly incorporated into their targets (i.e. the RCPs). Therefore, sNCB only contains an NC probe and it lights up upon binding with the target. The major contribution of this report is to demonstrate that the “embedded” G-rich sequence can work as the G-rich “overhang” in the fluorescence activation of silver clusters. The key advantages of the proposed sNCB/REEAD assay lie in lower cost (no need of probe purification and anti-fading medium), simpler preparation procedure (no need of stringency wash to remove the unbound probes, see ESI), and better imaging quality (resistance to photobleaching). We expect that the concept of sNCBs can be generally applied to a wide variety of isothermal amplification assays where the enhancer sequence can be embedded in the amplicons, such as NASBA,²⁹ SDA,³⁰ and LAMP.³¹

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

(A) NCB/REEAD detection scheme (not drawn to scale). The detection of enzymatic activity involves three steps: (1) enzyme-mediated (i.e. human topoisomerase I, hTopI) circularization of a synthetic DNA dumbbell hTopI substrate, (2) signal enhancement *via* an immobilized DNA primer and rolling circle amplification (RCA), and (3) hybridization of RCA products (RCPs) with activatable NanoCluster Beacons (NCBs) for detection. The right loop of the substrate is designed for primer binding while the left loop carries a guanine-rich enhancer sequence. (B) The conventional binary NCB consists of an NC probe (the cytosine-rich NC-nucleation sequence is shown in blue) and an enhancer probe (the enhancer sequence is shown in red). When NCB binds to a target, silver clusters interact with the enhancer sequence and turn on. (C) For the single NCB (sNCB) used in this study, the enhancer sequence is embedded in the RCPs. As a result, RCPs not only serve as a binding target but also an enhancer that turns on the fluorescence of bound sNCBs. In this configuration, only the NC probe is needed in an sNCB.

Fig. 2.

Comparison between the conventional REEAD assay and the proposed NCB/REEAD platform. (A) Fluorescence images of a conventional Rh/REEAD assay chip, a sNCB/REEAD chip, a control (no RCPs on the chip, stained with sNCBs), and a chip with *in situ* synthesized fluorescent Ag₂O particles. Images shown here are taken from frame 1 and frame 120 of a movie required under continuous illumination (frame rate is 1 Hz). (B) Fluorescence decay of representative RCPs labeled with rhodamine probes and sNCBs, respectively. (C) Low- and high-magnification STEM images of sNCB-labeled RCPs showing an average size of 1 μm with three-dimensional plate-like wavy structures.

Fig. 3. Fluorescence time traces of representative Ag_2O particles showed (A) blinking and (B) single-step photon bleaching. (C) On contrary, the time trace of an sNCB-labeled RCP exhibited a gradual fluorescence decay.

Fig. 4.

Detection of human topoisomerase I, hTopI, activity in crude human HEK293 cell extracts.

Top row: REEAD chips without any labeling. **Middle row:** the conventional Rh/REEAD assays. To facilitate the quantification of Rh-labeled RCPs, an anti-fading mounting medium was used here. **Bottom row:** the proposed NCB/REEAD assays (without the anti-fading medium). Samples were prepared at 3 different cell concentrations (10 cells per 5 μ l, 100 cells per 5 μ l and 1000 cells per 5 μ l).