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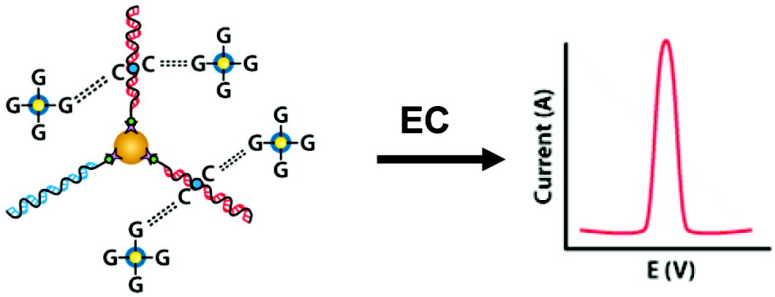

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Electrochemical Quantification of Single-Nucleotide Polymorphisms Using Nanoparticle Probes

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Abstract: We report a new approach for electrochemical quantification of single-nucleotide polymorphisms (SNPs) using nanoparticle probes. The principle is based on DNA polymerase I (Klenow fragment)-induced coupling of the nucleotide-modified nanoparticle probe to the mutant sites of duplex DNA under the Watson−Crick base pairing rule. After liquid hybridization events occurred among biotinylated DNA probes, mutant DNA, and complementary DNA, the resulting duplex DNA helixes were captured to the surface of magnetic beads through a biotin−avidin affinity reaction and magnetic separation. A cadmium phosphate-loaded apoferritin nanoparticle probe, which is modified with nucleotides and is complementary to the mutant site, is coupled to the mutant sites of the formed duplex DNA in the presence of DNA polymerase. Subsequent electrochemical stripping analysis of the cadmium component of coupled nanoparticle probes provides a means to quantify the concentration of mutant DNA. The method is sensitive enough to detect 21.5 attomol of mutant DNA, which will enable the quantitative analysis of nucleic acid without polymerase chain reaction preamplification. The approach was challenged with constructed samples containing mutant and complementary DNA. The results indicated that it was possible to accurately determine SNPs with frequencies as low 0.01. The proposed approach has a great potential for realizing an accurate, sensitive, rapid, and low-cost method of SNP detection.

Introduction

Single-nucleotide polymorphisms (SNPs) are point mutations that constitute the most common genetic variation, and they are often closely associated with susceptibility to various common diseases and individual differences in drug metabolism.[1,2] Numerous methods and technologies to identify point mutations have been reported to date, including oligonucleotide ligation,[3] primer extension,[4] allele-specific polymerase chain reaction (PCR),[5] microarray,[6] TaqManPCR,[7] and endonuclease digestion.[8] Quantifying SNPs is particularly important for estimating the frequency of SNP alleles in DNA pools.[9] To estimate very low SNP frequencies (such as <5%), an approach with high sensitivity, specificity, and reproducibility is necessary. Several quantitative approaches, including restriction fragment-length polymorphism, real-time pyrophosphate DNA sequencing, single-base extension with fluorescently labeled ddNTPs, 5′ nuclease allelic discrimination assay, and a minisequencing assay using matrix-assisted laser desorption ionization time-of-flight mass spectrometry have been developed.[10] Strategies have also been devised that rely on a gold nanoparticle probe[11] and the high specificity of enzymes[12] to allow SNP identification directly from genomic DNA. However, most of these methods and technologies in both quantification and qualitative SNP detection require target amplification, typically with PCR. Additional efforts are thus needed to create more broadly applicable methods that would allow accurate, sensitive, rapid, and low-cost SNP quantification without PCR amplification.

Recently, electrochemical detection of known or unknown SNPs has attracted considerable interest because of its high sensitivity, low cost, and use of a miniaturized and portable device.[13−17] Barton's group developed an electrocatalytic method for detecting single-base mismatches as well as DNA-base lesions in fully hybridized duplexes, based on charge transport through DNA films.[13] Willner used base-extension technology in connection with an enzyme label to detect DNA with known

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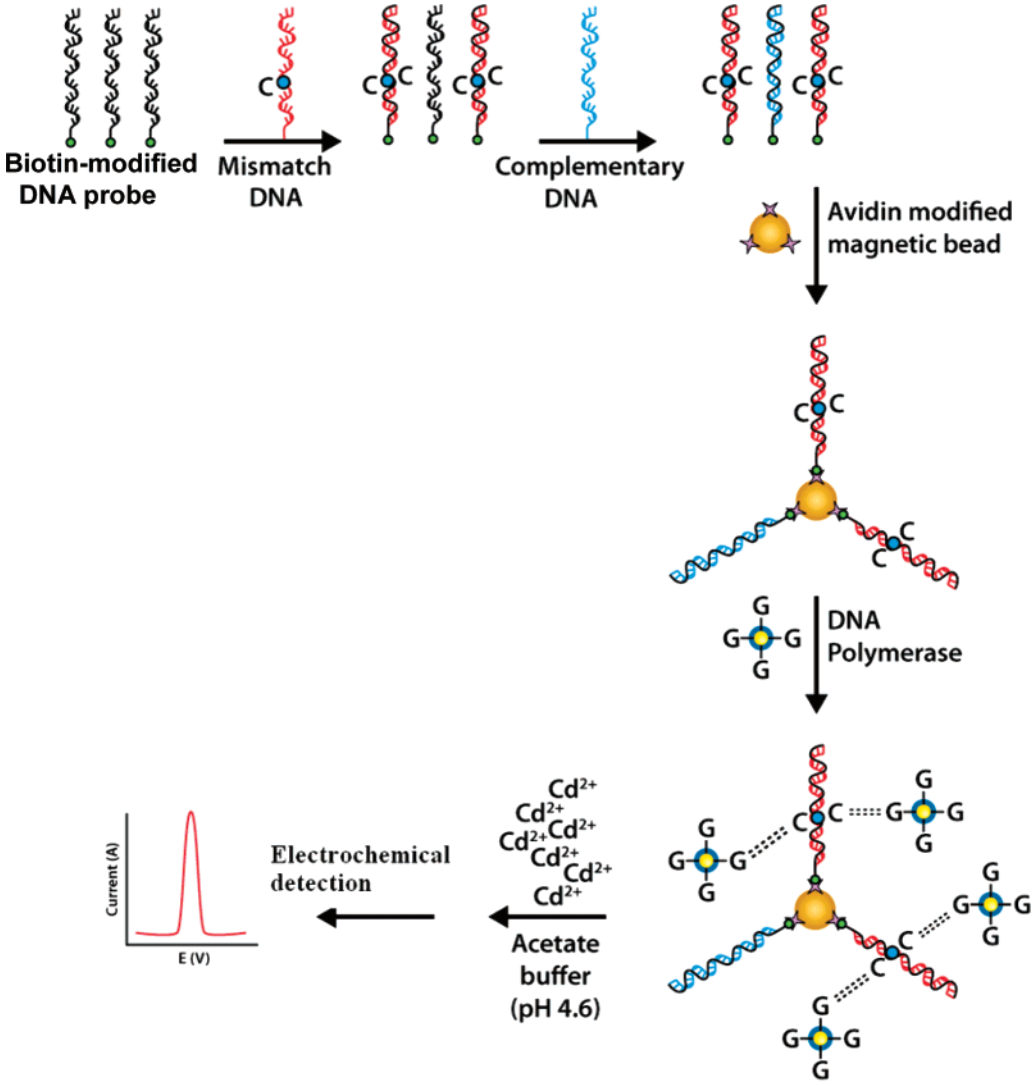
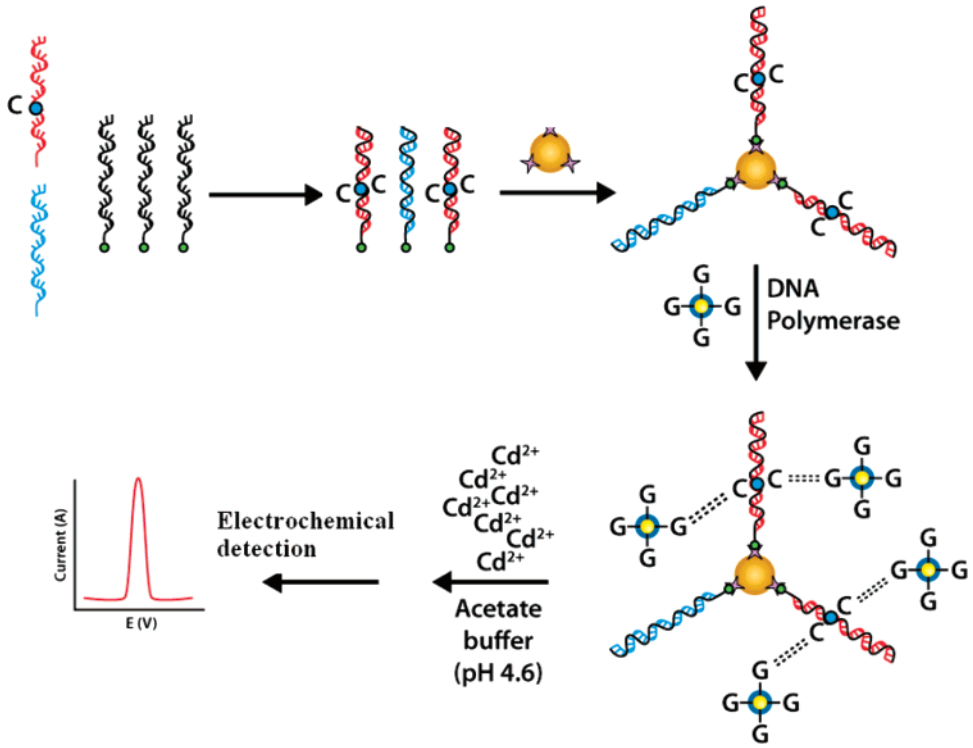
Scheme 1. Schematic of an Electrochemical SNP Quantitative Assay Based on a Nanoparticle Probe and a Sequential DNA Hybridization Reaction



point mutations with a detection limit of 1×10^{-14} mol/mL.[14] Recently, we and others reported a nanoparticle-based electrochemical coding technology to identify unknown SNPs.[16,17] Kerman et al.[16] used monobase-modified gold nanoparticles for electrochemical measurements of SNPs—a given unknown mutation was identified by four successive voltammetric measurements. Liu et al.[17] reported an effective nanocrystal-based bioelectronic approach for coding individual SNPs. Four nanocrystals, such as ZnS, CdS, PbS, and CuS, linked to the adenosine, cytidine, guanosine, and thymidine mononucleotides, respectively, were sequentially introduced to a magnetic-bead solution coated with a DNA duplex. Each mutation captures, via base pairing, different nanocrystal−mononucleotide conjugates and yields a characteristic multipotential stripping voltammogram whose peak potentials reflect the identity of the mismatch. The mismatch recognition events are being amplified by the metal accumulation feature of the stripping voltammetric transduction mode. The approach relies on multiple signals and leads to distinct multipotential fingerprints for specific SNPs in a single voltammetric run. Although it effectively identified unknown SNPs, this approach required a high concentration of mismatched DNA and could not be used to detect a low concentration of SNPs; the approach also failed to quantify the concentration of SNPs and thus estimate the frequency of SNP alleles in DNA pools.

In the present study, we report a new approach to demonstrate how to quantify the concentration of SNPs by using metallic nanoparticle probes and electrochemical detection. A general method is to use polymerase-induced coupling of the nucleotide-modified nanoparticle probe to the mutant site of duplex DNA and the subsequent electrochemical stripping detection of the captured nanoparticle probes on the duplex DNA. To demonstrate this concept, sequential DNA hybridization reactions among the biotin-modified DNA probe, mutated DNA, and complementary DNA were first applied to form the duplex DNA. Scheme 1 schematically presents the principle to quantify the concentration of SNPs in our initial study. It begins with liquid DNA hybridization between the biotin-modified DNA probe and mismatched DNA to form the duplex DNA. The excess DNA probes were hybridized by adding complementary DNA. Following magnetic capturing of the formed duplex DNA with avidin-modified magnetic beads, the excess of complementary DNA was removed by magnetic separation and washing steps. Nanoparticle probes modified with a base (here it is guanine) that is complementary to the mutation site (here it is cytosine) were coupled to the formed duplex DNA in the

Scheme 2. Schematic of an Electrochemical SNP Quantitative Assay Based on a Nanoparticle Probe and a One-Step DNA Hybridization Reaction



presence of DNA polymerase. The captured nanoparticle probes were detected with a disposable screen-printed electrode by electrochemical stripping analysis of metal components after they were released. The electrochemical signal (current density) is thus proportional to the concentration of mismatched DNA concentration in the sample solution. In this quantifying protocol, it is necessary to block the excess of biotin-modified DNA probes (to block the cytosine sites of unhybridized DNA probes) by adding complementary DNA, which provides the foundation of SNP quantification. In reality, considering a genomic sample with a low concentration of SNPs (low frequency of SNP alleles), which has a large number of complementary DNA probes, DNA hybridization reactions occur simultaneously among a biotin-modified DNA probe, mutated DNA, and complementary DNA after adding the biotin-modified DNA probes. So a one-step hybridization reaction among the biotin-modified DNA probe, mutated DNA, and complementary DNA was used to form the duplex DNA (Scheme 2). The new approach was also applied to estimate the SNP frequency with the constructed DNA samples, which were prepared by mixing a mismatched DNA target and a complementary DNA probe at different ratios.

Experimental

Apparatus. Square-wave anodic-stripping voltammetric measurements were performed with an electrochemical analyzer CHI 660A (CH Instruments, Austin, TX) connected to a personal computer. A disposable electrochemical screen-printed electrode consisting of a carbon working electrode, a carbon counter electrode, and an Ag/AgCl reference electrode was purchased from Alderon Biosciences, Inc. (cat. 0101, NC) for electrochemical measurements. A sensor connector (Alderon Biosciences, Inc., cat. 0012) allows for connecting the disposable screen-printed electrode to the CHI electrochemical analyzer. A centrifugal filter device (Amicon Ultra-15, 30 000 molecular weight cut off (MWCO), Millipore Corporation, MA) was used to concentrate the protein solution. A disposable PD-10 desalting column packed with Sephadex G-25 medium (exclusion limit 5000) was purchased from Amersham Bioscience Corp. (NJ) and used to purify a protein solution. A centrifuge was performed with a Sorvall@RC 26 Plus (Kendro Laboratory product). Magnetic capturing and separations were conducted on an MCB 1200 Biomagnetic Processing Platform (Sigris, CA).

Reagents. All stock and buffer solutions were prepared using autoclaved double-deionized water. The Tris-HCl buffer (0.1 M, pH = 8.0) was made from 1.0 M stock buffer, which was purchased from Sigma. Cadmium nitrate, Tween 20, and a solution of mercury atomic-absorption standard (1010 mg/L) was purchased from Aldrich. Apoferritin was purchased from Sigma. Sodium hydroxide, lithium chloride, sodium chloride, and guanosine 5′-monophosphate were purchased from Sigma. All chemicals were of analytical reagent grade. All experiments were conducted at room temperature.

The synthetic oligonucleotides were purchased from Integrated DNA Technologies, Inc. (IDT, Coralville, IA) and had the following sequences: DNA Probe: Biotin-5′-ATG TGG AAA ATC TCT AGC AGT-3′. Complementary DNA: 5′-ACT GCT AGA GAT TTT CCA CAT-3′. Mismatch DNA: 5′-ACT GCT AGA CAT TTT CCA CAT-3′.

The oligonucleotide stock solution (500 mg L^{-1}) was prepared with autoclaved water and was kept frozen. Proactive streptavidin-coated magnetic microspheres (0.83 μm diameter, CM01N, cat. 4725) were purchased from Bangs Laboratories (Fishers, IN).

Preparation of Cadmium Phosphate-Loaded Apoferritin (CPLA) Nanoparticle Probe. CPLA nanoparticles were prepared based on our recent published protocol.[18] An apoferritin solution (5 mg, equine spleen, Sigma) was prepurified on a PD-10 column to remove aggregates. The collected eluent fractions (0.1 M ammonium acetate, pH 7.0) were mixed and concentrated to 0.1 mL with a centrifugal filter device (Amicon Ultra-15) and were then washed twice with autoclaved water (3 mL) by using the same filter. Autoclaved water

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(2.9 mL) was then added. Cadmium nitrate (500 μL, 10 mM) was slowly added into the purified apoferritin solution (pH 8.0), and the mixture was continuously stirred for 1 h to allow the metal ions to diffuse into the cavity of the apoferritin. Subsequently, 0.2 M phosphate buffer (pH 7.0, 500 μL) was slowly introduced into the solution to form a metallic phosphate core inside the apoferritin. Excess metal cations remaining outside the apoferritin were precipitated with a phosphate buffer. After 30 min, the mixture was subjected to centrifugation at 10 000 rpm for 5 min. The supernatant was washed three times with 0.1 M Tris-HCl by using an Amicon filter (MWCO = 25 000). The metal phosphate-incorporated apoferritin nanoparticles were then reconstituted into a Tris-HCl solution (1 mL). The protein concentration was determined by using a bicinchoninic acid (BCA) assay with bovine serum albumin as the standard.

Preparation of Guanine-Modified CPLA (G−CPLA) Conjugate. G−CPLA conjugate was prepared by attaching a monobase, guanosine 5′-monophosphate, to the CPLA nanoparticles through their 5′ phosphate group via the formation of a phosphoramidite bond with the free amino groups of the apoferritin (protein shell). Guanosine 5′-monophosphate solutions were prepared using a 20 mM Tris-HCl buffer solution containing 20 mM NaCl (pH 7.0, tris-buffered saline (TBS)). Subsequently, 500 μL of guanosine 5′-monophosphate solution with a desired concentration was added to 500 μL of the CPLA nanoparticle suspension, and the mixture was shaken for 1 h, followed by separation with the PD-10 column. The collected eluent fractions were concentrated to 0.1 mL with a centrifugal filter device (Amicon Ultra-15) and were then washed twice with TBS (3 mL) by using the same filter. Finally, the purified G−CPLA conjugates were dispersed in TBS for the base-pairing without further alterations.

Procedure of Electrochemical Quantification of Single-Nucleotide Polymorphism. Electrochemical quantification of single-nucleotide polymorphism includes four steps:

Step 1. DNA Hybridization Reactions. For a sequential DNA hybridization reaction (Scheme 1), 25 μL of biotinylated DNA probes (1 nmol) and 25 μL of the desired concentration of mismatched DNA (mutated at a cytosine site) were added into a 1.5 mL centrifuge tube, and the mixture was incubated for 1 h with gentle mixing. Then 25 μL of complementary DNA (2 nmoL) was added, and the hybridization reaction was continued for another 1 h. A one-step DNA hybridization reaction (Scheme 2) was performed by mixing 25 μL of biotinylated DNA probes (1 nmol), 25 μL of the desired concentration of mismatched DNA, and 25 μL of complementary DNA (2 nmoL). The reaction was continued for 90 min.

Step 2. Magnetic Capturing of the Formed Duplex DNA. Magnetic capturing of the formed duplex DNA was carried out on the MCB 1200 biomagnetic processing platform using streptavidin-modified magnetic beads. Briefly, 5 μL of streptavidin-coated magnetic beads was transferred into a 1.5 mL centrifuge tube, and the beads were then washed twice with 95 μL of TTL buffer (100 mM Tris-HCl, pH 8.0, 0.1% Tween, and 1 M LiCl). After magnetic separation, the suspension was removed. The beads were resuspended above the DNA mixture (from Step 1) containing the formed duplex DNA and the excess of complementary DNA. The mixture was incubated for 30 min with gentle mixing. The magnetic beads, coated with the formed duplex DNA, were washed twice with 95 μL of TT buffer (250 mM Tris-HCl, 0.1% Tween 20) and blocked for 15 min with 100 μL of TT buffer containing 1% bovine serum albumin (BSA). The beads were washed twice with 95 μL of TT buffer and resuspended in 45 μL of 20 mM TBS (pH 7.8) with 60 mM KCl and 10 mM $MgCl_2$.

Step 3. Hybridization between Mismatched Sites of the Formed Duplex DNA and G−CPLA Conjugate. G−CPLA conjugate (5 μL) was added to the duplex DNA-coated magnetic bead solution in the presence of 0.5 U/μL DNA polymerase I (Klenow fragment), and the solution was mixed for 1 h at room temperature. After incubation, the magnetic-bead/DNA/CPLA assemblies were washed twice with 95 μL TT buffer to remove the excess solution and the nonspecifically bounded G−CPLA conjugates. The resulting magnetic-bead/DNA/CPLA assemblies were resuspended in 50 μL of 0.2 M acetate buffer (pH 4.6) containing 10 μg mL^{-1} mercury(II) atomic absorption standard solution. Cadmium phosphate was dissolved to release cadmium ions at pH 4.6 acetate buffer.[18] After 1 min of mixing and a subsequent magnetic separation, the acetate buffer (containing the dissolved cadmium ions) was transferred to a screen-printed electrode (SPE) for electrochemical stripping analysis.

Step 4. Electrochemical Detection. Dissolved cadmium ions were measured with square wave voltammetry (SWV) using an in situ plated mercury film on the SPE with a 1 min pretreatment at +0.6 V, followed by a 2 min accumulation at −0.9 V. After a 15 s rest period (without stirring), stripping was performed by scanning the potential from −0.9 to −0.5 V, with a step potential of 4 mV, an amplitude of 25 mV, and a frequency of 25 Hz. The curves that were obtained were baseline corrected with CHI 660A software.

SNP Frequencies Determination in Constructed DNA Samples. Constructed DNA samples were prepared by mixing mutant DNA and perfect matched DNA at different ratios ranging from 0 to 100%. Then, 25 μL of biotinylated DNA probes (1 nmol) was mixed with 50 μL of constructed DNA sample. The electrochemical signal of the artificial DNA sample was obtained by following the one-step hybridization procedures listed above (Step 1 to Step 4).

Results and Discussion

Preparation and Characterization of CPLA Nanoparticle Probe. The CPLA nanoparticle was prepared with an apoferritin template synthesis method, which was developed recently in our group.[18] Apoferritin is a native nanostructured protein composed of 24 polypeptide subunits that interact to form a hollow cagelike structure with a diameter of 12.5 nm; the interior cavity of apoferritin is 8 nm in diameter.[19] There are 14 channels that are formed at subunit intersections with diameters of 3−4 Å and connect the outside of apoferritin with its interior. Eight hydrophilic channels are thought to facilitate the passage of metal ions and small molecules of appropriate size into the cavity of the protein.[20] Figure 1A schematically illustrates the procedure of CPLA preparation. It includes two diffusion steps. Cadmium ions first diffuse into the apoferritin cage through channels at a pH of 8.0 and accumulate on the internal surface through the interaction between cadmium ions and the functional groups on the internal surface. After a 1 h balance, phosphate buffer is slowly introduced into the solution. Because the concentration of cadmium ions in the surface of the cavity is higher than that of the outside cavity, precipitation will first occur inside apoferritin to form cadmium phosphate seeds in the inner surface of the cavity. Once the cadmium phosphate seeds have formed in the cavity of apoferritin, the concentrations of cadmium ions and phosphate in the cavity will decrease; the cadmium ions and phosphate cations outside apoferritin will continue to diffuse into the cavity. The cadmium phosphate seeds will work as an autocatalyst, and metal phosphate will be formed quickly in the apoferritin cavity. This process will take 30 min to complete. Figure 1B presents a typical transmission electron microscopy (TEM) image of the stained CPLA sample. One can see that individual particles are clearly identifiable, and the dense cadmium phosphate cores with a diameter of approximately 8 nm appear black and are surrounded

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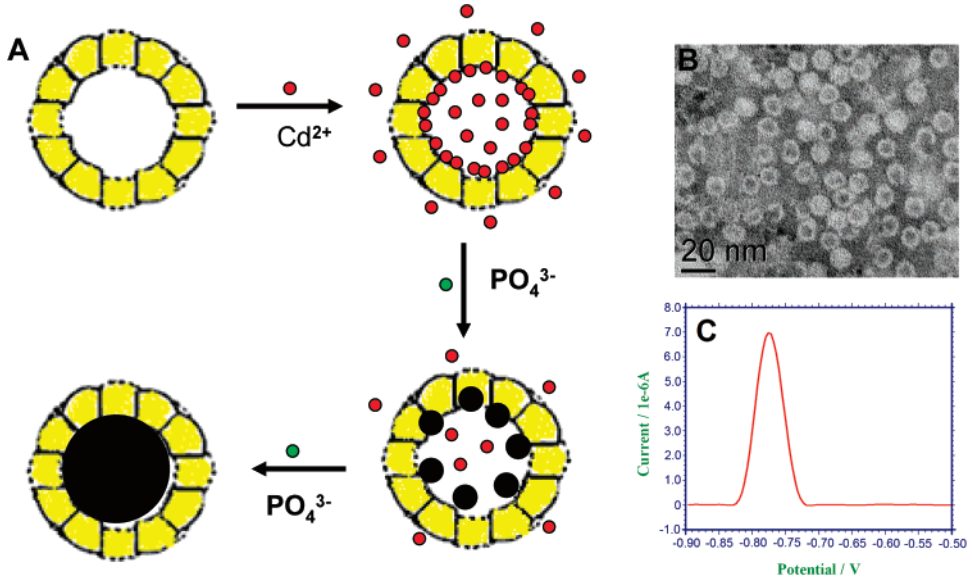

Figure 1. (A) Schematic illustration of the apoferritin-templated synthesis of $Cd_3(PO_4)_2$/apoferritin nanoparticle probes; (B) typical TEM image of a stained sample of $Cd_3(PO_4)_2$/apoferritin nanoparticles; (C) square-wave voltammogram of $Cd_3(PO_4)_2$/apoferritin nanoparticles in 0.2 M acetate buffer (pH 4.6) containing 10 μg mL^{-1} mercury. Voltammetric stripping readout with an in situ plated mercury-coated screen-printed electrode, using a 1 min pretreatment at 0.6 V, a 2-min accumulation at −1.4 V, a 15 s rest period, a square-wave voltammetric scan with a step potential of 50 mV, an amplitude of 20 mV, and a frequency of 25 Hz. The background correction was accomplished by using CHI 660A software.

by a protein shell (white). The electrochemical signal of the CPLA nanoparticle probe was first studied by measuring cadmium components in the CPLA nanoparticle using SWV. A well-defined cadmium peak was obtained by dispersing the nanoparticle suspension in a 0.2 M acetate buffer (pH 4.6) containing 10 μg mL^{-1} mercury after a 2 min accumulation (Figure 1C).

G−CPLA conjugate was prepared by modifying the CPLA−nanoparticle surface with monobase, guanosine 5′-monophosphate, through their 5′ phosphate group via the formation of a phosphoramidite bond with the free amino groups of the apoferritin (protein shell). The amount of guanine on the nanoparticle surface was estimated by measuring the concentrations of apoferritin and guanine. The apoferritin concentration was determined by the BCA assay (Pierce). The concentration of guanine was determined by electrochemically measuring the oxidation current of guanine after the digestion step with acid.[21] We optimized the ratio between guanine and CPLA by changing the concentration ratio between guanosine 5′-monophosphate and CPLA during the preparation. A low ratio (guanine to CPLA) would provide higher sensitivity because one G−CPLA will bound to one cytosine mutant site. After optimization, an average of two guanines were attached to a CPLA nanoparticle (results not shown).

Electrochemical Quantifying of SNP. The proof-of-principle was first demonstrated by using guanine−CPLA conjugate to detect the cytosine mutated DNA target under Scheme 1. Here, the liquid DNA hybridization reaction was employed to form the duplex DNA in the solution following magnetic capturing of the formed duplex. The CPLA nanoparticle probe was modified with guanine, complementary to the cytosine mutation site, and bound to the formed duplex DNA in the presence of DNA polymerase. Figure 2 shows the typical square-wave voltammograms of this electrochemical quantifying SNP assay

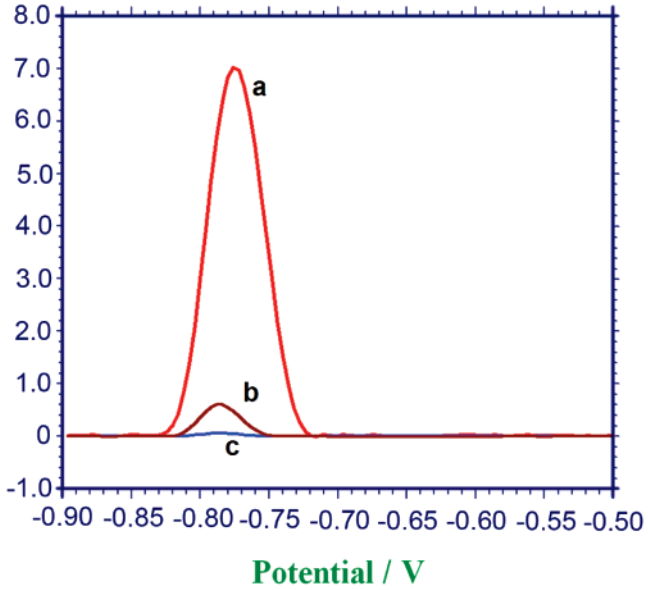

Figure 2. Typical square-wave voltammograms of 100 pM (Curve a) and 0 pM (Curve b and c) cytosine-mutated DNA with a sequential hybridization procedure (Scheme 1). Curve c was obtained with a BSA blocking step and a BSA-blocked CPLA nanoparticle probe. Electrochemical measurement conditions were the same as in Figure 1.

in the presence and absence of cytosine-mutated DNA. It can be seen that a well-defined cadmium stripping peak was observed in the presence of cytosine-mutated DNA (Curve a). The cadmium peak would be ascribed to the cadmium component of CPLA nanoparticle probes, which were bound to the cytosine cites of the formed duplex DNA (composed of a DNA probe and a cytosine-mutated DNA target) by base-paring (C−G). A significant small signal was also observed in the control experiment (in the absence of cytosine-mutated DNA, Curve b, control). Such a response would belong to the nonspecific adsorption of G−CPLA conjugate on the magnetic bead surface. It was found that blocking the magnetic bead surface (after

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magnetic capturing) with 1% BSA would reduce nonspecific adsorption; a negligible response was observed (Curve c in Figure 2). The delimitation of such nonspecific adsorption may be attributed to the shield effect of BSA, which was adsorbed on the surface of the magnetic beads. Such behavior also reflects the shielding of the magnetic beads and the efficient removal of unwanted constituents (including the excess of complement DNA and unbound nanoparticle probes) by magnetic effects.

In most of the magnetic-bead-based DNA hybridization assays, the hybridization reaction was performed with the DNA probe immobilized on the beads to form duplex DNA on the bead surface.[22] In the present work, the hybridization reaction is performed in the liquid solution, and then the duplex is captured on the beads. We compared the electrochemical responses of SNP with the magnetic-bead-based hybridization assay and proposed the liquid hybridization assay in the present work. It was found that the signal of the liquid hybridization assay was 1.3 times higher than that of the magnetic-bead-based assay (results not shown). Such a difference may come from the higher hybridization efficiency of the liquid hybridization reaction than in solid hybridization, leading to the formation of more DNA duplex, and then more nanoparticle probes are bound. The result is consistent with the reported literature.[23] Furthermore, the employed liquid DNA hybridization reaction in the present work also simplifies the experimental procedure and avoids many magnetic separation and washing steps.

Although the initial study demonstrated that the protocol can be used for the quantification of SNP, a genomic sample with a low concentration of SNPs (a low frequency of SNP alleles) in reality has a large number of complementary DNA. The DNA hybridization reaction will be performed simultaneously after adding the biotin-modified DNA probe. We studied the one-step hybridization reaction (see Scheme 2) with the biotin-modified DNA probe, complementary DNA, and mutated DNA instead of sequential DNA hybridization for electrochemical SNP detection. Figure 3A presents the electrochemical responses of a 50 pM cytosine-mutated target DNA with the sequential hybridization procedure and the one-step competitive hybridization procedure. Corresponding control experiments (in the absence of a mutant DNA target) were performed under the same conditions. It can be seen that the electrochemical response of mutant DNA under the one-step hybridization procedure is smaller than that of sequential hybridization. Such a difference may be attributed to the competitive hybridization of mutant DNA and complementary DNA with the limited amount of biotin-modified DNA probes. To improve the sensitivity of electrochemically quantifying SNPs, we optimized the DNA hybridization time in one-step competitive hybridization reactions. Figure 3B shows the effect of the hybridization reaction time on the electrochemical response of the 50 pM cytosine mutant target DNA. One can see that the electrochemical signal increases upon raising the hybridization time from 15 to 90 min, indicating an increase of the amount of cytosine mutant sites on the duplex DNA, leading to an increase in the amount of coupled CPLA nanoparticle probes. The response signal tends to be stable after 90 min, which was used as the optimal hybridization reaction time for most experiments.

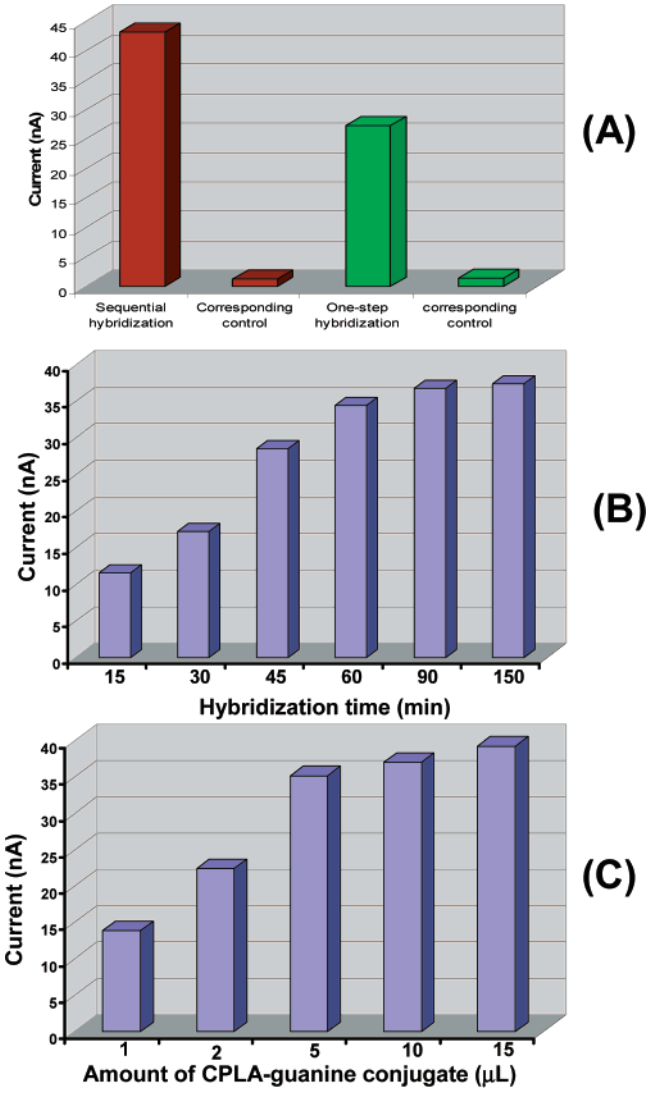

Figure 3. (A) Electrochemical responses of 50 pM and 0 pM cytosine-mutated DNA under sequential hybridization and one-step hybridization. Hybridization reaction time: 60 min. (B) Effect of one-step hybridization reaction time on the electrochemical response of 50 pM cytosine mutated DNA, amount of CPLA−guanine nanoparticle conjugate: 5 μL. (C) Effect of CPLA−guanine conjugate amount on the electrochemical response of 50 pM cytosine-mutated DNA, hybridization time: 90 min. Electrochemical measurement conditions were the same as in Figure 1.

In the present study, the response signal of the mutant DNA target depends on the amount of G−CPLA conjugates bound to the mutated sites of the formed duplex DNA (captured on the magnetic bead surface), which in turn corresponds to the amount of G−CPLA in the incubation solution. To obtain a maximum response using a minimum amount of G−CPLA conjugate, the optimal amount of G−CPLA in the incubation solution was estimated by incubating the duplex DNA-covered magnetic beads by increasing the amount of G−CPLA. The electrochemical response increases upon raising the amount of G−CPLA conjugate from 1 to 5 μL, and then it starts to level off, which corresponds to the saturation of the mutation sites (cytosine) of the formed duplex DNA on the magnetic bead surface. A 5 μL sample of G−CPLA was routinely used for these assays. Furthermore, a higher amount of G−CPLA would cause nonspecific adsorption to increase.

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Table 1. Comparison of Calculated and Observed SNP Frequencies for Costructured DNA Samples (Four Independent Measurements were Carried Out To Estimate the Error Rates, $n = 4$)

known frequency[a]	0.010	0.050	0.100	0.700
observed frequency[b]	0.01 ± 0.002	0.047 ± 0.005	0.097 ± 0.004	0.696 ± 0.010

[a] Frequency = Concentration of mutant DNA/(concentration of mutant DNA + concentration of complement DNA). [b] Frequency was obtained by calibrating with samples having known frequencies of 0, 0,30, 0.50, 0.90, and 1.0. Calibration was carried out using a regression line, and the SNP frequency was obtained by the regression equation.

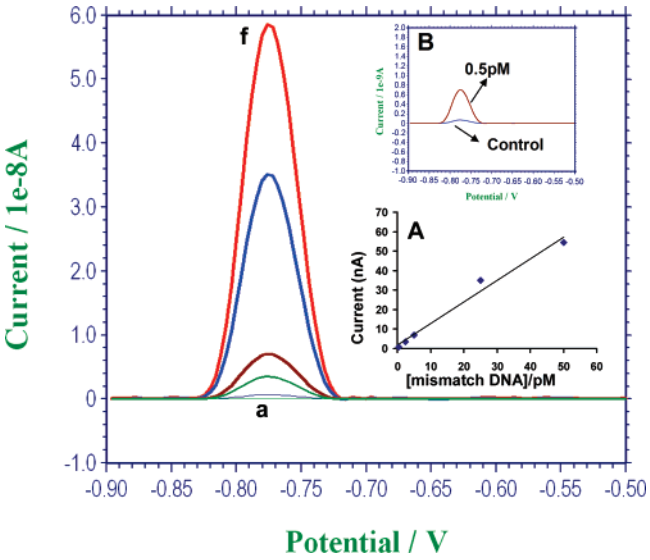

Figure 4. Square-wave voltammograms of 0, 0.5, 2.5, 5.0, 25.0, and 50.0 pM of the mismatched DNA target (a−f). Also shown (insets) are the resulting calibration plot (A) and the square-wave voltammograms of 0.5 pM (red curve) and 0 pM (control) mismatched DNA target (B). After a complete assay, magnetic-bead hybrids associated with nanoparticles were dispersed in 50 μL of 0.2 M acetate buffer containing 10 mg mL^{-1} Hg to release cadmium ions from the nanoparticles. After magnetic separation, the solution was transferred to a screen-printed electrode for SWV measurements. Electrochemical measurement conditions were the same as in Figure 1.

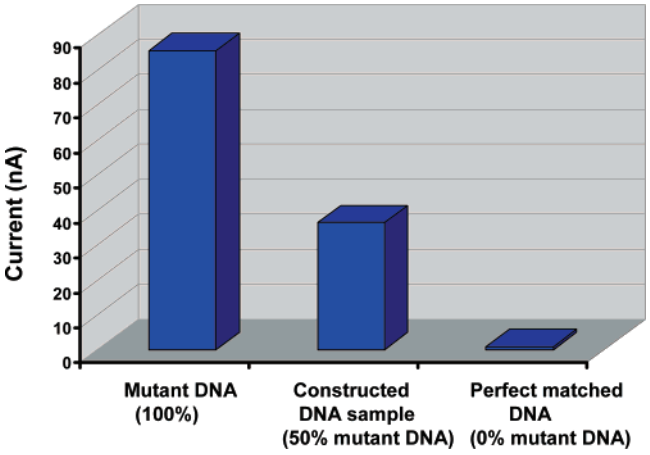

Figure 5. Typical electrochemical responses of mutant DNA (1 nmol), constructed DNA sample (1 nmol mutant DNA + 2 nmol perfect matched DNA), and perfect-matched DNA (2 nmol). Other experimental conditions were the same as in Figure 4.

After the above optimizations, the new SNP electrochemical quantification approach was challenged with different concentrations of the mismatched DNA. Figure 4 shows the electrochemical responses of different concentrations of cytosine-mutated DNA target. It can be seen that the voltammetric peaks of cadmium are well defined, and the current intensities increased with the increase of mismatched DNA concentrations. The resulting calibration plots are linear (inset A, correlation coefficients, 0.993). Also shown in Figure 4 (inset B) is the voltammetric signal for a 0.5 pM mutant DNA. For the lowest sensitivity, corresponding to 0.3 pM, the signal-to-noise ratio is >3. (The noise level is assumed to be the system's response upon analyzing 0 pM mismatched DNA following the same procedure.) This detection limit corresponds to 21.5 attomole in the 75 μL hybridization solution, which is comparable with that of a bioluminometric assay (14 attomol)[24] and a gold nanoparticle-enhanced surface plasmon resonance imaging measurement (1 pM).[25] This measurement is more sensitive than that of the enzyme tag (10 pM)[14] and colorimetric SNP discrimination (0.4 pmol).[26] Also, analyzing the contaminants (such as noncomplementary DNA) using this approach yields a negligible current signal, implying that the contaminants do not affect the analysis of the mismatched DNA (results not shown). The results presented in Figure 4 show excellent reproducibility over the entire concentration range. A series of six repetitive measurements of the 1 pM and 25 pM mismatched DNA targets yielded reproducible SWV peaks with a relative standard deviation of 2.9% and 2.3%, respectively (90 min hybridization; not shown).

Determination of SNP Frequencies in Constructed DNA Samples. One of the most important applications of the quantification of SNP is to estimate SNP frequency in DNA sample pools. To demonstrate the quantification of SNP frequencies, we used a cytosine-mutated DNA target as a mutant SNP allele and perfect-matched DNA as a wide-type SNP allele to construct an artificial DNA pool. Briefly, mutant DNA and perfect-matched DNA were mixed at different ratios and used as DNA samples. The SNP frequency was calculated with the following equation:

$$\text{SNP frequency} = \frac{I}{I_0 + I_{100}}$$

Here, I is the current intensity produced by the constructed DNA pool sample (containing mutant DNA and perfect-matched DNA), I_0 is the current intensity produced by the perfect-matched DNA sample (without mutant DNA), and I_{100} is the current intensity produced by the mutant DNA sample (without perfect-matched DNA). To start with, samples containing perfect-matched DNA, mutant DNA, and an equal molar mixture of perfect-matched DNA and mutant DNA were analyzed, and results are shown in Figure 5. As expected, a negligible signal was obtained with a perfect-matched DNA sample (0% mutant DNA); the response signal of the equal molar mixture of perfect-matched DNA and mutant DNA is smaller than that of mutant DNA (100%). The results were almost as expected, which

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(25) Li, Y.; Wark, A.; Lee, H. J.; Corn, R. M. *Anal. Chem.* **2006**, *78*, 3158−3164.
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indicates that the method is applicable for the SNP frequency analysis. A series of artificially prepared DNA samples with known SNP frequencies at 0.01, 0.05, 0.1, and 0.7, were used to challenge the current approach. The observed SNP frequencies in these constructed DNA samples were compared with known values and are listed in Table 1. Each measurement was repeated six times, and the results were highly reproducible. The observed frequencies were consistent with the known values, indicating that the new SNP quantitative approach is reliable.

Concluding Remarks

In conclusion, we have designed an electrochemical method based on nanoparticle probes for the specific quantification of single-nucleotide polymorphisms. The principle of the new SNP detection technology is based on DNA polymerase I (Klenow fragment)-induced coupling of the nucleotide-modified nanoparticle probe to the mutant sites of duplex DNA under the Watson−Crick base-pairing rule. Electrochemical stripping analysis is an effective and sensitive electronic transduction method for measuring metallic nanoparticle probes. The method is sensitive enough to detect 21.5 amol mutant DNA, which will enable the quantitative analysis of nucleic acid without PCR preamplification. It should be noted, however, that the measuring magnitudes of mutant DNA are controlled by the concentration of DNA probes and the efficiency of hybridization. The approach was challenged with constructed samples containing mutant and complementary DNA. The results indicated that it was possible to very accurately determine SNPs with frequencies as low 0.01. Further experiments that employ genomic DNA samples are under investigation. The proposed approach has a great potential for realizing an accurate, sensitive, rapid, and low-cost method of SNP detection.

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