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Ultrasensitive DNA Detection Using Oligonucleotide−Silver Nanoparticle Conjugates

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Oligonucleotide−gold nanoparticle (OGN) conjugates are powerful tools for the detection of target DNA sequences due to the unique properties conferred upon the oligonucleotide by the nanoparticle. Practically all the research and applications of these conjugates have used gold nanoparticles to the exclusion of other noble metal nanoparticles. Here we report the synthesis of oligonucleotide−silver nanoparticle (OSN) conjugates and demonstrate their use in a sandwich assay format. The OSN conjugates have practically identical properties to their gold analogues and due to their vastly greater extinction coefficient both visual and absorption analyses can occur at much lower concentrations. This is the first report of OSN conjugates being successfully used for target DNA detection and offers improved sensitivity which is of interest to a range of scientists.

Simple, ultrasensitive detection of specific DNA sequences has been the aim of many studies and technologies for decades. To achieve this, oligonucleotide probes have been created with a large range of reporting moieties that include fluorescent markers,[1] proteins,[2] radioactive elements,[3] and chemiluminescent compounds[4] to facilitate ultrasensitive analysis. Modification of oligonucleotides with gold nanoparticles was first reported over 10 years ago and opened up several new avenues of research.[5,6] They can be used as a novel nanostructured building material[7,8] and as an analytical tool to colorimetrically detect low concentrations of a target DNA sequence.[9] Visual differentiation between full complementarity and a single-nucleotide polymorphism when used in a sandwich assay format has been reported[10] and was possible due to the nanoparticle altering the melting characteristics of the DNA duplex, making the transition from double to single stranded much sharper when compared to unmodified oligonucleotides.[11] This sharper transition has been determined to be a cooperative melting phenomenon that results from multiple hybridization events occurring between conjugates and a decrease in the melting temperature as DNA strands dissociate due to a concomitant reduction in local salt concentration.[11] Modification of the DNA with nanoparticles means the melting temperature is not only dependent upon target oligonucleotide concentration and the ionic strength of the buffer medium as is normal but also on the size of the conjugated nanoparticle, the distance between the nanoparticles during hybridization, and the density of the oligonucleotide on the surface of the nanoparticle.[11] The melting transition can be monitored at multiple wavelengths, 260 nm (oligonucleotide maxima), 520 nm (unaggregated gold nanoparticle surface plasmon maxima), and 650 nm (aggregated gold nanoparticle surface plasmon maxima), via conventional absorption spectroscopy. The novel aspects of the nanoparticle label have allowed a wide variety of array-based detection methods to be used including use of a conventional flatbed scanner,[12] Raman spectroscopy,[13] and conductivity measurements.[14] All these techniques use a silver enhancement amplification methodology to coat the conjugate with silver metal and increase the visibility of the label or the generated signal.

Almost all the research focus has been concerned with using gold nanoparticles with very little work on other noble metal nanoparticles such as silver. This is due to the difficulty in the methods used for synthesis of silver nanoparticles which makes achieving repeatable size and optical characteristics problematic compared to gold nanoparticle synthesis. What work has been done utilizes direct conjugate−conjugate hybridization, hybridization to a functionalized surface, or hybridization solely to a target oligonucleotide.[15−19] At this time no research published has used

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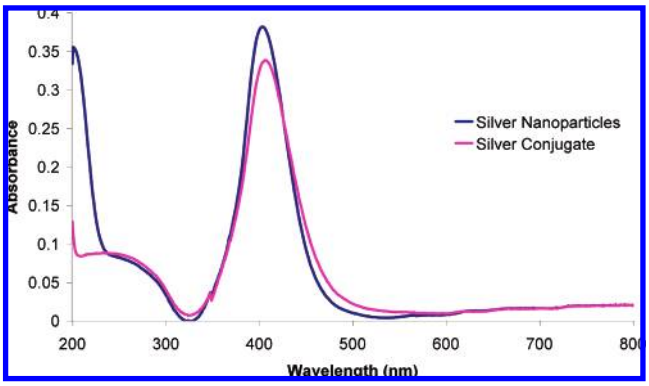

Figure 1. Combined UV−vis spectrum of silver nanoparticles and silver nanoparticles modified with a 5′-thiol-modified oligonucleotide (22 bases).

OSN conjugates in a sandwich assay to detect a target oligonucleotide. The benefit of using silver nanoparticles rather than gold is that the molar extinction coefficient is 100× greater therefore increasing sensitivity when using absorption spectroscopy, which leads to improved visibility due to the difference in optical brightness. In addition, silver nanoparticles provide much greater enhancements of Raman scattering than gold[20] and as such control over the aggregation of silver nanoparticles is of considerable interest to those looking for optimal sensitivity using surface-enhanced Raman scattering.

Here we report the first use of OSN conjugates in a sandwich assay to detect a target oligonucleotide with colorimetric oligonucleotide analysis to differentiate a single base mismatch.

RESULTS

Synthesis of Oligonucleotide−Silver Nanoparticle Conjugates. The attachment of oligonucleotide probes to silver nanoparticles was achieved by using a terminal alkyl thiol group that was situated at either the 3′ or 5′ terminus. The method used to create oligonucleotide−silver nanoparticle (OSN) conjugates is analogous to that used to synthesize oligonucleotide−gold nanoparticle (OGN) conjugates, and it was not necessary to alter the surrounding medium to facilitate attachment of the oligonucleotide.

Since the oligonucleotide bases also have reactive functional groups there is a tendency for any oligonucleotide strand attached to a metal nanoparticle to become associated with the surface. This problem is circumvented by the use of a "spacer" or "tether" of adenine bases.[19,21−23] This also has the secondary benefit of distancing the part of the sequence to be used for hybridization from the nanoparticle surface, increasing the hybridization efficiency by lessening any steric hindrance caused by competing oligonucleotide strands. Following the oligonucleotide conjugation there is a period of gradual curing, using a solution of phosphate-buffered saline (PBS) that is necessary for DNA hybridization. It is not possible to add this prior to the addition of DNA as the PBS would disrupt the citrate surface layer of the nanoparticle, causing irreversible aggregation. After centrifugation to concentrate the nanoparticle conjugates and remove any unreacted oligonucleotides, the conjugates were stable for up to 3 months at room temperature. The average yield of conjugate obtained compared to starting silver nanoparticle amount was 65%; however, this was not achieved for all conjugates. Oligonucleotides modified with an alkyl thiol group at the 3′ terminus produced conjugates of a lower yield when compared with those functionalized with the same concentration of 5′-modified oligonucleotide. At this time it is not clear what causes this lack of stability, but the alkyl chain of the 3′ thiol linker is three carbons shorter than the 5′ (six carbons) and no previous research has focused on this discrepancy. The difference could be that the alkyl chain increases the hydrophobicity to a greater extent leading to increased shielding of the nanoparticle from the disruptive ionic hybridization buffer.

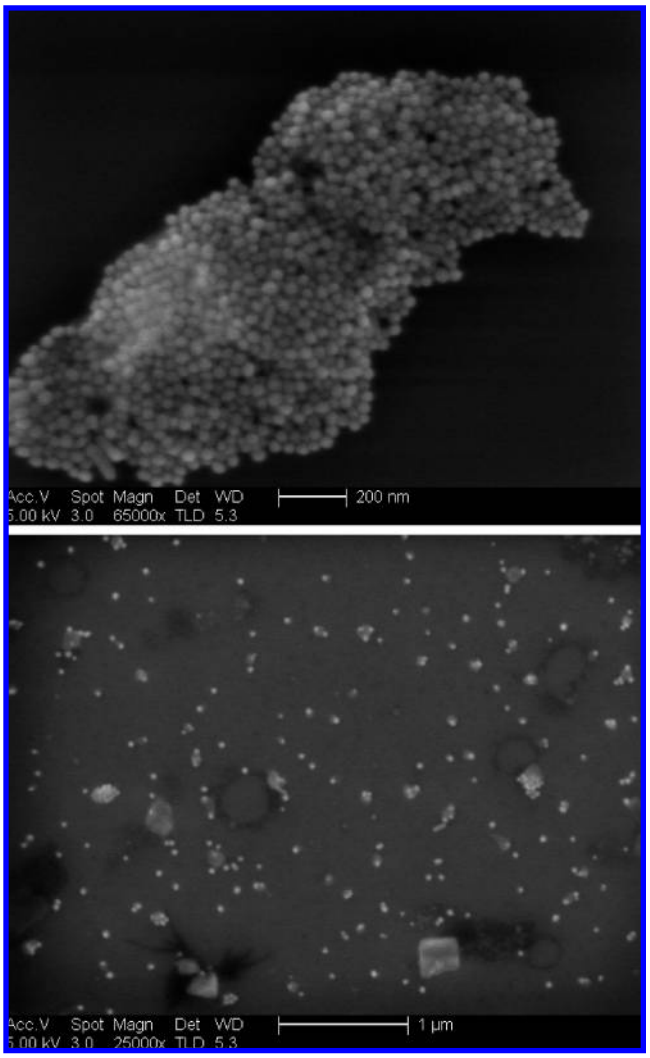

Figure 2. Scanning electron micrographs of OSN conjugates. The top image shows the large aggregates formed by hybridization of OSN conjugates to a complementary target. The sample in the bottom image contained a noncomplementary target and shows widely dispersed single nanoparticles and small agglomerates that are associated with crystals of the hybridization buffer.

Figure 1 shows the UV−vis spectrum obtained from unmodified silver nanoparticles and OSN conjugates and illustrates the

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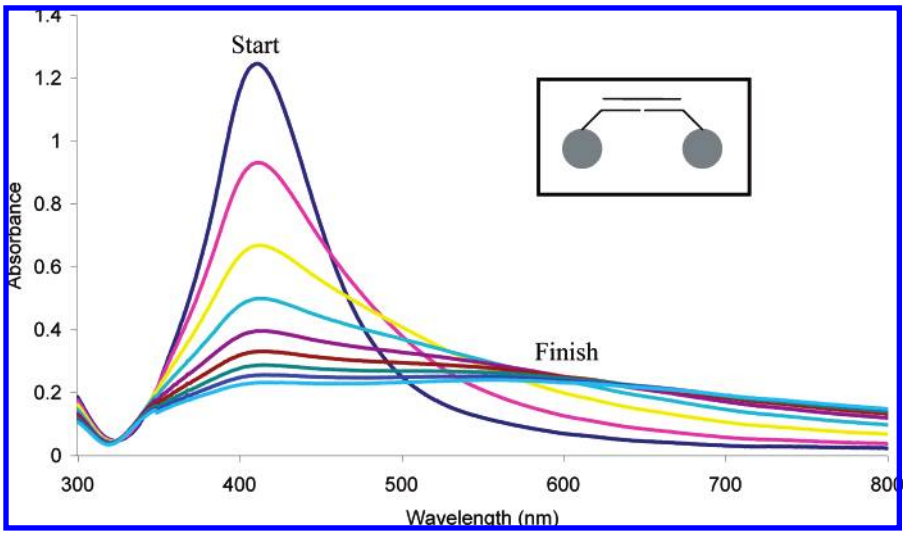

Figure 3. UV−vis spectra taken every 5 min of OSN conjugates (25 pM) hybridizing to a fully complementary target oligonucleotide (2.5 nM). Full spectrum scans were taken every 10 min for 80 min. The inset shows that the conjugates are hybridizing in the "tail-to-tail" juxtaposition.

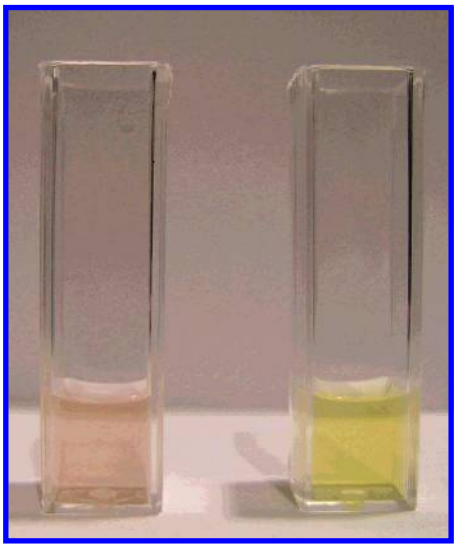

Figure 4. Visual image of OSN conjugates hybridized with a fully complementary target oligonucleotide (left) and a noncomplementary target (right) demonstrating the color change. The solution was 1 mL of PBS containing 30 fmol of each conjugate and 3 pmol of target oligonucleotide.

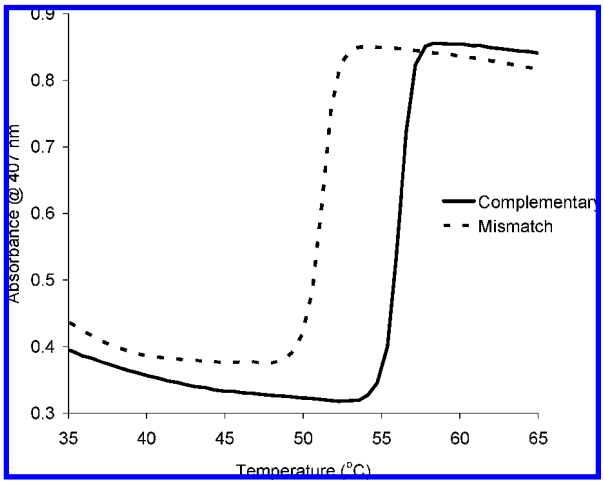

Figure 5. Melting curve of OSN conjugates (20 pM) showing the difference between a fully complementary target oligonucleotide (2 nM) and one containing a single base mismatch. The melting temperatures obtained were 56.0 and 51.2 °C showing a discrimination of 4.8 °C.

spectral red shift of ~7 nm and slight broadening that occurs in the silver surface plasmon upon conjugation with oligonucleotides. This mimics the observed shift in the surface plasmon of gold nanoparticles upon functionalization with DNA (518 nm → 523 nm).

The coverage of conjugated oligonucleotide on the silver nanoparticle surface was determined by using an oligonucleotide functionalized with a fluorescent molecule and an alkyl thiol modification. With the use of the previously published method[24] the coverage was determined to be 22.6 ± 2.4 pmol/cm^2 (30 nm diameter). The value reported for gold nanoparticles is 15 ± 4 pmol/cm^2 (13 nm diameter).[24]

Properties of Oligonucleotide−Silver Nanoparticle Conjugates. OSN conjugates have been previously synthesized, but their sandwich assay hybridization properties have never been reported or compared with OGN conjugates.[15−19] Hybridization of OSN conjugates was performed in a sandwich assay format where an unmodified target oligonucleotide sequence was added to a solution of conjugates that were each complementary to half of the target strand.

When hybridization of the conjugates to the target occurs the nanoparticles of the conjugates come into close proximity with each other, causing aggregation. This is sequence-specific and will not occur unless the target oligonucleotide is complementary to the probe sequence of the OSN conjugates. The aggregation caused by conjugates hybridizing to a complementary target can be visualized using scanning electron microscopy (SEM) (Figure 2).

Aggregation is accompanied with a simultaneous change in the surface plasmon of the nanoparticle that can be observed in solution by UV−vis spectroscopy (Figure 3) or visually (Figure 4). The UV−vis spectrum shows the surface plasmon peak broadens and decreases to a maxima at ~540 nm.

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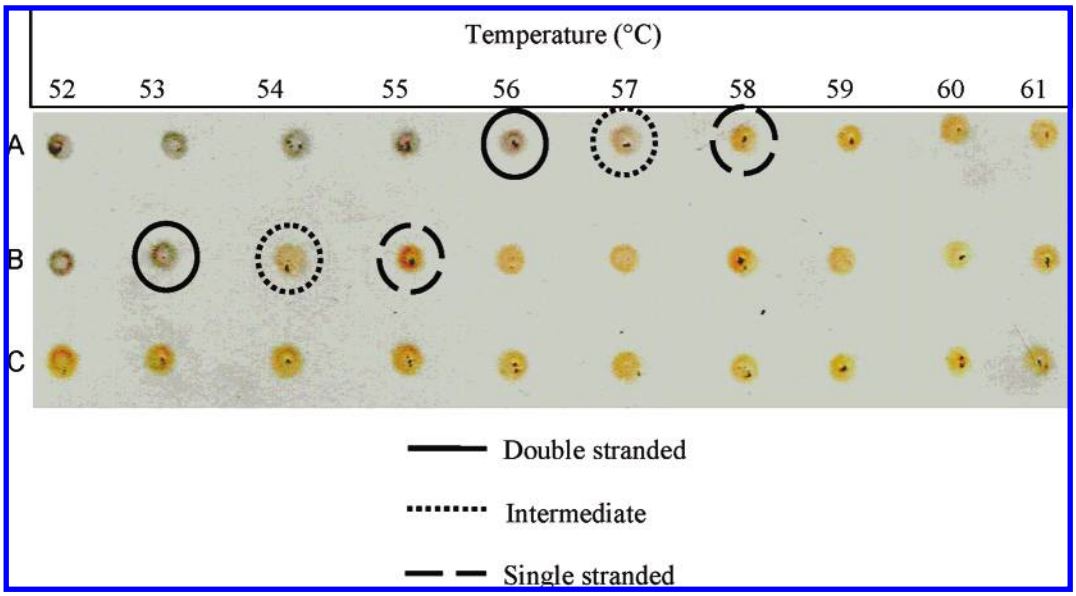

Figure 6. Visual image of a reversed-phase thin-layer chromatography plate spotted with OSN conjugates illustrating the difference between (A) fully complementary, (B) single base mismatch, and (C) noncomplementary targets with (A) and (B) showing colorimetry temperatures (T_c) of 54 and 57 °C, respectively. This is consistent with previous observations of the discrepancy between T_m and T_c (ref 10).

Single Base Mismatch Discrimination. When used in a sandwich assay, a target oligonucleotide that has a single base mismatch will still hybridize and aggregate due to its significant degree of complementarity to the conjugate sequences making it impossible to differentiate from a complementary strand without the use of precise temperature control. The placement and nature of the mismatch in the target strand is of considerable interest as previous research has shown that mismatches and imperfections such as deletions and insertions decrease the melting temperature.[10] Recent work has shown that certain mismatches actually increase the melting temperature indicating that nanoparticle-bound DNA has different hybridization behavior compared with free DNA.[25]

Analysis of single base mismatches by OSN conjugates were examined by visible analysis and by melting curve analysis. The melting transition is displayed in two ways: (1) as a UV−vis melting curve (Figure 5) and (2) via spotting aliquots of reaction mixture onto a reversed-phase thin-layer chromatography plate (Figure 6) that enabled visualization by capturing the hybridization state at that temperature.

Comparison with the analogous OGN conjugate system showed that the larger extinction coefficient of silver nanoparticles enables more sensitive analysis. Similar curves for OGN conjugates required a greater concentration of target oligonucleotide (Figure 7). Interestingly, if rather than taken as a concentration, the target oligonucleotide is taken as a multiple of conjugate concentration, then the lowest detectable hybridization efficiency is very similar. However, due to the larger molar extinction coefficient for silver nanoparticles, the minimum detectable target oligonucleotide concentration is 50× lower.

Factors Affecting the Melting Temperature. The melting temperature of OGN conjugates is affected by a number of factors such as target oligonucleotide concentration and interparticle distance. OSN conjugates were investigated to see if they shared the same properties. Although it was clear that target concentra-

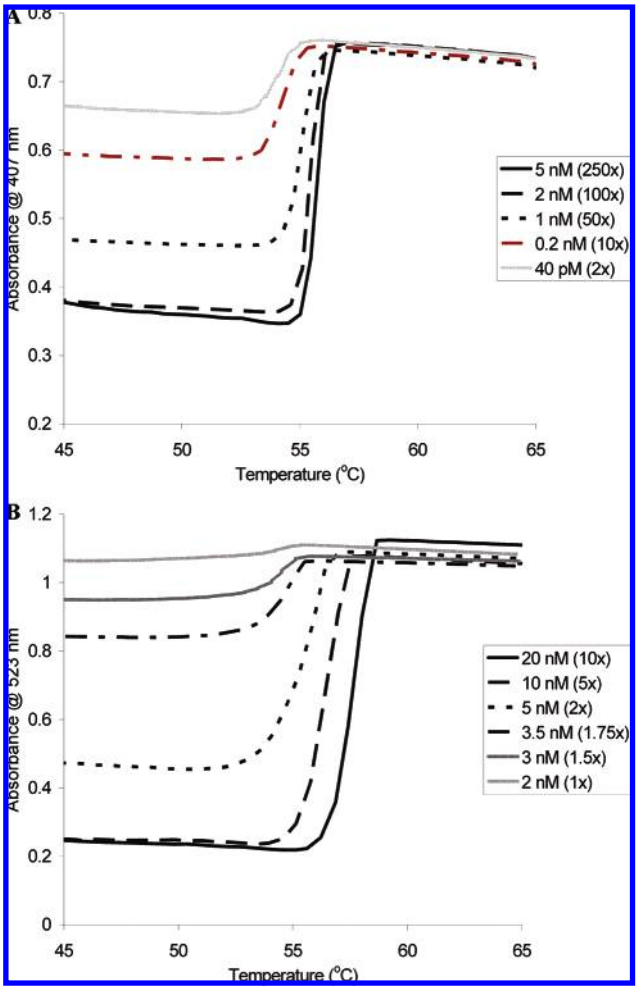

Figure 7. Melting curves of (A) OSN conjugates and (B) OGN conjugates showing the change in T_m due to exposure to lower concentration of target oligonucleotide. The minimum detectable target concentration using OGN conjugates is 2 nM. The corresponding minimum for OSN conjugates is 40 pM. The OGN conjugates were monitored at their respective unaggregated plasmon maximum (523 nm).

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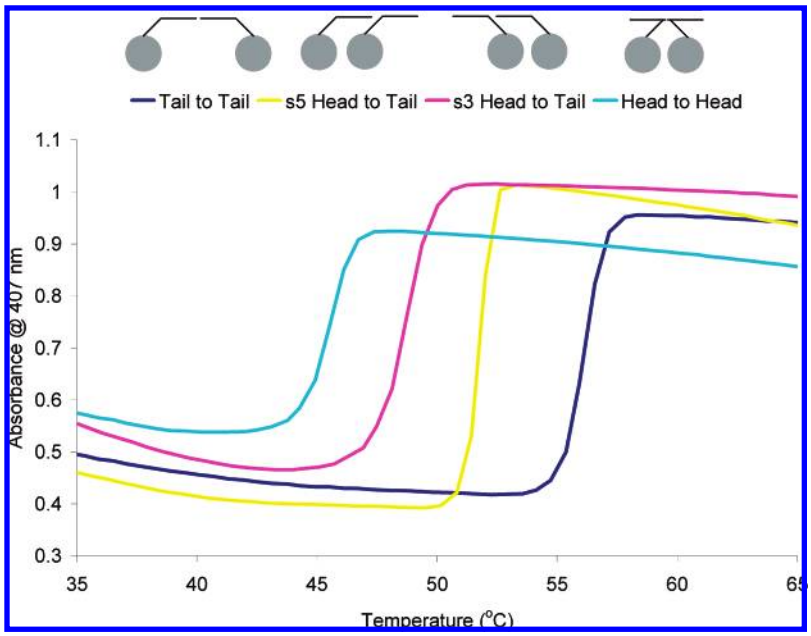

Figure 8. Melting curve comparison of OSN conjugates hybridized in the various geometries, including the two possible head-to-tail geometries with different alkyl thiol modification placement (tail-to-tail 56.0 °C, 5′ head-to-tail T_m = 51.4 °C, 3′ head-to-tail T_m = 48.3 °C, tail-to-tail 45.0 °C).

tion affects both rate of hybridization and melting temperature, analysis of interparticle distance produced an interesting outcome. It was clear that decreasing interparticle distance decreases hybridization rate, and melting temperature comparison of 3′- and 5′-modified OSN conjugates shows a difference when the nanoparticle orientation is the same (Figure 8).

This reinforces the observation made during synthesis that 3′-modified OSN conjugates are more unstable than their 5′ counterpart. The decrease in melting temperature suggests fewer sequences are hybridizing, which could be due to a decrease in the stability of the silver−thiol attachment caused by the shorter alkyl chain spacing the surface from the first oligonucleotide. The shorter alkyl chain also causes an increase in steric hindrance which also causes a decrease in melting temperature.

CONCLUSION

Oligonucleotide−silver nanoparticle conjugates have been proven to be as robust as their gold counterparts and enable detection of a lower concentration of a specific DNA sequence due to their larger extinction coefficient. Although the lowering of the detection limit does not yet yield PCR-less detection of genomic material, and rather than simply moving the existing oligonucleotide−nanoparticle conjugate chemistry to a different environment, it is hoped this work can be used to create new avenues of research exploiting the advantages of silver nanoparticles that are not currently possible using gold nanoparticles utilizing their enhancement properties in techniques such as surface-enhanced resonance Raman scattering.

EXPERIMENTAL SECTION

Oligonucleotide Sequences. The two oligonucleotide sequences used for functionalization of nanoparticles were 5′TCTCAACTCGTA and 5′CGCATTCAGGAT. They were modified by the incorporation of an alkyl thiol group to the desired terminus along with 20 adenine bases. The sequences of the target oligonucleotides used were 5′TACGAGTTGAGAATCCTGAATGCG, 5′TACGAGTTGAGCATCCTGAATGCG, and 5′GGAACAGTCCTTAGCCGAGCT for the fully complementary, single base mismatch, and noncomplementary targets, respectively. All reagents, monomers and CPG columns were purchased from Link Technologies (Bellshill, U.K.)

All sequences were synthesized using an Expedite 8909 synthesizer and purified using a PerSeptive Biosystems Inc. BioCAD SPRINT high-performance liquid chromatograph fitted with a ResourceQ (6.032 mL, 16 mmD, 30 mL) column.

Nanoparticle Synthesis. Silver nanoparticles were synthesized using a previously published method[26] and quantified using a molar extinction coefficient of $\epsilon = 2.87 \times 10^{10}$ M^{-1} cm^{-1}.

Oligonucleotide−Silver Nanoparticle Conjugate Synthesis. A solution of alkyl thiol-modified oligonucleotide (10 μM, 1 mL) was prepared in pH 8.5 phosphate buffer (60 mM) and 0.1 M dithiothreitol (DTT). This solution was added directly to a solution of silver nanoparticles (3 mL, 5.05×10^{-10} M) via a HiTrap Sephadex superfine desalting column. The solution was left to equilibrate for at least 24 h followed by the addition of 800 μL of pH 7 phosphate buffer (60 mM). There were two further periods of equilibration (24 h) between the additions of 123 and 126 μL of sodium chloride (2 M). The solution was centrifuged at 7000 rpm for 20 min and resuspended in 1000 μL of 0.3 M PBS (10 mM phosphate buffer pH 7, 0.3 M sodium chloride), then centrifuged and resuspended again.

Materials and Instrumentation. All materials and reagents (excluding those used in oligonucleotide synthesis) were reagent grade and purchased from Sigma Aldrich. All UV−vis spectra and

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melting curves were taken using a Varian Cary 300 Bio spectrophotometer fitted with a Peltier temperature controller. The reversed-phase thin-layer chromatography plate was imaged with a Hewlett-Packard PSc 1410 scanner/printer using Hewlett-Packard Image Zone version 5.0 software for data handling. All SEM images were taken using a FEI Sirion ultrahigh-resolution Schottky field emission scanning electron microscope with the assistance of Professor R. W. Martin and Dr. R. J. Stokes.

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