Sequence-specific analysis of oligodeoxynucleotides by precipitate-amplified surface plasmon resonance measurements

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1. ABSTRACT

A flow injection SPR spectrometer is used to detect DNA hybridization via amplification by an enzyme-catalyzed precipitation reaction. Hybridization of an oligodeoxynucleotide (ODN) target with a surface-confined ODN probe, followed by hybridization between the overhang on the resultant duplex and a biotin-tagged ODN, yields a "sandwich" complex (a three-component double-stranded). A streptavidin-horseradish peroxidase conjugate (SA-HRP) can be attached to the sandwich complex-covered surface via the biotin/streptavidin complexation. In the presence of H_2O_2, the HRP catalyzes oxidation of 4-chloro-1-naphthol (CN) to form a precipitate on the sensor surface. The precipitated film dramatically changes the refractive index at the metal/dielectric interface and significantly lowers the detection level. The method is shown to be reproducible, to possess high ODN sequence specificity and a high sensitivity allowing detection of ODN target concentration as low as 10 fM.

2. INTRODUCTION

Determinations of specific DNA sequences by DNA hybridization reactions have been carried out extensively in recent years for gene expression analysis and in diagnosis of diseases. Since many genes are expressed at very low levels in the cell, sensitive and selective DNA detection methods are desired for the quantification of gene expression. Thus far, a great number of methods have been developed for DNA assays. These methods include, but are not limited to, electrochemical methods (1-7) fluorescence microscopy (8-11), quartz crystal microbalance (QCM) (12-14), capillary electrophoresis (15, 16), and atomic force microscopy (17, 18). However, these methods either require prior labeling of the DNA sample for signal transduction (e.g., attaching an electroactive tag onto the DNA target (5)) or have certain limitations (e.g., photo-bleaching in fluorescence detection (19) and viscoelastic effect in QCM measurements).

As a powerful technique for the analysis of chemical and biological species, surface plasmon resonance (SPR) has been successfully used for gene analyses over the past decade (20). SPR is advantageous in that it is sensitive to small changes in the thickness or refractive index of species adsorbed onto the surface of a sensor (21-24). As a result, it is possible to detect DNA hybridization in a label-free format and in real time. However, conventional and commercial SPR spectrometers, while highly popular and becoming increasingly indispensable in many laboratories, cannot rival fluorescence measurements in terms of its sensitivity. The detection level is restricted to 1-10 nM for proteins with molecular weights in the 20-KDa range (25) and even higher for smaller molecules or species with low biomolecular affinities (26-28). Recently, we have shown that a highly sensitive SPR instrument (29, 30) based on a bicell detector is capable of quantifying orientation changes of self-assembled monolayers (SAMs) (31, 32) at the angstrom resolution. We also applied this instrument to DNA hybridization assays (33) and achieved a concentration detection level down to 54 fM. Using DNA-capped gold nanoparticles, it is demonstrated that detection levels for various SPR gene analyses can be significantly decreased (34, 35). A biocatalytic signal amplification approach for DNA hybridization analysis was developed (36, 37). Labeling DNA molecules with liposomes and the subsequent biocatalyzed precipitation were employed for electrochemical DNA hybridization assays and impedance measurements (38). A remarkable detection level (0.65 pM) was obtained for the electrochemical assay (39). Compared to amplification strategies by enzymatic reactions and attachment of DNA-capped gold nanoparticles, the enzyme-catalyzed precipitation reaction is simpler and does not require expensive bioreagents.

In this work, we used a commercial SPR instrument based on a position-sensitive detector to measure an enzyme-catalyzed precipitation reaction following DNA hybridization reactions. The instrument has a good sensitivity and has been shown to be capable of determining even heavy metal ions (Cd^{2+} and Hg^{2+}) bound to apo-metallothioneins pre-immobilized onto SPR substrates (40). Herein, we show that DNA assays can also be performed with this instrument and the sensitivity of the SPR detection is much improved by the precipitation reaction. The precipitate formed on the sensor surface significantly increases the refractive index at the DNA-covered substrate and enhances the weak hybridization signal that is too low to be measured by even a sensitive SPR spectrometer. By systematically investigating on the various analytical "figures of merit" of this approach, we demonstrate that the enzyme-catalyzed precipitation reaction can be straightforwardly implemented for sequence-specific SPR gene analysis.

3. MATERIALS AND METHODS

3.1 Chemicals and materials
Aminoethanol-HCl (AE), 11-mercaptoundecanoic acid (MUA), streptavidin-horseradish peroxidase (SA-HRP), 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimidehydrochloride (EDC), N-hydroxysuccinimide (NHS), and 4-chloro-1-naphthol (CN) were all obtained from Sigma. Hydrogen peroxide was purchased from Taopu Chemical Plant (Shanghai, China), and all the ODNs were acquired from Sangon Co., LTD (Shanghai, China). The 25-mer ODN capture probe with its 5' end modified by an amine group and the 10-mer ODN detection probe with its 3' end modified by biotin are 5'-amino-$(CH_2)_6$-TTT TTA GAC ATG CCC AGA CAT GCC C-3' and 5'-TCC AAC CGA G-biotin-3', respectively. To evaluate the selectivity of the method, four different target sequences (mismatching sequences underlined) were chosen: 5'-CTC GGT TGG ATT TGG GCA TGT CTG GGC ATG TCT-3' (complementary to the capture probe), 5'-CTC GGT TGG ATT TGG GCA TGT CTG TGC ATG TCT-3' (one base mismatching to the capture probe) , 5'-CTC GGT TGG ATT TGG GCA TGT CAT TTC ATG TCT-3' (four bases mismatching to the capture probe), 5'-AGG AGA GCT CAG GTT ACT AGT GCC TTT-3' (noncomplementary to the capture probe). All stock solutions were prepared daily with deionized water treated by a water purification system (Simplicity 185, Millipore Corp., Bedford, MA, USA). Other reagents were all from commercial sources with analytical purity and used as received.

3.2 Procedures
3.2.1 Solution preparation
ODN and SA-HRP solutions were prepared with phosphate-buffered saline (PBS buffer) containing 10.0 mM phosphate (pH = 7.0) and 0.1 M NaCl and stored at -7 °C. H_2O_2 solution was freshly prepared with deionized water. NHS/EDC solutions were prepared by mixing 0.1 M NHS and 0.4 M EDC in PBS buffer right before surface modification. The stock solution of 4-chloro-1-naphthol (CN) was prepared by dissolving CN in ethanol and then diluted with PBS buffer to a final concentration of 1.0 mM. The final solution was filtered through a 0.22 μ m-membrane filter prior to use.

3.2.2 SPR sensor surface modification
The SPR sensor fabrication and modification were carried out as follows. BK7 glass slides (Fisher Scientific, Tustin, CA, USA) were heated in a hot piranha solution (30% H_2O_2 /concentrated H_2SO_4 = 7:3, v/v) at 80 °C for 30 min. After cooling down to room temperature, the glass slides were thoroughly rinsed with water, and then sonicated in the solution of H_2O/NH_4OH/30% H_2O_2 (5:1:1,v/v/v) for 1 h. After rinsing thoroughly with water, the slides were stored in water for subsequent use. Gold films with a 50 nm thickness and a 2 nm Cr underlayer were deposited on the dried glass slides using a sputter coater (Model 108, Kert J. Lester Inc., Clairton, PA, USA). Prior to use, the gold film was annealed in a hydrogen flame to avoid surface contamination. MUA self-assembled monolayers (SAMs) were first formed by immersing the films in 4.0 mM MUA solution dissolved in ethanol overnight. The films were then washed thoroughly with ethanol and deionized water. The ODN capture probe was affixed onto the sensor surface by incubating the gold film in a mixed solution of 0.1 M NHS, 0.4 M EDC and 0.4 μM aminated ODN probe in a humid chamber for 1 h. After the ODN capture probe attachment, any possible
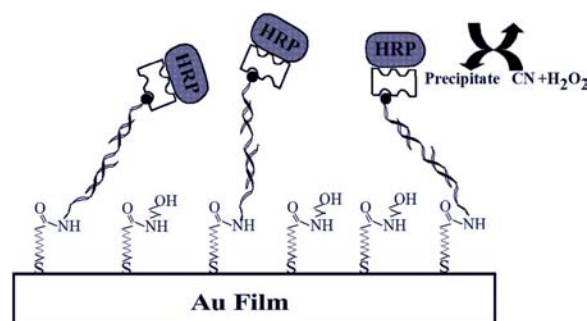
Figure 1. Schematic representation of the SPR detection of an ODN target amplified by the enzyme-catalyzed precipitation reaction. The SPR experiment was carried out by injecting a solution containing CN and H_2O_2 into the flow cell housing a sensor covered with an ODN duplex/SA-HRP conjugate. For clarity, alkanethiol, ODN, biotin, and SA-HRP are not drawn to scale.
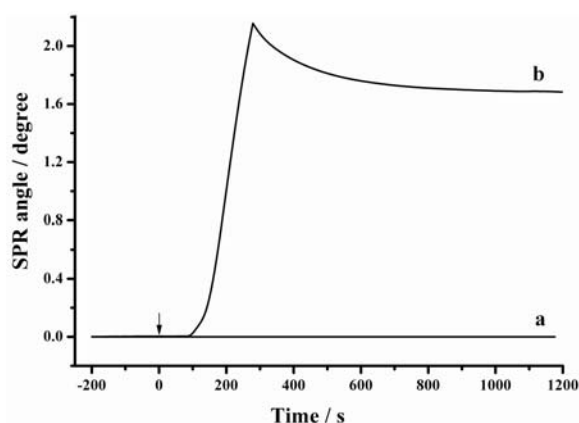
Figure 2. A SPR sensorgram acquired by injecting 50 μL of a solution containing 1 mM CN and 0.3 mM H_2O_2 onto a substrate covered with the ODN duplex/SA-HRP (curve b). Curve a was obtained upon injecting 5 pM ODN target solution into the flow cell housing an ODN duplex-covered sensor surface without being treated with the biotinylated detection probe solution. The arrow at 0 s indicated the time of injection.

nonspecifically adsorbed molecules were rinsed off the surface and the surface was dried under a stream of N_2. To block the unreacted sites, 1 M AE solution was cast onto the surface for 1 h, followed by thoroughly rinsing with water.

3.2.3 ODN target hybridization and SPR detection amplified by the enzyme-catalyzed precipitation

For each assay, 40 μL of PBS comprising a target ODN of a predetermined concentration was cast onto the capture probe-modified SPR substrate and the hybridization reaction was allowed to proceed for 2 h in a humid chamber. After the surface was thoroughly rinsed with PBS and water, 40 μL of PBS containing 4 μM biotinylated detection probe was cast onto the surface for 2 h. The films were then rinsed with PBS and water. To

attach SA-HRP onto the surface, 40 μL of PBS containing 0.01 mg ml^{-1} SA-HRP was pipetted onto the ODN duplex-covered SPR substrate and allowed to react for 1 h. After rinsing with PBS and water and drying with N_2, the film was assembled onto the SPR prism with an index-matching fluid (Type A immersion oil, Cargille Laboratories, Cedar Grove, NJ, USA). The mixed solution containing 1 mM CN and 0.3 mM H_2O_2 was then introduced into the flow cell and the SPR signal was recorded.

3.3 Flow injection-SPR apparatus

A BI-1000 SPR instrument (Biosensing Instrument, Tempe, AZ, USA), equipped with a position-sensitive detector and a flow injection (FI) system, was used throughout this work. For each experiment, the mixed solution containing 1 mM CN and 0.3 mM H_2O_2 was preloaded into a 50-μL sample loop on a six-port valve with a microsyringe. PBS was used as the running buffer delivered to the flow cell by a Genie Plus syringe pump (Kent Scientific, Torrington, CT) at a flow rate of 10 μL/min. The SPR angular shifts measured by the BI-1000 SPR instrument were calibrated using the ethanol calibration method (29, 41). All experiments were performed at room temperature (~25 °C) unless otherwise stated.

4. RESULTS

Figure 1 illustrates the sandwich DNA hybridization reaction and the follow-up precipitation reaction for amplified SPR detection of DNA. Briefly, a MUA SAM was first formed by immersing a gold film into 4.0 mM MUA solution. Amine-modified ODN capture probe molecules were immobilized onto the MUA SAM through the widely used EDC/NHS cross-linking reaction (42). Exposing the resultant film to an ODN target solution formed a duplex at the surface with a single-stranded DNA overhang. Biotinylated detection probe molecules were allowed to hybridize at the overhang. Subsequently, SA-HRP was attached to the duplex via interaction of the biotin tag on the detection probe with the streptavidin-HRP conjugate. In the presence of H_2O_2, HRP at the surface catalyzes the oxidization of CN to form the precipitate of benzo-4-chloro-hexadienone, which can be sensitively measured by FI-SPR. The thickness of the precipitate is dependent on the surface coverage of HRP molecules, which are quantitatively related to the number of DNA duplexes at the surface.

Figure 2 depicts a typical SPR sensorgram upon injecting 50 μL of a solution containing 1 mM CN and 0.3 mM H_2O_2 onto a substrate covered with the ODN duplex tagged with SA-HRP (curve b). The SPR dip shifted by 1.678 degree. As can be seen, a new baseline whose intensity was higher than the original one was established after the solution had been replaced by the carrier solution (at ~400s). The SPR dip shift was measured as the difference in the baseline SPR angles before and after the injection. Curve a was acquired at an ODN capture probe-covered substrate upon injecting 50 μL 5 pM complementary ODN target solution. The SPR dip shift remained essentially unchanged (less than 1 milli-degree),
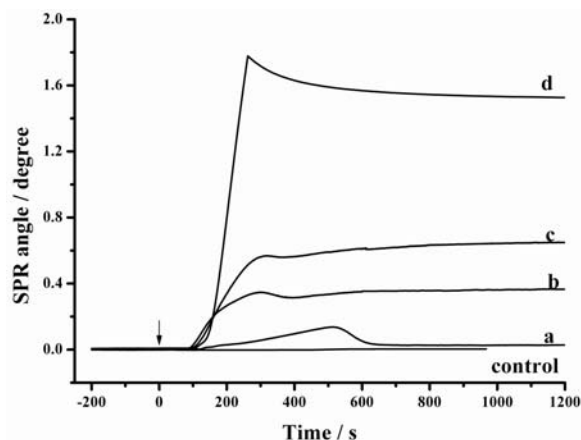
Figure 3. SPR sensorgrams acquired by injecting the CN/H$_2$O$_2$ solution onto substrates that had been treated with ODN targets of four different sequences: noncomplementary to the capture probe sequence (curve a), four bases mismatching to the capture probe sequence (curve b), one base mismatching to the capture probe sequence (curve c), and complementary to the capture probe sequence (curve d). The control experiment was acquired at an ODN duplex/HRP-covered substrate. All target solutions had a concentration of 50 fM. The arrow indicates the time of injection.
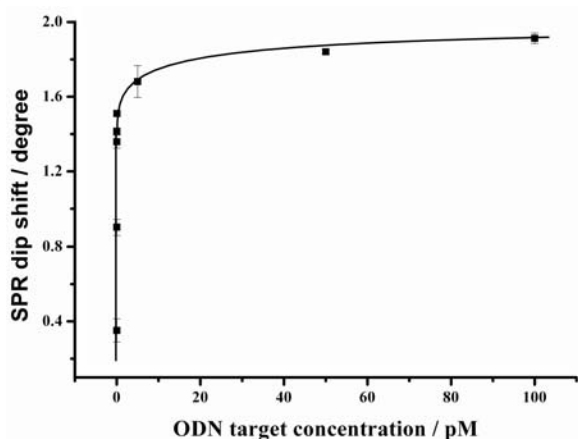

Figure 4. The dependence of SPR dip shift on the ODN target concentration. The relative standard deviations were deduced from at least three replicates and were shown as the error bars. The ODN target concentrations are 10 fM, 12 fM, 15 fM, 20 fM, 50 fM, 5 pM, 50 pM, and 100 pM.

suggesting that, without signal amplification, 5 pM would be close to the ODN detection limit of this SPR spectrometer. Curve b showed a considerable SPR dip change resulted from the refractive index change associated with the deposition of the precipitate onto the sensor surface. Since the dip shift in curve b is substantially greater than that in curve a, the enzyme-catalyzed precipitation is shown to be sensitive and effective for the detection of ODN hybridization.

Sequence-specific ODN analysis can also be realized. Figure 3 displays four SPR sensorgrams acquired by injecting the CN/H$_2$O$_2$ solution into the flow cell for contact with substrates that had been treated with four ODN targets of different sequences. The control curve was acquired by allowing the CN/H$_2$O$_2$ solution to be in contact with a substrate that was covered with the ODN duplex with the overhang (i.e., without the biotinylated detection probe) which had been exposed to the SA-HRP solution. The SPR signal returned to the original baseline upon elution of the CN/H$_2$O$_2$ solution. This indicates that the formation of a sandwich ODN structure is essential to the HRP attachment and the subsequent enzyme-catalyzed precipitation reaction. Furthermore, the SA-HRP conjugate does not appear to nonspecifically adsorb onto or can be easily rinsed off the surface. As can be seen from Figure 3, the SPR dip shift in curve d is approximately 1.510 degree, which is 2.3 times greater than that in curve c (~0.653 degree) and 4 times greater than that in curve b (~0.367 degree). Thus, sequence analysis down to a single-base mismatch is achievable. The relatively small change in curve b suggests that that with a four-base mutation, much less duplexes had formed at the sensor surface. When the hybridization reaction with a noncomplementary target at the same concentration (50 fM) was carried out, the dip shift was even more substantial (0.0240 degree in curve a). Thus, when there are duplexes and SA-HRP at the surface, the precipitation reaction will occur and the refractive index at the sensor/solution interface will be changed significantly. The biocatalyzed process was found to be instantaneous because an abrupt SPR dip shift was observed once the CN/H$_2$O$_2$ solution came in contact with the ODN duplex/SA-HRP-covered sensor surface. Even though all of the hybridization reactions were performed at ambient temperature, the number of base-pair mismatches still had a profound influence on the magnitude of the SPR dip shift.

Perhaps the most noteworthy aspect of our method is the remarkably amplified signals by the enzyme-catalyzed precipitation. We evaluated the dynamic range and detection levels of the method for DNA analysis by plotting of the extent of SPR dip shift as a function of the target concentration (Figure 4). In Figure 4, each point was averaged from at least three replicates. The relative standard deviations (RSD), shown as the error bars, are all less than 17%. These RSD values are quite reasonable given that multiple steps were involved in the assay and there exist slight variations of the experimental parameters (e.g., ambient temperature and thickness and uniformity of the sensor surface). The plot exhibits two regions with different slopes. The lower curve (from 10 fM to 5 pM) has a slope much steeper than that of the upper section (from 5 pM to 100 pM). The variations in the slopes (sensitivities) between the lower and upper regions may be interpreted on the basis of the surface coverage of the duplexes formed at different target concentrations. The surface density of the ODN duplexes in the lower region is scarce. Once the target DNA concentration exceeds 5 pM, the curve begins to level off, suggesting that most of the capture probes have formed duplexes with the target molecules. Note that a detection level of 10 fM of ODN target can be achieved.
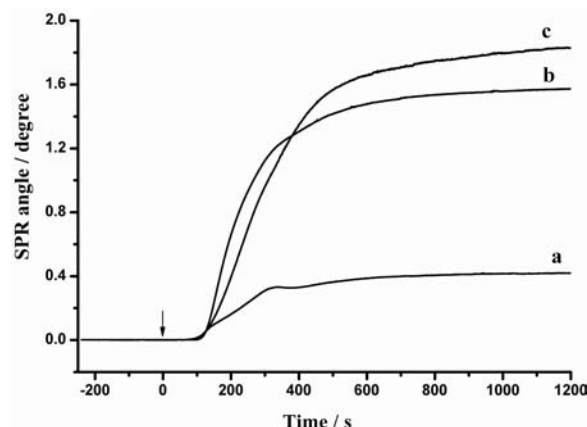
Figure 5. SPR sensorgrams acquired at different ODN hybridization temperatures. Curves a and b correspond to the SPR dip shifts resulted from hybridization reactions between the capture probe and ODN target at ambient temperature and 57 °C, respectively. In curve c, the hybridization between the capture probe and the target was performed at 57 °C and that between the overhang of the duplex and the detection probe at 32 °C. The concentration of the ODN target for all three cases was 10 fM.

Such a detection level is much lower than those reported by other methods (11, 43-45) and is comparable to that we achieved using the scheme based on signal amplification by ODN-capped gold nanoparticles (34).

As mentioned above, variations of some experimental parameters could cause noticeable SPR changes. We therefore examined the SPR dip shifts corresponding to DNA hybridization reactions performed at elevated temperatures. As shown in Figure 5, upon injecting the CN/H_2O_2 solution, a SPR dip shift of 0.423 degree was obtained from a sensor that had been treated with 10 fM complementary ODN target solution at ambient temperature (curve a). If the hybridization reaction between the capture probe and the target ODN was carried out at an elevated temperature (57 °C) but the subsequent hybridization between the overhang of the duplex and the detection probe was performed at ambient temperature, the SPR dip shift increased to 1.595 degree (curve b). In curve c, the first DNA hybridization occurred at 57 °C and the follow-up hybridization reaction with the biotinylated ODN detection probe proceeded at 32 °C. The SPR dip shift in curve c is approximately 1.885 degree, which is about 4.5 times greater than that shown in curve a. This result indicates that a higher hybridization temperature would allow a greater amount of enzyme to be attached to the surface, and consequently produce a thicker precipitate film. Notice that the dip shift in curve c is also slightly greater than that in curve b, suggesting that a small increase in the temperature for linking the biotinylated ODN detection probe to the duplex can further enhance the SPR signal. Although not thoroughly tested, conducting hybridization reactions at elevated temperatures is expected to facilitate the differentiation of ODN targets with a single base mutation. We thus demonstrate the potential of this method for single nucleotide polymorphism (SNP) analysis

and the amenability for detecting DNA targets present in a real sample at extremely low concentrations.

5. CONCLUSIONS AND PERSPECTIVES

In this work, an enzyme-catalyzed precipitation reaction has been successfully used in a sandwich gene assay format to amplify small signals detected by FI-SPR. The signal amplification originates from the precipitation onto the sensor surface of an insoluble product formed by the enzyme-catalyzed oxidation of 4-chloro-1-naphthol. The precipitate introduces an additional layer of a high refractive index. As a consequence, ODN targets can be measured at low femtomolar levels (a detection level of 10 fM for the 33-mer ODN target can be obtained). The analytical performance (sequence specificity, dynamic range, reproducibility, and temperature effect) of the method was evaluated. The method is shown to be reproducible and to possess high sequence specificity. Due to the compact and versatile design of the instrument and the simplicity of the immobilization scheme, our approach further expands the analytical utility of SPR while maintaining the unique features of the conventional SPR detection.

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Abbreviations: SPR: surface plasmon resonance, FI: flow injection, ODN: oligodeoxynucleotide, SA-HRP: streptavidin-horseradish peroxidase, CN: 4-chloro-1-naphthol, QCM: quartz crystal microbalance, SAMs: self-assembled monolayers, MUA: 11-mercaptoundecanoic acid, AE: aminoethanol-HCl, EDC: 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimidehydrochloride, NHS: N-hydroxysuccinimide, PBS: phosphate-buffered saline.

Key Words: SPR, FI, DNA hybridization, Enzyme-catalyzed precipitation

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