Magnetically assisted DNA assays: high selectivity using conjugated polymers for amplified fluorescent transduction

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ABSTRACT

We report a strategy for conjugated polymer (CP)-based optical DNA detection with improved selectivity. The high sensitivity of CP-based biosensors arises from light harvesting by the CP and the related amplified fluorescent signal transduction. We demonstrate that the use of magnetic microparticles significantly improves the selectivity of this class of DNA sensors. Compared with previously reported DNA sensors with CP amplification, this novel sensing strategy displays excellent discrimination against non-cognate DNA in the presence of a protein mixture or even human serum. We also demonstrate that the magnetically assisted DNA sensor can conveniently identify even a single-nucleotide mismatch in the target sequence.

INTRODUCTION

The detection of DNA hybridization is a topic of major scientific and technological interest. Application areas include clinical diagnosis, single-nucleotide polymorphism (SNP) genotyping, environmental studies, antiterrorism and forensic analysis (1). This utility has motivated the development of novel DNA sensors with optical (2–5), acoustic (6,7) or electronic (8–12) ‘read-out’, among which optical (fluorescent) detection methods have historically dominated state-of-the-art genosensors (1,13).

Aromatic, intercalating dyes, e.g. ethydium bromide (EB), served as the first-generation fluorescent DNA hybridization indicator; the fluorescence quantum yield of EB increases significantly upon intercalating into double-stranded (ds) DNA. However, because of the non-specific, hydrophobicity-driven binding of EB to single-stranded (ss) DNA, EB indicators show only limited selectivity against non-cognate DNA (14). In order to overcome this problem, fluorophore-tagged DNA probes were used for hybridization-based assays, which exhibited much improved detection selectivity over indicator-based detection (1). ‘Molecular beacon’ sensing strategy coupled fluorescence detection with stem–loop structured DNA probes, which further enhanced specificity for discrimination of single-nucleotide mismatches (15–17). More recently, novel materials ranging from inorganic nanocrystals (18,19) to rare earth elements (20) have been incorporated to further improve the performance of fluorescent DNA sensors. Conjugated polymer (CP)-based genosensors represent a new opportunity along these lines.

Conjugated polymers possess a unique combination of opto-electronic properties that have found use in a variety of areas (21). Relatively recently, the use of CPs as components in biosensors has stimulated significant research interest (3,22–25). In the biosensor application, CPs serve as a light-harvesting ‘antenna’. The light-harvesting feature originates from their very high absorption coefficients (as high as 10^6 M^-1 cm^-1) (23). Because CPs exhibit efficient energy migration along their delocalized backbones, the collected energy can be efficiently transferred to acceptors via either excited-state electron transfer or Förster resonance energy transfer (FRET) (23,25–27). The light-harvesting properties of CPs create an opportunity to amplify biosensor signals and thereby to develop highly sensitive optical biosensors (23).

Based on the so-called superquenching phenomenon arising as a result of rapid excited-state electron transfer from the polymer to quenchers, Chen et al. (22) have developed a novel CP-based biosensing strategy that can sensitively detect a variety of bio-recognition events (28–32).

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Gaylord et al. (3) demonstrated a different, FRET-based approach that employed CPs as the sensing element to detect specific DNA sequences. In their method, a fluorescein-tagged peptide nucleic acid (PNA) sequence served as the probe sequence. Hybridization of the neutral PNA probe to the negatively charged DNA target significantly creates an electrostatic attraction between the PNA/DNA and a cationic luminescent CP, thus bringing the tagged dye and the polymer sufficiently close to allow efficient FRET. This light-harvesting polymer-based FRET signal amplification provides a means to transduce DNA hybridization to optical sensing with improved sensitivity of more than an order of magnitude (3). A note of caution is that the use of the expensive PNA is key to this sensor (3). Replacement of PNA probes with fluorophore-tagged DNA probes leads to much worse selectivity as a result of the strong Coulomb attraction between the cationic luminescent polymer and the ssDNA probe (33).

In order to be able to quantitatively detect the specific sequences on target DNA, false positive signals that arise from non-specific interactions (i.e. other than the fundamental base pairing within dsDNA) must be eliminated. Thus, it is desirable to develop a DNA sensor that is essentially ‘signal-off’ in the unhybridized state. We note that the use of magnetic particles has attracted much recent attention in controlling bio-related systems owing to its operational convenience and separation efficiency (34,35). In the study reported here, by introducing magnetic particles to CP-based sensors, we developed a magnetically assisted DNA detection platform with polymer amplification that quantitatively identifies target DNA with perfect discrimination against non-cognate DNA; even a single-nucleotide mismatch in the target DNA sequence can be conveniently recognized. As a further step, we demonstrated the ability to detect specific sequences on DNA in the presence of either a mixture of proteins or human serum. This initial success suggests that this DNA sensor can be used for the detection of DNA hybridization in real samples.

**MATERIALS AND METHODS**

**Materials**

Oligonucleotides were obtained from TaKaRa Corporation and subsequently purified with either high-performance liquid chromatography or PAGE. Their sequences are listed in Table 1. Capturing probe 1 is terminated with a biotin that links it to streptavidin-coated magnetic microparticles (MMPs). Capturing probe 1 contains a 15-base probe sequence and a spacer (10 ‘T’s) that reduces surface effects from the MMPs (2). Signaling probe 2 is a 15-base sequence tagged with fluorescein (6-FAM) at the 5′-terminus. Target DNA 3 is a 40-base sequence which contains complementary sequences to 1 on one end and to 2 on the other end. DNA 4 contains a complementary sequence to probe 1 but includes a single-nucleotide mismatch to probe 2. DNA 5 is a random sequence that is non-complementary to either 1 or 2. Oligonucleotides 6–9 are related to the BRCA1 breast cancer gene and represent capture probe, signaling probe, target DNA and single-mismatched DNA, respectively.

Lysozyme (Lys), hemoglobin (Hb) and BSA were purchased from Sigma. The water-soluble polyfluorene (PF), Poly[(9,9-bis(3′-(N,N-dimethyl)-N-ethylammonium)-propyl)-2,7-fluorene]-alt-1,4-phenylene] Dibromide, was synthesized as previously reported (36). MagnetSphere® and streptavidin-coated MagnetSphere® paramagnetic particles (~1.0 μm diameter, 1 mg/ml) were obtained from Promega Corporation. All other reagents were of analytical grade. The buffer solutions were hybridization buffer (750 mM NaCl, 150 mM sodium citrate, pH 7.4), washing buffer (10 mM Tris–HCl, pH 7.4, 50 mM NaCl) and TTA buffer (250 mM Tris–HCl, pH 8.0, 0.1% Tween-20 and 5% BSA). All solutions were prepared using Milli-Q water.

**Spectroscopic measurements**

Absorption spectra were collected with a Zeiss UV-visible recording spectrophotometer, and fluorescence spectra were collected with a F-4500 fluorometer equipped with a xenon lamp excitation source (Hitachi). The excitation wavelength is 380 nm, and the spectra were recorded between 390 and 700 nm.

**The magnetically assisted DNA sensing strategy**

In our design, the DNA sensor is composed of three components: a cationic CP (PF, the light-harvesting polymer that serves as the signal amplification factor), a fluorescein-tagged DNA probe (signaling probe) and a streptavidin-coated MMP labeled with biotinylated DNA probes (capturing probe). Both the signaling probe and the capturing probe are designed to be complementary to different parts of the DNA target sequence, which forms the basis of ‘sandwich-type’ detection. The detection strategy is described in Scheme 1. In the presence of target DNA, the capturing probe binds to the target DNA, along with the signaling probe, proximal to the MMP. In contrast, in the absence of target DNA, the ‘sandwich’ complex cannot be formed, and thus the signaling probe is not attached to the MMP during the magnetic separation step. As a result, only in the presence of target DNA can the cationic luminescent polymer meet the signaling probe to form a FRET pair and emit amplified fluorescence from the fluorescein that reflects the quantity of target DNA.

**Preparation of capturing probe 1-labeled MMPs**

The streptavidin protein at MMP surfaces serves as a bridge to link biotinylated DNA to the MMP. MMPs from the stock

### Table 1. Oligonucleotide sequences

| Sequence                  |
|---------------------------|
| Capturing probe 1         |
| Target DNA 3              |
| Single-mismatched DNA 1   |
| Non-cognate DNA 5         |
| Capturing probe (BRCA1) 6 |
| Signaling probe (BRCA1) 7 |
| Target DNA (BRCA1) 8      |
| Single-mismatched DNA (BRCA1) 9 |

2,7-fluorene)-alt-1,4-phenylene] Dibromide, was synthesized as previously reported (36). MagnetSphere® and streptavidin-coated MagnetSphere® paramagnetic particles (~1.0 μm diameter, 1 mg/ml) were obtained from Promega Corporation. All other reagents were of analytical grade. The buffer solutions were hybridization buffer (750 mM NaCl, 150 mM sodium citrate, pH 7.4), washing buffer (10 mM Tris–HCl, pH 7.4, 50 mM NaCl) and TTA buffer (250 mM Tris–HCl, pH 8.0, 0.1% Tween-20 and 5% BSA). All solutions were prepared using Milli-Q water.

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solution were first washed with buffers as recommended by Promega (http://www.promega.com/tbs/tb246/tb246.pdf). Subsequently, a solution of biotinylated capturing probe 1 was added to the collected MMPs (at a ratio of 1.25 nmol probe 1 to 1 mg MMPs) and the mixture was incubated for ~10 min with gentle mixing. Note that the binding process of biotin–streptavidin at surfaces is fairly rapid (usually within 1 min) according to previous surface plasma resonance studies (37). The surface density of probe 1 was estimated to be 4–6 × 10¹¹ strands/cm² (http://www.promega.com/tbs/tb246/tb246.pdf). The MMP–1 complexes were then washed twice with TTA buffer and suspended in the hybridization buffer. After preparation, the MMP–1 complex was stored in a refrigerator for further use.

Detection of DNA hybridization

In a typical assay, both MMPs with capturing probe 1 (50 μg) and excess signaling probe 2 were added to solutions (1 ml) containing DNA sequences (3, 4, 5) and incubated for hybridization at 37°C (~20 min). The resulting MMPs were magnetically collected (~30 s), then washed twice with hybridization buffers. In the case of SNP detection, we washed MMPs with washing buffers. In order to avoid light scattering associated with these relatively large MMPs, we separated signaling probe 2 from the MMPs by rinsing the MMPs with 50 mM NaOH solution. Previous studies have demonstrated well that the use of this alkaline solution effectively denatures DNA duplexes within 1 min whereas biotin–streptavidin binding is essentially undisturbed under this condition (37). The rinsed solution containing both 2 and 3 was neutralized by adding an acid solution (50 mM HCl and 100 mM Tris–HCl, pH 7.4) of the same volume as the NaOH solution. Note that 2 and 3 re-form a duplex in this solution containing salts (see Supplementary Figure 1S). PF was added to form the FRET pair with the fluorescein tag at probe 2. Fluorescence measurements were performed by exciting PF at 380 nm. The spectra were normalized to the polymer emission, and background-subtracted fluorescence intensity was used as the quantitative index.

Detection of target DNA from the mixture

The artificial matrix was a 1.0 ml solution containing 1 nM target DNA 3, large amounts of random DNA 5 (100× more concentrated) and proteins that are often encountered in real applications (Lys, Hb and BSA, 1000× more concentrated). As a further step, we used human serum samples (1:10 dilution, a gift from Shanghai Orion Diagnostics Co. Ltd) containing 1 nM target DNA 3. All separation, collection and detection procedures were the same as described above, except that the MMPs were washed twice with TTA buffer after hybridization.
RESULTS

PF-amplified fluorescent transduction

Previous spectroscopic studies have proven that the emission of PF has sufficient spectral overlap with the fluorescein absorption that they form an excellent FRET pair (38). Consistent with this, we observed well-defined FRET signals upon addition of PF to the test solution containing probe 2 (Figures 1 and 2). The light-harvesting PF sensitized the emission of the fluorescein (the energy-transfer acceptor), leading to fluorescent signal amplification. In fact, we could still observe well-defined PF-sensitized fluorescein emission at probe 2 concentrations that were sufficiently dilute (100 pM) that fluorescence emission was negligible by direct excitation of fluorescein (Figure 1). These results verify that light-harvesting polymers do provide optical signal amplification and improve the detection sensitivity.

Detection of DNA hybridization

In our initial study, we employed a 40-base oligo 3 as the model target DNA sequence. Incubation of MMP–1 with both 2 and 3 in the hybridization buffer allows annealing of these DNA strands, resulting in the formation of the 'sandwich' complex at MMP surfaces. Upon applying the magnetic force, the MMPs were rapidly separated from the bulk solution (~30 s). After the addition of PF to the rinsed solution containing the duplex (formed by signaling probe 2 and target 3), we observed a strong FRET signal that increases in strength with the probe concentration. We then performed a quantitative assay for detection of target DNA. We observed that the intensity of PF-sensitized fluorescein emission was linearly proportional to the concentration of target DNA 3 in the range 0–10 nM (Figure 2). Note that the FRET signal can still be clearly identified from the background even for 100 pM target; thus we estimate that the limit of DNA detection is as low as 100 pM (100 fmol). This DNA sensor also shows excellent discrimination against non-cognate DNA. FRET signals were not observed when a non-cognate DNA 4 (5 μM) replaced the target 3. These indicate that we could selectively identify the target DNA sequence without interferences from excess non-cognate DNA.

Detection of a single-nucleotide mismatch

It is essential to identify a single-nucleotide mismatch in order to meet the strict requirement of certain applications, e.g. SNP genotyping. DNA duplex stability is known to be a function sensitive to the ionic strength of solutions. Because the DNA strands are negatively charged, cations are required to screen the Coulomb repulsion that would otherwise prevent the formation of the double helix (39). Mirkin and coworkers have demonstrated that mismatched DNA is much more susceptible to solutions of low ionic strength than perfectly matched DNA (40). Although Mirkin’s work was based only on DNA probes at the surface of gold nanoparticles, we observed that this also offered the opportunity to distinguish single-nucleotide mismatches by salt-stringency in our magnetically assisted DNA assays.

We performed a stringent washing step that was expected to destabilize dsDNA containing single-nucleotide mismatches. In this case, MMPs collected from the test solution were rinsed with the washing buffer of low ionic strength. We reason that DNA sequences containing a single-nucleotide mismatch can be largely removed during this stringent washing, while target DNA remains bound to the MMP surfaces. Indeed, we observed that the DNA sensor showed marked differences in FRET signals corresponding to perfectly matched DNA and single-nucleotide mismatched DNA (Figure 3), indicating that this DNA sensor exhibits excellent discrimination against even a single-nucleotide mismatch.

In order to demonstrate that this proposed strategy is applicable to real applications, we further employed the above-mentioned protocol to detect the BRCA1 breast cancer...
gene, a tumor suppressor gene. It is well known that women who inherit mutations of BRCA1 are highly susceptible to the development of breast cancer (41); thus it is important to detect mutations of the BRCA1 gene. By using the magnetically assisted DNA assay with CP amplifications, we could selectively detect 1 nM BRCA1-related oligonucleotide sequence. In contrast, we could not observe any FRET signal for 1 nM single-mismatched oligonucleotide (Supplementary Figure 4S).

**Detection of target DNA in complex samples**

In an attempt to test the applicability of this DNA sensor to detection in real samples, we prepared an artificial matrix containing 1 nM target DNA in a solution containing a 1000-fold higher concentration of proteins (Hb, Lys and BSA) and random DNA sequences. As a further step, we employed diluted human serum samples (1:10) containing either target DNA or non-complementary DNA. With the assistance of magnetic separation, we were able to observe FRET signals corresponding to the presence of target DNA. More importantly, we have shown that the FRET signal intensity is comparable to that obtained in pure DNA solutions (Figure 4), implying that the DNA detection is insensitive to non-specific species, and that it is possible to perform DNA detection even in blood samples.

**DISCUSSION**

The use of CPs as highly responsive optical reporters formed the basis of the highly sensitive CP-based DNA sensor proposed by Gaylord et al. (3). However, since the signal transduction of this sensor relies mainly on discrimination of electrostatic interactions between neutral PNA and the negatively charged PNA/DNA complex, the selectivity is imperfect. According to recent time-resolved spectroscopic studies (42), electrostatic interactions dominate in dilute PNA solutions, thus leading to fairly good selectivity between unhybridized PNA and hybridized PNA/DNA. In relatively concentrated PNA solutions, however, hydrophobic interactions between the PF backbone and PNA contribute to bringing the PF and fluorescein-tagged PNA within the FRET distance. As a result, non-specific FRET signals are observed even for hybridization with non-cognate DNA (42). Note that, even in dilute solutions, hydrophobic interactions exist, leading to relatively small, non-specific FRET signals (3,42). Moreover, replacement of PNA probes with DNA probes achieves only limited discrimination ability because of the relatively small difference in the Coulomb binding strength of ssDNA and dsDNA to PF (33).

The magnetically assisted sensor system represents an opportunity to develop high-performance CP-based DNA sensors even without the use of expensive PNA. The use of magnetic forces instead of simply relying on electrostatic forces is essential for the significant improvement of the sensor selectivity. As we have demonstrated with this magnetically assisted DNA sensor, target DNA leads to strong FRET emission whereas essentially no signal is observed for non-cognate DNA. In the present experimental setup, we could detect as little as 100 pM target DNA. Note that we could detect only 1 nM of fluorescein-tagged DNA without CP amplification. This clearly demonstrates that the use of CPs offers a signal amplification of an order of magnitude. This detection limit could be further lowered by using state-of-the-art fluorometers, or by finely tuning the backbone or side chains of CPs, which might lead to much improved amplification (43,44).

More importantly, single-nucleotide mismatches can be conveniently identified by a stringent wash with low ionic strength buffer with this strategy. Note that SNP detection is conventionally achieved by a thermal-stringency washing step, relying on the fact that the melting point of mismatched DNA is lower than that of the corresponding perfectly matched DNA. The salt-stringency washing first proposed by Mirkin and coworkers (40) is much more suitable for magnetically assisted assays. First, MMPs have only limited stability over high temperature (<65°C); second, salt-stringency washing steps can be done within a minute, whereas it is not convenient to perform thermal-stringency washing in the presence of the magnet (magnets are also susceptible to high temperature).
Other stringent conditions include the use of denaturing reagents of appropriate concentrations (e.g. urea) (45) or organic solvents (e.g. formamide) (46). Nevertheless, none of these approaches is as convenient as the salt-stringency approach employed here. Also of note is the fact that the current detection strategy might be generalized to multiplex detection, that is, distinguishing multiple targets in only one tube. A recent report by Liu et al. (47) has proved that it is possible to design CPs emitting several different colors, which is highly promising progress toward the goal of multiplexing.

Detection in real samples is more problematic for the previously established CP-based biosensor (3). Polymer fluorescence has proved susceptible to environmental changes, such as solvent environments (42) and non-specific interactions with proteins (48). This has largely prevented the use of CP-based biosensors in real applications. Wang et al. (48) reported that compensation for charges of CP significantly reduced non-specific signals arising from electrostatic interactions. However, in reality it is often difficult to identify all factors that lead to non-specific signals. We have demonstrated that the use of MMPs offers unprecedented advantages in this respect. MMPs are robust enough to allow repetitive washing under moderately stringent conditions, thus exhibiting the ability to efficiently remove non-specific species.

Very recently, Gaylord et al. (49) also reported SNP detection by combining their CP/PNA detection with an S1 nuclease enzyme. Excellent selectivity was offered by the S1 nuclease enzyme, which specifically digested all ssDNA and mismatched DNA in the absence of protection by the enzyme-resistant PNA. However, their assay strategy relies on enzyme digestion that requires incubation at 37°C for 1 h. In contrast, it typically takes <30 min to perform SNP detection with our magnetically assisted DNA assay. This takes advantage of rapid magnetic separation (~30 s), which is in sharp contrast to conventional, tedious bioseparation processes (usually hours), e.g. chromatography and centrifuging.

Based on fluorescence superquenching, Kushon et al. (31) reported the use of CP-coated polystyrene microspheres to detect DNA hybridization. Their system is also suitable for SNP detection by utilizing PNA probes as well as optimizing assay temperature (32). We note that both polystyrene microspheres and MMPs provide a nearly homogeneous environment that facilitates DNA hybridization processes, and at the same time possess the advantages of solid-state sensors (e.g. washability and reusability). Moreover, separation of polystyrene microspheres requires exhaustive dialfiltration, whereas MMPs can be separated from other species simply by applying the magnetic force. It is also possible to convert the present magnetically assisted DNA assay strategy to an automated chip-based assay format given the availability of microfluidic cells with MMP trapping abilities (50). We thus expect that such magnetically assisted assays should be highly generalizable to other biosensor systems with CP amplification and lead to a broad class of highly sensitive and highly selective CP-based biosensors.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at NAR Online.

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