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Highly sensitive detection of GG mismatched DNA by surfaces immobilized naphthyridine dimer through poly(ethylene oxide) linkers

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Abstract—Naphthyridine dimer is a unique molecule that strongly, and selectively, binds to the guanine–guanine mismatch in duplex DNA. We have synthesized naphthyridine dimers possessing a different length of poly(ethylene oxide) (PEO) linker, and immobilized them to CM5 sensor chip to carry out a surface plasmon resonance (SPR) assay of DNA duplexes containing a single base mismatch. The sensitivity of the sensor remarkably increased with increasing numbers of PEO units incorporated into the linker. With the sensor surface immobilized naphthyridine dimer for 1.5×10^3 response unit (RU) through three PEO units, the distinct SPR signal was observed at a concentration of 1 nM of the 27-mer G–G mismatch.

Discrimination of base mismatches from normal Watson–Crick base pairs in duplex DNA constitutes a key technology for the detection of single nucleotide polymorphisms (SNPs). To detect SNPs, a number of high throughput methods have been developed.[1–3] Most of these technologies use the difference in thermodynamic stability between mismatched and fully matched duplexes. We,[4–10] and others,[11–13] have pursued an alternative approach to SNPs detection by using small molecular ligands that can selectively bind to the mismatched base pairs. This is a modified method of heteroduplex analyses, which determines the presence of SNPs in testing sample DNAs by detecting the mismatched base pairs in heteroduplex produced between sample and the standard DNAs.[14,15] We have developed a novel sensor for a surface plasmon resonance (SPR) assay to detect the G–G mismatch duplexes by employing the surface where naphthyridine dimer **1** was immobilized.[4] Naphthyridine dimer **1** (Fig. 1) consisting of two 2-amino-7-methyl-1,8-naphthyridines having complementary surface of hydrogen bonding to a guanine and a linker connecting two chromophores binds selectively to the guanine–guanine (G–G) mismatch in duplex DNA.[4,5] In previous studies,[4] the sensor surface was synthesized by immobilizing **2** on a dextran matrix pre-coated on the gold surface of a Biacore CM5 sensor chip.[16] The SPR signals obtained by the sensor surface were much weaker than those expected from the molecular mass of the analyte DNA. It was anticipated that naphthyridine dimers immobilized with a short alkyl linker (e.g., **2**) would not be exposed on the surface, but would be folded back into the matrix. We have inserted hydrophilic poly(ethylene oxide) (PEO) units between **1**

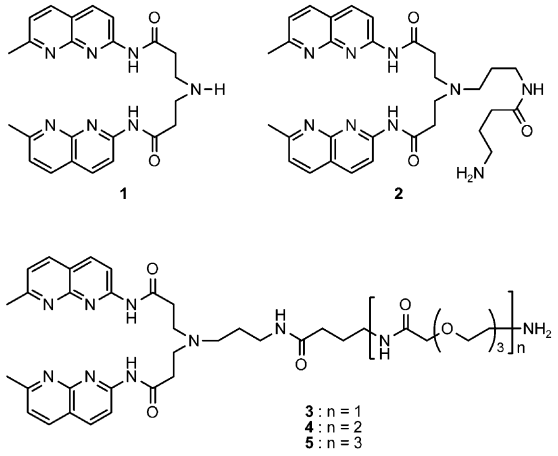

Figure 1. Structures of naphthyridine dimers with a different poly(ethylene oxide) linker length.

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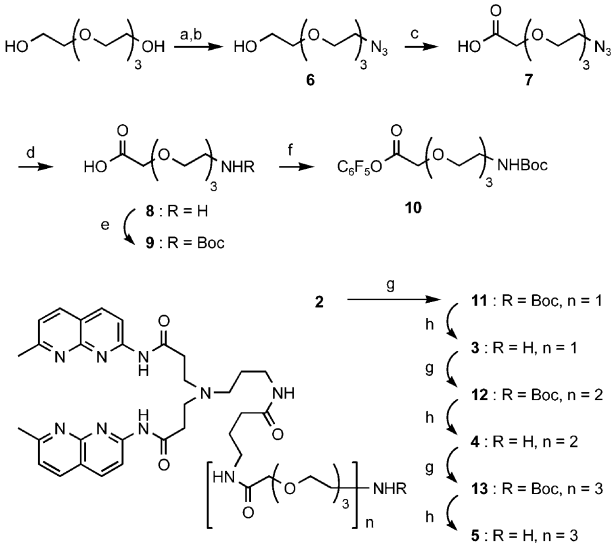
and the dextran matrix with the expectation that it would suppress undesirable folding, and so increase the fraction of ligand exposed on the surface. We here report that a new sensor surface of naphthyridine dimer having a long PEO spacer in the linker remarkably increased the sensitivity to the G–G mismatch 13 times that of the prototype. With the sensor surface immobilized naphthyridine dimer for 1.5×10^3 response unit (RU) through three PEO units, the distinct SPR signal was observed at a concentration of 1 nM of the 27-mer G–G mismatch.

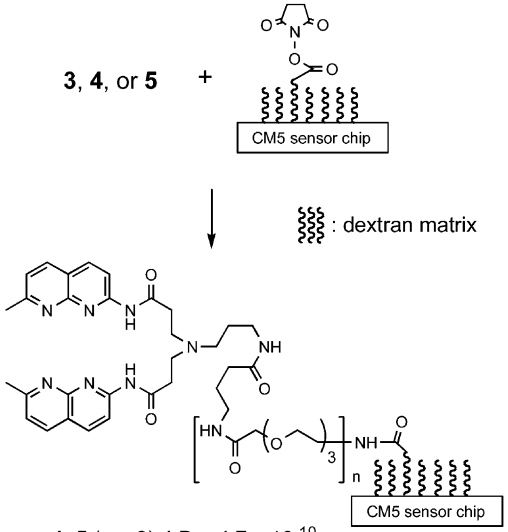
Naphthyridine dimers tethered to a PEO linker were synthesized as shown in Scheme 1. First, an activated amino acid of a PEO unit **10** was synthesized from tetraethylene glycol. Though the PEO unit related to **10** had been synthesized from triethylene glycol,[17,18] it was conveniently obtained by oxidation of mono-protected tetraethylene glycol. Mono mesylation of tetra(ethylene glycol) and a subsequent reaction with sodium azide produced ω-azide alcohol **6**, which was oxidized with Jones' reagent to carboxylic acid **7**. Hydrogenation of the azide **7** followed by protection of the resulting primary amino group in **8** produced *N*-Boc-amino acid **9**. The carboxylic acid of **9** was activated as a form of pentafluoro phenyl ester, giving the PEO unit **10**. Naphthyridine dimer tethered to an amino alkyl linker **2**[4] was reacted with **10**. The *N*-Boc group on the amino termini of the resulting **11** was deprotected to afford the naphthyridine dimer **3** having one PEO unit. The primary amine **3** was coupled with **10** giving **12**, which was deprotected to form **4** having two PEO units. Repetition of the coupling–deprotection sequence provided **5** having three PEO units.

Naphthyridine dimers tethered to a different length of PEO linker were immobilized on an activated carboxyl terminal attached to the dextran surface according to the procedure recommended by Biacore (Scheme 2).[16,19] We synthesized two **5**-immobilized sensor surfaces (**Sensors 1** and **2**), which differed in their ligand densities on the surface, one **4**-immobilized sensor surface (**Sensor 3**), and one **3**-immobilized sensor surface (**Sensor 4**). SPR detects changes in the refractive index on the surface layer caused by variation of the mass on the sensor chip surface, for example, when the analyte binds to the immobilized ligand on the surface. The change in SPR signal, termed the SPR response presented in response units (RU), is directly related to the change in surface concentration of biomolecules. SPR response of 1000 RU is equivalent to the change in surface concentration of 1 ng/mm^2. Thus, the density of immobilized ligands and analyte bound on the surface could be calculated by the difference in SPR response before and after the analysis.[16] **Sensor 1** was prepared by immobilizing **5** for 5.5×10^2 RU (1 RU = 1 pg·mm^{-2}, and it had a ligand density on the sensor surface of 4.7×10^{-10} fmol·nm^{-2}. **Sensor 2** was prepared by immobilizing **5** for 1.5×10^3 RU. The density of **5** on the surface was about 1.3×10^{-9} fmol·nm^{-2}. **Sensors 3** and **4** were prepared by immobilizing **4** and **3** for 4.6×10^2 and 5.4×10^2 RU, respectively. The ligand density of **Sensors 3** and **4** were 4.8×10^{-10} and 7.0×10^{-10} fmol·nm^{-2}, respectively, which are similar to the ligand density of **Sensor 1**.

The DNA oligomers used for the SPR studies were 27-mer duplex 5′-d(GTT ACA GAA TCT XYZ AAG CCT AAT ACG)-3′/3′-d(CAA TGT CTT AGA X′Y′Z′ TTC GGA TTA TGC)-5′ (1 μM) containing a G–G mismatch (XYZ/X′Y′Z′ = CGG/GGC represented a DNA duplex having the trinucleotide block) in the middle of the sequence. The effect of the linker length on the SPR intensity was assessed by measuring the specific binding of CGG/GGC to **Sensors 1**, **3**, and **4**, on



Scheme 1. Synthesis of naphthyridine dimers tethered to a different PEO linker. Reagents and conditions: (a) MsCl, NEt_3, dry diethylether; (b) NaN_3, EtOH, reflux, for 2 steps 46%; (c) Jones' reagent, acetone, 80%; (d) Pd–C, H_2, EtOH, quantitative; (e) $(Boc)_2O$, 2 M, NaOH, THF, 36%; (f) pentafluorophenol, EDCI, DMF, 80%; (g) **10**, Et_3N, THF, 90% for **11**, 78% for **12**, 74% for **13**; (h) HCl, AcOEt, $CHCl_3$, quantitative.



Scheme 2. Immobilization of naphthyridine dimers having different ligand density (LD, fmol·nm^{-2}) and lengths of a PEO linker on to the dextran coated gold surface.

which the naphthyridine dimer was covalently bound through 3, 2, and 1 PEO units, respectively. The buffer used for the analysis was 10 mM of HEPES (pH = 7.4) containing 150 mM of NaCl. The measurements were carried out at 25 °C. Because the degree of immobilization of the ligand on the surface was different for **Sensors 1**, **3**, and **4**, the SPR intensity was normalized, and was quoted in units of mass of DNA bound to a unit per immobilized ligands (pg of DNA per pmol of an immobilized ligand) (Fig. 2). A prototype of the sensor where **2** was immobilized showed a signal intensity of 94 pg·pmol^{-1} after a period of 180 s after the injection of **CGG/GCC**. As can be seen from Figure 2, the SPR intensity increased to 1.5×10^2, 4.9×10^2, and 1.2×10^3 pg·pmol^{-1} as increasing the number of PEO units incorporated in the linker from one to three. The signal obtained for **Sensor 1** is about 13-fold stronger in intensity than that obtained for **2**-immobilized sensor. Further incorporation of the PEO unit in the linker did not produce remarkable signal enhancement.

Having found that the incorporation of three PEO units significantly increased the SPR intensity, we then determined the detection limit of the G–G mismatch duplex by using **Sensor 2**. A distinct SPR signal was observed for CGG/GGC at a concentration of 1 nM, whereas duplexes containing a G–A mismatch (XYZ/X′Y′Z′ = CGG/GAC), a G–T mismatch (XYZ/X′Y′Z′ = CGG/GTC), and a G–C match (XYZ/X′Y′Z′ = CGG/GCC) base pair did not show any significant signal (Fig. 3). Further lowering of the concentration of CGG/GGC resulted in a loss of signal. By using a refined linker with three PEO units for the immobilization of the naphthyridine dimer and the Biacore 2000 instrument, a concentration of 1 nM for the 27-mer duplex was found to be the lower limit for the detection of the G–G mismatch. Because the SPR intensity increased with increasing molecular weight of the analyte, the detection limit of longer duplexes containing the G–G mismatch would be below 1 nM.

The effect of the sequence flanking to the G–G mismatch on the SPR detection by the **1**-immobilized surface was investigated by analyzing 10 G–G mismatches by **Sensor 1**. The sensorgrams were obtained by applying 1 μM of XYZ/X′Y′Z′ to Sensor 1. The SPR signals for all the G–G mismatches were clearly detected and

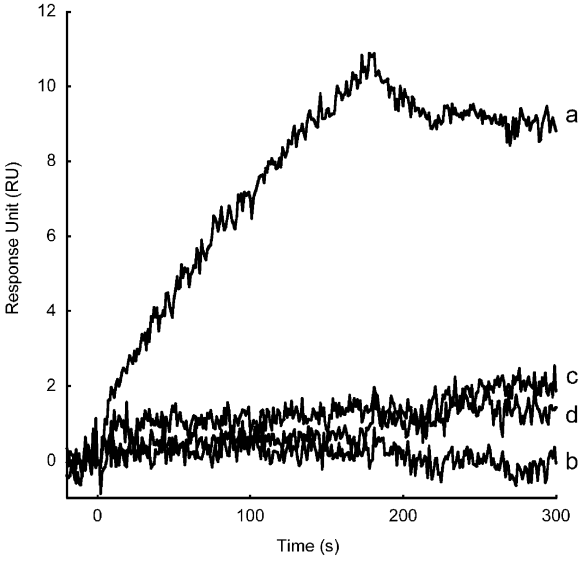


Figure 3. Detection of mismatched DNAs at a concentration of 1 nM by **Sensor 2**. Key: (a) CGG/GGC, (b) CGG/GAC, (c) CGG/GTC, and (d) CGG/GCC.

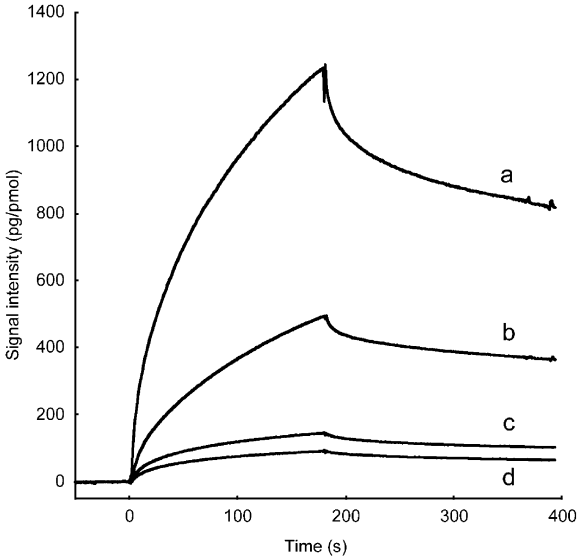


Figure 2. Binding of 27-mer duplex 5′-d(GTT ACA GAA TCT **XYZ** AAG CCT AAT ACG)-3′/3′-d(CAA TGT CTT AGA **X′Y′Z′** TTC GGA TTA TGC)-5′ (1.0 μM) containing the G–G mismatch (XYZ/X′Y′Z′ = CGG/GGC) to the naphthyridine dimer-immobilized sensors (**Sensors 1**, **3**, and **4**) possessing a different length of PEO unit. Key: (a) **Sensor 1**, (b) **Sensor 3**, (c) **Sensor 4**, and (d) **2**-immobilized sensor. The signal intensity represents an amount of DNA (pg) bound to a unit amount (pmol) of each immobilized ligand on the surface.

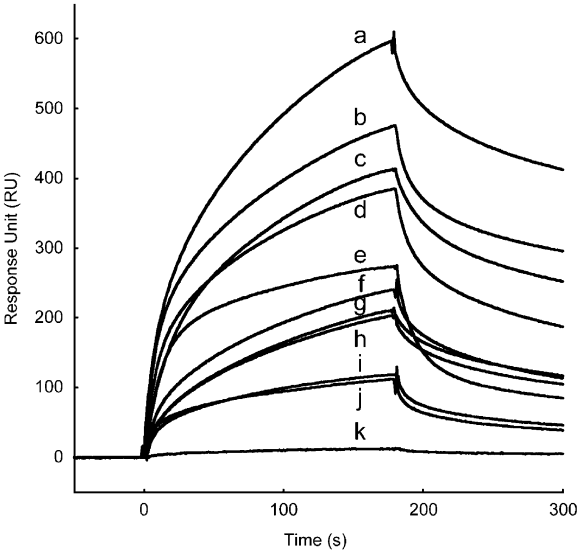


Figure 4. Sensorgrams obtained for the binding of XYZ/X′Y′Z′ to **Sensor 1** at a concentration of 1 μM duplex. Binding was measured for 180 s and dissociation for 120 s. Key: (a) GGC/CGG, (b) CGG/GGC, (c) CGC/GGG, (d) TGG/AGC, (e) TGA/AGT, (f) AGG/TGC, (g) TGC/AGG, (h) AGC/TGG, (i) AGA/TGT, (j) AGT/TGA, (k) CGG/GAC (GA mismatch).

sequence dependent on the flanking base pairs (Fig. 4). The strongest SPR signal was observed for the GGC/CGG, whereas the weakest signal was observed for AGT/TGA. The difference in SPR intensity between these two G–G mismatches is about 6 folds. The G–G mismatches flanking G–C base pairs binds to the **1**-immobilized surface stronger than those flanking A–T base pairs. These observations are in good agreement with the results obtained by DNase I foot print titration and UV-melting studies of duplexes containing the G–G mismatch in the presence of **1**.[15] In contrast, the SPR signal observed for the G–A mismatch was quite weak as compared to those of the G–G mismatch. These results showed that **Sensor 1** is capable of detecting the G–G mismatch in duplex DNA irrespective of the sequence flanking to the mismatch.

Unlike most SPR studies using the surfaces where macromolecules were immobilized, the drug-immobilized surface was extremely sensitive to the way of drug immobilization due to a small molecular size relative to the surface and its hydrophobic character. The studies described here reveal that incorporation of three PEO linker units between naphthyridine dimer and the dextran surface is especially effective in increasing the SPR sensitivity without losing the mismatch specificity.

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