Specific and sensitive detection of nucleic acids and RNases using gold nanoparticle–RNA–fluorescent dye conjugates†

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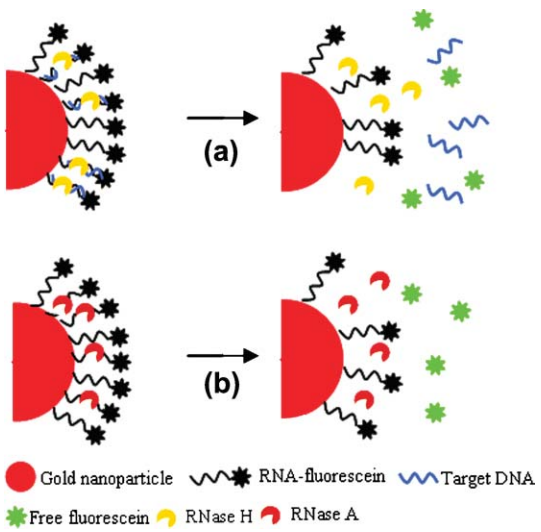
Gold nanoparticles were modified with RNA and utilized to detect specific DNA sequences and various RNA nucleases.

Gold nanoparticle (GNP)-based assays have been used to detect biomolecules such as DNA and proteins. These assays rely on the ability of GNPs to undergo colorimetric changes or induce fluorescence quenching of proximal dyes.[1] The highly efficient energy transfer between GNPs and fluorophores provides a basis for novel assay designs and multiplexing potential.[2] Here we describe our use of GNPs conjugated to fluorescently labeled RNA molecules for the detection of specific DNA sequences and of two RNA metabolizing enzymes, RNase H and RNase A.[3,4]

GNPs (13 nm diameter) were synthesized by sodium citrate reduction of $HAuCl_4$ and modified with RNA (details in ESI†). The RNA, (5′-SH$(CH_2)_6$ AAACGCACUC AGCACCGCAA GAUUCU-3′ $(CH_2)_6$-fluorescein) was obtained from Dharmacon (Lafayette, CO, USA). These conjugates were then used in two assays for (i) detecting an RNA nuclease such as RNase A, and (ii) identifying and quantitating a specific sequence of DNA by utilizing the enzyme RNase H. RNase A and other non-specific nucleases degrade the conjugates,[4] leading to an increase in fluorescence.[1] To identify a DNA strand, we utilized the unique property of RNase H for cleaving the RNA phosphodiester bonds only when it is contained in an RNA–DNA heteroduplex, while not digesting the DNA within the heteroduplex, nor single- or double-stranded RNA or DNA.[3] Thus, assays designed to utilize these properties of RNase H should be hybridization and sequence-dependent. Further, as the DNA strand becomes unhybridized upon RNA degradation, another intact RNA molecule can hybridize, leading to linear signal amplification.

Scheme 1 shows a cartoon of our detection approach using the enzymatic degradation of our GNP–RNA–dye conjugates. The fluorescein-labeled RNA molecule in an intact conjugate exhibits very low fluorescence due to the proximity of the quenching moiety, the GNP. However, upon RNA degradation and diffusion of the fragments into solution, a strong increase in fluorescence intensity is seen. This method provides a basis for the detection of a specific sequence of DNA (Scheme 1(a)) and for RNase A (Scheme 1(b)) by monitoring RNA cleavage using a standard fluorimeter.

We used our GNP–RNA–dye conjugates to detect a complementary single-stranded DNA target (5′-AGAATCTTGC GGTGCTGAGT GCGTTT-3′) (Fig. 1(a)). The selective enzymatic digestion of the RNA–DNA heteroduplex by RNase H was carried out with a customized reaction buffer (1 nM GNP–RNA–dye conjugate, 50 mM Tris-HCl, 75 mM KCl, and 8 mM $MgCl_2$, pH 8.2, 10 μL total volume) containing RNase H (43 μunits per μL, Promega) for one hour at 37 °C. RNase H buffers generally include dithiothreitol or mercaptoethanol as a protein-disulfide reducing agent for maximum activity. However, because the thiol–gold bond is unstable to ligand exchange by thiols,[4] we did not include this reagent; small amounts of reducing agent were present in the enzyme storage buffer (future GNP–RNA–dye designs may utilize a less labile linker). We note that Lima *et al.* reported relatively unperturbed RNase H activity over at least one hour in the absence of a reducing agent.[5] Thus, we kept reaction times to a minimum to maintain optimal enzymatic activity. Complementary target DNA was detected at 10 pM with a signal-to-background ratio of 1.8, while non-complementary DNA (5′-GGTAGCCCAT TACTCTAGCA CACTCTCTAG GGTG-3′) yielded a



Scheme 1 Cartoon showing RNA–dye modified GNPs for detecting DNA, RNase H, and RNase A. Due to the enzymatic digestion of the RNA portion of the conjugates by either RNase H or RNase A, fluorescein diffuses away from the GNP beyond distances of efficient energy transfer, leading to a detectable fluorescent signal. The GNPs were modified with a 26-base RNA molecule containing a 5′-hexylthiol and a 3′-fluorescein. DNA is detected through hybridization to the RNA portion of the probes followed by RNase H digestion. RNase H can be detected by pre-forming an RNA–DNA heteroduplex and monitoring probe degradation. RNase A is detected using the single-stranded conjugates.

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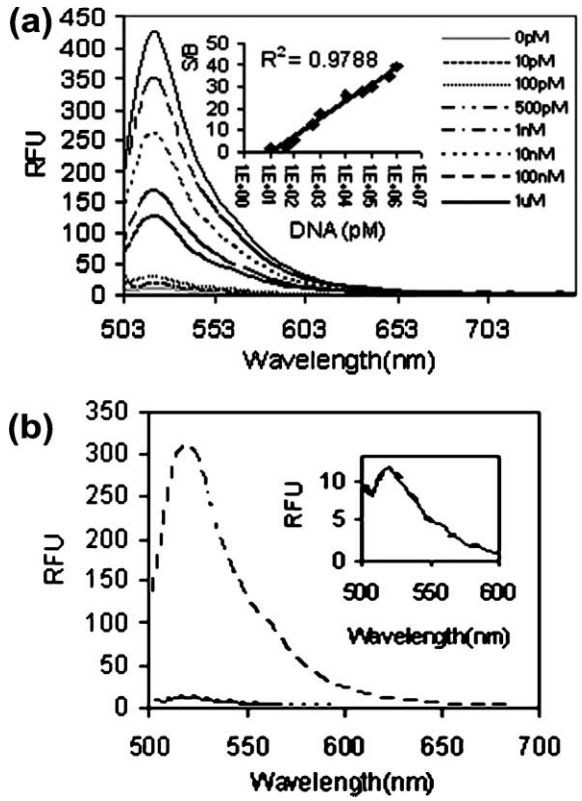

Fig. 1 Target DNA detection using RNase H. (a) Increasing the concentration of DNA with fixed GNP–RNA–dye and RNase H concentrations shows a detection limit of 10 pM. (b) Sequence specificity is displayed by fluorescence before (solid line) and after (dashed line) complementary DNA of 100 nM is added; inset shows similar conditions for before and after 100 nM non-complementary DNA was added to the GNP–RNA–dye and RNase H.

fluorescence intensity nearly identical to that generated using the enzyme without any DNA (Fig. 1(b)). This confirms that the assay can be used to detect a specific DNA target sequence over a non-complementary sequence and we are currently pursuing more challenging mismatch discrimination.

Our GNP–RNA–dye conjugates can also be used to detect low levels of various RNA degrading enzymes. For example, RNase H activity is seen in the retroviral reverse transcriptase which is a validated drug target against HIV.[6] Thus, our conjugates could be used for high-throughput screens for RNase H and/or HIV reverse transcriptase inhibitors. RNase A is found in nearly all organisms and is important for cellular RNA metabolism. RNase A has been extensively studied as a model system to understand protein stability and enzymatic mechanisms.[7] The cytotoxic activity of RNase A provides the basis for its potential use in cancer treatment.[4] Thus, rapid and sensitive assays for these and other RNA degrading enzymes are under investigation.[8–11] Three types of such assays have been described: radiometric, spectrophotometric, and fluorescent. The most sensitive and commonly used type of assay is fluorescence-based and can detect 7.3 fM enzyme.[11] However, more sensitive methods would be useful, particularly for clinical applications. Our conjugates also enable the detection of RNase H and RNase A using either prehybridized DNA–(GNP–RNA–dye conjugates) or just the GNP–RNA–dye conjugates, respectively.

RNase H was detected using our GNP–RNA–dye conjugate with excess complementary DNA to form the heteroduplex substrate (Scheme 1(a)). RNase A was detected directly using the single-stranded conjugate (Scheme 1(b)). Fig. 2(a) shows the results from a 140 second time course. The detection sensitivity for RNase H (Fig. 2(b)) was 43 μunits in a 10 μL reaction volume with a one hour incubation at 37 °C. RNase A was detected at 7.3 pM after one hour of incubation at 37 °C (Fig. 2(c)) and 0.73 fM after a two hour incubation.

The quenching efficiency within the GNP–RNA–dye conjugate is an important factor for determining the overall signal-to-noise ratio. Quenching efficiency was determined from comparing the fluorescence signal of both attached and released fluorescein (details in ESI†). In our constructs, fluorescein molecules are coupled to GNPs by chemisorption of a monolayer of thiolated 26-base single-stranded RNA probe molecules. The fluorescence for the isolated GNP–RNA–dye was ~3% of that obtained from just the RNA–dye component of the conjugate (at the same total dye concentration). Our conjugates contain ~70 RNA–dye components per GNP, similar to previous reports for GNP–DNA conjugates (details in ESI†).

In a previous report, ~70% quenching was observed between 1.4 nm GNPs and a single fluorescein molecule positioned 15 base

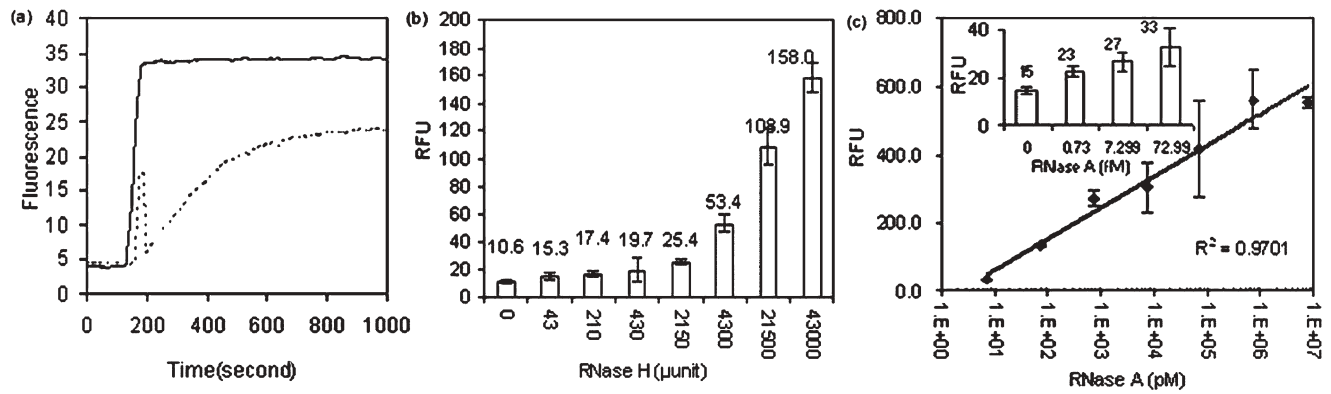

Fig. 2 RNase assays. (a) Time-driven detection of RNase H (dotted line) and RNase A (solid line). After about 140 seconds, RNase H and RNase A were injected. (b) Sensitivity of detection for RNase H. (c) Sensitivity of detection for RNase A. Reaction volumes and incubation times for the assays in (b) and (c) were 10 μl and one hour at 37 °C. The inset of (c) shows the sensitivity of detection for RNase A with a two hour incubation.

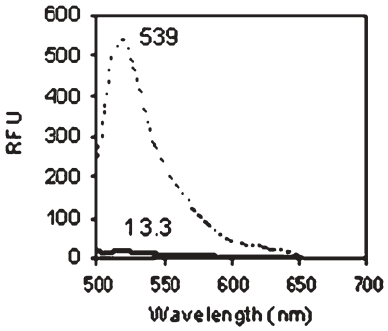

Fig. 3 Fluorescence change using RNase A. Quenched GNP–RNA–dye conjugate (solid curve) shows a fluorescence intensity increase of ~40-fold when incubated with RNase A (dotted line).

pairs away; both labels were at the ends of a double-stranded DNA molecule.[12] Here we use a single-stranded nucleic acid with a reduced persistence length compared to the double stranded nucleic acid[10] used previously, increasing the compactness of the conjugate and the efficiency of energy transfer. Trubetskoy and co-workers reported that fluorescein exhibits >50% self-quenching for a system in which the dye–dye distance was ~2.1 nm using labeled plasmid DNA and assuming a rigid double-stranded scaffold.[8] In our system, the distance between two neighboring fluorescein molecules is similar at ~1.4–2.9 nm (details in ESI†). We attribute the ~97% quenching efficiency to a combination of the non-radiative energy transfer to the GNP and from dye–dye quenching between fluorescein molecules which are in proximity. Treatment of the GNP–RNA–dye conjugate with RNase A results in a ~40-fold increase in fluorescence (Fig. 3), demonstrating that this enzyme efficiently degrades the single-stranded RNA attached to the GNPs, leading to a significant change in distance between the two moieties through diffusion.

Nucleic acid biosensing applications are increasingly important in both fundamental and clinical settings. GNP–RNA–dye conjugates show promise in the detection of a target DNA sequence using nuclease discrimination. We determined a sensitivity limit of 10 pM DNA similar to a report by Dubertret and co-workers using a GNP-quenched molecular beacon approach (sensitivity of 67 pM).[1] In our case, an additional signal amplification is provided by the enzymatic cycling of RNase H (the DNA target is not degraded by the enzyme and may form a heteroduplex with many RNA probes during the incubation time). RNase H has been used in various DNA detection strategies,[13] although to our knowledge this combination of homogeneous solution-phase GNPs is unique. A recent report utilizing RNase H degradation with enzymatic cycling on a self-assembled monolayer of RNA on a gold film was capable of detecting 1 fM DNA with surface plasmon resonance imaging. We anticipate that the further optimization of our assay may provide for high sensitivity optical detection in a homogeneous solution phase, effectively combining molecular beacon and surface cycling strategies. Important parameters for optimization include the surface density of the probe, position of the dye within the strand, colloidal stability, quenching efficiency with distance, and even the source of the RNase enzymes. We expect this novel method for sensitive and specific detection of nucleic acids to be useful for fundamental studies regarding the effects of nanoparticle surface composition on enzyme activity, and for nuclease and DNA detection.

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