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Fluorescent silver nanocluster DNA probes for multiplexed detection using microfluidic capillary electrophoresis†

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DNA-stabilized fluorescent silver nanoclusters (AgNC DNA) are a new class of fluorophore that are formed by sequence specific interactions between silver and single-stranded DNA. By incorporating both target-binding and fluorescent-reporting sequences into a single synthetic DNA oligomer, AgNC DNA probes eliminate the need to conjugate dye or quencher molecules. In this study, we modify a AgNC DNA probe to demonstrate single-color multiplexed detection of DNA targets. We show that appending different lengths of poly-dT to the probe sequences tunes the electrophoretic mobility of AgNC DNA probes without affecting their fluorescence spectra. We use this to introduce a set of AgNC DNA probes selective for Hepatitis A, B and C target sequences that can be processed together in a simple, single-step protocol and distinguished with a resolution of 3.47 and signal to noise ratio of 17.23 in under 10 seconds by microfluidic capillary electrophoresis.

Introduction

The growing need for highly specific and sensitive molecular probes for nucleic acids has led to research and development of fluorescent probes that take advantage of the inherent selectivity of short synthetic DNA oligos[1]. For example, molecular beacons (MBs) are a popular nucleic acid based probe for DNA detection composed of a synthetic hairpin DNA covalently labeled with an organic fluorophore and a quencher molecule[2,3]. MBs rely on the probe undergoing a conformational change upon binding to its DNA target. This conformational change increases the distance separating the fluorophore and quencher to produce an increase of fluorescence signal. However, these probes often suffer from high background fluorescence and issues with binding as a result of interactions between bound dyes and target[4]. Furthermore, conjugating two different molecules to each MB requires purification steps that result in high costs with low yields compared to unmodified DNA synthesis. (A standard molecular beacon can cost $500 for a guaranteed yield of 10 nmol.) Additionally, for applications that require multiplexing, a multi color approach using MBs with fluorophores of distinct emission spectra, adds potentially unwanted complexity to optical detection hardware.

As an alternative to using fluorophores and quenchers for nucleic acid probes, the facility of using fluorescent silver nanoclusters has recently been demonstrated. Fluorescent silver nanoclusters (AgNC-DNA) self-assemble on single stranded DNA template strands and can have photophysical properties rivaling those of MBs[5–9]. AgNC-DNA are few-atom silver clusters stabilized by the nucleobases of a synthetic DNA host strand[10,11]. They are easily synthesized and can be tuned to exhibit bright fluorescence emission across the visible spectrum via the nucleotide sequence of the host DNA, making them ideal candidates as novel fluorophores[12,13]. The prospect of incorporating sequences for both AgNC stabilization and DNA target binding selectivity into a single oligomer has led to a growing number of novel detection strategies[14]. Some methods of DNA detection using AgNCs rely on spectral shifts[15,16], while others produce 'turn-on' probes[5–7], providing a simple and robust method for fluorescence based DNA detection.

Many of these approaches are intended to produce modular probes, where the target binding portion of the sequence can easily be substituted. The standard approach to multiplexing involves distinguishing between probes by color[17,18]. For the case of AgNC-DNA probes, this means changing both the target binding sequence and the AgNC forming sequence[19]. Although there exists a varied palette of AgNC-DNAs to choose from[13], their synthesis conditions and formation times are often just as varied and can change spectra once incorporated into a larger probe strand often with a reduction in color variation[19]. Recent advances have shown that machine learning algorithms combined with batch processing of oligomers can aid in the discovery of new sequences that stabilize AgNCs[20], but many have similar emission spectra, presenting a challenge for colorimetric multiplexing[21]. Therefore, an alterna-

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tive to color differences between the AgNC-DNA probes is needed for robust multiplexing.

To address this need, we show that by appending poly-dTs to probe sequences, it is possible to easily and rapidly separate and identify different AgNC-DNA probes for nucleic acid targets. These poly-dT regions are easily appended to probes during oligo synthesis and do not require the additional steps, purifications or cost required of a base or end label modification, making this approach particularly well suited for several AgNC based probes[5,7]. The strategy of appending poly-dT is not well suited to typical, end-labelled MBs because it would increase the distance between fluorophore and quencher, resulting in higher background fluorescence levels. Our separation-based approach of AgNC-DNA probes dramatically increases the potential degree of multiplexing by demonstrating an alternative to typical multi-color methods. Multi-color multiplexing is limited by the finite bandwidth resulting from wide emission spectra and the increasing complexity of filter sets needed to distinguish overlapping spectra. Our approach can be easily extended to multiplex tens of species with a high resolution CE separation by simply appending poly-dTs. The DNA oligos for these probes cost $60 for a guaranteed yield of 30 nmole, making them an affordable alternative to multiplexing using spectrally distinct MBs or trying to adapt the approach to MBs using base modifications as opposed to end-tagged strands. This approach In this proof-of-principle work, we have generated single-color probes to detect DNA targets for Hepatitis A, B and C from a single sample, a formidable task using standard PCR techniques[22]. We then identify these probes from a pre-mixed sample using microfluidic capillary electrophoresis (mCE).

Results and Discussion

Probe Design and Synthesis

To demonstrate a multiplexed approach towards using luminescent AgNC-DNA probes we modify a hairpin DNA that was previously characterized for nucleic acid detection of a Hepatitis B surface antigen gene[7] (HBV) to create turn-on probes for two additional nucleic acid targets: Hepatitis A (HAV) and Hepatitis C (HCV). This hairpin based AgNC probe was previously characterized and revealed to have a superior signal to background ratio, linear fluorescence response to target DNA concentration and a limit-of-detection of 3 nM[7]. We chose this probe design because of its simple, single-stranded, modular design, which we hoped would translate into a straightforward process of adapting it to different target DNA sequences without having to optimize the AgNC forming sequence for each probe. Each probe consists of a length of synthetic DNA divided into 4 sequence regions: i) a fluorescent AgNC nucleation region (12 bases), ii) a probe region (30 bases), iii) a AgNC-blocking region (7 bases), and iv) a mobility modifying region (10-20 bases). The structure and behavior of the probes are outlined schematically in Fig. 1. In the native 'off' state, the AgNC nucleation region hybridizes to the blocking region of the probe. While it is hybridized, the silver is unable to access the fluorescence-inducing sequence[12] and formation of the AgNC is inhibited. When the probe hybridizes to its target, the hairpin opens and exposes the AgNC- stabilizing region of the probe. Silver ions bind to the newly exposed nucleobases and become fluorescent nanoclusters upon chemical reduction with sodium borohydride. The exposed blocking region is short and not expected to stabilize a fluorescent AgNC[7].

Tailoring a probe to different targets involves replacing the 30 base probe region with a sequence complementary to the desired target. For the HAV and HCV probes, we replaced the probe region with sequences complimentary to conserved 30 base regions in the 5'UTR of the HAV and HCV genome[23,24], which are commonly used for detection[25,26]. For optimal function of the AgNC-DNA probe, neither probe nor target sequences should form fluorescent AgNCs themselves. Since AgNC-DNA typically form on cytosine rich strands, especially those with significant secondary structures[27], we selected probe-target sequences with few cytosine and guanine repeats, and little or no secondary structure within the probe region sequence because secondary structure in either probe or target sequences could also have a negative impact on probe-target hybridization.

Using this strategy, we found sequences that produced viable fluorescent probes for HAV and HCV that were spectrally similar to the original HBV probe (See Table S1). Each probe has a stable predicted hairpin conformation with melting temperatures above 45 °C that opens in the presence of complimentary target with an estimated melting temperature above 50 °C, as expected[28,29] (See Figures S5, S6 and Table S3).

However, despite adhering to this design strategy, we had to try several HCV probe sequences before finding one that produced a viable probe. These unsuitable sequences were complementary to various regions of the HCV's 5' UTR but yielded poor probes that either did not fluoresce upon hybridization or produced fluorescent clusters in their native hairpin state (See Table S2, Fig. S1). The predicted secondary structures of these problematic probes contain some secondary structure within their hairpin loops (Figure S5), which may contribute to why some of the probes themselves stabilized fluorescent AgNCs, however, their lower melting temperatures suggests that the structures within the loop should not be very stable. Once hybridized to target, these non-functional probes have estimated melting temperatures comparable to the functional probes. We conclude that the ability of a probe to stabilize a fluorescent AgNC is sensitive not only to the AgNC region sequence, but also to interactions with bases