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DNA-Binding Small-Ligand-Immobilized Surface Plasmon Resonance Biosensor for Detecting Thymine-Related Single-Nucleotide Polymorphisms

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Abstract: A surface plasmon resonance (SPR) biosensor that carries DNA-binding small ligands has been developed for the detection of single-nucleotide polymorphisms (SNPs). 3,5-Diaminopyrazine derivatives, with a hydrogen-bonding profile fully complementary to the thymine base, were utilized as recognition elements on the sensor surface, and a target single-stranded DNA sequence was hybridized with a DNA probe containing an abasic site to place this site opposite a nucleobase to be detected. In a continuous flow of sample solutions buffered to pH 6.4 (0.25 M NaCl), the 3,5-diaminopyrazine-based SPR sensor can detect an orphan nucleobase in the duplex with a clear selectivity for thymine over cytosine, guanine, and adenine (5'-GTT GGA GCT GXG GGC GTA GGC-3'/3'-CAA CCT CGA CNC CCG CAT CCG-5'; X = abasic site, N = target nucleobase G, C, A, or T). The SPR response was linear in the concentration range 10–100 nM. Allele discrimination is possible based on the combination of different binding surfaces in a flow cell of the SPR system, which is demonstrated for the analysis of the thymine/cytosine mutation present in 63-meric polymerase chain reaction (PCR) amplification products (*Ha-ras* gene, codon 12, antisense strand). Comparison with a bulk assay based on 3,5-diaminopyrazine/DNA binding shows that the immobilization of 3,5-diaminopyrazine derivatives on the SPR sensor allows more sensitive detection of the target DNA sequence, and binding selectivity can be tuned by controlling the salt concentration of sample solutions. These features of the DNA-binding small-molecule-immobilized SPR sensor are discussed as a basis for the design of SPR biosensors for SNP genotyping.

Keywords: analytical methods · biosensors · DNA recognition · ligand design · surface plasmon resonance

Introduction

Surface plasmon resonance (SPR) has become a promising optical technique for studying biomolecular interactions. A wide range of applications have been enabled by SPR biosensors, including drug screening, kinase analysis, and antibody development.[1,2] Of particular interest to us is the development of SPR biosensors for gene detection, especially single-nucleotide polymorphism (SNP) genotyping. Most approaches to this end have employed hybridization adsorption, in which complementary probe DNA molecules are immobilized on the sensor surface to detect single-base mutations in DNA.[3–6] Significant efforts have been devoted to achieve sufficient selectivity and sensitivity, based on a combination of coupled bioconjugates,[4] DNA-modified gold nanoparticles,[5] and/or surface enzyme reactions.[6] On the other hand, we have taken a different approach to the design of SPR biosensors for SNP genotyping, which is based on the use of DNA-binding small molecules immobilized on the sensor surface. Although there are a few reports on this class of SPR biosensor for gene detection, some novel examples have been recently reported by Nakatani et al.[7] In their approach, small molecules that target mismatched base pairs in DNA duplexes were utilized as recognition elements, and SPR biosensors that detect G/G, G/A, and C/C mismatches in hetroduplexes or trinucleotide repeats were successfully developed. Herein, we utilized synthetic small molecules designed to recognize an orphan nucleobase opposite an abasic (AP) site in DNA duplexes (Figure 1 A). In our approach, a target single-stranded DNA sequence is hybridized with a DNA probe containing an abasic site to place this site opposite the nucleobase to be detected, and the immobilized ligand selectively binds to the orphan (target) nucleobase in the duplex of the target DNA and probe DNA sequences. The binding event on the sensor surface can be clearly monitored through a change in SPR signals, thus enabling the detection of the single-base mutation. The key to the present sensor is that the mutation nu-

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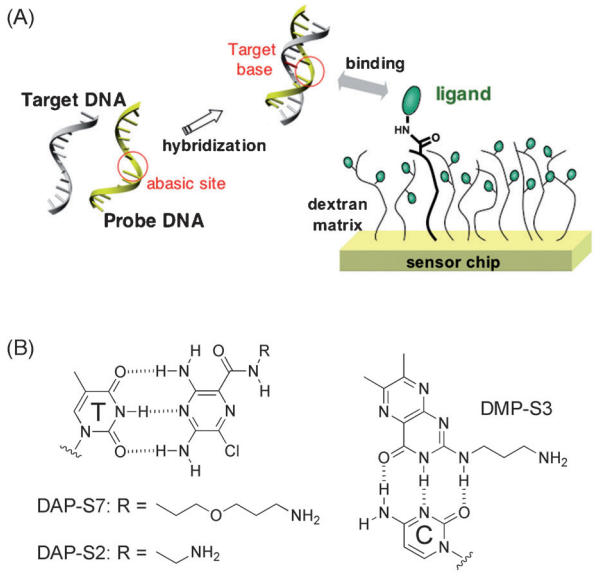

Figure 1. A) Illustration of ligands that bind abasic sites immobilized on the sensor surface. Ligands with an active amino group are immobilized covalently by coupling to a carboxylic acid group of the dextran matrix on the surface. For SNP detection, a DNA probe containing an abasic site is hybridized with a target DNA sequence to place the abasic site facing a target nucleotide, thus providing a binding pocket for ligands to recognize intrahelical target nucleotides. B) Chemical structures of ligands that bind abasic sites utilized in this study.

cleobase is directly detected by binding with ligands immobilized on the sensor surface. We prepared 3,5-diaminopyrazine derivatives with an active amino group for covalent immobilization onto the sensor surface (Figure 1B), and the resulting SPR sensor is capable of detecting the orphan nucleobase in the duplex with a clear selectivity for thymine over cytosine, guanine, and adenine. The sensor is, therefore, applicable to the detection of a thymine-related single-base mutation. Allele discrimination is demonstrated for the analysis of the thymine/cytosine mutation, for which a cytosine-selective sensor that carries a dimethylpteridine derivative (DMP-S3; Figure 1B) is coupled with the thymine-selective sensor. Significantly, as compared with the bulk assay based on 3,5-diaminopyrazine/DNA binding, the immobilization of 3,5-diaminopyrazine derivatives on the SPR sensor allows more sensitive detection of the target DNA sequence, and binding selectivity can be tuned by controlling the salt concentration of sample solutions. Herein, we present these features of the DNA-binding small molecule-immobilized SPR sensor for SNP detection.

Results and Discussion

In our previous reports,[8–10] we found a series of fluorescent aromatic ligands that can selectively bind to an orphan nucleobase opposite the abasic site in DNA duplexes (compare with Figure 1A). In contrast to typical DNA-binding ligands capable of targeting double-stranded DNA sequences by intercalation or groove binding, the identified aromatic ligands typically targeted non-Watson–Crick base-pairing sites in DNA duplexes, in which the binding was promoted selectively by pseudo-base pairing along the Watson–Crick edge of the intrahelical target nucleobases. The analysis of a single-base mutation could be performed based on the binding-induced fluorescent signaling, for which a target single-stranded DNA sequence was hybridized with a DNA probe containing an abasic site to place this site opposite the nucleobase to be detected. As for thymine binding and sensing,[9] 3,5-diaminopyrazine derivatives, with a hydrogen-bonding profile fully complementary to thymine (Figure 1B), were found to work with a micromolar range of binding affinity (dissociation constant for 3,5-diamino-6-chloro-2-pyrazine carbonitrile $K_d = 2.6\ \mu M$[9b]).

Given such an ability to bind to the thymine base, we prepared two 3,5-diaminopyrazine derivatives DAP-S2 and DAP-S7 (Figure 1B), in which an active amino group was introduced to the pyrazine ring through spacers of different length for covalent immobilization onto the sensor surface. These ligands were synthesized by reaction of methyl 3,5-diamino-6-chloropyrazine-2-carboxylate with ethane-1,2-diamine or 3-(3-aminopropoxy)propan-1-amine. The ligands thus prepared were immobilized onto the sensor chip (CM5; Biacore) by coupling to the carboxylic acid group on its surface.

First, we examined the effect of spacers attached to the pyrazine ring on SPR responses. Figure 2 shows typical responses to 21-meric DNA duplexes containing an abasic site, as measured in a continuous flow of sample solutions with the Biacore 3000 system. As expected from the binding properties of 3,5-diaminopyrazine derivatives in bulk solution, both sensors exhibit a selective response to thymine over cytosine, guanine, and adenine, thus indicating that the immobilized ligands are working on the sensor surface. However, the magnitude of the SPR responses strongly depends on the spacer of the ligands. The response of the

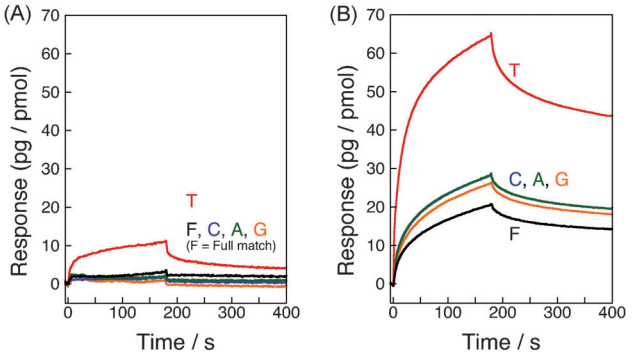

Figure 2. SPR responses to 21-meric DNA duplexes containing abasic sites (1.0 μM, 90 μL) obtained by ligand-immobilized sensors: A) DAP-S2 and B) DAP-S7. The response represents the amount of DNA (pg) bound to a unit amount of each ligand (pmol) immobilized on the sensor surface. Measurements were carried out at 5°C in a continuous flow (30 $\mu L\,min^{-1}$) of a running buffer (pH 7.4, 0.01 M HEPES, 3.0 mM EDTA, 0.005% surfactant P20, 0.15 M NaCl).

DAP-S2-immobilized sensor is only moderate (11 pg mol^{-1} at 180 s after injection of DNA), even for a relatively high concentration of the DNA sample (1.0 μM; Figure 2A). In contrast, an effective response up to 64 pg mol^{-1} is obtained for thymine by another sensor based on DAP-S7, which has a longer spacer (Figure 2B). The binding event on the sensor surface is, therefore, highly sensitive to the spacer length, probably for steric reasons, which has been discussed for the SPR sensors with immobilized naphtyridine dimers for the detection of the G/G mismatch.[7d]

Interestingly, the response properties of the DAP-S7-based sensor can be tuned by controlling the salt concentration of the solutions of DNA. Again, considerable responses are observed for guanine, cytosine, and adenine, thus resulting in relatively moderate selectivity for thymine in solutions containing 0.15 M NaCl (Figure 2B). At higher salt concentrations, however, the selectivity becomes better, which is accompanied by a decrease in the SPR responses (see Figure S1 in the Supporting Information). Significantly, the sensor shows a highly selective response to thymine in solutions containing 0.50 M NaCl (Figure 3A), whereas useful selectivity and sensitivity are obtained in solutions containing 0.25 M NaCl (Figure 3B). At a salt concentration of 0.25 M, a selective response to thymine is effectively obtained, even at a concentration of 10 nM of the sample solution (responses at 180 s after an injection of DNA (measured in resonance units; RU): (11±0.6), (4.8±0.2), (3.8±0.4), and (3.3±0.1) RU for T, G, A, and C, respectively; fully matched duplex: (1.6±0.3) RU; $n=3$), and the SPR signal is linear in the concentration range 10–100 nM (Figure 3B). The observed sensitivity is almost comparable to that of unamplified imaging SPR based on DNA or RNA hybridization adsorption (detection limit: 10 nM for 18-meric oligos)[3d] and is superior to that of unamplified scanning SPR for DNA detection (detection limit: 150 nM for 143-meric/101-meric duplex).[3c]

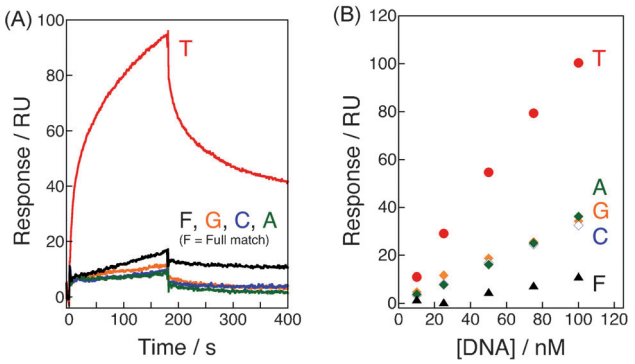

Figure 3. Effect of NaCl concentration on SPR responses of a DAP-S7-immobilized sensor to 21-meric DNA duplexes containing abasic sites. Measurement were carried out at 5°C in a continuous flow (30 μL min^{-1}) of a running buffer (pH 6.4, 1/15 M phosphate buffer, 3.0 mM EDTA, 0.005% surfactant P20). A) Sensorgram for 1.0 μM duplexes obtained in solutions containing 0.50 M NaCl. Ligand-immobilized: 2180 RU. B) Concentration dependence of SPR responses (at 180 s after injection of DNA) obtained in solutions containing 0.25 M NaCl. Ligand-immobilized: 1590 RU.

It is also of interest to note that this sensitivity has not been obtained in homogeneous solutions for the 3,5-diaminopyrazine/DNA binding,[9] in which an effective fluorescent response was obtained in the concentration range roughly equivalent to the dissociation constant (that is, in the micromolar range). The amidation of 3,5-diaminopyrazine derivatives has little effect on the binding affinity, which is confirmed for methyl 3,5-diamino-6-chloropyrazine-2-carboxylate and 3,5-diamino-6-chloropyrazine-2-carboxamide. In solutions buffered to pH 7.0 (110 mM Na^+, 5°C), the dissociation constants of these ligands are 1.0 and 1.9 μM, respectively, when binding to orphan thymine in the duplex (5'-GTGTG CGTTG ATA TGGAC GCAGA-3'/3'-CACAC GCAAC TXT ACCTG CGTCT-5'; X = abasic site). The binding affinities are also comparable to the affinity of 3,5-diamino-6-chloro-2-pyrazine carbonitrile (K_d = 2.6 μM).[9b] Thus, the relatively high sensitivity we obtained in the present heterogeneous assay may be assisted by an inherent property of the SPR system to sense a change in surface mass concentration on the sensor chip (1000 RU ≈ 1 ng mm^{-2} for most biomolecules),[2b] and the binding of higher molecular weight analytes would give a larger response in RU. In addition, it is likely that a characteristic feature of surface-binding events in the continuous-flow system works effectively here, resulting in an apparent increase in the binding affinity of the immobilized 3,5-diaminopyrazine derivatives. This effect, one of the so-called mass-transport effects,[2a] can be seen from the dependence of the sensorgram on the surface-binding capacity (Figure 4). A box-shaped response to thymine is observed with a sensor chip that carries low amounts of ligand (79 RU; Figure 4A), thus indicating that a rapid interaction is taking place to reach the steady state at any DNA concentration. The resulting titration curve is well explained by 1:1 binding with K_d = (73±12) μM (Figure 4B; $n=3$). As the amount of ligand is increased (Figure 4C), the shape of the sensorgram response changes, at which the rate for the interaction seems to be slower due to limited analyte transport from bulk solution to the sensor surface. Under such mass-transport limiting conditions, the apparent binding affinity for thymine is roughly estimated to be K_d = 130 nM (Figure 4D; ligand-immobilized: 1590 RU), and a sensitive detection becomes possible with a linear response at a lower concentration range (compare with Figure 3B).

Further studies were carried out to demonstrate and evaluate the performance of the DAP-S7-based sensor for potential applications. First, an examination of the effect of flanking nucleobases (5'-qXz-3'/3'-q'Tz'-5'; X = abasic site) on SPR responses reveals that the response depends on the nucleobases that flank the abasic site (see Figure S2 in the Supporting Information), thus indicating that the binding affinity to thymine is influenced by nucleobases adjacent to the abasic site. When 21-meric DNA duplexes (200 nM, 60 μL) were analyzed by the sensor chip carrying 1640 RU of DAP-S7, the largest SPR response up to 563 RU is ob-

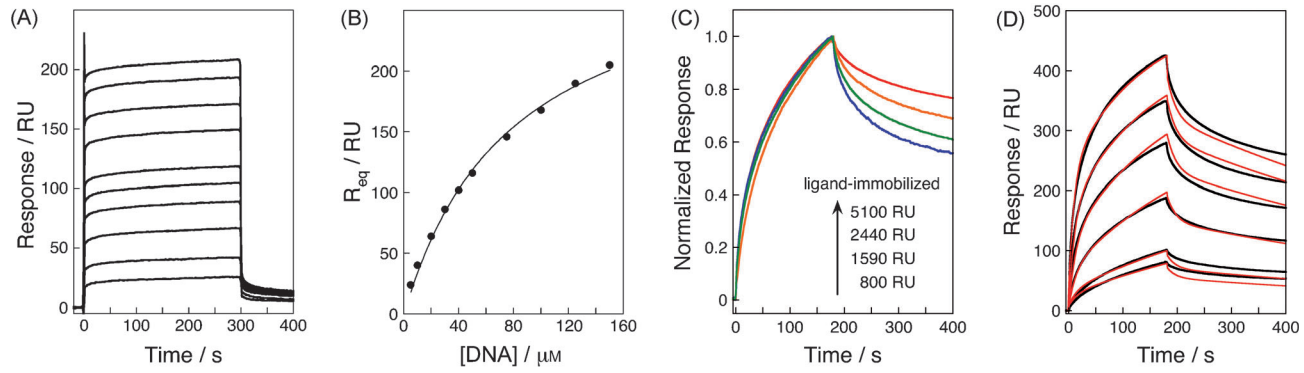

Figure 4. A) SPR responses of DAP-S7-immobilized sensor (79 RU) to thymine in the 21-meric DNA duplex containing an abasic site (5.0–150 μM). Measurements were carried out at 5 °C in a continuous flow (30 μL min^{-1}) of a running buffer (pH 6.4, 1/15 M phosphate buffer, 3.0 mM EDTA, 0.005 % surfactant P20, 0.25 M NaCl). DNA duplex: 5'-GTT GGA GCT GXG GGC GTA GGC-3'/3'-CAA CCT CGA CTC CCG CAT CCG-5' (X = abasic site; C3 spacer). B) Binding isotherm for the DAP-S7/thymine interaction on the sensor surface. The steady-state binding level in RU (RU_{eq}) was plotted as a function of DNA concentration, and the resulting titration curve was analyzed by a nonlinear fitting based on a 1:1 binding model. C) Effect of the amount of immobilized DAP-S7 on SPR responses to thymine in the 21-meric DNA duplex containing an abasic site (250 nM). Experimental conditions are same as given in Figure 4 A. D) Analysis of SPR responses to 21-meric DNA duplexes (75, 100, 250, 500, 750, 1000 nM) obtained by the DAP-S7-immobilized sensor (1590 RU). Black lines: measured, red lines: fitted. The observed SPR responses were analyzed by using BIAevaluation with a two -state reaction model.

tained for 5'-TXG-3'/3'-ATC-5', whereas the response is the smallest for 5'-GXC-3'/3'-CTG-5' (236 RU). However, the sensor keeps the thymine selectivity for both duplexes, and the SPR signal is distinct enough to detect the target thymine base with considerable reliability (see Figure S3 in the Supporting Information). Thus, irrespective of the nucleobases that flank the abasic site, the present sensor is suitable for the selective detection of thymine in DNA duplexes containing an abasic site. An examination of the effect of pH value of the sample solutions (see Figure S4 in the Supporting Information) reveals that between pH 5.6 and 8.0, the magnitude of the SPR response is maintained for thymine without a loss of the response selectivity. Thus, the sensor can be used in this pH range, and it can be combined with other ligand-immobilized sensors for allele discrimination (see below). The lifetime of the present sensor is also good for repeated measurements (see Figure S5 in the Supporting Information). A preliminary check reveals that during 20 cycles of continuous measurements no decrease in the SPR responses was observed for the DNA samples, and there was no indication of instability of the ligand molecules immobilized on the sensor surface.

The thymine-selective DAP-S7-based sensor was coupled with a cytosine-selective sensor that carries a derivative of dimethylpteridine, DMP-S3 (Figure 1 B), as a DNA recognition element. It was previously demonstrated that a parent dimethylpteridine moiety selectively bound to guanine opposite the AP site in the DNA duplexes (K_d = 160 and 3000 nM for G and C, respectively, in 110 mM Na^+ at pH 7.0 and 5 °C),[8d] and the guanine-related single-base mutation was successfully analyzed by using this ligand.[8g] Interestingly, the introduction of an aminopropyl group at the C2 amino nitrogen atom of the parent pteridine species results in an enhancement of binding affinity for cytosine in bulk solutions (K_d = 240 and 450 nM for G and C, respectively, in 110 mM Na^+ at pH 7.0 and at 5 °C), and a DMP-S3-immobilized sensor shows clear selectivity for cytosine over other nucleobases (see Figures S6 and S7 in the Supporting Information). Examination of the salt dependence of the SPR responses (Figure S6) and the effect of pH value of the sample solutions (Figure S7) shows that the DMP-S3-based sensor works well in solutions buffered to pH 5.8 (0.25 M NaCl). Therefore, the DMP-S3-based sensor can be used simultaneously with the DAP-S7-based sensor under identical experimental conditions. Here, two different binding surfaces, that is, flow channel (FC), were prepared in the microfluidic pathway of Biacore 3000, in which DMP-S3 and DAP-S7 were immobilized in FC2 and FC3, respectively (Figure 5 A). The resulting sensor that carries both DMP-S3 and DAP-S7 indeed works well in solutions buffered to pH 5.8 (0.25 M NaCl) and is applicable to the detection of the thymine/cytosine mutation. Here, four types of 21-meric DNA duplex (T homozygote, C homozygote, C/T heterozygote, and a fully matched duplex) were analyzed as a model system. For the FC3 that carried DAP-S7, the largest response was obtained for the T homozygote (T/T) and the response dropped on the order of heterozygote > C homozygote (C/T > C/C; Figure 5 B). In contrast, the order of the SPR response is reversed for the FC2 immobilised with the cytosine-selective DMP-S3 (i.e., C/C > C/T > T/T). By plotting these SPR responses in a scatter plot (Figure 5 C), three separated clusters are obtained, thus representing the three genotypes of samples C/C, C/T, and T/T. The sensor that carries DMP-S3 and DAP-S7 was finally applied to the analysis of the single-base mutation present in 63-meric DNA sequences (*Ha-ras* gene,[11] codon 12, antisense strand) obtained by asymmetric PCR amplification. In addition to the wild-type (GCC) and 3 mutant-type samples (GGC, GAC, GTC), a total of 12 samples (3 T homozygotes, 3 C homozygotes, 3 heterozygotes, and 3 controls) were prepared for

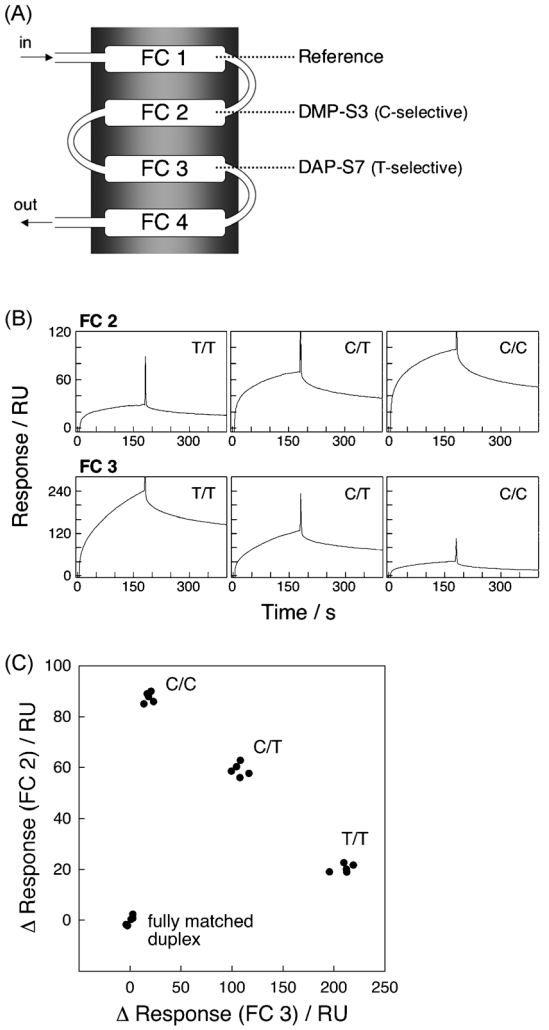

Figure 5. A) Illustration of the microfluidic pathway of Biacore 3000. Flow-channel 1 (FC1) was used as a reference, and DMP-S3 and DAP-S7 were immobilized in FC2 and FC3, respectively. B) SPR responses to 21-meric DNA duplexes (500 nM). Measurements were carried out at 5°C in a continuous flow (30 μL min^{-1}) of a running buffer (pH 5.8, 1/15 M phosphate buffer, 3.0 mM EDTA, 0.005% surfactant P20, 0.25 M NaCl). Ligand-immobilized: 260 and 1230 RU for DMP-S3 and DAP-S7, respectively. DNA duplexes containing abasic sites: 5'-GTT GGA GCT GXG GGC GTA GGC-3'/3'-CAA CCT CGA CNC CCG CAT CCG-5' (X = abasic site; C3 spacer, N = T or C), fully complementary duplex: 5'-GTT GGA GCT GAG GGC GTA GGC-3'/3'-CAA CCT CGA CTC CCG CAT CCG-5'. Each type of sample (C/C, C/T, or T/T) was independently injected into the microfluidic pathway, and the time was set at 0 s when the sample was loaded into each flow channel (FC2 or FC3). C) Scatter plot of SPR responses (at 180 s) to the 21-meric DNA duplexes. A total of 20 model samples (5 T homozygotes, 5 C homozygotes, 5 C/T heterozygotes, and 5 fully matched duplexes) were analyzed. The experimental conditions are same as given in Figure 5B.

the detection of the thymine/cytosine mutation. These PCR samples were purified to decrease nonspecific responses due to the biological background and were analyzed at 5°C in a continuous flow (20 μL min^{-1}) of a buffer solution (pH 5.8) containing 0.25 M NaCl and 500 nM 20-meric DNA probe containing AP sites. The DAP-S7-based sensor exhibits a selective response to a T-containing mutant-type sequence (GTC) over the other sequences (GCC, GAC, GGC; Figure 6A), whereas a selective response to a C-containing wild-type sequence (GCC) is obtained for the DMP-S3-based sensor (Figure 6B). As was demonstrated for the model system (Figure 5C), three genotypes of samples C/C, C/T, and T/T can be clearly distinguished in a scatter plot of SPR responses of the sensor with DMP-S3 and DAP-S7 (Figure 6C). In contrast to conventional hybridization-based SPR assays, the immobilized ligands ensure the selective detection of the single-base mutation, and therefore the present system decreases the labor needed to design probe DNA sequences and to provide careful control of the temperature for the probe-hybridization step.

Conclusion

In summary, a SPR sensor that carries DNA-binding small ligands has been developed for the analysis of single-nucleotide polymorphisms. We utilized one type of ligand that binds an abasic site as a recognition element and found that the 3,5-diaminopyrazine derivative DAP-S7-immoilized sensor could detect a thymine base with sufficient affinity and selectivity. Allele discrimination was demonstrated for the thymine/cytosine mutation present in 63-meric PCR amplification products (*Ha-ras* gene, codon 12, antisense strand), for which a cytosine-selective sensor that carries a derivative of dimethylpteridine DMP-S3 was coupled with the thymine-selective sensor. In contrast to conventional SPR biosensors based on hybridization adsorption, the selectivity and sensitivity of the present sensor originated from surface binding events of immobilized ligands that could recognize a nucleobase to be detected, with the help of DNA probes containing abasic sites to provide a binding pocket in the resulting duplexes. Therefore, this study has presented the use of synthetic small molecules as a tool for the design of SPR biosensors for SNP genotyping, and this approach offers considerable design flexibility when used in combination with currently available techniques, such as DNA-modified gold nanoparticles to increase the response sensitivity. We are now undertaking further studies to prepare a set of ligands that bind abasic sites that are suitable for SPR-based SNP genotyping systems.

Experimental Section

Materials: DNA samples used in the present study were custom synthesized and purified by using HPLC (>97%) by Nihon Gene Research Laboratories Inc. (Sendai, Japan). For the synthesis of DNA probes containing abasic (AP) sites, a propyl linker (C3 spacer) was utilized. The concentration of DNA was determined from the molar extinction coefficient at $\lambda = 260$ nm.[12] Unless otherwise mentioned, the SPR binding studies were done with 21-meric duplexes containing AP sites (5'-GTT GGA GCT GXG GGC GTA GGC-3'/3'-CAA CCT CGA CNC CCG CAT CCG-5', X = AP site, N = G, C, A, or T) or fully complementary duplexes (5'-GTT GGA GCT GAG GGC GTA GGC-3'/3'-CAA CCT CGA CTC CCG CAT CCG-5'). Water was deionized (≥18.0 MΩ cm specific resistance) by using an Elix 5 UV Water Purification System and

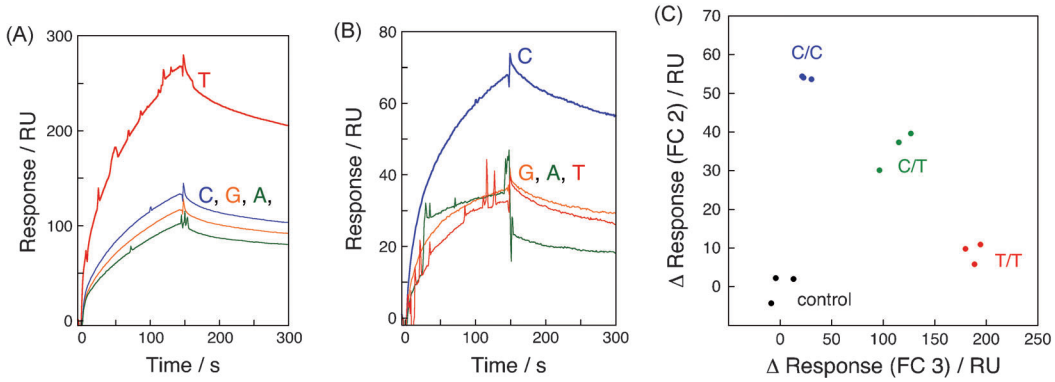

Figure 6. Analysis of PCR products (*Ha-ras* gene, codon 12, antisense strand) by ligand-immobilized sensors: A) DAP-S7 (1300 RU) and B) DMP-S3 (104 RU). C) Scatter plot for C > T genotyping (*Ha-ras* gene, codon 12, antisense strand) based on SPR responses of the sensors carrying DMP-S3 (FC2) and DAP-S7 (FC3). A total of 12 samples (3 T homozygotes, 3 C homozygotes, 3 C/T heterozygotes, and 3 controls) were analyzed. Each type of sample (C/C, C/T, T/T, or control) was independently injected into the microfluidic pathway, and SPR responses at 150 s in each FC are plotted. Ligand-immobilized: 243 RU (DMP-S3) and 1370 RU (DAP-S7).

a Milli-Q Synthesis A10 system (Millipore Corp., Bedford, MA, USA). The other reagents were commercially available, analytical grade, and used without further purification.

Synthesis of 3,5-diamino-*N*-(2-aminoethyl)-6-chloropyrazine-2-carboxamide (DAP-S2): This compound was synthesized according to a reported procedure.[13] A mixture of methyl 3,5-diamino-6-chloropyrazine-2-carboxylate (610 mg, 3.0 mmol) and ethane-1,2-diamine (10 mL) was heated at 100 °C for 24 h. The excess diamine species was removed under vacuum, and the residue was purified by recrystallization from 2-propanol to give the product as a pale brown solid (420 mg, 61 %). ^{1}H NMR ([D$_6$]DMSO, 500 MHz): δ = 7.88 (t, 1 H, J = 6.0 Hz), 6.96 (s, 2 H), 3.18 (q, 2 H, J = 6.5 Hz), 2.64 ppm (t, 2 H, J = 6.5 Hz); HRMS (ESI) for $C_7H_{12}ClN_6O$: calcd: 231.0761 [M+H$^+$]; found: 231.0756.

Synthesis of *N*-[3-(3-aminopropoxy)propyl]-3,5-diamino-6-chloropyrazine-2-carboxamide (DAP-S7): This compound was synthesized analogously to **DAP-S2** from methyl 3,5-diamino-6- chloropyrazine-2-carboxylate (100 mg, 0.50 mmol) and 3-(3-aminopropoxy)propan-1-amine (1 mL). The excess diamine species was removed under vacuum, and the residue was purified by recrystallization from 2-propanol to give the product as a pale brown solid (18 mg, 12 %). ^{1}H NMR ([D$_6$]DMSO, 500 MHz): δ = 7.93 (t, 1 H, J = 6.0 Hz), 6.96 (s, 2 H), 3.42–3.38 (m, 4 H), 3.25 (q, 2 H, J = 6.5 Hz), 2.58 (t, 2 H, J = 6.5 Hz), 1.70 (m, 2 H), 1.58 ppm (m, 2 H); HRMS (ESI) for $C_{11}H_{20}ClN_6O_2$: calcd: 303.1336 [M+H$^+$]; found: 303.1331.

Synthesis of 2-(3-aminopropylamino)-6,7-dimethylpteridin-4(3*H*)-one (DMP-S3): This compound was synthesized according to a previous report.[14a] A mixture of 4-hydroxy-2-methylthio-6,7-dimethylpteridine (270 mg, 1.2 mmol)[14b,c] and 1,3-diaminopropane (1 mL) was heated at 100 °C for three days. Diethyl ether (7 mL) was added to the mixture, and the resulting precipitate was washed with diethyl ether. The precipitate was further purified by recrystallization from water to give the product as a pale yellow solid (80 mg, 7 %). ^{1}H NMR (D_2O, 270 MHz): δ = 3.38 (t, 2 H, J = 6.3 Hz), 2.89 (t, 2 H, J = 6.8 Hz), 2.38 (s, 3 H), 2.37 (s, 3 H), 1.80 ppm (quin, 2 H); HRMS (ESI) for $C_{11}H_{17}N_6O$: calcd: 249.1464 [M+H$^+$]; found: 249.1458.

Preparation of ligand-immobilized sensor chips and SPR measurements: SPR measurements were performed with a Biacore 3000 system (Biacore, Uppsala, Sweden). In this system, the SPR signal was a measure of the SPR angle and the response was expressed in resonance units (RU; 1000 RU is approximately equivalent to a change of 1 ng mm^{-2} in surface-protein concentration).[2b] Immobilization of ligands on the sensor chip CM5 (carboxymethylated dextran surface; Biacore) was carried out by using an amine-coupling kit (Biacore). A typical procedure was as follows: A solution (50 μL) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC; 0.4 M) and *N*-hydroxysuccinimide (NHS; 0.1 M) was injected in a continuous flow (5 μL min^{-1}, 20 °C) of HEPES-buffered saline with ethylenediaminetetraacetic acid (EDTA) and surfactant P20 (HBS-EP) buffer (10 mM HEPES, pH 7.4, 3.0 mM EDTA, 0.005 % surfactant P20, 0.15 M NaCl). A solution (40 μL) of ligand (250 μg mL^{-1}) in sodium acetate buffer (10 mM; 50 % dimethyl sulfoxide) was injected onto the activated surface of the sensor chip. Ethanolamine hydrochloride (50 μL) was injected to block the activated carboxymethylated dextran surface, followed by repeated injections of a solution of guanidine hydrochloride in water (5 μL, 6 M) to wash the sensor surface. This immobilization process was monitored by SPR measurements, and the amount of immobilized ligands on the sensor surface was modulated by the reaction period of the immobilization. For SPR-binding experiments, all the measurements were performed at 5 °C in a continuous flow of a running buffer (pH 5.6–8.0; 30 μL min^{-1}) containing NaCl (150–500 mM). Before the measurements, the sample solutions were annealed as follows: heating at 75 °C for 10 min and gradually cooling to 5 °C (3 °C min^{-1}). Unless otherwise stated, regeneration of the sensor surface was done with guanidine hydrochloride (5 M).

Preparation and analysis of PCR amplification products: Asymmetric PCR[15] amplification of 63-meric antisense strands of the *Ha-ras* gene (codon 12)[11] was carried out with a 20-meric forward primer (5'-GACGG AATAT AAGCT GGTGG-3') and a 20-meric reverse primer (5'-TGGAT GGTCA GCGCA CTCTT-3'). The reaction solution (100 μL) contained deoxyribonucleotide triphosphates (dNTPs; 0.2 mM each), 10 × PCR buffer (10 μL; TaKaRa), forward primer (20 pmol), reverse primer (300 pmol), template (0.5 ng each), and Taq (2.5 U; TaKaRa). The conditions of the PCR process: holding at 94 °C for 5 min followed by 50 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s, and 72 °C for 7 min. Afterward, the product solution was kept at 4 °C. PCR product: 5'-TGGAT GGTCA GCGCA CTCTT GCCCA CACCG NCG GCGCC CACCA CCACC AGCTT ATATT CCGTC-3' (N = G, C, A, or T). After PCR amplification, the PCR product was purified three times by centrifugal filtration (1500 × *g*, 5 min; Microspure-EZ with Microcon YM-10, Millipore) and the filtrate was freeze-dried. A PBS-EP (phosphate-buffered saline with EDTA and surfactant P20) buffer was added to the freeze-dried filtrate (95 μL; pH 5.8, 0.067 M phosphate buffer, 3.0 mM EDTA, 0.005 % surfactant P20) containing NaCl (0.25 M) and a solution of 20-meric DNA probe containing abasic sites in water (5 μL, 10 μM; 5'-GTG GGC GCC GXC GGT GTG GG-3'; X = abasic site; C3 spacer). The reaction mixture was annealed as follows: heating at 95 °C for 20 min and gradually cooling to 5 °C (3 °C min^{-1}). A control sample was a 63-meric antisense strand (N = T; PCR amplification product) that was hybridized with a 21-meric DNA probe (5'-GTG GGC GCC GAC GGT GTG GG-3'). An aliquot of the sample solution (50 μL) was analyzed in a continuous flow of solution (20 μL min^{-1}) at 5 °C with a Biacore 3000 system equipped with the ligand-immobilized sensor chip.

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