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Label-Free Colorimetric Detection of Specific Sequences in Genomic DNA Amplified by the Polymerase Chain Reaction

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Abstract: We document the surprising result that single-stranded DNA adsorbs on negatively charged gold nanoparticles (Au-nps) with a rate that depends on sequence length and temperature. After ss-DNA adsorbs on Au-nps, we find that the particles are stabilized against salt-induced aggregation. These observations can be rationalized on the basis of electrostatics and form the basis for a colorimetric assay to identify specific sequences and single nucleotide polymorphisms on polymerase chain reaction (PCR)-amplified DNA. The assay is label-free, requires no covalent modification of the DNA or Au-np surfaces, and takes on the sensitivity of PCR. Most important, binding of target and probe takes place in solution where hybridization occurs in less than 1 min. As an example, we test PCR-amplified genomic DNA from clinical samples for single nucleotide polymorphisms (SNPs) associated with a fatal arrhythmia known as long QT syndrome.

Introduction

Highly selective detection of specific DNA sequences is increasingly important in clinical diagnosis, pathology, and genetics.[1] Nearly all assays for DNA sequences use the polymerase chain reaction (PCR) to amplify specific sequence segments from as little as a single copy of DNA to easily detected quantities.[2] The use of PCR not only addresses sensitivity issues but also effectively purifies samples to ameliorate the effects of large quantities of DNA that may not be of interest for a given assay. These features presently make the use of PCR nearly indispensable for the analysis of genomic DNA despite the development of a wide variety of innovative sensing approaches such as surface plasmon resonance (SPR),[3] fluorescent microarrays,[4] assays based on semiconductor or metal nanoparticles,[5−9] and water-soluble conjugated polymer-based sensors.[10] These techniques have been demonstrated mostly on purified synthesized oligonucloetides, but it may be possible to adapt some of them to be compatible with PCR-amplified samples. Once PCR amplification is utilized, however, the merit of an assay is primarily determined by its simplicity rather than its sensitivity because additional amplification is straightforward. Most of the above approaches require expensive instrumentation or involve time-consuming synthesis to modify DNA, substrates, or nanoparticles. In addition, it is usually necessary to conduct hybridization in the presence of substrates that introduce steric hindrance, which leads to slow and inefficient binding between probe and target. As a result, the postprocessing of PCR-amplified samples is expensive and time-consuming.[11]

Complexes between DNA and negatively charged Au-nps have been studied for many years,[12] and many creative schemes have exploited Au-nps covalently functionalized with DNA sequences to bind specific target DNA sequences either for nano-assembly or for oligonucleotide sensing.[6−9,12−15] It has been widely assumed that the negative phosphate backbone of

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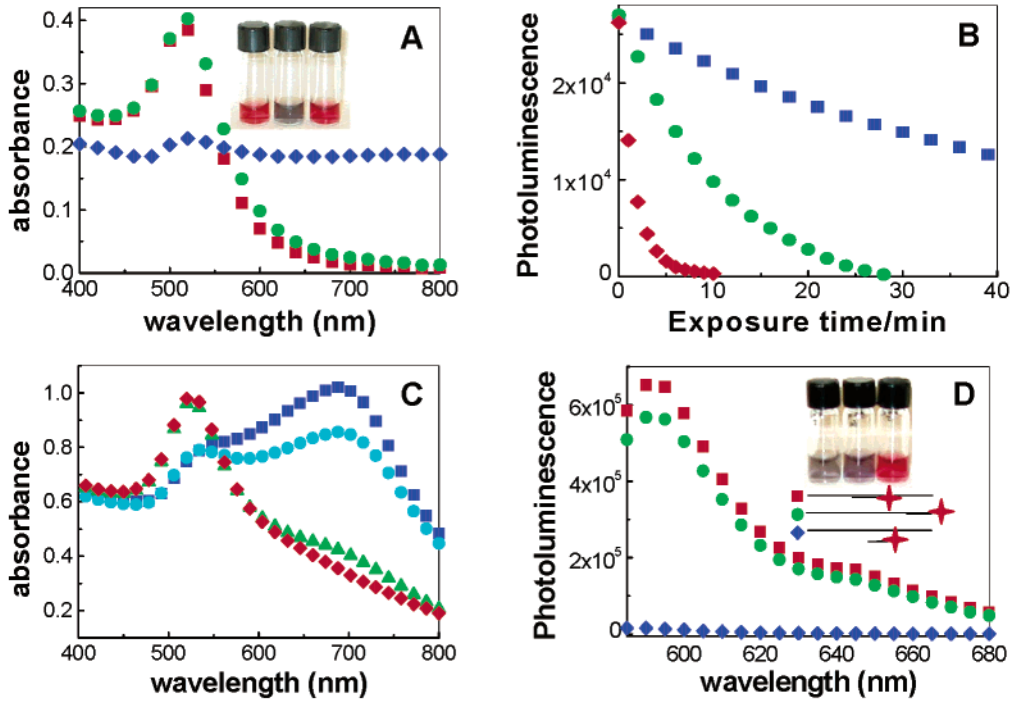

Figure 1. Adsorption of ss-DNA to gold nanoparticles. (A) Absorption spectra of 300 μL of gold colloid and 100 μL of deionized water (red), 100 μL of 10 mM PBS (0.2 M NaCl) (blue), 300 pmol of 24-base ss-DNA first, then 100 μL of 10 mM PBS (0.2 M NaCl) (green). (B) Photoluminescence intensity versus time following the addition of 4 pmol of rhodamine red-tagged ss-DNAs to 1000 μL of gold colloid. 10 mer (red), 24 mer (green), and 50 mer (blue). (C) Absorption spectra of the mixture of 200 pmol of ss-DNA (50 mer) and 300 μL of gold nanoparticles heated at different temperature for 2 min, followed by the addition of 300 μL of 10 mM PBS (0.2 M NaCl). 22 °C (blue), 45 °C (cyan), 70 °C (green), and 95 °C (red). (D) The fluorescence spectra of the hybridized solutions of rhodamine red-labeled 15 mer ss-DNA and 50 mer ss-DNA and gold colloid, the 15 mer binding to the 50 mer in the middle (red), at the end (green) and not at all (blue). The lower inset schematically illustrates the binding positions between the 15 mer and 50 mer. The upper inset contains color photographs of the corresponding mixtures (from left to right) with no fluorescent label on the 15 mer. See the Supporting Information for experimental details.

DNA repels negatively charged Au-nps and makes nonspecific attachment negligible.[7,16] In the present work, we show that single-stranded DNA (ss-DNA) has strong attractive electrostatic interactions with citrate-coated Au-nps. In fact, ss-DNA adsorbs efficiently on these Au-nps with a rate that depends on sequence length and temperature. Moreover, the adsorption of ss-DNA can prevent the Au-nps against salt-induced aggregation.[17]

On the basis of these observations, we have designed a rapid colorimetric assay for specific sequences and SNPs in PCR-amplified genomic DNA that is based on electrostatics and requires no labeling or surface functionalization chemistry. Detection of single nucleotide polymorphisms (SNPs) in genomic DNA is particularly challenging but is at the forefront of diagnostic technology because it has been associated with a number of hereditary conditions and cancers and is likely to be responsible for many more.[18,19] In our assay, we use the aggregation of unmodified Au-nps as a hybridization indicator. The analytes, PCR product without additional processing, are hybridized with probes under normal physiological conditions prior to exposure to the Au-nps. This avoids the steric constraints associated with hybridization on functionalized surfaces[6] and allows the entire assay including hybridization to be completed in less than 10 min. The result is determined visually and requires no detection instrumentation. As an example, we demonstrate the application of the assay to screen for the presence of SNPs associated with a fatal arrhythmia known as long QT syndrome in clinical samples of genomic DNA.

Results and Discussion

The color of gold colloid is very sensitive to the degree of aggregation of Au-nps in suspension,[20,21] and the aggregation can be easily induced with electrolytes such as salt.[17] This phenomenon can be easily monitored by absorption spectroscopy or by visual observation. Au-nps (13 nm in diameter) in aqueous solution are stabilized against aggregation by a negatively charged coating of citrate ions.[22] As individual particles, Au-nps have a surface plasma resonance absorption peak at 520 nm (Figure 1A: red) and appear pink (Figure 1A, inset: left vial). Immediate aggregation of the Au-nps occurs when enough salt is added to screen the electrostatic repulsion between the ion-coated Au-nps. The result is a broad featureless absorption spectrum (Figure 1A: blue) and blue-gray color (Figure 1A, inset: middle vial) characteristic of the surface plasma resonance of Au-np aggregates.[20,21]

We find that moderate amounts of salt (total concentration $\approx$ 0.05−0.2 M) no longer cause aggregation of the Au-nps if

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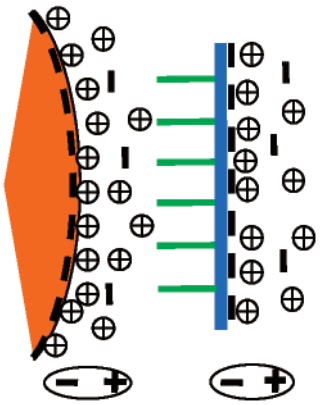

Figure 2. Schematic of the interaction between negatively charged Au-np and ss-DNA. The orange wedge represents the Au-np, the green lines represent the DNA bases, and the blue line represents the phosphate backbone.

enough ss-DNA (of order 10 strands per nanoparticle for 24 mers) is added to the gold colloid before addition of the salt that would otherwise cause aggregation. Under these circumstances, the gold colloid retains its absorption spectrum and color (Figure 1A, green; and inset: right vial). The reason for the stabilization of the colloid is that the oligonucleotides adsorb and add negative charges to the Au-nps that enhance their repulsion. This assertion is confirmed by fluorescence quenching experiments using rhodamine red-tagged ss-DNA (Figure 1B). When the oligonucleotide sticks to the Au-np, the attendant proximity of the dye to the gold leads to fluorescence quenching.[7,8] The fluorescence quenching experiments also show that the adsorption rate depends on sequence length, with shorter sequences sticking much more rapidly to the Au-np (Figure 1B). In addition, we have found that increasing temperature also results in faster adsorption (Figure 1C). To the best of our ability to determine with a sampling of oligonucleotide sequences, there is no sequence dependence for the results of Figure 1. Both the ss-DNA adsorption on Au-np and the Au-np aggregation inferred from the data in Figure 1A−D are irreversible.

The adsorption of ss-DNA on negatively charged Au-nps is contrary to the conventional wisdom[7,16] because, in its native configuration, ss-DNA is coiled so that the hydrophilic negatively charged phosphate backbone is most exposed to the aqueous solution.[23] We can rationalize the fact that ss-DNA sticks to Au-np, as well as the dependence on sequence length and temperature, with a simple picture derived from the theory of colloid science.[17] Both the gold and the ss-DNA attract counterions from the solution and are well described by electrical double layers as depicted schematically in Figure 2. In every case, there are attractive van der Waals forces between the oligonucleotide and the nanoparticle. The electrostatic forces are due to dipolar interactions and depend on the configuration and orientation of the ss-DNA. When transient structural fluctuations permit short segments of the ss-DNA to uncoil and the bases face the Au-np, attractive electrostatic forces cause ss-DNA to adsorb irreversibly to the gold. The requisite fluctuations are more prevalent in short sequences because there is less of the chain remaining to enforce the coiled morphology. Hence, short ss-DNA oligonucleotides adsorb more quickly. Similarly, increases in temperature facilitate fluctuations that expose the bases and unwind the coiled structure to make the adsorption faster. Increases in temperature will also serve to break secondary structure in longer DNA chains, thereby making the geometry of Figure 2 more easily accessible.

The length-dependent adsorption can be exploited to develop an assay appropriate for the detection of PCR-amplified DNA sequences that are typically several hundred base pairs in extent.[2] We can design short oligonucleotide "probes" with the idea that, when these are hybridized to the long strands, they will not adsorb rapidly on Au-np. They will therefore be unable to prevent salt-induced aggregation, and the attendant color changes when there is a sequence match between the probe and part of the long strand. Alternatively, if we fluorescently label the short probes, their fluorescence will be quenched by adsorption on the Au-np unless they are "tied up" by hybridization to the long target strand. Figure 1D illustrates the proof of principle for each of these assays with synthesized 50-base oligonucleotide targets and rhodamine-labeled 15-base probe.

Following these model experiments, we have designed simple colorimetric assays that address the critical issues that arise in the analysis of PCR-amplified DNA. First, we can ascertain whether the amplified DNA contains the desired sequence by evaluating hybridization with our probes. Second, we show that it is straightforward to identify SNPs in the amplified sequences. All of our experiments are performed on PCR product obtained from a clinical diagnosis laboratory without further purification. The sequence we probe derives from genomic DNA taken in patient studies of a fatal cardiac arrhythmia called long QT syndrome.[24] This condition has been associated with a mutation in the KCNE1 gene.[25]

Current assays for point mutations on PCR-amplified sequences involve time-consuming procedures, expensive instrumentation, or both.[2,11] Our method takes less than 10 min to verify amplification of the appropriate sequence and test for SNPs with the same thermal cycler used to do the PCR. To confirm amplification of the desired sequence, we follow the protocol illustrated schematically in Figure 3A. We choose two ss-DNA probes with sequences complementary to the desired PCR product that have melting temperatures lower than the primers and add these to the PCR product solution. The PCR-amplified ds-DNA is dehybridized at 95 °C to produce ss-DNA. These mixtures are annealed below the probe melting temperature so that the probes can hybridize with the PCR-amplified sequence if it is present. At the same time, the unconsumed primers also bind to the PCR product because they have melting temperatures higher than those of the probes. As in the PCR process itself, competition for binding locations from rehybridization of the PCR-amplified complement is negligible because it is slower for steric reasons. When gold colloid is exposed to this mixture, the salt in the hybridization solution causes immediate Au-np aggregation and a color change if the probes have hybridized to the amplified DNA target (Figure 3B, left vial). When the PCR product is not complementary to the probes or the PCR amplification fails altogether, the probes adsorb to the Au-nps and prevent aggregation (Figure 3B, right vial).

Single base-pair mismatch detection requires a slightly different protocol because a single base mismatch still permits hybridization of the probe to the target sequence. We use the

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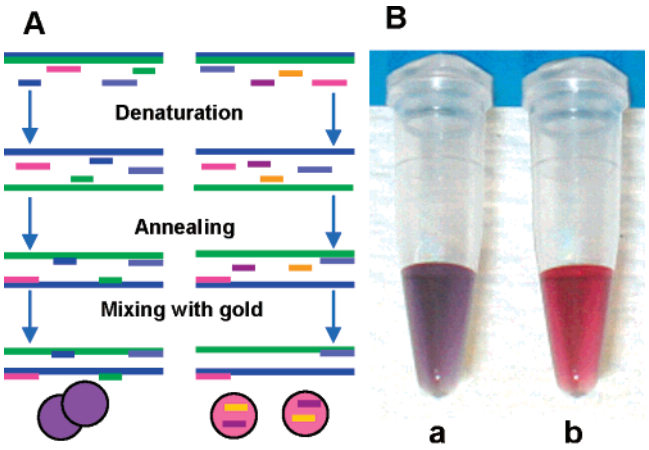

Figure 3. Identification of PCR-amplified DNA sequences. (A) Schematic of the detection protocol. The mixture of PCR product and probes is denatured and annealed below the melting temperature of the complementary probes followed by addition of gold colloid. The long blue and green lines represent the PCR-amplified DNA fragments, and the pink and light blue medium bars represent the excess PCR primers. The short blue and green bars are complementary probes that bind, resulting in Au-np aggregation (purple color). The short purple and orange bars are noncomplementary probes that do not bind and adsorb to the Au-np, preventing Au-np aggregation and leaving the solution pink. (B) Color photographs of the resulting solutions with complementary probes (a) and noncomplementary probes (b). We used 8 μL of PCR product, 3.5 pmol of probe, and 70 μL of gold colloid in each vial.

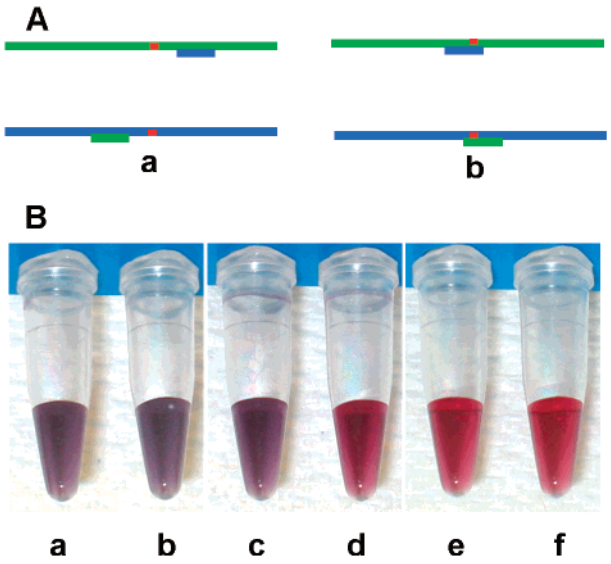

Figure 4. Single base-pair mismatch detection. (A) Detection strategy. The red spots on the long green and blue lines represent positions of a potential SNP. The long green and blue lines are the complementary sequences of the PCR-amplified DNA fragment. The short green and blue bars are probes complementary to parts of the wild-type sequence of the PCR-amplified DNA fragment as illustrated. (B) Detection of a single base-pair mismatch. Vials b, d, and f contain PCR product with probes overlapping the single-base mismatch, while a, c, and e contain PCR product with probes not overlapping the single base-pair mismatch. Photographs are taken of the mixtures annealed at 50 °C (a, b), 54 °C (c, d), and 58 °C (e, f). We used 8 μL of PCR product, 3.5 pmol of probe, and 70 μL of gold colloid in each vial.

same concept as that used for specific sequence detection with the strategy depicted in Figure 4A. Four probes are selected that have the same melting temperature, lower than that of the PCR primers. The sequences are chosen to be complementary to the wild-type sequence of the target. Two of the probes bind and overlap the position of the possible point mutation, while two are used as controls and bind at locations that do not overlap the SNP under study. If a mutation exists on the target sequence, the probes covering the mutation will dehybridize at lower temperature than the control probes situated elsewhere in the sequence that are designed to be perfectly matched. At a temperature below the melting point of either sequence, the probes remain attached to the PCR-amplified DNA and cannot prevent salt-induced Au-np aggregation (Figure 4B: a, b). Above the melting temperature of both perfect and mismatched sequences, dehybridization occurs for either type of sequence and Au-np aggregation is prevented (Figure 4B: e, f). At temperatures above where the mismatched sequence dehybridizes but below where the perfectly matched sequence dehybridizes, color differences indicating the presence of a SNP are detected (Figure 4B: c, d). Of course, if one wanted to determine specifically which mutant base was present at the location of the potential SNP, experiments analogous to those of Figure 4 could be done using probes with all three possible mismatched bases.

Conclusions

We have demonstrated that ss-DNA adsorbs to Au-np with a rate that is length- and temperature-dependent. In addition, adsorption of ss-DNA on Au-np can effectively stabilize the colloid against salt-induced aggregation. We have utilized these observations to design a simple, fast colorimetric assay for PCR-amplified DNA based strictly on the electrostatic properties of DNA. Our approach obviates the need for gel electrophoresis and other complex sequencing procedures. It can be implemented with inexpensive commercially available materials in less than 10 min, and no instrumentation beyond the programmable thermal cycler used for PCR is required. An important feature of the method is that, unlike chip-based assays[26] or other approaches that utilize functionalized nanoparticles,[6−9] hybridization occurs under optimized conditions that can be regulated independent of the assay. We have applied the assay to clinical samples of genomic DNA that screen for SNPs associated with a hereditary cardiac arrhythmia known as long QT syndrome. We believe that our approach can replace some traditional analytical methods for the postprocessing of PCR-amplified DNA and that it will find broad application.

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Supporting Information Available: Experimental details of the materials synthesis, DNA sequences, and mixing protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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