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ORIGINAL PAPER

Electroactive chitosan nanoparticles for the detection of single-nucleotide polymorphisms using peptide nucleic acids

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Abstract Here we report an electrochemical biosensor that would allow for simple and rapid analysis of nucleic acids in combination with nuclease activity on nucleic acids and electroactive bionanoparticles. The detection of single-nucleotide polymorphisms (SNPs) using PNA probes takes advantage of the significant structural and physicochemical differences between the full hybrids and SNPs in PNA/DNA and DNA/DNA duplexes. Ferrocene-conjugated chitosan nanoparticles (Chi-Fc) were used as the electroactive indicator of hybridization. Chi-Fc had no affinity towards the neutral PNA probe immobilized on a gold electrode (AuE) surface. When the PNA probe on the electrode surface hybridized with a full-complementary target DNA, Chi-Fc electrostatically attached to the negatively-charged phosphate backbone of DNA on the surface and gave rise to a high electrochemical oxidation signal from ferrocene at ~0.30 V. Exposing the surface to a single-stranded DNA specific nuclease, Nuclease S1, was found to be very effective for removing the nonspecifically adsorbed SNP DNA. An SNP in the target DNA to PNA made it susceptible to the enzymatic digestion. After the enzymatic digestion and subsequent exposure to Chi-Fc, the presence of SNPs was determined by monitoring the changes in the electrical current response of Chi-Fc. The method provided a detection limit of 1 fM (S/N=3) for the target DNA oligonucleotide. Additionally, asymmetric PCR was employed to detect the presence of genetically modified organism (GMO) in standard Roundup Ready soybean samples. PNA-mediated PCR amplification of real DNA samples was performed to detect SNPs related to alcolohol dehydrogenase (ALDH). Chitosan nanoparticles are promising biometarials for various analytical and pharmaceutical applications.

Keywords Electrochemical DNA biosensor · Chitosan nanoparticles · Peptide nucleic acids · Single-nucleotide polymorphism detection · Nuclease S1

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Introduction

Nanoparticles formed from biomaterials provide a promising platform for the development of biosensors. Here, we applied chitosan as the biomaterial in the preparation of electroactive bionanoparticles. Chitosan is the deacetylated form of chitin. Chitin, which is found in the exoskeletons of crustaceans, is the second most abundant natural polysaccharide in nature. Chitosan consists of two subunits, D-glucosamine and *N*-acetyl-D-glucosamine, linked together by β(1,4)glycosidic bonds. In particular, the nanoscale structures of this hydrophilic biopolymer, termed "chitosan nanoparticles," have intrigued the scientists in pharmaceutical, biochemical, and environmental fields [1–4]. Chitosan nanoparticles have been well defined, and have characteristics that include biodegradability, biocompatibility, low

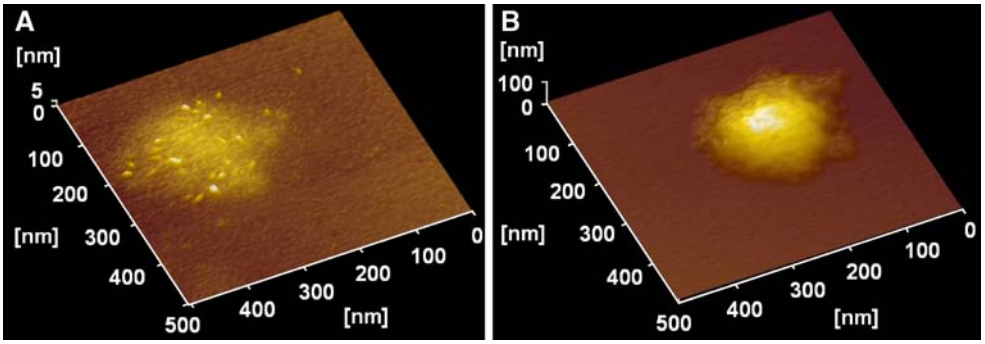


Fig. 1 AFM images of chitosan nanoparticles (**a**) and ferrocene-conjugated chitosan nanoparticles (Chi-Fc, **b**) on a bare mica surface

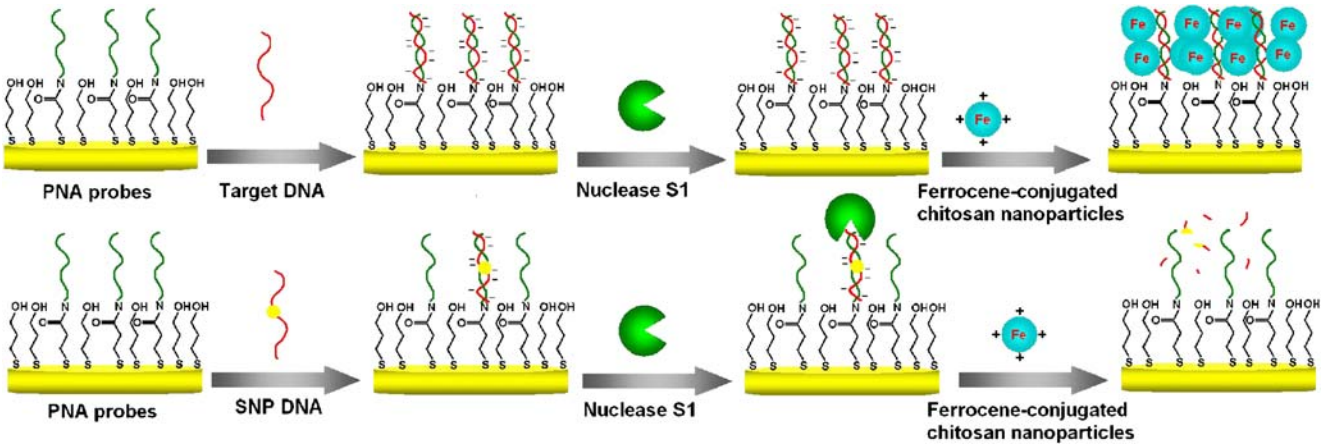
immunogenicity and minimal cytotoxicity [5–7]. The positively charged structure of chitosan in an aqueous environment enables its interaction with negatively charged molecules, such as DNA. The complexation of DNA with chitosan provides protection from degradation by nucleases [8] and is therefore raising much interest in relation to the development of DNA-based vaccines [9, 10]. We have exploited this interaction between the positively charged glucosamine units of chitosan nanoparticles and the negatively charged phosphate backbone of DNA in this report in order to create a highly specific electrochemical DNA hybridization detection protocol.

Peptide nucleic acid (PNA) is a synthetic DNA analog in which the phosphate and deoxyribose backbone of DNA is replaced by a polypeptide [11, 12]. The neutral polypeptide backbone of PNA leads to increased strength of the PNA/DNA base-pairing [13]. The lack of charge repulsion strongly facilitates the hybridization between complementary DNA and PNA as compared to DNA to DNA. Thus, PNA-based biosensors have become of particular interest for single-nucleotide polymorphism (SNP) detection [14–20].

Chitosan nanoparticles (Chi) were synthesized with complex coacervation method using poly(oxyethylene) (20) sorbitane monolaurate (Tween 20) and poly(oxyethylene) (20) sorbitane monooleate (Tween 80) at room temperature (Fig. 1a) [21]. Then ferrocene carboxylic acid was covalently attached to the free amino groups using the coupling reagents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). Interestingly, we observed that the attachment of ferrocene molecules onto the chitosan backbone enhanced the compactness of the spherical form of the nanoparticles (Fig. 1b), as ascertained using atomic force microscopy (AFM).

As shown in Scheme 1, ferrocene-conjugated chitosan nanoparticles (Chi-Fc) were used as the electroactive hybridization indicator. Chi-Fc had no affinity towards the neutral PNA probe immobilized on the gold electrode (AuE) surface. When the PNA probe on the electrode surface hybridized with the full-complementary target DNA, Chi-Fc electrostatically attached to the negatively charged phosphate backbone of DNA on the surface, giving rise to a high electrochemical oxidation signal from ferrocene at ~0.30 V. Upon exposing the surface to single-stranded DNA specific nucleases, nuclease S1 was found to be very effective at removing the nonspecifically adsorbed DNA. An SNP in the target DNA to PNA made it susceptible to the enzymatic digestion. After the surface had been rinsed, SNPs were determined by monitoring the electrical current response of Chi-Fc.

Demidov et al. [22] reported that DNA oligonucleotides were efficiently protected from nuclease S1 when they were



Scheme 1 Schematic illustration for the principle of the electrochemical detection of SNPs using PNA probes with nuclease S1 digestion and ferrocene-conjugated chitosan nanoparticles (Chi-Fc) as the electroactive label

bound to the complementary PNA. Nuclease S1 could not recognize DNA in these fully matching DNA/PNA duplexes. It is worth noting that the structure of the DNA/PNA duplex is perturbed at the position of a SNP [23] so that nuclease can competitively react with the phosphodiester linkage at this site and hydrolyze it. This primary digestion forms small oligonucleotides which are easily separated from PNA because of the thermodynamic instability of their duplexes. Thus, the free single-stranded oligonucleotides become prone to further digestion into smaller forms [24]. This pioneering work prompted a series of studies of the effects of the presence of a SNP in a DNA/PNA duplex on its digestion by nucleases [24, 25]. Therefore, the PNA probe's significant resistance to nucleases was essential for avoiding the deterioration of the full-hybrid duplex, thus providing a very promising platform for the detection of SNPs.

Experimental

Apparatus

Electrochemical studies were performed in conjunction with differential pulse voltammetry (DPV) using an Autolab PGSTAT 100 electrochemical analysis system (Eco Chemie, Utrecht, The Netherlands). The three-electrode system was purchased from Bioanalytical Systems Inc. (West Lafayette, IN, USA) and consisted of a gold disk electrode (i.d. 1.6 mm, AuE) used as the working electrode, a reference electrode (Ag/AgCl), and a platinum wire used as the auxiliary electrode.

Atomic force microscopy (AFM) was performed in air with a commercial unit (SPA400-SPI3800, Seiko Instruments Inc., Chiba, Japan) equipped with a calibrated 20-μm x-y scan-range and 10-μm z-scan-range PZT scanner. A silicon nitride tip (SI-DF40, spring constant=42 N/m, Seiko Instruments Inc.) was used. Mica substrates fused or AFM measurements were purchased from Furuuchi Chemical Co. (Tokyo, Japan). Transmission electron microscopy (TEM) was performed using a Hitachi H-9000 NAR electron microscope (Tokyo, Japan) with an accelerating potential of 100–300 kV.

Reagents

The synthetic oligonucleotides were purchased from Fasmac Co. (Kanagawa, Japan), and had the following sequences:

- PNA or DNA probe: (5′) Cys-O- ACC ACC ACT TC -NH_2 (3′), where Cys and O denote a cysteine residue and an ethylene glycol unit, respectively.
- DNA target: 5′- GGT TTC GAA GTG GTG GTC TTG -3′
- PNA target: H- GAA GTG GTG GT -NH_2
- Guanine SNP: 5′-GGT TTC GAA G*G*G GTG GTC TTG -3′
- Cytosine SNP: 5′ -GGT TTC GAA G*C*G GTG GTC TTG -3′
- Adenine SNP: 5′ -GGT TTC GAA G*A*G GTG GTC TTG -3′
- Noncomplementary (NC): 5′-TAG GAC CCT GGA GGC TGA ACC CCG TGC T-3′

The primers for the detection of Roundup Ready soybeans were determined by Feriotto et al. [26] and were purchased from FASMAC (Kanagawa, Japan). The amplified PCR product was 139 bp in length.

- RupR1 (forward): 5′-TGT ATC CCT TGA GCC ATG TTG-3′
- RupR2 (reverse): 5′-CGC ACA ATC CCA CTA TCC TTC-3′
- GMO capture DNA and PNA probes (P35S): (5′) Cys-O-GGC CAT CGT TGA-NH_2 (3′)

The oligonucleotide stock solutions (100 μM) were prepared in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE), and stored at −20 °C. Ferrocene carboxylic acid was purchased from Fluka (Tokyo, Japan). Nuclease S1 (*Aspergillus orzyae*) was purchased from Life Technologies (Carlsbad, CA, USA) and Promega (Tokyo), respectively. Chitosan 10 (5–20 cP) with a low molecular weight (1.2 kDa), chitosanase (EC 3.2.1.132), poly(oxyethylene) (20) sorbitane monolaurate (Tween 20) and poly(oxyethylene) (20) sorbitane monooleate (Tween 80) were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were obtained from Dojindo Laboratories (Kumamoto, Japan). Ultrapure water (18.3 MΩ-cm) from Milli-Q (Nihon Millipore, Tokyo, Japan) was used in all preparations.

Procedure

Preparation of chitosan nanoparticles

Chitosan solution was prepared as a mixture of chitosan (0.15–0.30%, w/v),and acetic acid (2%, v/v) with Tween-20 or Tween-80 (2–4%, w/v). After the chitosan was totally dissolved, 10% (w/v) sodium sulfate solution was slowly titrated into the chitosan mixture under constant stirring at 300 rpm. After the addition of sodium sulfate, the solution was stirred for another 30 min. The suspension was centrifuged and the nanoparticles were collected and washed twice with Milli-Q water. The nonconjugated chitosan nanoparticles were kept in Milli-Q water at 4 °C until use. The chitosan nanoparticles were then dispersed to form a 1% (w/v) chitosan nanoparticle suspension in PBS (pH 7.4) under shaking for 3 h at room temperature. Then

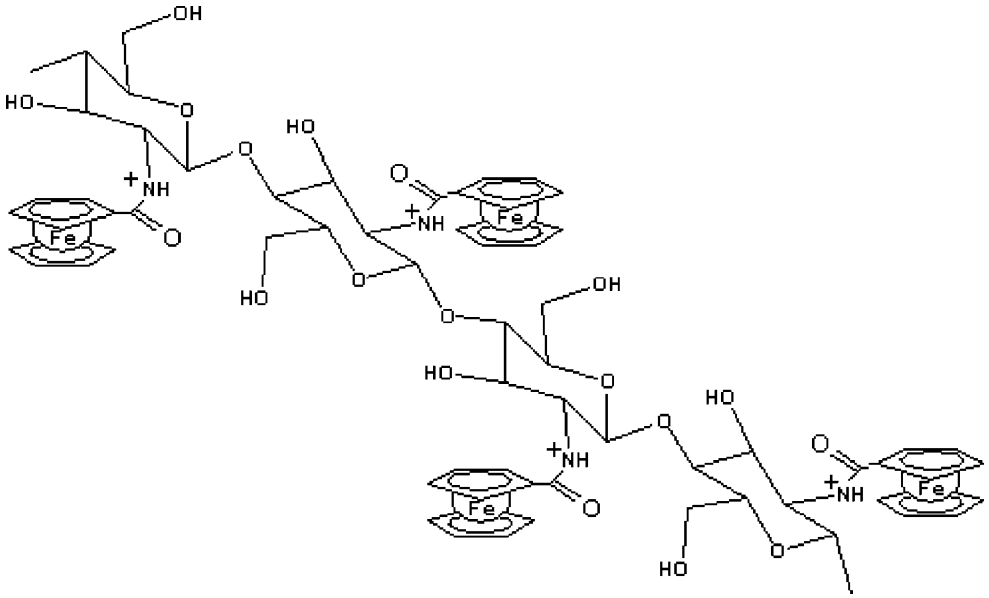


Fig. 2 Chemical structure of ferrocene-conjugated chitosan nanoparticles (Chi-Fc)

an aqueous solution of ferrocene–carboxylic acid at a final concentration of 10 mM was added to the covalent agents, 5 mM EDC and 8 mM NHS in PBS. Covalent activation was allowed for 1 h. Then the covalently functionalized ferrocene molecules were reacted with the chitosan nanoparticles to form ferrocene-conjugated chitosan nanoparticles (Chi-Fc), as depicted in Fig. 2.

The size of the Chi-Fc nanoparticles were regulated with extrusion by passing them through 2- and then 0.4-μm polycarbonate filters ten times and then performing gel filtration and dialysis to remove the free, nonconjugated ferrocene. Chi-Fc preparations were stored in PBS at 4 °C until use. Chi-Fc during storage was found to be stable in PBS at 4 °C for three months.

Extrusion of the nanoparticles through polycarbonate filters was found to reduce the size heterogeneity, as recently reported by Ho and coworkers [27]. The characteristics of the Chi-Fc are given in Table 1.

We calculated that there were 26±4 Chi-Fc/μL and that each Chi-Fc contained ~2.5×10^4 molecules of ferrocene by assuming that the ferrocene concentration inside the chitosan nanoparticles was equal to the original solution used and by comparing the voltammetric responses of hydrolyzed Chi-Fc to that of standard ferrocene solutions. Chi-Fc was hydrolyzed using chitosanase. Chitosanase is defined as the enzyme that performs endohydrolysis of β(1,4)-linkages between D-glucosamine residues in partly acetylated chitosan. The enzymatic hydrolysis of Chi-Fc was performed at pH 5.5 for 10 min while heating at 65 °C. The apparent K_m for chitosan substrate (Sigma) was 29 μg/ml, while k_{cat} was 727.5/min. An aliquot (20 μL) of Chi-Fc was hydrolyzed and the amount of ferrocene molecules released was measured with a relative standard deviation (RSD) of 1.6% for five replicates. The RSD value indicated that the variance of the number of ferrocene molecules in each Chi-Fc was negligible, yielding a uniform size distribution of the population in the bulk.

AFM observation of chitosan nanoparticles

Images were taken in dynamic force mode (DFM mode) at optimal force in a moisture control box at room temperature and 30–40% humidity. The electrostatic binding event between chitosan and the negatively charged mica surface was utilized for the attachment of nanoparticles. Chitosan nanoparticles (100 μL) were deposited onto the mica directly from the stock solution. After 10 min of incubation at room temperature, the mica disk was rinsed twice with 100 μL of ultrapure water and dried with a gentle stream of nitrogen gas. Figures S1A and S1B (see the "Electronic Supplementary Material") show a cross-sectional analysis of the AFM images.

Table 1 Characteristics of the chitosan nanoparticles (Chi) and ferrocene-conjugated chitosan nanoparticles (Chi-Fc)

Characteristic	Value
Mean diameter (nm) of chitosan nanoparticles	358
Mean diameter (nm) of ferrocene-conjugated chitosan nanoparticles	263
Population of chitosan nanoparticles (number/μL)	15±8
Population of ferrocene-conjugated chitosan nanoparticles (number/μL)	26±4

TEM observation of chitosan nanoparticles

Chi-Fc (2 μL) were deposited onto the amorphous carbon grid directly from the stock solution. After evaporation at RT, the carbon grid was further dried under low atmospheric pressure overnight. The dried samples were then used for TEM characterization. Figure S2 (see the "Electronic Supplementary Material") shows a TEM image of Chi-Fc.

Electrochemical procedure

All experiments were conducted at room temperature except if otherwise stated. For statistical evaluations, the current data from five different sensors in five similarly prepared solutions were taken into consideration (n=5), except if otherwise stated.

Probe immobilization

Gold disk electrodes (AuE, 2 mm^2 area, Bioanalytical Systems (BAS), West Lafayette, IN, USA) were used for all of the experiments. The electrodes were polished with wet alumina slurry (Wako Pure Chemical Industries, Ltd., Japan) on a pad for 3 min and rinsed repeatedly with water and finally cleaned in a sonicator. Mixed monolayer surfaces containing PNA probe and 1 mM 6-mercapto-hexanol (6MH, Dojindo Laboratories, Japan) were prepared by immersing the polished AuE into a 1 μM solution of PNA probes in 50 mM phosphate buffer solution (PBA, pH 7.4) for 2 h, rinsing with 20 mM Tris buffer solution (TBS, pH 7.0), immersing into 6MH aqueous solution at room temperature for 1 h, and finally rinsing with TBS and drying at room temperature. All of the electrodes were stored in water at 4 °C until use. PNA probes on a solid surface are resistant to nuclease digestion and can be utilized for ten successive applications.

Hybridization

Hybridization was performed by exposing the PNA/DNA probes to the desired concentration of DNA/PNA target strands in TBS containing 20 mM NaCl (pH 7.4) for 30 min. Background blanks were measured in TBS containing no target DNA/PNA under conditions identical to those used for the hybridization reactions. After the hybridization, the hybrid-modified AuE was rinsed with TBS.

Enzymatic digestion

The digestion of the hybrid molecules on the electrode surface by nuclease S1 (or Mung Bean nuclease) was carried out using 30 mM acetate buffer solution that included 1.0 mM zinc acetate and 5% (v/v) glycerol (EDB, pH 4.6). After a predetermined time (10–30 min) at room temperature (RT), EDTA was added at a final concentration of 10 mM to stop the reaction. The surface was then rinsed with blank EDB.

Labeling with ferrocene-conjugated chitosan nanoparticles (Chi-Fc)

After the enzymatic digestion, Chi-Fc in 30 mM acetate buffer solution (ABS) was added onto the electrode surface at RT, and the electrodes were incubated for 10 min.

Transduction

Cyclic voltammetry (CV) was performed at a scan rate of 100 mV/s and a step potential of 2 mV. Differential pulse voltammetry (DPV) was performed in blank PBS while scanning from +0.10 to +1.20 V with an amplitude of 25 mV and a step potential of 5 mV. The raw voltammograms were treated using Savitzky–Golay smoothing (level 4) and "moving average" baseline correction with a peak width of 0.005 V.

Asymmetric PCR (a-PCR) for genetically modified organisms (GMOs)

GMO detection was carried out according to the PCR conditions reported by Feriotto et al. [26]. The protocols were adjusted to AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). The amplicon from the standard PCR was used instead of genomic DNA.

A standard sample of Roundup Ready soybeans including 5% GMO was used as genomic DNA. Standard PCR was performed in a final volume of 50 μL containing 1× Gold Buffer and 75 mM $MgCl_2$ using 1.25 U/reaction of AmpliTaq Gold DNA polymerase (Applied Biosystems), 8 mM dNTPs, 25 μM forward and reverse primers, and 50 ng of genomic DNA. Fifty PCR cycles were performed under the following conditions: denaturation, 30 s, 95 °C; annealing, 30 s, 60 °C; elongation, 30 s, 72 °C. The length of the Roundup Ready PCR product was 139 bp.

Asymmetric PCR (a-PCR) was also carried out using the product from the standard PCR and the same procedure as described above, except that the reverse PCR primer concentration was 5 μM. Since a forward-to-reverse primer ratio of 5:1 was used, the majority of the final PCR product was single-stranded DNA (ssDNA).

Agarose gel electrophoresis for GMOs

Gel electrophoresis was carried out in 3% agarose (Takara, Japan). The amplicon was diluted with loading buffer (bromphenol blue). Running buffer was 1×TAE buffer.

Electrophoresis was carried out at a constant 100 V for 20 min. Amplisize (Bio-Rad, Mississauga, ON, Canada) was used as the ladder. The gel was then stained with incubation in 200 mL 1×TAE buffer with 6 μL ethidium bromide. The gel was visualized using a Printograph (Atto, Tokyo, Japan).

Electrochemical detection of GMOs

The a-PCR products were diluted 20 times with TBS containing 20 mM NaCl, and then used as the target molecule. The DNA/PNA capture probe was first hybridized with the PCR product on the sensor surface for 30 min. After the hybridization, the sensor was rinsed briefly with TBS. Another amplicon obtained from the PCR of alcohol dehydrogenase [20] was also used as the noncomplementary PCR control (nc-PCR). After rinsing with blank TBS, enzymatic digestion and labeling with Chi-Fc were performed as described above.

PNA-mediated PCR amplification of SNP samples related to alcohol dehydrogenase (ALDH)

PNA-mediated PCR amplification of alcohol dehydrogenase (ALDH) fragments, including the mutation site, was performed using human genomic DNA. PCR amplification of a 176-bp fragment on exon 12 and upstream of intron 12 of the ALDH gene was performed as described by Chen et al. [29] using the following primer pairs:

- DNA primer 1: 5′- CAA ATT ACA GGG TCA ACT GCT ATG -3′
- DNA primer 2: 5′- GCC GCG CCC GCC GCC CCG CGC CCC CCC GCC CGC CCC GCG CTC CAC ACT CAC AGT TTT CAC-3′
- PNA probe for PCR clamping: H- CCA CAC TCA CAG TTT TC -NH_2
- $ALDH^1$ PNA/DNA target: (5′) H- GCA TAC ACT GAA GTG AA -NH_2 (3′)
- $ALDH^2$ PNA/DNA target: (5′) H- GCA TAC ACT AAA GTG AA -NH_2 (3′)
- $ALDH^1$ PNA/DNA capture probe: (5′) H- TTC ACT TCA GTG TAT GC -NH_2 (3′)
- $ALDH^2$ PNA/DNA capture probe: (5′) H- TTC ACT TTA GTG TAT GC -NH_2 (3′)

Human genomic DNA was extracted from human hair samples of consenting Caucasian and Japanese donors according to the standard protocols of ISO-HAIR™ (Nippon Gene Co. Ltd., Toyama, Japan). PCR was performed using a thermal cycler system (Takara Inc., Tokyo, Japan) as follows: a preheating (95 °C for 5 min) step was followed by 35 cycles (at 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min) and a final extension at 72 °C for 15 min in 50 μL of reaction mixture containing 50 ng genomic DNA, 1× buffer, 1.5 mM $MgCl_2$, 10 pmol of each primer and PNA probe for PCR clamping, 0.25 mM dNTPs, and 1.25 U of AmpliTaq (Perkin-Elmer, Norwalk, CT, USA). PCR products of length 176 bp were run on 4% (w/v) agarose gels prestained with ethidium bromide (0.5 μg/ml). Gels were run at 100 V in 1×TE buffer (100 mM Tris, 2 mM EDTA) for 180 min, examined under ultraviolet illumination, and gel electrophoresis images were taken.

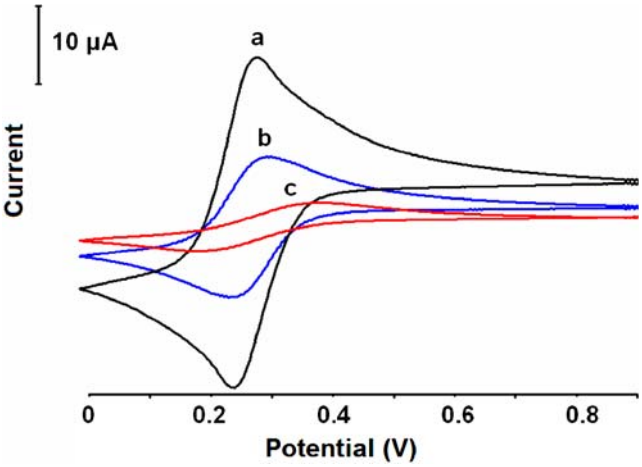


Fig. 3 Cyclic voltammograms of 2 mM $[Fe(CN)_6]^{4-/3-}$ in PBS at (**A**) a bare AuE, (**B**) a PNA probe–6MH mixed monolayer immobilized on AuE, (**C**) a PNA probe immobilized on AuE at 100 mV/s

DNA primers were added together at a high concentration of 1 μM to prevent duplex formation. After binding these oligonucleotides, the SNP-containing mutant amplicons (m-PCR) were applied to the PNA or DNA probe-modified GCE surface (20 μl) and left for 10 min to hybridize. In addition, blank PCR solution and another amplicon obtained from a PCR of a genetically modified organism (GMO) were used as the negative controls. After a stringent washing process with blank TBS salt, the changes in current response were monitored.

Results and discussion

The PNA probe used in this study was linked to a cysteine (Cys) via an ethylene glycol linker (O). The PNA probes formed a self-assembled monolayer (SAM) on the AuE

Table 2 Binding constants of Chi-Fc to a DNA probe- and a DNA/DNA hybrid-immobilized electrode surface

$[Na^+]$ (mM)	*K* for DNA probe	*K* for DNA–DNA hybrid
0	$(1.7\pm0.5)\times10^6$	$(1.4\pm0.3)\times10^6$
10	$(2.2\pm0.2)\times10^5$	$(2.8\pm0.5)\times10^5$
100	$(2.1\pm0.3)\times10^4$	$(3.9\pm0.6)\times10^4$

Table 3 Binding constants of Chi-Fc to a PNA probe and a PNA/DNA hybrid-immobilized electrode surface

$[Na^+]$ (mM)	*K* for PNA probe	*K* for PNA–DNA hybrid	*K* for PNA–PNA hybrid
0	$(1.5\pm0.5)\times10^6$	$(1.7\pm0.7)\times10^6$	$(1.2\pm0.4)\times10^6$
10	$(1.6\pm0.9)\times10^6$	$(4.9\pm0.3)\times10^6$	$(1.5\pm0.3)\times10^6$
100	$(1.8\pm0.8)\times10^6$	$(7.9\pm0.5)\times10^6$	$(1.7\pm0.4)\times10^6$

surface in 2 h. In order to make the SAMs more permeable and to increase the hybridization efficiency, we used a short probe immobilization time and mixed the probes with 6MH, as reported by Herne and Tarlov [29–31]. Figure 3A shows a cyclic voltammogram (CV) measured with a bare AuE using 2 mM $[Fe(CN)_6]^{4-/3-}$ in PBS. Figure 3B shows the CVs measured with PNA-probe-modified AuE in a mixed monolayer with 6MH. When we did not use 6MH during SAM formation, the electron transfer between the electroactive marker and the electrode was significantly suppressed due to the steric and electrostatic hindrances arising from the PNA SAM on the surface (Fig. 3C). It should also be noted that these are individual traces in a single plot. We calculated that 5.3 $(\pm0.08)\times10^{12}$ molecules/cm^2 was the optimum surface coverage for PNA and DNA probes on AuE. The surface coverage values were determined using the CV of $[Fe(CN)_6]^{4-/3-}$ [29–31]. The modification of the AuE surface with DNA and PNA affects the well-described CV characteristics of $[Fe(CN)_6]^{4-/3-}$. The change in the charge measured from CV cycles corresponds to the amount of nucleic acid immobilized on the AuE surface.

The binding constants of Chi-Fc, *K*, in PBS to DNA/PNA probes and hybrids immobilized on the AuE surface were determined from a Langmuir analysis of the adsorption isotherm data obtained under various ionic strength conditions (Tables 2 and 3). The full coverage values of Chi-Fc were 2.5 and 1.3 μC/cm^2 for DNA/DNA and DNA/PNA hybrid-immobilized surfaces, respectively. The increase in the redox molecule coverage from DNA probe to DNA/DNA hybrid was almost double the former value, which was attributed to the probe and the target having the same base-pair length, so that hybrid formation doubled the number of binding sites on the surface (Table 2). PNA probes and PNA/PNA hybrids were not affected significantly by the ionic strength effect (Table 3).

Discriminations between full-complementary target DNA and SNP using nuclease S1 were monitored with differential pulse voltammetric signals after rinsing the enzymatic digests from the surface (Fig. 4A). When Chi-Fc was bound to the DNA/PNA hybrids, a high current response was obtained (Fig. 4A-a). Since DNA/PNA hybrids were sufficiently protected from the enzymatic digestion, the remaining hybrids on the electrode surface attached to Chi-Fc, resulting in a high current response (Fig. 4A-a). On the other hand, SNP-containing DNA oligonucleotides (Fig. 4A-b) and noncomplementary target DNA (Fig. 4A-c) were easily digested down to mononucleotides by the nuclease. There was no negative charge in the PNA/PNA hybrid, so a low current response was obtained (Fig. 4A-d). When no molecule was attached to the PNA probe in blank PBS, no current response was obtained (Fig. 4A-e). The current signals from all of the SNP combinations were clearly superimposed on each other, and so we obtained similarly low responses for all of them (Fig. 4B, A-G, A-C, and A-A).

The role of Chi-Fc in the protection of DNA oligonucleotides from enzymatic digestion on the AuE surface was observed. In this experiment, a DNA/DNA hybrid was formed on the electrode surface. Then Chi-Fc was exposed to the hybrid-modified AuE. Due to electrostatic affinity, the Chi-Fc bound to the DNA/DNA hybrid. A DPV mea-

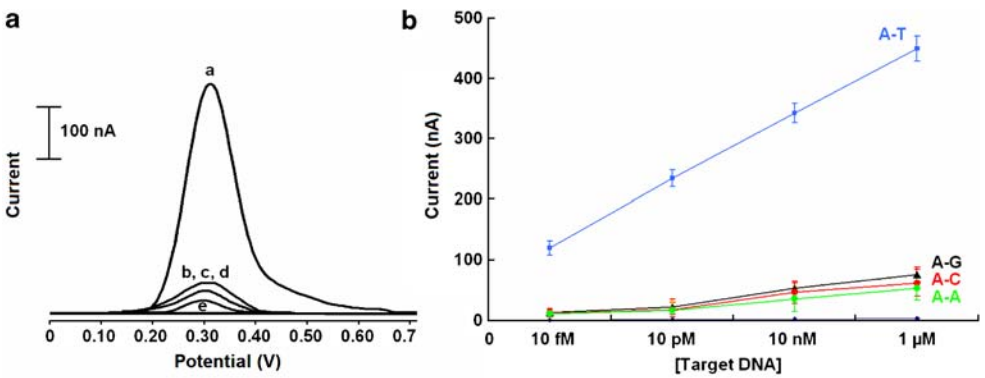


Fig. 4 **A** Differential pulse voltammograms for the detection of SNPs using Chi-Fc and PNA probes after hybridization with (*a*) target DNA, (*b*) A-G SNP, (*c*) noncomplementary DNA, (*d*) target PNA, (*e*) PBS only; **B** plot of the dependence of the peak current signal on the target DNA and SNP concentrations

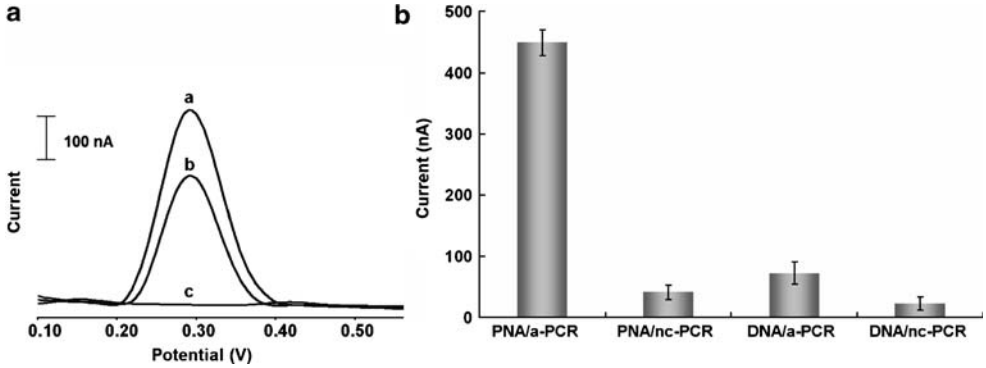


Fig. 5 **A** Differential pulse voltammograms for the protection of DNA molecules from nuclease digestion using Chi-Fc and DNA probes after hybridization with target DNA in the absence of nuclease S1 (*a*), (*b*) after nuclease S1 digestion, (*c*) after hybridization with A-G SNP oligonucleotide; **B** Columns for the peak current signal after the hybridization of PNA and DNA probes with an asymmetric PCR product (a-PCR) related to GMO, and a noncomplementary PCR product (nc-PCR) related to alcohol dehydrogenase, with error bars shown (n=5)

surement was taken without performing the enzymatic digestion process. Since the duplex structure of the DNA/DNA hybrid creates more negative charge on the surface, which attracts Chi-Fc, a significantly higher peak current response was obtained from the DNA/DNA hybrid electrodes compared to the PNA/DNA hybrid-modified ones (Fig. 5A-a). When the surface was exposed to nuclease S1, a high signal was still obtained due to the strong protection of DNA molecules resulting from the presence of Chi-Fc (Fig. 5A-b). When the DNA/A-G SNP hybrid was not treated with Chi-Fc, the DNA molecules were digested in their entirety, and no current response was obtained (Fig. 5A-c). The detection limit for DNA target was determined as 1 fM (S/N=3).

We have also carried out experiments using PCR products (Fig. 5B). Asymmetric PCR (a-PCR) amplicons related to genetically modified organisms (GMO) were hybridized with PNA probe immobilized on an AuE surface. A high current response was obtained although the PNA/a-PCR hybrid was treated with nuclease S1. A small current response was observed from the PNA/noncomplementary PCR (nc-PCR) product related to alcohol dehydrogenase, as the digestion left only the PNA probe on the surface. DNA probes were more susceptible to nuclease S1 digestion, and low current responses were obtained from hybridization with a-PCR and nc-PCR products. As the long single-stranded PCR that was hybridized with the short probe on the surface was targeted by the nuclease, no Chi-Fc could accumulate on the surface to give a high current response.

PNA probes were also used to block PCR amplification in this report because PNA cannot function as a primer for DNA polymerase. PNA-mediated PCR clamping is a specific technique that allows the detection of two alleles that differ by only one SNP. We used this technique to detect the alcohol dehydrogenase (ALDH) SNP in real human DNA samples (Fig. 6). Many people from Far Eastern countries lack the ALDH activity responsible for the oxidation of acetaldehyde produced during ethanol metabolism, and thus suffer from an alcohol-flush reaction. Crabb et al. [32] found that the mutation in the inactive enzyme was a substitution of lysine for glutamate at

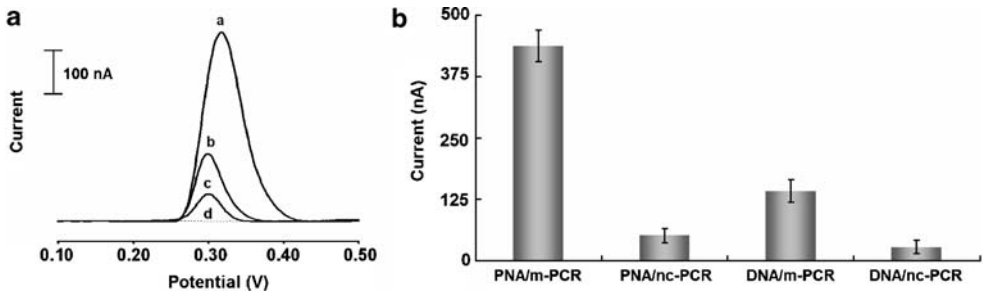


Fig. 6 **A** Differential pulse voltammograms for the detection of SNP related to alcohol dehydrogenase (ALDH) using Chi-Fc and PNA probes after hybridization with (*a*) mutant PCR product (m-PCR), (*b*) nc-PCR, which was amplified from GMO samples; DNA probes with the same recognition sequence as the PNA ones after hybridization with (*c*) m-PCR, (*d*) nc-PCR, which was amplified from GMO samples; **B** Columns for the peak current signals after hybridization of PNA and DNA probes with m-PCR and nc-PCR, with error bars shown (n=5)

position 487. The allele ($ALDH^2$) encoding the mutant (m) subunit was dominant. We have recently reported the development of a biosensor for the detection of ALDH SNP using $[Co(NH_3)_6]^{3+}$ as the electroactive metal complex [20]. In this report, we have tested the real samples from Caucasian and Japanese donors for the ALDH mutation in connection with PNA-mediated PCR and Chi-Fc. When a SNP exists in the gene, the PNA probe from PCR clamping does not bind to that region and so PCR takes place, resulting in the amplification of the double-stranded mutant DNA amplicons (m-PCR). The m-PCR amplicon is exposed to thermal denaturation, resulting in single-stranded DNA. Then the PNA capture probe on the surface binds to its complementary gene sequence on the m-PCR product, and the subsequent accumulation of Chi-Fc on the sensor surface results in a high current signal (Fig. 6A-a). The nc-PCR amplicons related to genetically modified organisms (GMO) were hybridized with PNA probe for ALDH that was immobilized on an AuE surface. A low current response was obtained after the treatment with nuclease S1 (Fig. 6A-b). A small current response was also observed from the hybridization of the DNA probe for ALDH with m-PCR (Fig. 6A-c). As expected, the hybridization of the DNA probe for ALDH with nc-PCR showed the lowest response (Fig. 6A-d), because the nuclease S1 treatment denatured all of the nucleic acids from the surface, leaving no binding target for Chi-Fc. Figure 6B shows a bar chart that compares the current responses for these hybridization reactions, along with their error bars (n=5).

Conclusions

The present method of SNP detection in DNA using PNA probes takes advantage of the significant structural and physicochemical differences between the full hybrids and SNPs in PNA/DNA and DNA/DNA duplexes. Dictated by this difference, single-stranded DNA-specific enzymes selectively choose these SNP sites and hydrolyze the DNA molecules. The present method should be also applicable to high-throughput genotyping of multiple SNPs. In combination with array technology, amplified DNA fragments in multiplex reactions can be captured by specific PNA probes and then subjected to enzymatic digestion. The undigested PNA/DNA duplexes would be detected using Chi-Fc in connection with highly sensitive electrochemical techniques.

References

1. Martin CR, Kohli P (2003) Nat Rev Drug Discov 2:29–27
2. Koerner H, Price G, Pearce NA, Alexander M, Vaia RA (2004) Nat Mater 3:115–120
3. Roy K, Mao HQ, Huang SK, Leong KW (1999) Nat Med 5:387–391
4. Kerman K, Saito M, Morita Y, Takamura Y, Tamiya E (2004) Anal Chem 76:1877–1884
5. Janes KA, Calvo P, Alonso MJ (2001) Adv Drug Deliv Rev 47:83–97
6. van der Lubben IM, Konings FA, Borchard G, Verhoef JC, Junginger HE (2001) J Drug Target 9:39–47
7. Cui Z, Mumper RJ (2001) J Control Rel 75:409–419
8. Illum L, Jabbal-Gill I, Hinchcliffe M, Fisher AN, Davis SS (1999) Int J Pharm 178:231–243
9. Illum L, Jabbal-Gill I, Hinchcliffe, M, Fisher, AN, Davis, SS (2001) Adv Drug Deliv Rev 51:81–96
10. van der Lubben IM, Verhoef JC, van Aelst AC, Borchard G, Junginger HE (2001) Biomaterials 22:687–694
11. Nielsen PE, Egholm M, Berg RH, Buchardt O (1991) Science 54:1497–1500
12. Egholm M, Buchardt O, Nielsen PE, Berg RH (1992) J Am Chem Soc 114:1895–1897
13. Wittung P, Nielsen PE, Buchardt O, Egholm M, Nordén B (1994) Nature 368:561–563
14. Lucarelli F, Tombelli S, Minunni M, Marrazza G, Mascini M (2008) Anal Chim Acta 609:139–159
15. Odenthal KJ, Gooding JJ (2007) Analyst 132:603–610
16. Kerman K, Ozkan D, Kara P, Erdem A, Meric B, Nielsen PE, Ozsoz M (2003) Electroanalysis 15:667–670
17. Kerman K, Matsubara Y, Morita Y, Takamura Y, Tamiya E (2004) Sci Technol Adv Mater 5:351–357
18. Komiyama M, Ye S, Liang X, Yamamoto Y, Tomita T, Zhou J-M, Aburatani H (2003) J Am Chem Soc 125:3758–3762
19. Ren B, Zhou J-M, Komiyama M (2004) Nucleic Acids Res 32: e42
20. Kerman K, Vestergaard M, Nagatani N, Takamura Y, Tamiya E (2006) Anal Chem 78:2182–2189
21. Yanagawa H, Ogawa Y, Furuta H, Tsuno K (1989) J Am Chem Soc 111:4567–4570
22. Demidov V, Frank-Kamenetskii MD, Egholm M, Buchardt O, Nielsen PE (1993) Nucleic Acids Res 21:2103–2107
23. Eriksson M, Nielsen, PE (1996) Nat Struct Biol 3:410–413
24. Ratilainen T, Holmén A, Tuite E, Nielsen PE, Nordén B (2000) Biochemistry 39:7781–7791
25. Ray A, Nordén B (2000) FASEB J 14:1041–1060
26. Feriotto G, Borgatti M, Mischiati C, Bianchi N, Gambari R (2002) J Agric Food Chem 50:955–962
27. Viswanathan S, Wu L-C, Huang M-R, Ho J-AA (2006) Anal Chem 78:1115–1121
28. Chen CY, Shiesh SC, Wu SJ (2004) Clin Chem 50:481–489
29. Herne TM, Tarlov MJ (1997) J Am Chem Soc 119:8916–8920
30. Steel AB, Herne TM, Tarlov MJ (1998) Anal Chem 70:4670–4677
31. Steel AB, Herne TM, Tarlov MJ (1999) Bioconjugate Chem 10:419–423
32. Crabb DW, Edenberg HJ, Bosron WF, Li TK (1989) J Clin Invest 83:314–316