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Sequence-Specific Detection of Femtomolar DNA via a Chronocoulometric DNA Sensor (CDS): Effects of Nanoparticle-Mediated Amplification and Nanoscale Control of DNA Assembly at Electrodes

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Abstract: We herein report a novel nanoparticle-based electrochemical DNA detection approach. This DNA sensor is based on a "sandwich" detection strategy, which involves capture probe DNA immobilized on gold electrodes and reporter probe DNA labeled with gold nanoparticles that flank the target DNA sequence. Electrochemical signals are generated by chronocoulometric interrogation of $[Ru(NH_3)_6]^{3+}$ that quantitatively binds to surface-confined capture probe DNA via electrostatic interactions. We demonstrated that the incorporation of a gold nanoparticle in this sensor design significantly enhanced the sensitivity and the selectivity. Nanoscale control of the self-assembly process of DNA probes at gold electrodes further increased the sensor performance. As a result of these two combined effects, this DNA sensor could detect as low as femtomolar (zeptomoles) DNA targets and exhibited excellent selectivity against even a single-base mismatch. In addition, this novel DNA sensor showed fairly good reproducibility, stability, and reusability.

Introduction

Sequence-specific detection of DNA targets associated with either genetic or pathogenic diseases has become increasingly important in molecular diagnostics.[1,2] Polymerase chain reaction (PCR), an enzyme-based DNA amplification technology, is often employed toward these applications.[3] Nevertheless, while PCR is extremely sensitive, it remains to be improved from the practical point of view. Its disadvantages include relatively long assay time, high assay cost, and error-prone nature that occasionally leads to "false-positive" signals.[4−6] Hybridization-based DNA detection is intrinsically more specific than PCR arising due to the extremely high specificity of DNA base pairing. While traditional hybridization-based Southern blotting (DNA blotting) relies on hazardous radioactive labels, more recently developed DNA biosensors that takes the advantage of modern optoelectronics have shown great promise for rapid, sensitive, reliable, and cost-effective DNA detection in clinical diagnostics.[7−9]

A variety of signal transduction techniques have been incorporated in the biosensor design, such as optical,[8,10−13] electrochemical,[14−18] and piezoelectric transducers.[19,20] Due to the fact that electrochemical detectors are simple, portable, and inexpensive, electrochemical DNA sensors are widely recognized to be a promising solution for point-of-care diagnostics as well as many other important applications, including antiterrorism and environmental monitoring.[9,18,21] A typical electrochemical DNA sensor involves a solid electrode and surface-confined capture probe DNA; upon hybridization of the immobilized probes to the sequence-specific target DNA, redox labels that either intercalatively bind to the hybridized double-stranded DNA or are covalently tagged to DNA strands generate corresponding electrochemical signals.

Apparently, the detection sensitivity of a DNA sensor is determined by signal variation amplitude of a hybridization event. As a result, various strategies have been proposed to

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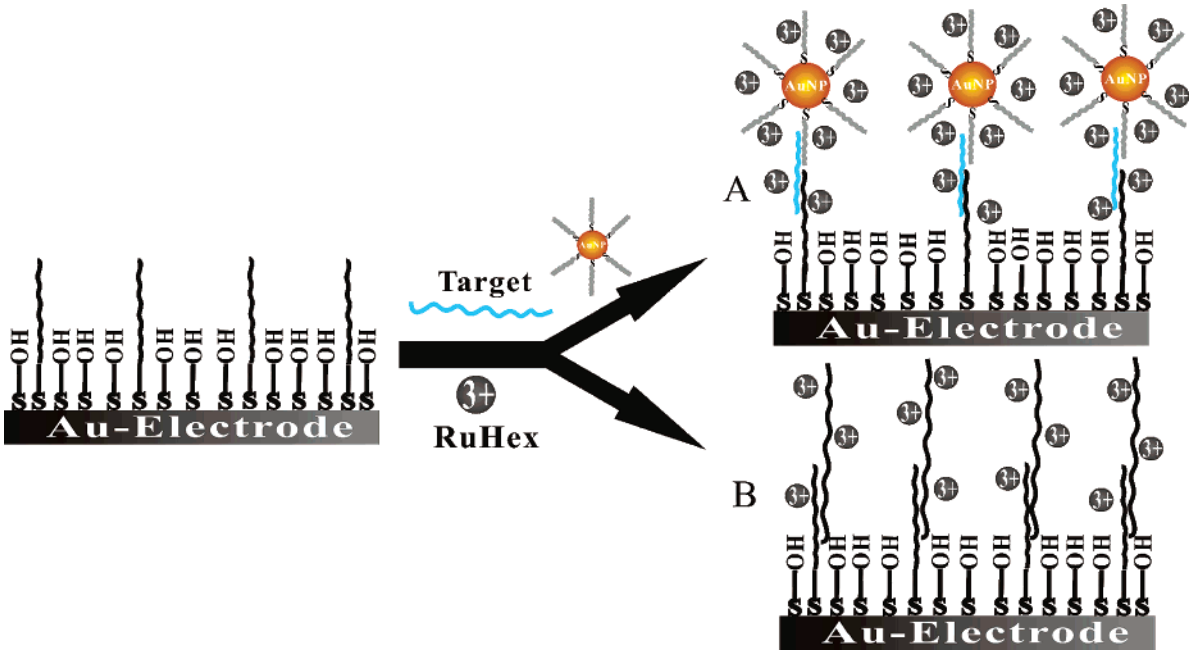
Scheme 1. CDS Strategy for DNA Detection[a]



[a] RuHex cations electrostatically bind to negatively charged DNA strands in a stoichiometric approach. Chronocoulometric interrogation of the redox reaction of RuHex quantitatively reflects the amount of DNA strands localized at the electrode surface. (A) AuNPs amplified detection: in the presence of target DNA, detection probes loaded on AuNPs form "sandwich" complexes with targets and capture probes anchored at the electrode surface. Note that AuNPs heavily loaded with negatively charged DNA strands offer a great amplification for signal transduction. (B) Nonamplified detection: hybridization brings target DNA to the electrode surface. Note that the increased RuHex redox charge only arises due to the hybridized targets.

increase electrochemical turnovers in corresponding DNA hybridization. For example, previous reports involve either enzyme-based incorporation of many redox labels in a single DNA strand[22,23] or replacement of small organic redox molecules with redox enzymes that can translate a single hybridization event into over 10 000 enzyme turnovers.[15,24] However, commercialization of these sensors might be hampered by either high cost or relatively poor stability of enzymes. Inorganic nanoparticles can also serve as redox labels for DNA detection,[25,26] taking the advantage of their high stability, low cost, and labeling convenience.[27] In the present work, we report a novel nanoparticle-based electrochemical DNA sensor by exploiting the signal amplification feature of gold nanoparticles (AuNPs) as well as controlled interfacial assembly of DNA probes at gold electrodes.

A "sandwich-type" detection strategy is employed in our design, which involves capture probe DNA self-assembled at gold electrodes and reporter DNA loaded on AuNPs (Scheme 1), both of which flank the DNA target sequence. As a result, in the presence of target DNA, the capture probe brings the target DNA, along with the reporter DNA labeled with AuNPs, proximal to the electrode surface. Since a single AuNP is loaded with hundreds of reporter DNA strands, this offers a significant amplification for the detection of target DNA. In contrast, in the absence of target DNA, the sandwich complex cannot be formed, leaving the surface-confined capture probe unhybridized. An electroactive complex, $[Ru(NH_3)_6]^{3+}$ (RuHex), serves as the signaling molecule, which binds to anionic phosphate of DNA strands in a stoichiometric approach. Previous studies have well demonstrated that binding of RuHex to DNA is completely through electrostatic interaction while free of any duplex intercalation.[28,29] As a result, redox charge of RuHex is a direct function of the amounts of DNA strands localized at electrode surfaces.[29] By employing this strategy, we demonstrate that this prototype DNA sensor is reproducible, stable, reusable, and can sensitively detect femtomolar target DNA with excellent differentiation ability for single-nucleotide polymorphisms (SNPs).

Materials and Methods

Materials. All oligonucleotides (Table S1 of the Supporting Information) were synthesized and purified by Sangon Inc. (Shanghai, China). Their concentrations were quantified by OD260 based on their individual absorption coefficients. Probes 1 and 2 were thiolated with a $-(CH_2)_6-$ spacer at either the 5′ or 3′ end, respectively. Target DNA 3 is a 38-base sequence that contains complementary sequences to both 1 and 2. DNA 4 is the mismatched counterpart of 3, which contains a single-nucleotide mismatch to probe 1. DNA 5 is a random sequence that is noncognate to either 1 or 2. Oligonucleotides 6−9 have the analogous meanings, while their sequences are associated with BRCA-1, a well-known breast cancer gene.[30]

AuNPs (20 ± 3 nm, ∼0.01% $HAuCl_4$) were obtained from Sigma, the concentration of which was estimated by assuming that a nanoparticle was an ideal ball tightly packed with Au atoms. Ethylenediaminetetraacetic acid (EDTA), mercaptohexanol (MCH), hexaammineruthenium(III) chloride ($[Ru(NH_3)_6]^{3+}$, RuHex), and tris(2-

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carboxyethyl)phosphine hydrochloride (TCEP) were from Sigma. Tris(hydroxymethyl)aminomethane was from Cxbio Biotechnology Ltd. The buffer solutions employed in this study are as follows. DNA immobilization buffer: 10 mM Tris-HCl, 1 mM EDTA, 10 mM TCEP, and 0.1 M NaCl (pH 7.4). Hybridization buffer: phosphate buffered saline (PBS, pH 7.4) with 0.25 M NaCl and 10 mM phosphate buffer. Buffers for both electrochemistry and electrode washing are 10 mM Tris-HCl solutions (pH 7.4). All solutions were prepared with MilliQ water (18 MΩ·cm) from a Millipore system.

Electrochemical Measurements. All electrochemical measurements were performed with a CH 650 electrochemical workstation (CH Instruments Inc., Austin). A conventional three-electrode configuration was employed all through the experiment, which involved a gold working electrode, a platinum wire auxiliary electrode, and an Ag/AgCl reference electrode. Cyclic voltammetry (CV) was carried out at a scan rate of 50 mV/s, and chronocoulometry (CC) at a pulse period of 250 ms and pulse width of 700 mV. The electrolyte buffer was thoroughly purged with nitrogen before experiments.

Functionalization of AuNPs with Reporter Probe DNA. AuNPs are readily functionalized with thiolated DNA strands via the well-known gold−sulfur chemistry. It has been well documented that self-assembly of thiolated DNA on AuNPs significantly increases the stability of AuNPs.[10] Conjugates of oligonucleotide DNA−AuNPs were synthesized following the published protocol.[10] Briefly, DNA−AuNPs were synthesized by incubating reporter probes (DNA 2 or 7, 3.0 μM) in 1 mL of 20 nm AuNPs solution (~1.2 nM). After standing for 16 h, the DNA−AuNP conjugates were "aged" in salts (0.1 M NaCl, 10 mM phosphate, pH 7.0) for 40 h. Excess reagents were removed by centrifuging at 15 000 rpm for 30 min. The red oily precipitate was washed, re-centrifuged, and then dispersed in 1 mL solution (10 mM phosphate buffer with 0.25 M NaCl, pH 7.0).

DNA Self-Assembly and Hybridization at Gold Electrodes. Gold electrodes (2 mm in diameter, CH Instruments Inc., Austin) were first polished on microcloth (Buehler) with Gamma micropolish deagglomerated alumina suspension (0.05 μm) for 5 min. Residual alumina powder was removed by sonicating electrodes in ethanol and water for 5 min, respectively. Then electrodes were electrochemically cleaned to remove any remaining impurities.[17] After being dried with nitrogen, electrodes were immediately used for DNA immobilization.

Electrodes with DNA self-assembly monolayers (SAMs)[29] of different surface density were interrogated in this work. Low-density surfaces (1.2×10^{12} molecule/cm^2) were obtained by incubation of electrodes with 0.2 μM of capture probes (1 or 6) in the immobilization buffer for 30 min. Medium-density (6.0×10^{12} molecule/cm^2) and high-density (1.2×10^{13} molecule/cm^2) surfaces were prepared by incubation of electrodes with 2 and 5 μM of capture probes in the immobilization buffer with 1 M NaCl for 1 h, respectively, The DNA-modified electrodes were further treated with 1 mM MCH for 2 h to obtain well-aligned DNA monolayers. The DNA surface density as well as DNA hybridization efficiency was quantitatively measured with CC as previously described.[29,31]

Target DNA was pre-annealed with reporter probes loaded on AuNPs at 37 °C for 30 min in the hybridization buffer, and then 4 μL of the solution was placed on gold electrodes with DNA SAMs for 1 h at room temperature. Since the pre-annealing step was performed in a quasi-homogeneous condition (hybridization at nanoparticle surfaces), which is usually faster than heterogeneous hybridization at the electrode surface, we expect that this pre-annealing/on-electrode hybridization process is more efficient than the two-step on-electrode hybridization process. After hybridization, electrodes were extensively rinsed with washing buffer and dried under a stream of nitrogen prior to electrochemical characterization.

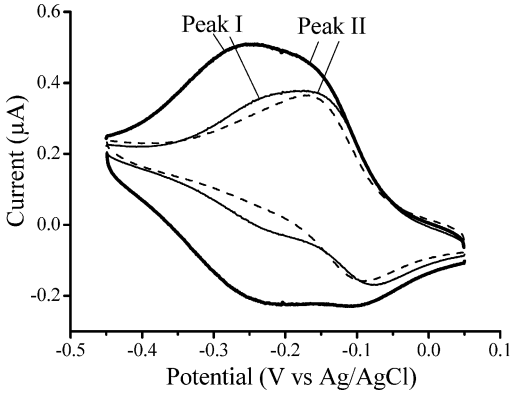

Figure 1. Cyclic voltammograms of gold electrodes modified with capture probe **1** in 10 mM Tris buffer (pH 7.4) with 50 μM RuHex before (dash line) and after hybridization with 1 μM target **3** in the absence of AuNP amplification (solid line) and 10 pM target **3** in the presence of AuNP amplification (thick line). Scan rate = 50 mV/s.

Results and Discussion

AuNP-Amplified Detection of DNA Hybridization. We employed RuHex as the signaling molecule in this chronocoulometric DNA sensor (CDS). CV was first employed to characterize the electrochemistry of RuHex at gold electrode surfaces with DNA/MCH monolayers. Consistent with previous studies, two pairs of peaks were observed (Figure 1): one (peak pair I) arose due to the redox reaction of RuHex electrostatically bound to the phosphate backbone of DNA, while the other (peak pair II) was ascribed to RuHex diffused to the MCH portion in the mixed SAM of DNA/MCH.[29,31] Therefore, the peak pair I reflected the amount of DNA strands localized at the electrode surface. Interestingly, in the absence of AuNP amplification, only slight increase in the peak current was observed even after hybridization with 1 μM DNA target; in contrast, with AuNP amplification, we observed significant enhancement of peak I only with 10 pM DNA target. These CV curves provided an intuitive impression of the amplification effect of AuNPs, implying that one could use AuNPs to realize DNA detection with high sensitivity.

We then employed CC to characterize the redox process of RuHex (detailed procedures are included in the Supporting Information). We have previously demonstrated that the RuHex/DNA/electrode system generated significantly more intense signal in chronocoulometry than in voltammetry.[31] This is possibly because a large portion of RuHex molecules entrapped in the heterogeneous film are kinetically *electroinactive* during "dynamic" voltammetric scans, while nearly all RuHex molecules are *electroactive* in the "static" chronocoulometric measurements.[31] Of note, we observed an amplification effect in CC experiments similar to that in CV (Figure S1 of Supporting Information).

Nanoscale Control of DNA Assembly and Hybridization. DNA hybridization efficiency at surfaces is a sensitive function of surface density of immobilized DNA capture probes.[32,33] We thus prepared a series of DNA SAMs with different surface density, by varying probe concentration, self-assembly time, and/or ionic strength in the immobilization buffer (see Materials and Methods). As shown in Figure 2, DNA hybridization

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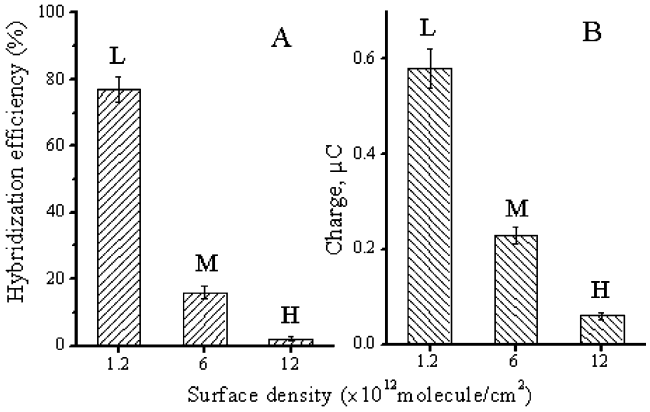

Figure 2. (A) Comparison of hybridization efficiency for gold electrodes with different DNA surface density. DNA hybridization was performed by incubation of electrodes in 1 μM solution of target DNA for 60 min. (B) Comparison of signal intensity for gold electrodes with different DNA surface density. DNA hybridization was performed by incubation of electrodes in 10 pM solution of target DNA in the presence of AuNP amplification. Redox charges of RuHex bound to DNA were obtained from chronocoulometric measurements. Signal was defined as the difference in the redox charge of RuHex after and before hybridization (signal = $Q_{after} - Q_{before}$, $Q_{before} = 0.090 \pm 0.005$ μC). Error bars show the standard deviations of measurements taken from at least three independent experiments. In the figure, L, M, and H stand for low-density (1.2×10^{12} molecule/cm^2), medium-density (6.0×10^{12} molecule/cm^2), and high-density (1.2×10^{13} molecule/cm^2) surfaces. Estimated intermolecular distances for electrodes with different surface density are as follows: low density (9.2 nm); medium density (4.1 nm); high density (2.8 nm).

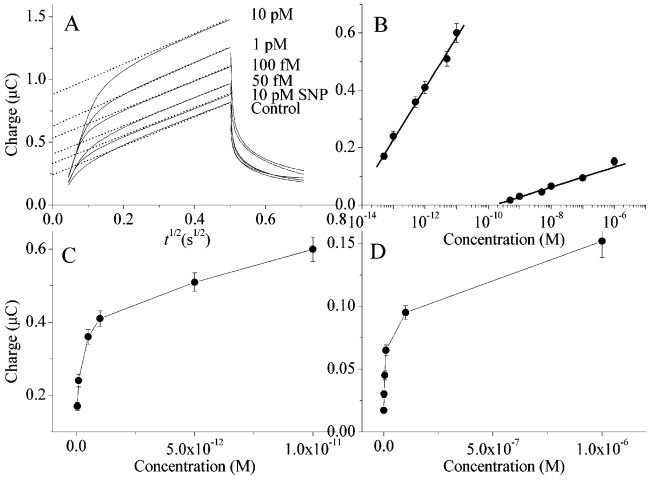

Figure 3. (A) Chronocoulometry curves for electrodes with capture probe **1** hybridized with target DNA at a series of concentrations (50 fM, 100 fM, 1 pM, and 10 pM), and with 10 pM single-nucleotide mismatched (SNP) DNA. Note that reporter DNA labeled with AuNPs is always present during the hybridization, including the control experiment that is free of target DNA ("control" curve). The electrolyte is 10 mM Tris buffer (pH 7.4) containing 50 μM RuHex. Pulse period = 250 ms; pulse width = 700 mV. Intercepts at $t = 0$ in chronocoulometric curves represent redox charges of RuHex bound to DNA. (B) Logarithmic plot of signal versus target DNA concentration, where the definition of signal is the same as that in Figure 2. The left half represents the plot for detection in the presence of AuNP amplification, while the right half represents the plot for detection in the absence of AuNP amplification. Error bars show the standard deviations of measurements taken from at least three independent experiments. (C) and (D) are the original plots (without logarithmic conversion) for detection in the presence and in the absence of AuNP amplification, respectively.

efficiency decreased along with the increased surface density, with highest efficiency (~80%) at low-density surfaces (1.2×10^{12} molecule/cm^2) and lowest efficiency (<5%) at high-density surfaces (1.2×10^{13} molecule/cm^2). We then challenged CDS with 10 pM target DNA as well as reporter DNA labeled with AuNPs. Consistently, we observed that the CDS signal for the surface with low density was approximately 10 times more intense than that with high density. This clearly showed that control of DNA assembly was essential for sensitivity improvement, and that low-density DNA monolayers were optimal for high-sensitivity DNA detection.

Sensor Performance of CDS. We observed that redox charges of RuHex increased upon hybridization with complementary DNA 3, and the intensity was cognate to the target concentration. We then evaluated the detection performance of CDS by exposing the sensor to a series of target DNA concentrations. We found that signal intensity was logarithmically related to target concentrations across the range of 50 fM to 10 pM (Figure 3). The limit of detection was estimated to be 10 fM (3 times signal-to-noise ratio). Note that we could only detect 0.5 nM target DNA in the absence of AuNP amplification (Figure 3), the sensitivity of which is more than 4 orders of magnitude lower than that of CDS. This clearly showed the large amplification effect of AuNPs. In addition, the employed "on-electrode" hybridization approach allowed the use of a small sample volume as low as 4 μL, equating to an absolute detection limit of 40 zmol (approximately 24 000 molecules/μL). Also of note, the CDS was also fairly reproducible, with small sensor-to-sensor variations (RSD ranging from 5.0 to 7.1% for different target concentrations).

Differentiation of single mismatches is of significant interest for a variety of important applications, including SNP genotyping.[34] We tested a mismatched DNA 4, which contained a single-base mismatch in the middle of the capture probe binding section. As shown in Figure 3, the mismatched DNA could be readily distinguished from the perfectly matched DNA. We found that the signal for mismatched DNA (10 pM) was only <15% of that for complementary DNA of the same concentration while comparable to that for complementary DNA in the femtomolar range, which clearly showed that CDS could satisfactorily perform SNP assays (>6:1).

To further demonstrate the applicability of CDS for SNP discrimination, we employed a model system associated with a breast cancer gene, BRCA-1. It has been well documented that women inheriting BRCA-1 mutations are highly susceptible to the development of breast cancer.[30] We then challenged the CDS with a series of BRCA-1 mutations, that is, DNA 9−11 containing different base mismatches (T, C, and G) in the middle of the capture probe binding section. As shown in Figure 4, only the perfectly matched DNA produced prominent signals, while signals corresponding to all mismatched DNA were not significant (<20%).

Besides sensitivity and selectivity, reusability is also an important feature for biosensors. We found that CDS could be conveniently regenerated by incubation of modified electrodes in hot water (80 °C) for 5−10 min, which completely removed hybridized DNA via thermal denaturation. Importantly, CDS almost retained original performance after three regeneration cycles, with negligible loss of probes and without sacrificing hybridization efficiency (Figure S2 of Supporting Information). In addition, CDS is also fairly robust at normal storage conditions. After sensor preparation, gold electrodes with DNA

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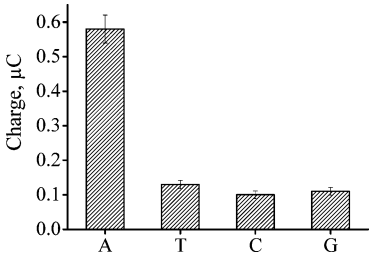

Figure 4. Comparison of signal intensity for gold electrodes hybridized with a series of targets associated with BRCA-1 at 10 pM: the perfectly matched DNA (A) and single-nucleotide mismatched DNA (T, C, G). The definition of signal is the same as that in Figure 2. Error bars show the standard deviations of measurements taken from at least three independent experiments.

SAMs could be stored in the refrigerator with minimal loss of DNA SAMs for at least a week. Nevertheless, we noticed that significant loss of DNA SAMs (>40%) occurred under more stringent conditions, such as 1 h incubation at boiling temperature, suggesting that the stability of CDS remains to be improved.

Discussion

Tarlov and co-workers first proposed an effective way to quantitatively measure DNA surface density by using RuHex as a redox probe.[29] This method is nevertheless not of sufficient sensitivity (∼nanomolar) for DNA detection (Figure S2 of Supporting Information). Recently, Kelley and co-workers realized picomolar DNA detection sensitivity by incorporation of nanoelectrodes and electrocatalysis that coupled the redox reaction of RuHex with the chemical reaction between RuHex and ferricyanide.[35,36] In the present work, we report a novel CDS approaching femtomolar detection sensitivity for target DNA. This sensitivity exceeds that of conventional fluorescent DNA detection (nanomolar to picomolar) by at least 2 orders of magnitude. We also note that this femtomolar sensitivity is comparable to or even better than the sensitivity of the nanoparticle-based solid-state DNA sensors (scanometric or electrical) developed by Mirkin and co-workers.[10,12] In addition, both assay speed and operation convenience are significantly improved due to the optimized DNA assembly and the absence of a silver-staining step in their approaches.[10,12] Very recently, Hansen et al. reported an electrochemical approach for DNA detection by using metal sulfide nanoparticles. They could detect as few as 33 fM DNA targets, which was nevertheless rather time-consuming (∼8 h).[37]

The high sensitivity of CDS arises due to two combined effects. First, capturing of a single target DNA strand also brings an AuNP loaded with a few hundred DNA strands proximal to the surface, while each strand contains 25 RuHex binding sites (the reporter probe contains 25 bases/phosphate ions). As a result, a single hybridization event is translated into more than 10^3 redox events, leading to signal amplification by at least 3 orders of magnitude. Second, nanoscale control of DNA assembly offers additional signal amplification. Since the diameter of ssDNA is also at the nanometer scale (approximately 1 nm), hybridization might be significantly hampered by the steric effect within a dense monolayer of DNA probes. In fact, we have demonstrated that efficiency of DNA hybridization is highly dependent on intermolecular spacing within the DNA SAMs. When the surface density decreases from 1.2×10^{13} to 1.2×10^{12} molecule/cm^2, the distance between DNA strands increases from 2.8 to 9.2 nm (Figure 2). Such a precise control of DNA surface density in CDS leads to a signal amplification factor of 10.

CDS with femtomolar sensitivity (and zmoles in absolute detection limit) is among the most sensitive solid-state DNA sensors ever reported[27] and satisfactorily meets the requirement of genetic analysis and clinical diagnostics in many cases. Despite this, the CDS sensitivity is still not comparable to those of PCR[3] and several solution-phase DNA assay methods.[38] This is partially associated with limited diffusion rates of target DNA from bulk solutions to electrode surfaces.[39] Indeed, as shown in Figure 3D, the apparent dissociation constant (K_d) for this heterogeneous hybridization is in the high nanomolar range, which suggests that the hybridization at the surface is largely influenced by mass transport. To realize high-end applications, such as direct, unamplified pathogen detection, further optimization of the system and incorporation of a variety of existing technologies, such as electrocatalysis,[40] high-affinity locked or peptide nucleic acid (LNA or PNA) probes,[41,42] and/or magnetically or electrically facilitated hybridization,[43] might lead to further signal amplification by several orders of magnitude.

The CDS selectivity compares favorably with previously reported DNA detection methods.[10] The excellent selectivity of CDS may also benefit from the use of AuNPs. Pioneering work from Mirkin group has well demonstrated that the AuNP−DNA ensemble features extremely sharp denaturation transition over both temperature and ionic strength gradients.[10,12,27] In particular, mismatched DNA and its target are forced apart under sufficiently low ionic strength due to the existence of strong coulomb repulsion between the two negatively charged DNA strands.[12] Interestingly, the solution of low ionic strength (10 mM TE buffer free of salts) employed in this work has dual roles. One is to avoid screening the electrostatic binding between RuHex and DNA, and the other is to stringently wash off mismatched DNA. Indeed, CDS can reliably differentiate SNPs associated with BRCA-1 at room temperature, which is a significant improvement compared to conventional SNP assays that rely on thermal stringency via precise temperature control. It should be mentioned that the CDS selectivity might be further increased by using highly specific PNA or LNA probes.[41,42] It is worthwhile to point out that we observed the nonspecific adsorption of a small amount of AuNPs to the sensor surface. The nonspecific signal arising due to the adsorption of AuNPs is approximately 0.021 ± 0.010 μC, which is reasonably small compared to the AuNP-amplified signal but does increase the background signal. Design of surfaces that are completely

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resistant to AuNP adsorption might decrease the background signal and further increase the detection sensitivity.

Conclusion

We report a novel approach to DNA detection via CDS, a solid-state chronocoulometric DNA sensor exhibiting very high sensitivity and selectivity. These features, as well as its operation convenience, stability, and reusability, make it a promising alternative to conventional fluorescent DNA detection methods. Since CDS is electrochemistry-based, one might expect the design of an integrated, portable, and low-cost device for DNA detection based on the proof-of-concept approach described in this work.

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Supporting Information Available: Detailed description of the experiments and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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