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Amplified Voltammetric Detection of DNA Hybridization via Oxidation of Ferrocene Caps on Gold Nanoparticle/Streptavidin Conjugates

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Gold nanoparticle/streptavidin conjugates covered with 6-ferrocenylhexanethiol were attached onto a biotinylated DNA detection probe of a sandwich DNA complex. Due to the elasticity of the DNA strands, the ferrocene caps on gold nanoparticle/streptavidin conjugates are positioned in close proximity to the underlying electrode modified with a mixed DNA capture probe/hexanethiol self-assembled monolayer and can undergo reversible electron-transfer reactions. A detection level, down to 2.0 pM (10 amol for the 5 μL of sample needed) for oligodeoxynucleotide samples was obtained. The amplification of the voltammetric signals was attributed to the attachment of a large number of redox (ferrocene) markers per DNA duplex formed. The ferrocene oxidation current increased with the target concentration and began to level off at a target concentration of 10 nM. An Excellent linearity was found within the range between 6.9 and 150.0 pM and reasonable relative standard deviations (between 3.0 and 13.0%) were obtained. The amenability of this method to the analyses of polynucleotides (i.e., PCR products of the pre-S gene of hepatitis B virus in serum samples) was also demonstrated. The method is shown to be simple, selective, reproducible, and cost-effective and does not require labeling of the DNA targets.

The use of functional metal nanoparticles for biosensing has generated a great deal of interest.[1−15] For gene analysis, the incorporation of metal (e.g., Au) and polymeric nanoparticles was found to improve the sensitivity[4−15] and sequence specificity[4] of a given method. For example, oligodeoxynucleotide (ODN)-capped Au nanoparticles, coupled with the follow-up silver-staining process, have allowed scanometric[5] and calorimetric[6] DNA detection schemes to be developed. These particles have also been shown to result in signal enhancements for quartz crystal microbalance,[7−9] surface plasmon resonance,[10] and electrochemical measurements of DNA hybridization events.[11−15]

Electrochemical methods, when compared to the conventional methods for DNA analysis (e.g., fluorescence microscopy[16,17] and radioactivity[18] measurements) and some of the aforementioned methods, are straightforward and sensitive and do not require sophisticated instrumentation.[19−24] Consequently, they are suitable for the development of inexpensive and portable devices for disease diagnoses.[19−21] Typically, the DNA hybridization is detected by electrochemical reactions of a redox marker or reporter on the target (e.g., ferrocenylated ODNs[25,26]), nucleotide bases such as guanines and adenines,[27,28] electroactive products gener-

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ated by the tags on the duplexes (e.g., an enzyme[29−33] or a mediator[34,35]), and indicator molecules (e.g., intercalating[36,37] or cationic metal complexes[38−40]). Among these approaches, ferrocene (Fc) derivatives attached onto DNA strands for gene sequencing has attracted broad attention,[12,25,26,36,37,41,42] owing to the reversibility of the Fc oxidation reaction and the versatility in functioning Fc derivatives for various cross-linking reactions. For example, Fc tags are used in a new hand-held CMS eSensor system manufactured by Motorola Inc. for simultaneous electrochemical detection of different DNA sequences.[41] Willner and coworkers reported an amplified bioelectrocatalytic detection of viral DNA by introducing a large number of Fc groups through the replication of Fc-labeled DNA by DNA polymerase.[42] Wang et al. recently showed that "electroactive beads", polystyrene microspheres loaded with Fc molecules, can be used to tag the target DNA and, upon separation and bead dissolution, be released into a solution and conveniently detected with chronopotentiometry.[12] Heller and co-workers[29,30] and Azek et al.[31] demonstrated that enzyme-labeled DNA detection probes could be used in conjunction with screen-printed carbon electrodes for inexpensive and amplified sandwich assays of oligonucleotide or polynucleotide targets. The enzyme molecules catalyze the redox reactions of electroactive substrates and consequently enhance the detection of DNA hybridization.

Despite many studies based on the Fc-labeled DNA or posthybridization attachments of Fc-containing species or particles, direct voltammetric detection of Fc-capped nanoparticles linked to DNA duplexes that are confined to an electrode surface has not been demonstrated. We report a novel voltammetric assay using Fc-capped gold nanoparticle/streptavidin conjugates. Owing to the large number of Fc moieties present at each gold nanoparticle, the voltammetric signals were greatly amplified, resulting in a low detection level and a desirable selectivity for both oligonucleotide and polynucleotide analyses.

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EXPERIMENTAL SECTION

Materials. Potassium perchlorate, ethylenediaminetetracetic acid (EDTA), ethanol, Tris-HCl, NaCl, KH_2PO_4, and K_2HPO_4 were purchased from Beijing Chemical Reagents Co. (Beijing, China). 1-Hexanethiol (HT) was obtained from Aldrich. 6-Ferrocenylhexanethiol was synthesized according to the literature procedure,[43] and gold nanoparticle/streptavidin conjugates were acquired from Sigma. The washing buffer is 0.1 M phosphate buffer (pH 7.0) with 0.1% Tween. Serum samples containing hepatitis B virus (HBV) were provided by Xiehe Hospital (Wuhan, China). ODN capture probes with their 5′ ends modified with hexylthiol groups and the biotinylated detection probes were both obtained from Shanghai Shenggong Co. (Shanghai, China). The capture and detection probes have sequences of SH-$(CH_2)_6$ 5′-TTT TTG GAG CAC CCA CGT GTC CTG GCC-3′ and 5′GCT CAG TTT ACT AGT GCC ATT TGT TTT T-biotin 3′, respectively, and are both complementary to the pre-S gene of HBV. For the sandwich assay of ODN targets, four different sequences (mismatching sequences underlined) were chosen to evaluate the selectivity of the method: 5′ ACT AGT AAA CTG AGC ATA CTG GCC AGG ACA CGT GGG TGC 3′ (complement to the capture probe), 5′ACT AGT AAA CTG AGC ATA CTG GCC AGG ACA TGT GGG TGC 3′ (single-base mutant to the capture probe), ACT AGT AAA CTG AGC ATA CTG GTC CGG ACA TGT GGG TAC3′ (four base-pair mismatch to the capture probe), and 5′GAA TTC GAG CTC GGT ACC CGG GGA TCC TCT ACT GGC CGT CGT TTT AC 3′ (noncomplement to the capture probe). For real sample analysis, PCR products of the HBV gene from serum samples and mitochondrial DNA were determined using the same capture and detection probes. The two primers used to generate the PCR products have sequences of 5′-GGA CTT CTC TCA ATT TTC TAG-3′ and 5′-CAA ATG GCA CTA GTA AAC TGA GC-3′. The sequence of the HBV pre-S gene is

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                    Eco72 I   Bal I  Hae III
                       |       ||
GGACTTCTCTCAATTTTCTAGGGGGAGCACCCACGTGTCCTGGCCAAAATTCG
CAGTCCCCAACCTCCAATCACTCACCAACCTCTTGTCCTCCAATTTGTCCTGG
   Fok I
    |
CTATCGTTGGATGTGTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCT
ATGCCTCATCTTCTTGTTGGTTCTTCTGGACTACCAAGGTATGTTGCCCGTTTG
TCCTCTACTTCCAGGAACATCAACTACCAGCACGGGACCATGCAAGACCTGC
                                          Rsa I
                                           |
ACGATTCCTGCTCAAGGAACCTCTATGTTTCCCTCTTGTTGCTGTACGAAACC

TTCGGACGGAAACTGCACTTGTATTCCCATCCCATCATCCTGGGCTTTCGCAA
Hae III                                        Spe I
 |                                              |
AATTCCTATGGGAGTGGGCCTCAGTCCGTTTCTCCTGGCTCAGTTTACTAGTG
CCATTTG
```

Preparation of 6-Ferrocenylhexanethiol-Capped Gold Nanoparticle/Streptavidin Conjugates. The gold nanoparticle (diameter, 10 nm)/streptavidin conjugates were modified with 6-ferrocenylhexanethiol by first diluting 200 μL of a conjugate solution to 400 μL with a PBS buffer (pH 7.2). The resulting solution was then mixed with 0.5 mL of hexane containing 5.0 mM 6-ferrocenylhexanethiol for 24 h on a vortex stirrer. At the hexane/water phase boundary, the 6-ferrocenylhexanethiol molecules adsorbed onto the gold nanoparticles and the resultant

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modified gold nanoparticles remained in the aqueous phase. The solution was centrifuged, and the hexane phase containing 6-ferrocenylhexanethiol was decanted. The gold nanoparticle/streptavidin conjugates capped with 6-ferrocenylhexanethiol were thoroughly rinsed with hexane, resuspended in a PBS buffer (pH 7.2), and diluted 10-fold prior to use.

Determination of the Surface Coverage of 6-Ferrocenylhexanethiol on Gold Nanoparticles and the Gold Nanoparticle/Streptavidin Ratio. To determine the average number of 6-ferrocenylhexanethiol molecules per gold nanoparticle, the Fc-capped Au nanoparticle/streptavidin conjugates were treated with a HNO_3/HCl solution (v/v 3:1), diluted with water, and analyzed by inductively coupled plasma mass spectrometry (ICPMS, Elan 6100DRC, Perkin-Elmer/Sciex Corp.). The average number of Fc groups (monitored as the Fe signal) per Au nanoparticle was determined to be 127 ± 18. To characterize the streptavidin/Au nanoparticle conjugates, an UV/visible spectrum of the conjugate solution was measured, which showed adsorption peaks for streptavidin at ~280 nm and for Au nanoparticles at 520 nm. The gold content in the conjugates was also quantified by ICPMS. The ratio between Au nanoparticles and a streptavidin molecule was determined to be 1.7 by comparing the absorbance value of the spectrum to that of a standard streptavidin solution and the ICPMS signals to those of standard gold solutions. Given that protein molecules have a strong tendency to adsorb onto metallic surfaces,[44] it is reasonable to assume that all the Au nanoparticles are linked to the streptavidin molecules. Thus, the value of 1.7 suggests that some of conjugates are of Au nanoparticle/streptavidin ratio of 1, whereas others may have two gold nanoparticles attached to a single streptavidin molecule.

Extraction of the HBV DNA and the PCR Experiment. A 20-μL serum sample and 30 μL of a 0.5% NP-40/0.5% Tween-20 solution were added to a centrifuge tube. Upon standing for 10 min, the mixture was centrifuged at 12 500 rpm for 10 min at 4 °C. Finally, 20 μL of the supernatant comprising the HBV gene was transferred into another centrifuge tube for the PCR experiment. To generate the PCR products, the extract from the serum samples was mixed with the primers, dNTPs, and Taq DNA polymerase (2 units). After an initial heating at 95 °C for 5 min, 35 PCR cycles, consisting of a denaturation step at 94 °C for 45 s, an annealing step at 50 °C for 45 s, and an extension step at 72 °C for 45 s, were performed. The final mixture was allowed to stand at 72 °C for 10 min. The PCR products were checked using agarose gel electrophoresis.

Probe Immobilization and Hybridization Procedures. *Immobilization of Thiolated ODN Probes onto Au Electrodes.* Before modification, a gold disk electrode (2 mm in diameter) was polished successively with 0.3- and 0.05-μm alumina slurries (Buehler, Lake Bluff, IL). The electrode was then sonicated in water for 5.0 min. This cleaning step was followed by casting 10 μL of TE (10 mM Tris-HCl and 1.0 mM EDTA) solution containing 1.0 μM capture probe onto the electrode. To avoid water evaporation, the electrode was held upside down in a humidified Styrofoam chamber. Upon immobilization overnight, the electrode was washed with water and soaked in an aqueous solution containing 0.1 mM hexanethiol solution for 5 min. Estimation of the surface coverage of the capture probe microgravimetrically was conducted either with a homemade quartz crystal microbalance or with a CHI-440 electrochemical quartz crystal microbalance (CH Instruments, Austin, TX). Details about the instrumental setup and the typical surface cleaning and immobilization procedures have been given in our published procedures.[45−47]

DNA Hybridization and Subsequent Attachment of the Fc-Capped Gold Nanoparticle/Streptavidin Conjugates. For each assay, 5.0 μL of TNE solution (TE + 0.1 M NaCl) containing a given concentration of the target DNA was cast onto the electrode covered with a probe/HT mixed self-assembled monolayer (SAM) and the hybridization reaction was allowed to proceed for 2 h in the humidified Styrofoam chamber. After the surface was thoroughly rinsed with a washing buffer, the electrode was further exposed to a TNE solution containing 1.0 μM biotinylated probe for another 2 h. Most hybridization reactions were performed at room temperature in the humidified chamber. For the experiments related to HBV gene analysis, the electrode covered with the capture probe was immersed in a sealed small centrifuge tube containing 100 μL of the target sample and the hybridization steps took place in a thermobath (62 °C for the duplex formation between the capture probe and the target and 50 °C for that between the target and the detection probe). Upon completion of the two-step hybridization reaction, the electrode was rinsed with a washing buffer and water and the Fc-capped gold nanoparticle/streptavidin conjugates were attached by soaking the electrode in a conjugate solution for 1 h.

Electrochemical Measurements. The electrochemical experiments were carried out with a CHI-615 electrochemical analyzer (CH Instruments) with a Ag/AgCl electrode as the reference electrode and a platinum wire as the auxiliary electrode; 1.0 M $KClO_4$ solution was used as the supporting electrolyte.

RESULTS AND DISCUSSION

The scheme for DNA hybridization detection based on electrochemical oxidation of Fc caps on the gold nanoparticle/streptavidin conjugates is shown in Figure 1. The thiolated capture probe/HT mixed SAM[48] was first formed. Surface coverage of the capture DNA probe had been measured to be $\sim 2.62 \times 10^{-12}$ mol/cm^2 by a quartz crystal microbalance. Upon hybridization with an ODN or a polynucleotide target, a second hybridization with the biotinylated ODN detection probe was carried out. Finally, Fc-capped Au nanoparticle/streptavidin conjugates were attached to the electrode surface via the biotin/streptavidin complexation. Because of the elasticity of the DNA strands and the relatively large mass of the nanoparticle conjugates, the Fc moieties are positioned in close proximity to the underlying electrode, allowing facile electron transfer to occur. Since each Au nanoparticle is covered with a large number of 6-ferrocenylhexanethiol molecules (127 ± 18), the voltammetric signal is significantly amplified. The surface coverage of 6-ferrocenylhexanethiol on a gold nanoparticle surface was estimated to be 5.3×10^{13} molecules/cm^2 (the spherical cap area of the gold nanoparticle linked to the streptavidin molecule was excluded in the calculation). Compared to the

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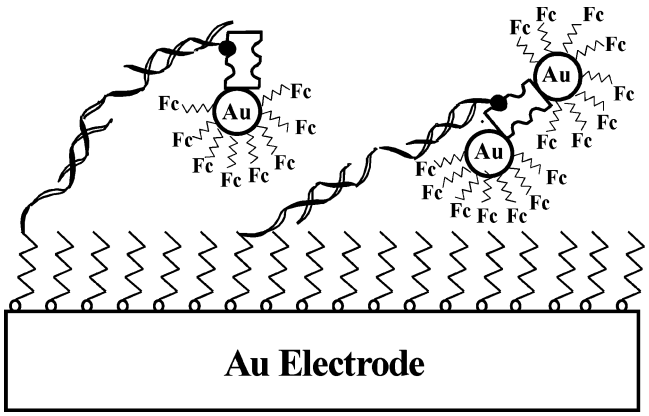

Figure 1. Schematic representation of the amplified electrochemical detection of DNA hybridization via oxidation of the ferrocene caps on the gold nanoparticle/streptavidin conjugates. For clarity, 1-hexanethiol, DNA, streptavidin, and 6-ferrocenylhexanethiol molecules are not drawn to scale. The scheme pictorially reflects the fact that one streptavidin molecule could be linked to one or two ferrocenylalkanethiol-modified Au nanoparticles.

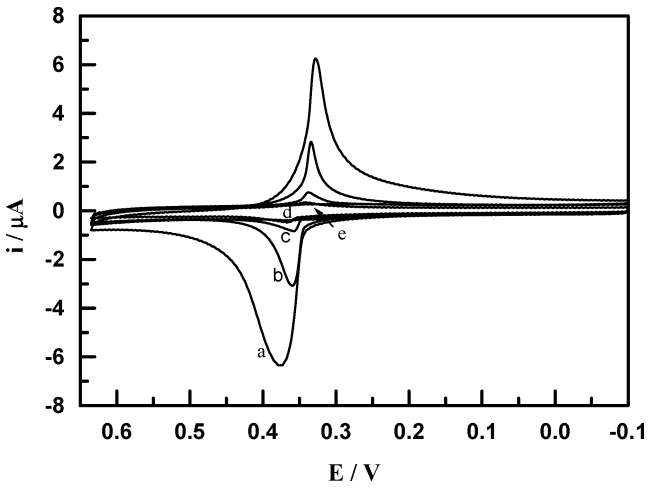

Figure 2. CVs acquired at electrodes covered with a 27-mer capture probe and hexanethiol SAM after room-temperature hybridization in a 10 mM Tris-HCl/0.1 M NaCl buffer containing (a) l.4 nM complementary 39-mer target, (b) 0.068 nM complementary 39-mer target, (c) 1.4 nM 39-mer target with a single-base mismatch, (d) 1.4 nM 39-mer with a four-base mismatch, and (e) 1.0 nM noncomplementary 47-mer target, followed by the second hybridization with a biotinylated 28-mer detection probe and the Fc-capped Au nanoparticle attachment. The scan rate was 0.3 V/s, and 0.1 M $KClO_4$ was used as the electrolyte solution.

surface coverage of a ferrocenylalkanethiol SAM on a planar gold surface,[49] such a value corresponds to ~20% of a monolayer. Another aspect is that more than one gold nanoparticle might be attached to a single streptavidin molecule, and consequently, the voltammetric signal will be further enhanced. As mentioned in the Experimental Section, the average ratio between gold nanoparticle/streptavidin is 1.7, suggesting that some of the streptavidin molecules are linked to one gold nanoparticle and many more are linked to two gold nanoparticles. This value is reasonable given the comparable sizes between streptavidin (8.4 nm × 8.4 nm × 4.65 nm)[50] and the gold nanoparticle (diameter, 10 nm).

The electrochemical responses at electrodes modified with a 27-mer capture probe after hybridization with a 39-mer complement of two concentrations (curves a and b) are shown in Figure 2, together with those arising from hybridization reactions with single-base (curve c) and four-base (curve d) 39-mer mutants and a 47-mer noncomplement (e). The difference in the peak currents between curves a and b suggests that the intensity of the Fc oxidation signal is dependent on the target concentration. The peak-to-peak separation (ΔE_p) of curves a and b are 46 and 26 mV, respectively, and the anodic peak currents (i_{pa}) were found to be proportional to the scan rates within the range of scan rates examined (0.1−1.0 V/s). These characteristics suggest that the Fc moieties can undergo facile electron-transfer reactions with the underlying Au electrode.[51] It can be seen from Figure 2 that the voltammograms exhibit a characteristic diffusional "tailing" of the peaks and the tailing and ΔE_p are more serious or greater at higher target concentrations. It is known that introduction of repulsive interaction between the electroactive centers confined on the electrode surface causes the peaks to become broader.[52] This is conceivable, because, when the Fc moieties are oxidized, the adjacent gold nanoparticles become positively charged and may repel one another.[53] In addition, the positively charged gold electrode[43,54] can also electrostatically repel the positively charged gold nanoparticles. Such explanations are drawn on the observation and interpretation of the electrostatic interactions within electrochemically oxidized Fc-terminated SAMs at gold electrodes.[53]

Peaks in curves c−e are much diminished or absent. i_{pa} in curve c is only 6.2% of that in curve a. Moreover, for the single-base 39-mer mutant, when the hybridization was performed at an elevated temperature (e.g., 62 °C), essentially no peaks were observed after the completion of the second hybridization and the nanoparticles' attachment. However, a comparable voltammetric response for a complementary target at the same temperature could be observed (data not shown). Thus, it is apparent that sequence-specific DNA analysis can be conducted.

The sensitivity and dynamic range of this method were estimated. The plot of i_{pa} against the target concentration between 6.9 pM and 28 nM is given in Figure 3. The error bars (RSD ranging from 3.0 to 13.0%) were relatively small, indicating that the method is quite reproducible. Around 10 nM, the curve began to level off, suggesting that most of the capture probes have been hybridized with the target. The i_{pa} values were found to linearly increase with the target concentration over a wide concentration range (between 6.9 pM and 0.15 nM, as shown in the inset). The slope of the plot (inset) is 32.5 μA/nM or 6.5 μA/fmol (5 μL of a target solution was used for each assay). Based on $3s/m$ (s is the standard deviation of the measurements of noncomplementary target solutions and m is the slope of the plot in the inset of Figure 3), the limit of detection (LOD) of the method was estimated to be less than 2.0 pM (or 10 amol for 5 μL of sample used for the

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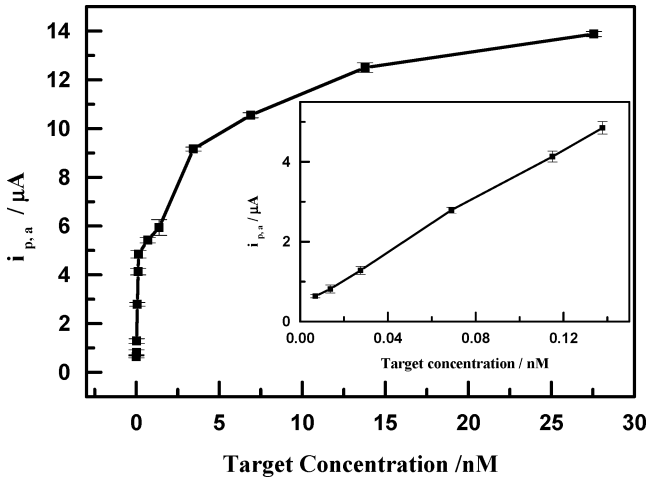

Figure 3. Plot of faradaic currents against the 39-mer target concentration. Inset: the enlarged portion of the plot in the target concentration range of 6.9 pM−0.15 nM; The regression equation is i_{pa} (μA) = 32.5 [target] (nM) + 0.41 (r^2 = 0.999).

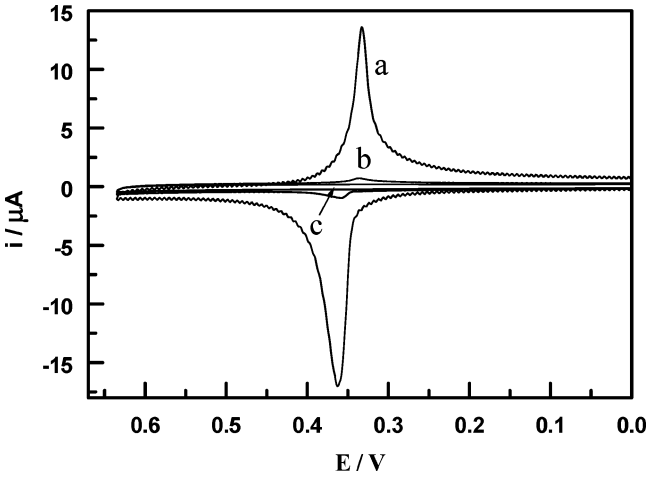

Figure 4. CVs collected at electrodes covered with a 27-mer HBV pre-S gene capture probe and hexanethiol SAM after hybridization in a 10 mM Tris-HCl/0.1 M NaCl buffer containing (a) 3.2 nM HBV pre-S gene, (b) 0.16 nM HBV pre-S gene, and (c) mitochondrial DNA, followed by the second hybridization with a biotinylated 28-mer detection probe and the attachment of the Fc-capped Au nanoparticle/streptavidin conjugates. The two-step DNA hybridization occurred at 62 and 50 °C, respectively. The scan rate was 0.3 V/s, and 0.1 M $KClO_4$ was used as the electrolyte solution.

assay). Such a value is lower than those of electrochemical detections of DNA indicators,[38,39] ferrocenylated ODN target,[26] and direct oxidation of guanines[27] and compares well with that of indirect oxidation of guanines with electrogenerated ruthenium tris(bipyridine) trications.[34] Moreover, this voltammetrically based method offers an LOD that is also comparable to those of fluorescence measurements[16] as well as to those of the enzyme-amplified amperometric detection of oligonucleotide targets.[29,30] We attribute the improvement of the LOD to the signal amplification associated with the attachment of a large number of Fc moieties to each duplex formed. Although the previous methods based on the use of gold nanoparticles,[11,15] Fc-encapsulated microspheres,[12] or biocatalytic reactions involving Fc[42] or enzyme-tagged DNA targets,[31−33] and other electroactive mediators[35] for amplified DNA detections also possessed excellent sensitivities, they were either indirect detections or require multiple separation or sample pretreatments.[24] The amplified voltammetric detection scheme described herein is simpler and obviates sample pretreatments.

To demonstrate the feasibility of the approach for real sample analysis, PCR products of hepatitis B virus pre-S gene extracted from serum samples were measured. As can be seen from Figure 4, appreciable faradaic currents could be observed when the samples contained the HBV pre-S gene (curves a and b). The magnitude of the current was found to be dependent on the HBV gene concentration. When a mitochondrial DNA target, whose sequence mismatches that of the HBV pre-S gene capture probe, was analyzed, essentially no peaks appeared in the CV (curve c). Therefore, sequence-specific polynucleotide analysis can also be performed with this method.

CONCLUSION

A novel gold nanoparticle/streptavidin conjugate covered with a large number of ferrocenylalkanethiol molecules was synthesized and applied to amplified sandwich assays of both oligonucleotide and polynucleotide samples. The analytical performance of this method (e. g., dynamic range, reproducibility, selectivity, and detection limits) was evaluated through the analyses of a complementary ODN target and targets with varying numbers of mismatching bases. The voltammetric method for trace DNA analysis is simple, selective, and reproducible and obviates the need of labeling the target samples. A remarkable feature is the LOD achieved through signal amplification by Fc-capped gold nanoparticle/streptavidin conjugates (down to 10 amol). Such a LOD is lower than those of many other electrochemical DNA assays and compares well to fluorescence and radioactivity measurements or other amplified voltammetric detection methods. Extension of this methodology to antigen−antibody and DNA/protein analysis is currently in progress.

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