DNA Detection

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# Surface-Plasmon-Enhanced Fluorescence Spectroscopy for DNA Detection Using Fluorescently Labeled PNA as "DNA Indicator"

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A rapid detection of the presence/absence of a specific DNA sequence is crucial for the identification of genetically modified organisms (GMOs) in foods, the diagnosis of infections, and the monitoring of the environment. Most available methods rely on the immobilization of DNA probes onto sensor surfaces and their subsequent hybridization with the target DNA from the solution. Recently, several researchers demonstrated that it is possible to use conjugated polymers[1] or DNA-functionalized nanoparticles[2] for homogeneous DNA detection, thus avoiding the difficulties associated with DNA-probe immobilization. However, the proposed strategies involve several process steps, which makes them unsuitable for practical DNA detection.

Herein, we report on the possibility of using labeled peptide nucleic acids (PNAs) as probes for homogeneous DNA detection. The PNA molecules are DNA analogues in which an uncharged pseudopeptide chain replaces the negatively charged sugar-phosphate backbone of natural DNA, thus resulting in an achiral and neutral mimic.[3] When used as the probe strand in a DNA sensor, PNA has been reported to have many advantages over DNA, such as higher stability,[3c,4] higher selectivity,[5] and low requirements for a particular buffer solution.[3c,6] The different electrostatic features of PNA and DNA make the two molecules show an unequal affinity towards a positively charged surface. Figure 1 shows surface-plasmon-enhanced fluorescence spectra after the adsorption of either PNA or DNA on a plasma-polymerized allylamine (ppAA) surface. It is apparent that the amount of DNA strands adsorbed onto the ppAA surfaces is much higher than that of PNA. The small fluorescent signal observed when injecting PNA may be caused by nonspecific interactions.

When the fluorescently labeled PNA hybridizes with the DNA in solution, a negatively charged PNA/DNA hybrid is formed, which can adsorb onto a positively charged surface. Here, thin ppAA films were used as the positively charged surface. The ppAA polymer was deposited onto a thiol-SAM-modified gold substrate (SAM = self-assembled monolayer) by using a 100-W radio-frequency (rf) input power to initiate the polymerization. The plasma polymerization of allylamine has been discussed in some of our previous publications[7] and will not be treated further here. The modified surfaces proved to be highly efficient for the immobilization of negatively charged DNA.[7c,d] High-power deposits of allylamine do not only provide a certain number of amine groups on the surface (see Figure S1 of the Supporting Information), but also a good stability in aqueous solution (see Figure S2 of the Supporting Information).

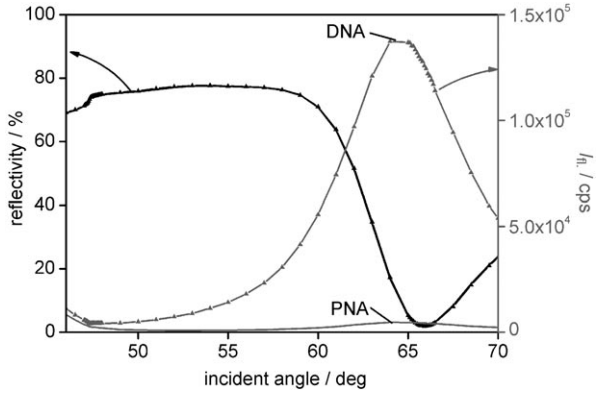


***Figure 1.*** SPFS measurements after the adsorption of Cy5-labeled PNA (namely, P1) and DNA on a ppAA surface. PNA and DNA have the same sequences (that is, ACATGCAGTGTTGAT-Cy5). (The amount of PNA and DNA in the solutions was 1 nmol.)

The fluorescent label of the surface-attached PNA/DNA hybrid can be read out by surface-plasmon-enhanced fluorescence spectroscopy (SPFS), which is a combination of surface-plasmon resonance (SPR) and fluorescence spectroscopy.[8] All experiments were carried out at room temperature and below the melting temperature of the hybrid. The excitation of a surface-plasmon mode at the gold/dielectric interface results in an enhanced optical field, which can excite fluorophores located within the evanescent tail of the surface-plasmon mode, thus resulting in a very strong fluorescent signal. Moreover, the SPR evanescent field decays exponentially into the dielectric medium—with a penetration depth of approximately $L = 150$ nm—which gives SPFS its characteristic surface sensitivity. Only those fluorophores adsorbed, adhered, or bound to the surface will be excited, while those in the bulk solution will not. Scheme 1 illustrates the concept of the present DNA detection method. The labeled PNA is neutral and does not adsorb onto the positively charged surface (Scheme 1a). As a consequence, no fluorescent signal can be detected by SPFS. On the other hand, as shown in Scheme 1b, the specific hybridization of the target DNA with the labeled PNA results in a negatively charged hybrid, which will adsorb onto the positively charged surface (the ppAA films in this case) by means of electrostatic interactions. The dye associated with PNA will thus be excited and can be detected by using SPFS. In this way, the presence of a

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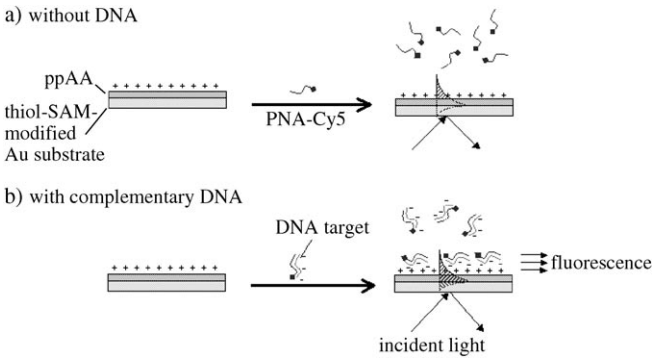


***Scheme 1.*** DNA detection by using SPFS and fluorescent PNA molecules as DNA probes.

particular target DNA can be detected without the necessity to label it. Here, the labeled PNA does not only serve as a DNA catcher (which recognizes a particular target DNA), but also as a fluorescent indicator.

In a typical experiment for detecting different DNA sequences, the fluorescently labeled PNA (that is, P1) was firstly added to different target DNA solutions, namely, T1 (fully complementary) and T2 (with one base-pair mismatch). The PNA/DNA mixture was shaken for 0.5 hours—to achieve the hybridization of PNA and DNA—and then introduced into the flow cell of the SPF spectrometer. Figure 2a shows the real-time SPFS detection of the fluorescent signal upon injection of the two different solutions. A solution of P1 (1 nmol) in phosphate-buffered saline (PBS) is used here as reference. The fluorescent intensity increases slightly after introducing P1. In the case of single-mismatch DNA (that is, T2/P1), the fluorescent signal only shows a very small increase (which is comparable to that observed for the pure P1 injection and is most likely the result of an unsuccessful hybridization between T2 and P1). On the other hand, a strong increase in the SPFS signal can be observed for the fully complementary DNA target (namely, T1/P1). This result clearly shows that the T1/P1 hybrid (now a negatively charged entity) is able to bind to the surface. Figure 2b shows the SPF spectra after injection of various sample solutions. The hybrid between PNA and the fully complementary DNA target (namely, T1/P1) exhibits a strong fluorescent signal, while that between PNA and a DNA strand with a single mismatch (namely, T2/P1) shows a fluorescent signal, which is only slightly higher than that of the reference PNA solution. It is apparent that this DNA detection method can discriminate effectively between different DNA sequences with one base-pair mismatch.

The regeneration of a sensor surface is very important for the success of a practical sensing device. As shown in Figure 2a, the fluorescent signal returned to the baseline after rinsing the sensor surface with 50 mM NaOH, which indicates that all the PNA/DNA hybrids were removed from the ppAA surface. The SPFS measurements were repeated twice on each sample and a good reproducibility could be observed, which also shows that the ppAA surfaces are reusable after regeneration.

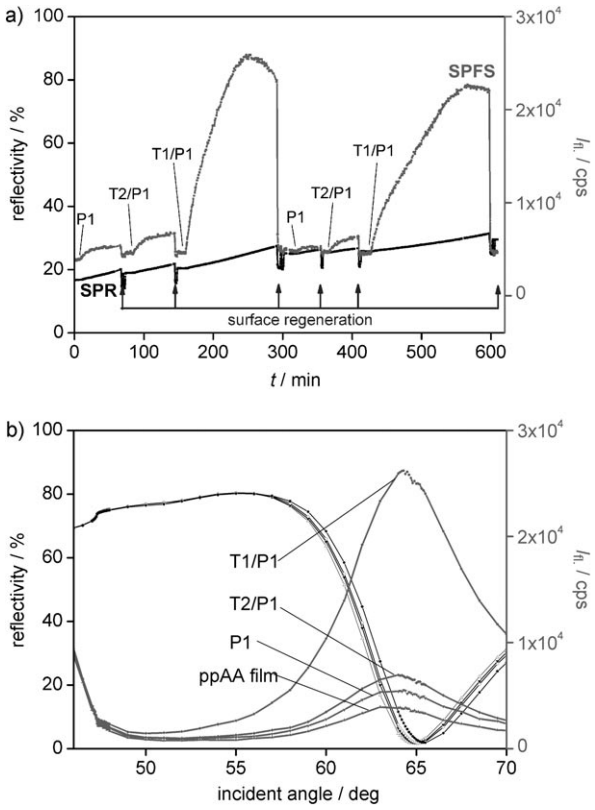


***Figure 2.*** a) SPFS kinetic measurements carried out during the addition of 1 nmol of P1, T1/P1, and T2/P1. The surface was regenerated by treating it (for 2 min) with a 50 mM NaOH solution and rinsing it with a PBS buffer. b) SPFS measurements after the adsorption of various species. The SPF spectrum of the bare ppAA surface in the PBS buffer is given as a reference.

To identify the limit of detection (LOD) of the present method, a series of T1/P1 solutions (with concentrations ranging from 100 pM to 1 nM) was tested, thereby using a 500 pM P1 solution as reference (Figure 3). Compared to the SPFS background, no additional fluorescent signals could be observed in these cases, which indicates that the nonspecific adsorption of PNA is negligible at such low concentrations. On the other hand, a 200 pM T1/P1 solution was sufficient to generate a clear increase in the SPFS signal (see the insert of Figure 3). This means that the lowest concentration that can be detected by using the present method is 200 pM. At this concentration, the initial hybridization process seems to be controlled by the diffusion of the DNA target from the bulk to the surface, thus showing a linear signal increase over time.

Since electrostatic interactions are nonspecific, other negatively charged species in the sample solution would definitely interfere with the adsorption of a specific PNA/DNA duplex on the ppAA surface. In extreme cases, such negatively charged species could occupy all the positive sites on the ppAA surface and inhibit the adsorption of the PNA/DNA complex of interest. To address this issue, a T1/P1 sample solution (1 nmol) was mixed with a negatively charged competitor DNA (a species with two base mismatches with respect to PNA). The resulting fluorescent intensity, obtained

  

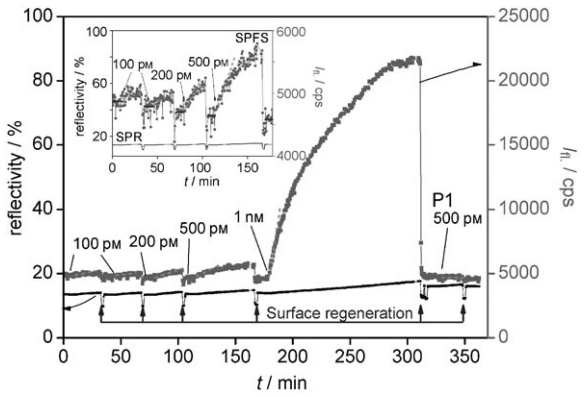


***Figure 3.*** SPFS kinetic measurements carried out during the addition of T1/P1 solutions of various concentrations (a 500 pM PNA solution was used as reference). The insert shows a magnification. The solid (——) and dashed (-----) lines represent the baseline and the slope (that is, the fluorescence increase over time), respectively, and are added as a guide for the eye. A 200 pM T1/P1 solution leads to a clear increase of the SPFS signal (this signal remains almost constant in the PBS buffer).

by using SPFS, is shown in Figure 4. An increase from 1 to 10 nmol in the concentration of the competitor DNA has

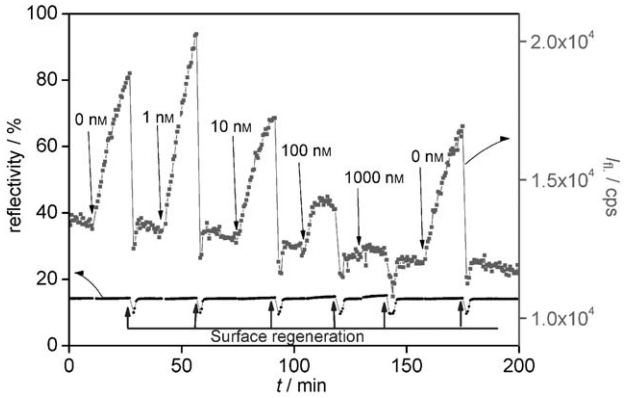


***Figure 4.*** SPFS results obtained for a mixture of T1/P1 (1 nmol) with different amounts of a competitor DNA (1–1000 nmol).

almost no effect on the SPFS signal. However, if the concentration of the competitor DNA is further increased (to 100 nmol), the fluorescent intensity decreases. Finally, when the concentration of the competitor DNA reaches 1000 nmol, the fluorescent signal almost disappears, thus indicating that the positive sites of the ppAA surface have been occupied by the competitor DNA. After a regeneration step, a PNA solution (1 nmol) was injected into the flow cell again. Interestingly, the resulting fluorescent intensity was almost the same as that observed after the first injection of PNA. It is apparent that the adsorbed DNA can be removed by the regeneration solution, thereby leaving a surface that is still active for DNA detection.

We have demonstrated a new homogeneous method for DNA detection, which takes advantage of the surface sensitivity of SPFS and the different electrostatic properties of PNA and DNA. Several advantages are offered by this method: 1) It avoids the step of immobilization of DNA probes on sensor surfaces, thus reducing the complexity and costs of sensor preparation. 2) Since commercially available PNAs are used not only as DNA probes but also as fluorescent indicators, no labeling of the target DNA is required. 3) DNA/PNA hybridization occurs in solution and is thus faster than that occurring on the sensor surface[9] (consequently, the present approach promises a rapid identification of specific genes). 4) The method has all the advantages associated with using PNA as a DNA probe, which include high affinity, specificity, and easy requirements for the buffer solution.

Since the LOD of the method is 200 pM, polymerase chain reaction (PCR) amplification of the target DNA is still needed. However, PCR amplification of the DNA target has become a routine process in DNA analysis. We believe that the present method would be particularly suitable for detecting specific genes in GMO, viral, and pathogen DNA. The detection of PCR products will be tested in the future, with a focus on the direct ascertainment of double-stranded DNA targets, by using the present concept. Moreover, multiplexed detection of oligonucleotide targets may be achieved, because a very large number of probes can be designed by using quantum dots as narrow-band spectroscopic fingerprint labels.

## *Experimental Section*

Fluorescently labeled PNA (P1: ACATGCAGTGTTGAT-Cy5) was purchased from Applied Biosystems (Foster City, CA). The single-stranded DNA (ssDNA) species used in this work—including the complementary target DNA (T1: 3′-ATCAACACTGCATGT-5′), the one-mismatch target DNA (T2: 3′-ATCAACACTACATGT-5′), and the competitor DNA (3′-ATCAACTGAACATGT-5′)—were purchased from MWG Biotech (Ebersberg, Germany). All binding reactions were carried out using a PBS solution (0.01 M) with 0.0027 M KCl and 0.137 M NaCl at pH 7.4 and 25 °C. PNA/DNA hybridization was carried out by adding 100 nmol of P1 into solutions of T1 and T2 (100 nmol). The mixture was shaken at room temperature for 0.5 hours, thus allowing a complete PNA/DNA hybridization. The substrates used for the SPFS measurements were gold-coated (50 nm, thermally evaporated) LaSFN9 slides. The allylamine monomer was purchased from Sigma–Aldrich (Germany). Deposition of ppAA films was carried out in a home-built plasma reactor under continuous-wave conditions.[7e] The plasma input power was 100 W. The monomer vapor pressure was 0.1 mbar. The soluble part of the PPAA films was removed by ethanol extraction (for 15 h).[7e] To achieve an optimized fluorescent signal in the SPFS measurements—and avoid losing too much intensity as a result of the energy transfer to the metallic substrate—the chromophores had to be sufficiently separated from the substrate surface.[8a] Here, the thickness of the ppAA film was set to 40 nm to achieve an optimum signal. The SPFS measurements were carried out at room temperature with a home-built setup, as described before.[8] The whole fluorescence detection unit was mounted on a goniometer so that its position could be fixed relative to that of the sample. The ppAA-coated slides were attached to a flow cell (made of stainless steel), which was covered by a quartz-glass window with a low intrinsic fluorescence background. A reference experiment was carried out by injecting 1 nmol of fluorescent PNA into the SPFS flow cell. Then, mixtures of PNA and the DNA target were sequentially added. The fluorescent signal

  

was recorded in real time. A 50 mM NaOH solution was added to regenerate the sensor surface.

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