Homogeneous, Nanoparticle-Based Quantitative Colorimetric Detection of Oligonucleotides

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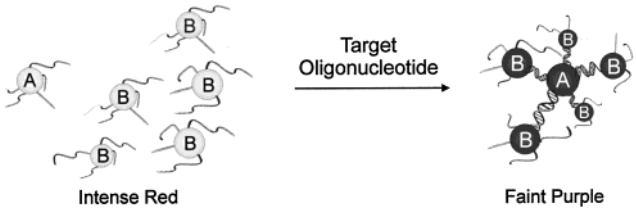
The *homogeneous* detection of oligonucleotides is essential for monitoring and quantifying the amount of product generated by PCR.[1] In general, homogeneous assays are attractive detection formats since they offer the potential to be monitored in real time, are amenable to automation, reduce the risk of contamination, and eliminate time-consuming washing steps.[2,3] Currently, most homogeneous oligonucleotide detection formats rely on fluorescence,[4,5] and although highly sensitive, they require relatively expensive probes and monitoring equipment. Recently, a *heterogeneous* colorimetric oligonucleotide detection system based on alkylthiol-functionalized oligonucleotide-modified 13 nm gold probes was developed in this laboratory.[6] In this system, dispersed oligonucleotide-modified gold nanoparticles are assembled into aggregated polymeric networks via hybridization events with complementary target oligonucleotides and spotted onto a reverse phase silica gel support as a function of temperature. The oligonucleotide-induced aggregation occurs with a concomitant red-to-blue color change that is easily visualized on the support. The attributes of this spot test are: (1) ultrahigh selectivity, (2) ability to be monitored by the naked eye, and (3) low cost. With respect to using a similar strategy for the homogeneous detection of oligonucleotides in solution, the drawbacks of the system are: (1) a detection limit of 1 nM, which requires it to be used in a post PCR *non-quantitative* format, and (2) the sedimentation properties of the nanoparticle networks, which precludes the possibility of obtaining acceptable endpoints. Herein, we describe a new strategy that employs the use of 50 nm gold particle probes and the generation of smaller aggregate structures through non-unity probe (Scheme 1) ratios to demonstrate the concept of a quantitative colorimetric homogeneous assay for oligonucleotides with definitive endpoints.

To increase the sensitivity of nanoparticle based assays, we have developed methods for modifying and stabilizing 50 and 100 nm gold particles using dithiane epiandrosterone functionalized oligonucleotides.[7] It was recently shown that 13 nm particles modified with dithiane epiandrosterone-functionalized oligonucleotides are substantially more stable to thiols than ones modified with alkylthiol-functionalized oligonucleotides. Since the molar extinction coefficients for the plasmon bands associated with the particles are 10^8, 10^{10}, and 10^{11} M^{-1} cm^{-1} for 13, 50, and 100 nm gold particles, respectively;[8] we hypothesized that the use of 50 and 100 nm gold particles (rather than 13 nm particles) would lead to higher sensitivity assays and open the possibility for the development of a homogeneous, quantitative colorimetric detection format based upon gold nanoparticles.

Scheme 1



The surface modification of the 50 and 100 nm gold particles with dithiane androsterone-functionalized oligonucleotides was accomplished using a procedure similar to the one used for modifying 13 nm gold particles with alkylthiol-functionalized oligonucleotides (Supporting Information).[9] To maximize hybridization kinetics and improve the overall stability[10] of the modified gold nanoparticles, dithiane androsterone-functionalized oligonucleotides with dA[20] tethers were used in conjunction with the fifteen base-recognition sequences.[11] In the presence of a target oligonucleotide[12] that is complementary to the recognition sequences of the oligonucleotides attached to the gold probes (1:1 probe ratio), hybridization occurs, leading to nanoparticle aggregation and a concomitant decrease in molar extinction of the surface plasmon bands, Figure 1A and B.[13] Note that the breadth of the plasmon bands associated with the dispersed 50, and especially the 100 nm particles, makes them less desirable for use in a spot test since pre- and post-test (aggregation) colors are not easily distinguishable. However, the large extinction changes in the plasmon region of the spectra that accompany target-induced particle aggregation make these probes ideal for use in a homogeneous detection system for oligonucleotides. These aggregation processes are reversible; upon heating the gold nanoparticle aggregate solutions above the thermal denaturation temperature of the oligonucleotide duplex interconnects, the particles redisperse as evidenced by UV−vis spectroscopy.[14] These melting transitions occur over a very narrow temperature range (the fwhm[15] of the first derivatives of the melting transitions

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(9) (a) Gold nanoparticles (50 and 100 nm) were purchased from Vector Labs, Burlingame CA. The actual particle sizes are 51.9 ± 3.5 and 99.5 ± 4.5 nm as determined by the supplier via TEM. (b) The oligonucleotide modification procedures for the 50 and 100 gold particles with dithiane androsterone-functionalized oligonucleotides have been deposited as Supporting Information.
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(11) 5′-dith-epiandro-$[A]_{20}$-TAA-CAA-TAA-TCC-CTC-3′; 5′-dith-epiandro-$[A]_{20}$-ATC-CTT-ATC-AAT-ATT-3′. The 15 base recognition sequences are complementary to a 30 base section of a PCR product from *Bacillus anthracis*, the 141 base pair anthrax protective antigen.
(12) 5′-GAG-GGA-TTA-TTG-TTA-AAT-ATT-GAT-AAG-GAT-3′.
(13) All hybridization experiments were performed in a buffer solution of 0.3 M NaCl, 10 mM phosphate pH 7.0, and 0.01% (w/w) sodium dodecyl sulfate.
(14) Thermal denaturation profiles of 50 and 100 nm gold particle aggregates have been deposited as Supporting Information.
(15) Full width at half-maximum.

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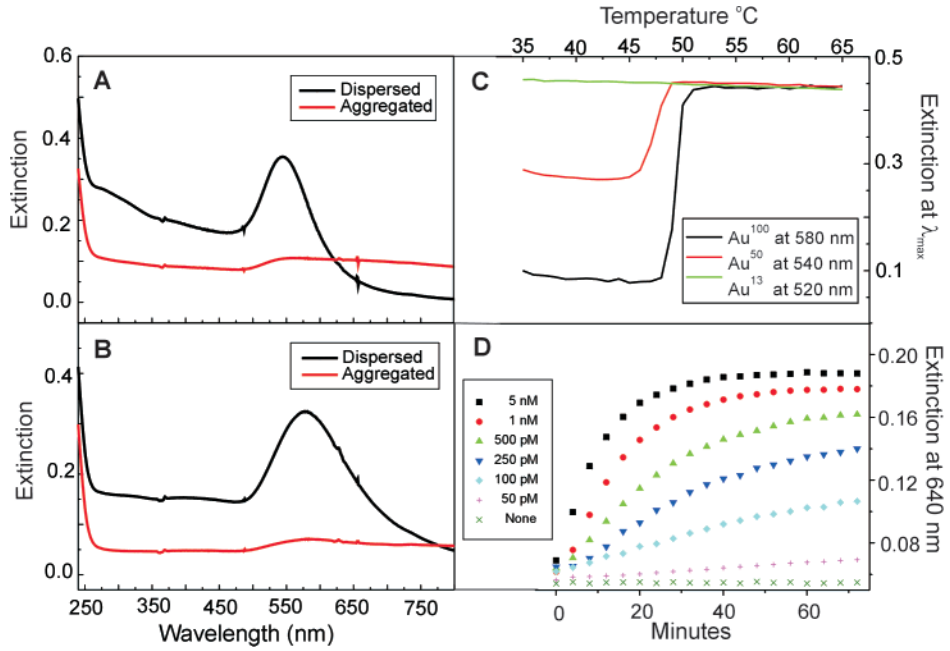

Figure 1. UV−vis spectra prior to and after thermal denaturation for aggregates of (A) 50 nm and (B) 100 nm Au nanoparticles. (C) Thermal denaturation profiles for aggregated 13 (ΔAbs. 0%), 50 (ΔAbs. 57%) and 100 nm (ΔAbs. 395%) Au nanoparticle solutions (1:1 probe ratios).[16] (D) Target oligonucleotide (50 pM−5 nM) induced changes in extinction at 640 nm due to Au nanoparticle aggregation (5:1 probe ratio).

are ∼2.0 °C), which is emerging as a general feature of gold nanoparticle probe/target aggregates. Assays based on these sharp transitions have higher target selectivities than assays based on oligonucleotide probes with conventional melting profiles.[6]

To demonstrate the importance of increasing particle size to increase assay sensitivity, we studied three solutions consisting of 13, 50, and 100 nm diameter Au probes, respectively. All three solutions had 1:1 probe ratios and concentrations such that the extinctions at their respective λ_{max} values were identical (see inset Figure 1C).[16] Target (500 fmol) was added to each solution (1 mL), and melting analyses were carried out by monitoring λ_{max} in the visible region for each probe solution, Figure 1C. The solution containing the 13 nm particles shows no detectable response, while the 50 and 100 nm particle probes exhibit typically sharp melting profiles with T_m's of 47.4 and 49.4 °C, respectively. For the 13 nm system, the amount of cross-linking target oligonucleotide added to the Au nanoparticle solution is not sufficient to induce optically detectable Au nanoparticle aggregation. However, both the 50 and 100 nm systems show significant extinction dampening of their surface plasmon bands due to oligonucleotide hybridization-induced nanoparticle aggregation as evidenced via melting analyses, Figure 1C (red and black traces). By increasing particle size and keeping oligonucleotide surface coverage constant, the increased sensitivity will scale approximately according to the increase in plasmon intensity divided by the ratio of particle radii squared (Supporting Information).

Taking advantage of the increased extinction values of the plasmon resonances associated with the larger particle probes, we have developed a proof-of-concept colorimetric homogeneous assay for the detection of oligonucleotides. While the 100 nm gold probes are more sensitive to lower oligonucleotide concentrations than the 50 nm probes, adequate endpoints were not obtainable with the 100 nm probes due to aggregate sedimentation. Consequently, the 50 nm probes were used for this colorimetric homogeneous assay. By adjusting the ratio of the two sequence specific 50 nm gold probes to 5:1, we can promote the formation of smaller gold nanoparticle aggregates[17] that do not precipitate over the course of the experiment. This leads to a colorimetric detection system with definitive endpoints in the visible region of the spectrum, Figure 1D. Using this protocol, we studied a series of target concentrations ranging from 50 pM to 5 nM. In a typical experiment, 5 μL of target oligonucleotide was added to a 1 mL aliquot of probe solution,[13,18] and changes in the UV−vis spectrum were monitored as a function of time. At 640 nm distinct endpoint extinction values are attained for the six different target concentrations studied.

This study is significant for several reasons. (1) It shows that nanoparticle probes can be used for the homogeneous colorimetric detection of oligonucleotides and, therefore, offers an attractive alternative to more expensive fluorescence-based methods. (2) It maps out the important chemical considerations in using nanoparticle probes for such assays. (3) It shows that, although larger particle probes and reduced oligonucleotide surface coverages can lead to higher sensitivities and larger detection ranges, particle sedimentation and probe ratios must be taken into account in designing any nanoparticle-based detection system for oligonucleotides where quantification is desired. Current work is aimed at evaluating the stability of these systems under PCR conditions.

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Supporting Information Available: Structure of dithiane androsterone; dithiane androsterone-functionalized oligonucleotide modification procedures for the 50 and 100 nm gold particles, their thermal denaturation profiles upon aggregation, and their first derivatives; TEM images of aggregates generated from 1:1 and 5:1 probe ratios and an analysis of the relationship between probe size and sensitivity (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) Absorbance of 0.47 at λ_{max} of 520, 540, and 580 nm prior to aggregation; for 13, 50, and 100 nm gold nanoparticles (2 nM, 35 pM, and 3.8 pM, respectively) and a target concentration of 500 pM were used for the melting studies. Prior to melting these solutions were annealed at 40 °C for 2 h.

(17) This was verified by dynamic light scattering and TEM (Supporting Information) 2 h post target addition, through the comparison of a 1:1 to a 5:1 ratio of gold nanoparticle probe solutions. The solutions were ∼30 pM in gold nanoparticle probe and 500 pM in target oligonucleotide. The 1:1 ratio of probes had an effective aggregate diameter of ∼300 nm after 60 min, while the 5:1 ratio of probes had an effective diameter of only 130 nm.

(18) The total concentration of gold probes was 30 pM.