Surface plasmon field-enhanced fluorescence spectroscopy in PCR product analysis by peptide nucleic acid probes

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ABSTRACT
Surface plasmon field-enhanced fluorescence spectroscopy (SPFS) was recently developed for PCR product analysis, which allowed for real-time monitoring of hybridization processes and for the detection of trace amounts of PCR products, with a detection limit of 100 fmol on the peptide nucleic acid (PNA) probe surface, and 500 fmol on the DNA probe surface. By selectively labeling the strands of PCR-amplified DNA, it was shown that the heat denaturation process in combination with the application of low-salt condition substantially reduced the interference from the antisense strands and thus simplified the surface hybridization. Furthermore, SPFS was demonstrated to be capable of quantitatively discriminating the difference induced by single nucleotide substitution, even within one minute of contact time.

INTRODUCTION
The recent development of DNA biosensors with the merits of high sensitivity and selectivity has been accelerated greatly by the achievements in the Human Genome Project (HGP). A number of biosensors, based on surface plasmon resonance (1,2), acoustic waves (3), electrochemistry (4) or fiber optics (5), have demonstrated excellent performances in oligonucleotide analysis by enabling real-time monitoring of hybridization kinetics and mismatch discrimination. However, great challenges still exist for detecting PCR-amplified DNA or genomic DNA. As analytes, PCR products differ from oligonucleotides mainly in two aspects. First, oligonucleotide targets used in most of the biosensing studies are usually <30 bases in length, while PCR products vary from 100 bp to several kb in length. The vast amount of bases is accompanied by a significantly enhanced sequence complexity and imposes tremendous difficulties in PCR product analysis. For instance, the non-selective fragments of PCR products may potentially interfere with the specific hybridization by partially matching with the surface-attached probes or by physical adherence, which usually results in high background signals in DNA biosensors (6) and genechips (7). Steric hinderance arising from the solid support and neighboring bound species is also expected to be enhanced in the presence of non-selective fragments, which considerably decreases the binding efficiency of PCR products. These length-dependent effects are presumed to account for the difficulties in biosensor applications for PCR product detection. Second, the double-stranded nature of PCR products is another major problem, because it renders the recognition unit of the sense strand inaccessible to the surface-attached probes. As a consequence, the sensitivities reported for PCR product detection are so far rather poor (mostly in the nanomolar range) (8,9), which is generally several orders of magnitude lower than that reported for oligonucleotides (in the pico-, or femtomolar range) (10). At present, only endpoint results can be offered for most of the reported techniques (11–14). These hinder an in-depth investigation of the hybridization mechanism and quantitative detection of single nucleotide polymorphisms (SNPs).

In this paper, surface plasmon field-enhanced fluorescence spectroscopy (SPFS) is employed for investigating the interfacial hybridization of PCR products, in combination with the application of peptide nucleic acids (PNAs) probes. Several advantages are expected in this study. First, combining the enormous surface plasmon field enhancement with the sensitive fluorescence technique, SPFS could be a powerful tool in detecting SNPs and investigating hybridization kinetics. Second, as mentioned earlier, the presence of the antisense strand may interfere with the hybridization of the sense strand with the surface-attached probes. By selectively labeling the sense or/and antisense DNA strand, the influence of the antisense strand can be possibly explored. Third, decorated at the N-terminus by nine ethylene glycol units and a biotin moiety, the PNA probes are immobilized on a streptavidin (SA) modified gold surface via biotin/SA interaction. According to the molecular dimensions of the SA molecule (4.5 × 4.5 × 5.3 nm³) (15), the bound PNA molecules are laterally spaced. Thus, this surface is desirable to alleviate the steric hinderance induced by neighboring sites to some extent. Finally, the uncharged pseudopeptide backbone of PNA allows for

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Nucleic Acids Research, Vol. 32 No. 22 © Oxford University Press 2004; all rights reserved

Published online December 14, 2004
the application of low salt condition (16), in which both the inter- and intra-molecular structure of PCR products can be largely eliminated after the pretreatment of heat denaturation process. In view of these factors, our work will be focused on the quantitative detection of SNPs, elucidating the hybridization phenomenon, and systematic study of the limit-of-detection (LOD) of PCR products by employing SPFS.

MATERIALS AND METHODS

Surface functional multilayer assembly

Figure 1 describes the multilayer architecture employed for the PNA probe assembly. The biotinylated thiols and OH-terminated thiols were synthesized in our group and purchased from Sigma, respectively. A mixed thiol solution was prepared in absolute ethanol with a total concentration of 0.5 mM (0.45 mM OH-terminated thiol and 0.05 mM biotinylated thiol). The gold substrates were immersed in the thiol solution for >6 h at room temperature. They were then subjected to repeated rinsing in ethanol. After being dried with a stream of nitrogen gas, the substrates can be stored in nitrogen at 4°C for several days.

SA was purchased from Sigma. Exposing the binary thiol modified gold substrates to a 1 μM SA solution, a monolayer of the tetrameric protein was attached within minutes via the interaction with the biotin moiety. The biotinylated PNA P2, PNA-11 (synthesized in our group), or DNA P2 (MWG-Biotech) can then be assembled onto the SA layer (cf. Table 1). The buffer condition used in this work is 10 mM phosphate buffer (PB, pH 7.4), containing 0.005% Tween-20 and 2 mM EDTA. High sodium concentrations were obtained by adding sodium chloride.

PCR amplification

The PCR protocol was optimized on a thermocycler (Biometra) by designing the primer sequences and adjusting the annealing temperature and concentration of each component. Recombinant plasmids (~4 kb) were used as templates for the PCR amplification. The forward and reverse primers were purchased from MWG-Biotech. As listed in Table 1, both are 30-base oligonucleotides with both calculated melting temperatures being close to each other. Unlabeled or labeled by Cy5 at the 5° end, primers were employed as needed. Unless otherwise stated, the Cy5-labeled forward primer and unlabeled reverse primer were used routinely for amplifying the PCR products with only the sense strand labeled.

All PCRs were carried out under the same conditions. Reagents for each 50 μl reaction volume included: 5 U of Taq polymerase (Amersham Biosciences), 1× PCR buffer (Amersham Biosciences), 60 pmol of the forward primer and 80 pmol of reverse primer, 0.2 mM dNTPs (Fermentas) and 100 ng of the plasmid DNA. After an initial denaturation step at 96°C for 1 min, each of the 30 cycles of amplification consisted of 30 s of template denaturation at 96°C, 30 s of primer annealing at 50°C and 30 s of primer extension at 72°C.

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Sequence</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>PNA</td>
<td>NH₃(egl)₉-TGTAATACACAATCT-GO-H</td>
<td>Biotin (N-terminus)</td>
</tr>
<tr>
<td>PNA-11</td>
<td>PNA</td>
<td>NH₃(egl)₂-GAGTCACAAGTT-COOH</td>
<td>Biotin (N-terminus)</td>
</tr>
<tr>
<td>P2</td>
<td>DNA</td>
<td>5’-TTT-GTACTCATCACAATCT-3’</td>
<td>Biotin (5’ end)</td>
</tr>
<tr>
<td>Forward primer</td>
<td>DNA</td>
<td>5’-GTACCGGACGCTGGATCCACTAGTACCGCC-3’</td>
<td>Cy5 (5’ end) if needed</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>DNA</td>
<td>5’-GCCCGGATGATCAGATGTGATCCCTCAGAAT-3’</td>
<td>Cy5 (5’ end) if needed</td>
</tr>
<tr>
<td>Sense strand of T1</td>
<td>DNA</td>
<td>5’- ... TAGTTTGAGCATCA ... -3’</td>
<td>Cy5 (5’ end)</td>
</tr>
<tr>
<td>Sense strand of T2</td>
<td>DNA</td>
<td>5’- ... TAGTTGTGAGCATCA ... -3’</td>
<td>Depending on the forward primer</td>
</tr>
<tr>
<td>Sense strand of T3</td>
<td>DNA</td>
<td>5’- ... TAGTTGTGAGCATCA ... -3’</td>
<td>Cy5 (5’ end)</td>
</tr>
</tbody>
</table>
Preparation of the analytes

The resulting 196 bp PCR products (T1, T2 and T3, cf. Table 1) were identified by agarose electrophoresis (2%). As shown in Table 1, the three PCR products contain a 15-base recognition sequence present in the middle of the sense strand approximately, which fully matches or exhibits a one-base/two-base mismatch with PNA P2, respectively. Ethanol precipitation was performed afterwards in order to remove the salts, dNTPs, excess primers and enzymes from the PCR sample. For 50 μl of PCR sample, 125 μl ethanol (Sigma) and 5 μl of sodium acetate (3 M) were added. The mixture was shaken, and then kept at −20°C for >6 h to induce precipitation. The PCR products were collected by centrifugation at 16 000 g for ~0.5 h. The ethanol precipitation was empirically examined to effectively purify the PCR products, even at a large sample scale.

Since all the PCR products used in this paper are end-labeled by Cy5 in the sense or/and antisense strand, their concentrations can be determined by recording the adsorption of Cy5 at λ ~650 nm by the following equation:

\[
[\text{Cy5}] = (A_{650})/250000
\]

assuming a molar extinction coefficient of ε = 250 000 M⁻¹ cm⁻¹, as provided by the manufacturer (Amersham Biosciences).

The PCR products underwent the following denaturation process prior to the biosensing studies, unless otherwise stated: the heat denaturation is composed of three steps, heating the DNA sample (in 10 mM PB buffer) at 96°C for several minutes, quickly quenching it to 0°C in an ice-water mixture and returning to room temperature. LOD assessments

The detection limit of PCR products was assessed on the PNA probe layer. Figure 3 presents the hybridization of PCR T2 to a PNA P2 surface over a wide range of concentrations. Each injection of the sample solution was followed by a certain period of time for interaction and then by a surface regeneration step by rinsing the cell with a 10 mM NaOH solution for ~1 min. The probe surface demonstrated excellent robustness, sustaining repetitive regeneration for at least 30 times. At such low target concentrations, the initial binding process is limited by the target diffusion from the bulk to the surface, displaying a linear signal increase with time (at a constant sample solution flow). The slope of the fluorescence signal (R) increase is...
proportional to the analyte concentration $c_0$, given by the following equation:

$$\frac{dR}{dt} = k_m c_0^2$$

where $k_m$ is the mass-transport rate constant. A calibration curve is hence obtained by plotting the binding slopes versus the analyte concentrations. The LOD is reached if the calibration curve intersects with the horizontal line, given by the baseline stability. The baseline stability is defined as three times the standard deviation of measurements of the time-dependent fluorescence intensity at $c_0 = 0$, which is typically $\sim 10$ cps min$^{-1}$ for our present SFS system. The calibration curve intersects with the baseline deviation level and gives the detection limits of PCR T2 at 100 fmol. This value is quite comparable with that of oligonucleotide target (data not shown), although the PCR product deserves a slower mass-transport rate and hence a poorer detection limit. The unexpected low LOD of the PCR product probably benefits from the largely reduced surface quenching effect. As both theoretically (18) and experimentally (19) demonstrated, the dyes carried by oligonucleotide targets are still subjected to metal-induced surface quenching upon hybridization, as a result of the short sequence length and the limited separation distance ($\sim 5–6$ nm) imposed by the biotin-SA multi-layer structure. In contrast, labeled at the end of the 196-base sense strand of PCR T2, the dye can be spaced away from the metal surface with a considerably larger distance, and thereby deserves a higher fluorescence yield because of less quenching.

As a comparison, the LOD of PCR products was also assessed on the DNA probe layer. As presented in Figure 4, a sodium concentration of 450 mM was applied in the hybridization of PCR products on the DNA P2 surface in order to overcome the electrostatic barrier of DNA/DNA strands. The extrapolated LOD was $\sim 500$ fmol, higher than that obtained on the PNA P2 surface ($\sim 100$ fmol). We attribute it to the promoted re-annealing effect in the high salt condition. The re-annealing of PCR products depletes the effective sense DNA strands for surface hybridization and accounts for the ascended LOD concentration. The re-annealing effect was also reflected by the kinetic curves in Figure 4. The fluorescence signals were shown to be equilibrated after the linear increase in a short period of time, as a result of the reduced concentration...
of the free sense strand. All these again revealed the importance of low salt condition in PCR product hybridization.

SNP discrimination

Figure 5 [(A) and (B)] presents the hybridization of PCR T2 and T1 to the PNA P2 surface at 10 mM sodium concentration, respectively. Upon equilibrium, the fluorescence signal obtained by the fully complementary sequence is higher than that with one-base mismatch (A/C mismatch) by a factor of ∼4.5 (∼7 by subtracting the signal from the non-selective binding). The binding kinetics suggests that the SNP can be possibly distinguished even after only 1 min of contact time, with the signals differing by a factor of ∼3.5 with the non-selective binding subtracted (cf. inset of Figure 5). The signal change induced by the one-base substitution occurring in the recognition unit is rather pronounced and reproducibly detected.

The two-base mismatched situation was checked by the hybridization of PCR T3 with PNA P2, shown in Figure 5C. The resulting hybridization signal was found to be slightly higher than that obtained by the control experiment (cf. Figure 5D), in which PCR T2 was allowed to hybridize with non-complementary PNA-11. This is in accordance with that observed in oligonucleotide hybridization (17), i.e. the affinity of the recognition sequence was substantially reduced by the two-base mismatch and even close to that of the non-specific binding. The non-specific binding of PCR products arises not only from physical adhesion, but also possibly from the partial hybridization of non-selective DNA fragments with the surface probes. It has the tendency to be enhanced with the increase in sequence length and complexity, which, in most cases, can be largely eliminated by performing the hybridization reactions at a higher temperature (∼30–40°C) (16).

CONCLUSIONS

In this work, SPFS fulfilled the requirements for real-time and in situ analysis of surface hybridization of PCR products with an excellent sensitivity. Working under mass-transport controlled hybridization conditions, the detection limits of PCR products were assessed to be 100 fmol for PNA probes and 500 fmol for DNA probes, respectively. Moreover, SPFS demonstrates rapid and quantitative identification of SNPs, which holds the potential for the development of a new generation of DNA biosensors.

The outstanding sensitivity of the SPFS technique gives new insights into the molecular details of the hybridization behavior of PCR products, which is of significance in fundamental research and largely unexplored so far. In contrast to DNA probes, the neutral backbone of PNA probes allows for the application of a relatively low salt condition. By labeling the sense and/or the antisense strand, it was revealed that only a minority of the antisense strand participated in the surface hybridization, with the low salt condition applied (10 mM Na+). This provides us with a clear hybridization profile, and holds the promise for quantitative interpretation of the corresponding hybridization kinetics in the future.

ACKNOWLEDGEMENTS

This work was supported by an EU grant (QLKI-2000-31658, DNA-track) and by the Deutsche Forschungsgemeinschaft (KN 224/13-1).

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