Peptide nucleic acid modified magnetic beads for intercalator based electrochemical detection of DNA hybridization

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Abstract

The specific binding of peptide nucleic acid (PNA) to its complementary DNA target is combined with magnetic separation to enable discrimination against single nucleotide polymorphisms (SNP). PNA probes with biotin label at 5′-end were attached to strepavidin coated superparamagnetic iron oxide beads. PNA modified beads were then challenged with non-complementary, SNP containing and perfect-match DNA targets. PNA probe showed no affinity towards non-complementary DNA. The non-specific binding of SNP containing DNA target was suppressed by the washing step of the beads by using sodium dodecylsulfate in blank buffer solution. Then, an electro-active intercalator, 7-dimethyl-amino-1,2-benzophenoxazinium salt (Meldola’s blue, MDB) was introduced to the beads. MDB intercalated between the double-helix of the hybrid molecules on the beads. After removing the excessive MDB, the beads were collected from the solution by immersing a biotin modified carbon paste electrode into the solution. Specific hybridization between PNA probe and DNA target was determined by monitoring the voltammetric peak of MDB. Numerous factors affecting the MDB signal, such as target DNA concentration, intercalator concentration and accumulation time were investigated. MDB signal indicated a detection limit of 2 pM in connection with 20 min hybridization time.

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1. Introduction

DNA biosensors based on hybridization detection are under development towards the goal of rapid and inexpensive testing of genetic and infectious diseases [1]. Electrochemical transducers offer promising candidates for converting the hybridization event into an analytical signal [2]. Nowadays, electrochemical biosensors are getting increasingly attractive for DNA hybridization detection, due to their high sensitivity, small dimensions, low cost, and compatibility with microfabrication technology [3–6].

Peptide nucleic acid (PNA) is a structural DNA mimic with a neutral N-(2-aminoethyl) glycine-based pseudopeptide backbone, which has been reported to form Watson–Crick complementary duplexes with DNA [7]. PNA has recently demonstrated remarkable hybridization properties towards complementary oligonucleotides [8], but was originally synthesized as a gene-targeting antisense drug [9]. Inhibition of gene expression inside cells by PNA was also reported [10,11]. In comparison with DNA/DNA hybrids, PNA/DNA duplexes have higher thermal stability, and can be easily formed at low ionic strength conditions [12]. The neutral peptide-like backbone of PNA eliminates the interstrand electrostatic repulsion, and thus provides the basis for the probe to hybridize to target DNA sequences with high affinity and specificity [13]. Ratilainen et al. [14] characterized the binding of PNA to DNA in terms of binding affinity (perfectly matched duplexes), and sequence-specific binding (one-base mismatch) using absorption hypochromicity, melting curves, and isothermal titration calorimetry.

Tomschik et al. [15] investigated the redox behavior of PNA and DNA oligomers. A quartz crystal microbalance in connection with thiol-derivetized PNA probes enabled sensitive detection against one-base mismatches in the p53 gene [16]. Factors influencing the immobilization of PNA probe onto carbon paste electrode (CPE) surface, its hybridization to the p53 target DNA sequence, and the chronopotentiometric detection step
by using [Co(phen)$_3$]$^{3+}$ as an intercalator were also reported [17,18]. Methylene blue based electrochemical detection of DNA hybridization was also reported by using PNA probe immobilized electrodes [19–21]. Kerman et al. [22] has recently reported a label-free electrochemical method to detect point mutations by using PNA probes. Kuhn et al. [23] reported that the stemless PNA beacon hybridized rapidly to the complementary oligonucleotides, and was insensitive to the change of ionic strength, providing the basis for effective detection of DNA targets under various conditions.

Recently, Komiyama et al. [24] combined PNA with single-stranded DNA specific nucleases. Mismatched DNA molecules in DNA/PNA duplexes were hydrolyzed by these nucleases, whereas fully complementary hybrids were kept intact. This event was detected by using 3,3'-diethylthiadiocarbocyanine dye, which changed its color from blue to purple upon binding to DNA/PNA hybrid. Höök et al. [25] reported that thiolated PNA and DNA immobilized via a sulphur group directly on a bare-Au surface were less efficient as probes for hybridization than were biotinylated PNA and DNA coupled onto a streptavidin coated substrate.

Magnetic beads play a major role in bioaffinity assays [26], and have recently been utilized for electrochemical detection of DNA hybridization. Wang et al. [27,28] and Palecek et al. [29] have shown that important errors associated with non-specific adsorption could be suppressed by conducting the hybridization and transduction steps at different surfaces such as magnetic beads and bare electrode surfaces, respectively. Streptavidin coated beads were used in connection with biotinylated DNA probes hampering label-free detection of DNA hybridization based on guanine oxidation signal [27]. The whole procedure took about 45 min by using chronopotentiometric adsorptive stripping analysis with a detection limit of 160 pM [27]. An alkaline-phosphatase-based electrochemical detection of DNA hybridization was also performed by using magnetic beads [28]. Despite the low detection limit (500 pg target DNA in 50 µl sample), the whole procedure took about 80 min. Palecek et al. [29] performed DNA hybridization at magnetic beads, and electrochemical detection at mercury or solid mercury amalgam electrodes. The voltammetric signal of purine bases released from DNA by acid treatment provided specific detection of hybridization, and the whole procedure took about 70 min [29].

In this paper, biotinylated PNA probes with streptavidin coated magnetic beads in combination with a redox-active intercalator Meldola’s blue (MDB) are reported. The chemical structure of MDB is shown in Scheme 1. The intercalation mechanism of MDB has recently been reported by Reid et al. [30].

The specific binding event between PNA and target DNA was further supported by using the magnetic separation, and surfactant washing steps. Non-specifically adsorbed single nucleotide polymorphism (SNP) containing and non-complementary DNA could successfully be separated from the beads by holding the beads in a magnetic field, and removing the remaining supernatant solution. Using surfactant agents to remove SNP containing target DNA was shown to be an effective method [31,32]. In this paper, SNP containing PNA/DNA duplex was found to be weak against surfactant separation, by increasing the negative charge on the SNP containing duplex, which in turn resulted in the separation of the mismatched DNA from the PNA probe on the bead. Detection of DNA hybridization was achieved, where MDB intercalated into the PNA/DNA couple, and where significant quantities of the intercalator were associated with the hybrid molecules on the bead surface. Monitoring the changes in the voltammetric waves of the intercalator enabled the specific and rapid detection of PNA/DNA hybridization in about 50 min. Three powerful elements for electrochemical detection of DNA hybridization are combined in this paper for the first time, which are sequence-specific PNA probes, streptavidin coated magnetic beads, and washing steps with surfactant agents in connection with biotin modified carbon paste electrode (BCPE). The features of the method are described and discussed in the following sections.

2. Experimental

2.1. Apparatus

Square wave voltammetry (SWV) was performed with a CV-50W Voltammetric Analyzer (BAS, Bioanalytical Systems, West Lafayette, IN). A Hitachi U-3010 spectrophotometer in connection with UV Solutions software (Tokyo, Japan) was used to determine the DNA concentration of samples [33]. The electrochemical detection was carried out in a 2.0 ml cell containing a CPE, (3.0 mm i.d.), the Ag/AgCl reference electrode (BAS, Bioanalytical Systems, West Lafayette, IN) and a platinum wire as the auxiliary electrode. A magnetic stirrer provided the convective transport.
2.2. Chemicals

The oligonucleotides were purchased from Fasmac (Kanagawa, Japan); their base sequences are as follows:

PNA probe: Biotin–NH₂CO(Gly) ACC ACC ACT TC NH₂
Complementary target DNA: 5'-GGT TTC GAA GTG GTC TTG-3'
SNP containing DNA: 5'-GGT TTC GAA GGG GTG GTC TTG-3'
Non-complementary DNA: 5'-GGGGC ACGTT TATCC GTCCC TCCTA GTGGC GTGCCC-3'

The complementary base sequence in target DNA and mismatched base in SNP containing DNA are shown in bold letters. PNA stock solution (100 mg/l) was prepared with 50 mM phosphate buffer solution (pH 7.40) and kept frozen. DNA stock solutions (100 mg/l) were prepared with 10 mM Tris–HCl, 1 mM EDTA (pH 8.00, TE) and kept frozen. More dilute solutions of probe were prepared using 0.50 M acetate buffer containing 20 mM NaCl (pH 4.80, ABS). More dilute solutions of targets were prepared using 20 mM Tris–HCl buffer solution containing 20 mM NaCl (pH 7.00, TBS).

2.3. Procedure

Experimental procedure is illustrated in Scheme 2. The duration of the whole procedure is about 50 min, which is promising in comparison with other magnetic bead-based electrochemical SNP detection procedures [27–29]. All experiments were conducted at room temperature (unless otherwise stated, 25 ± 0.5 °C).

2.3.1. Biotinylated PNA probe modification onto strepavidin coated beads

The bead labeled probes were prepared using a modified procedure reported by Wang et al. [27,28]. Biotinylated PNA probe at 10 nM concentration were added and incubated for 15 min with gentle mixing. The beads were subsequently washed twice with TTL buffer (20 mM Tris–HCl, pH 7.00, 0.1% Tween, 0.50 M LiCl), and suspended in hybridization solution (20 mM Tris–HCl, pH 7.00 with 750 mM NaCl). Step 1 shows the PNA probe modified magnetic beads in a vial.

2.3.2. Hybridization in solution

After resuspending PNA probe modified beads in 100 µl hybridization solution, target DNA containing hybridization solution was added. The hybridization proceeded for 20 min (unless otherwise stated) at room temperature with gentle mixing. The same procedure was also applied with SNP containing or non-complementary oligonucleotides. Target and non-complementary DNA are represented as dark and light gray lines, respectively. Step 2 shows the specific hybridization reaction between PNA probes and target DNA (dark gray lines) for 20 min. Since, PNA hybridized specifically with target DNA,
non-complementary DNA remained unattached to the beads (light gray lines).

2.3.3. Magnetic separation
PNA/target DNA attached magnetic beads were collected at the bottom of the vial by placing a magnet under it for 3 min as shown in Step 3. The unattached non-complementary DNA remained in the solution, and was easily removed by pipetting the buffer solution away. Non-specifically attached non-complementary DNA was further removed from the beads by washing with 0.30 M NaOH and 1% w/v sodium dodecyl sulfate (SDS) containing TBS for three times (Step 3-a). The beads were resuspended in SDS containing TBS, and left for 1 min, then separated from the solution by applying a magnetic field for 3 min. The buffer solution containing the removed non-complementary DNA was taken from the vial by pipetting.

2.3.4. MDB binding to the hybrid
MDB was accumulated onto hybrid modified beads by addition of the intercalator into the beads solution. Hybrid modified beads were interacted with MDB for a desired time with gentle mixing. MDB intercalated between the PNA/target DNA hybrids with the help of its planar aromatic ring. After the intercalation process, the MDB attached hybrid modified beads were once again separated from the buffer solution by applying a magnetic field for 3 min as shown in Step 4. The unbound MDB molecules were removed from the beads by pipetting out the remaining solution from the collected beads.

2.3.5. Preparation of BCPE
A portion of the carbon paste (30/70% (w/w) graphite/oil) was mixed with biotin, so that the final quantity was 5% (w/w). The biotin modified carbon paste mixture was tightly packed into a Teflon electrode holder and polished to a smooth finish.

2.3.6. Bead accumulation onto BCPE
MDB attached beads were then resuspended in blank TBS, and a BCPE was inserted into the solution with gentle mixing as shown in Step 5. Streptavidin modified beads were strongly attached onto the BCPE. The binding of hybrid molecules on the beads directly onto the CPE surface with the help of the strong streptavidin/biotin attraction provided easy collection of the beads from the solution in 10 min as shown in Step 6. Both physical adsorption of PNA/DNA hybrids on the electrode surface, and streptavidin/biotin interaction played a role in this collection step.

2.3.7. Electrochemical transduction
Bead attached BCPE was transferred into electrochemical cell containing blank TBS. The reduction signal of MDB was measured by using square wave anodic stripping voltammetry (SWV, Step 7) by scanning from +0.10 to −0.40 V with an amplitude of 25 mV and a step potential of 4 mV at 15 Hz. The raw voltammograms were treated by using the least square smoothing (level 21) and linear baseline correction of the electrochemical software. Repetitive measurements \((n = 3)\) were carried out by renewing the BCPE surface, and repeating the above assay formats.

3. Results and discussion
The new electrochemical DNA hybridization sensor system couples an efficient magnetic separation with sequence-specific PNA probes. Fig. 1 shows the square wave voltammograms for the reduction signal of MDB at around −0.25 V obtained from PNA modified magnetic beads attached to BCPE after hybridization with (a) target DNA, (b) SNP containing DNA, and (c) non-complementary DNA. The reduction signal of MDB was greatly enhanced, when the perfect-match target DNA was present in the hybridization solution. With the help of its planar phenoxazine ring, MDB could intercalate between the duplex structures of hybrid on the bead, and led to a high signal (Fig. 1a). When there was only SNP containing DNA in the solution, hybridization between the PNA probes and SNP containing DNA occurred at a lesser extent, and the washing step with SDS removed a significant amount of SNP containing DNA from the beads (Fig. 1b). PNA probes did not show any affinity towards the non-complementary DNA, thus only a small MDB signal could be observed (Fig. 1c).

Fig. 2 shows the histograms for the mean and the standard deviation of the MDB signal obtained from PNA probe modified magnetic beads after hybridization with perfect-match target DNA (target), SNP containing DNA...
(SNP), non-complementary DNA (NC), without any target DNA in the solution (probe). MDB signal was not found to be increasing in the presence of only PNA modified beads (probe). Thus, it was concluded that MDB could selectively intercalate only between the duplex structures of the hybrid. MDB signal without any PNA modified beads or DNA (MDB only) was also observed to be as small as the probe signal, indicating that direct attachment of MDB on the beads was suppressed by the multiple washing steps of magnetic separation.

Fig. 3 shows the calibration plot for the dependence of MDB signal upon increasing the concentration of target DNA (target), and SNP containing DNA (SNP). The concentration of perfect-match target DNA was increased from 5 to 30 pM (target). MDB signal increased linearly until 20 pM, and then leveled off. Thus, 20 pM of target DNA was used in further experiments. The hybridization signal indicated a detection limit of 2 pM in connection with 20 min hybridization time. Increasing the concentration of SNP containing DNA did not cause a significant enhancement in the MDB reduction signal (SNP), indicating that weakly attached SNP containing DNA could successfully be removed from neutral PNA probes by multiple washing steps of SDS in connection with magnetic separation.

Fig. 4 shows the calibration plot for the dependence of MDB signal on the concentration of MDB added to the hybrid modified beads containing TBS solution. As the concentration of MDB increased, the amount of the intercalated molecule between the double-stranded hybrid reached a saturation level at 10 μM, and then almost remained constant. Thus, the intercalator concentration of 10 μM was applied for further experiments.

The accumulation time for the intercalator also had an effect on the MDB signals. Fig. 5 shows the calibration plot for the accumulation time dependence on the MDB signal obtained from hybrid modified beads in the presence of 10 μM MDB. After the addition of MDB, an accumulation time of 10 min was found necessary to obtain the saturation of the intercalation sites on the beads.

A series of five repetitive measurements of 20 pM target DNA was used for estimating the precision (not shown). This series yielded a mean peak potential current of 507 nA.
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