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The SPR Sensor Detecting Cytosine−Cytosine Mismatches

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Abstract: We have synthesized the first surface plasmon resonance (SPR) sensor that detects cytosine−cytosine (C−C) mismatches in duplex DNA by immobilizing aminonaphthyridine dimer on the gold surface. The ligand consisting of two 2-aminonaphthyridine chromophores and an alkyl linker connecting them strongly stabilized the C−C mismatches regardless of the flanking sequences. The fully matched duplexes were not stabilized at all under the same conditions. The C−T, C−A, and T−T mismatches were also stabilized with a reduced efficiency. SPR analyses of mismatch-containing 27-mer duplexes were performed with the sensor surface on which the aminonaphthyridine dimer was immobilized. The response for the C−C mismatch in 5′-GCC-3′/3′-CCG-5′ was about 83 times stronger than that obtained for the fully matched duplex. The sensor successfully detects the C−C mismatch at the concentration of 10 nM. SPR responses are proportional to the concentration of the C−C mismatch in a range up to 200 nM. Aminonaphthyridine dimer could bind strongly to the C−C mismatches having 10 possible flanking sequences with association constants in the order of 10^6 M^{-1}. The facile protonation of 2-aminonaphthyridine chromophore at pH 7 producing the hydrogen-bonding surface complementary to that of cytosine was most likely due to the remarkably high selectivity of **1** to the C−C mismatch.

Introduction

Surface plasmon resonance (SPR) detects changes in the refractive index caused by variation of the mass on the sensor chip surface, e.g., when the analyte binds to the immobilized ligand on the surface.[1] In most SPR studies, macromolecules such as proteins and DNAs are immobilized on the sensor surface to detect protein−protein[2,3] and protein−DNA[4,5] interactions. We have studied the SPR detection of mismatched base pairs by using the sensor surface where a small molecular weight ligand that specifically binds to the mismatched base pairs was immobilized.[6−10] This technique would be useful for the high-throughput heteroduplex analyses of single nucleotide polymorphisms (SNP). Heteroduplex analyses[11,12] detect the mismatched base pairs in heteroduplexes produced by cross-hybridization of a sample and the standard DNAs.[13−15] When a sample DNA is identical to the standard DNA in base sequences, cross-hybridization of two DNAs produces a fully matched duplex but not a heteroduplex containing a mismatch site. Therefore, the heteroduplex analyses need analytical methods that discriminate the mismatch-containing duplexes from the fully matched duplexes. Conventional low-throughput methods for the detection of mismatched base pairs in heteroduplex analyses were enzymatic and chemical cleavage at the mismatched sites,[16−18] gel electrophoresis,[12,19] and selective capture by mismatch-binding proteins.[20,21] Heteroduplex analyses are applicable to both SNP discovery (mapping) and detection (typing). In the SNP mapping, mismatched base pairs produced in a heteroduplex will be identified by the subsequent sequencing. In contrast, a predetermined mismatched base pair is produced in SNP detection because the sequence of the standard DNA, the site of mutation, and the type of mutation have been identified. We report here the first SPR sensor detecting C−C mismatches in duplex DNA on which novel ligand molecule aminonaphthyridine dimer **1** was immobilized on the surface. The ligand strongly stabilized the C−C

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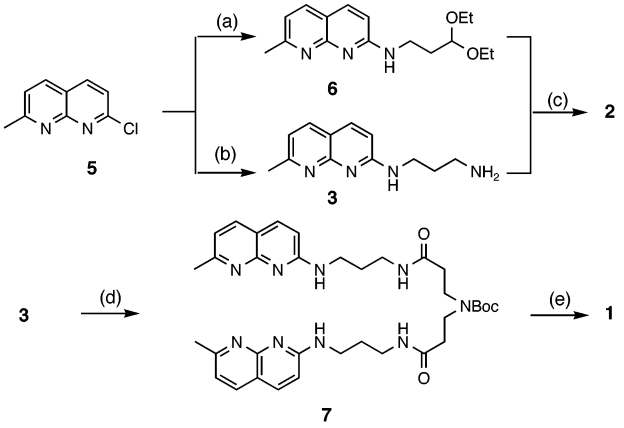
(1) *Biacore User's Manual*; Pharmacia-Biosensor: Piscatway, NJ, 1990.
(2) Williams, C. *Curr. Opin. Biotechnol.* **2000**, *11*, 42−46.
(3) Quinn, J. G.; O'Neill, S.; Doyle, A.; McAtamney, C.; Diamond, D.; MacCraith, B. D.; O'Kennedy, R. *Anal. Biochem.* **2000**, *281*, 135−143.
(4) Jayarajah, C. N.; Thompson, M. *Biosens. Bioelectron.* **2002**, *17*, 159−171.
(5) Willis, R. C. *Mod. Drug Discovery* **2002**, *5*, 26−33.
(6) Nakatani, K.; Sando, S.; Saito, I. *Nat. Biotechnol.* **2001**, *19*, 51−55.
(7) Nakatani, K.; Sando, S.; Kumasawa, H.; Kikuchi, J.; Saito, I. *J. Am. Chem. Soc.* **2001**, *123*, 12650−12657.
(8) Nakatani, K.; Sando, S.; Saito, I. *Bioorg. Med. Chem.* **2001**, *9*, 2381−2385.
(9) Smith, E. A.; Kyo, M.; Kumasawa, H.; Nakatani, K.; Saito, I.; Corn, R. M. *J. Am. Chem. Soc.* **2002**, *124*, 6810−6811.
(10) Nakatani, K.; Hagihara, S.; Sando, S.; Sakamoto, S.; Yamaguchi, K.; Maesawa, C.; Saito, I. *J. Am. Chem. Soc.* **2003**, *125*, 662−666.
(11) Landers, J. P. *Anal. Chem.* **2003**, *75*, 2919−2927.
(12) Nataraj, A. J.; Olivos-Glander, I.; Kusukawa, N. *Electrophoresis* **1999**, *20*, 1177−1185.
(13) Syvänen, A.-C. *Nat. Rev. Genet.* **2001**, *2*, 930−942.
(14) Kwok, P. Y. *Annu. Rev. Genomics Hum. Genet.* **2001**, *2*, 235−258.
(15) Schafer, A. J.; Hawkins, J. R. *Nat. Biotechnol.* **1998**, *16*, 33−39.
(16) Myers, R. M.; Larin, Z.; Maniatis, T. *Science* **1985**, *230*, 1242−1246.
(17) Rowley, G.; Saad, S.; Giannelli, F.; Green, P. M. *Genomics* **1995**, *30*, 574−582.
(18) Roberts, E.; Deeble, V. J.; Woods, C. G.; Taylor, G. R. *Nucleic Acids Res.* **1997**, *25*, 3377−3378.
(19) White, M. B.; Carvalho, M.; Derse, D.; O'Brien, S. J.; Dean, M. *Genomics* **1992**, *12*, 301−306.
(20) Fazakerley, G. V.; Qignard, E.; Woisard, A.; Guschlbauer, W.; van der Marel, G. A.; van Boom, J. H.; Jones, M.; Radman, M. *EMBO J.* **1986**, *5*, 3697−3703.
(21) Smith, J.; Modrich, P. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4374−4379.

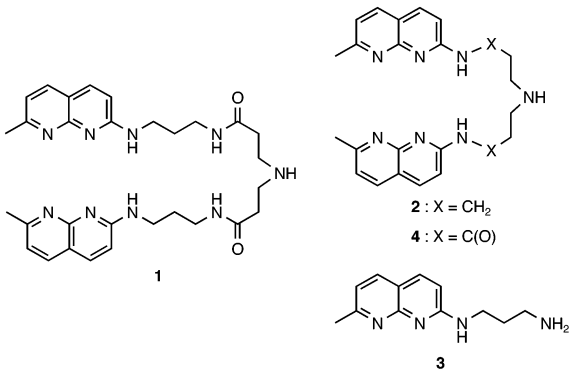
Scheme 1. Synthesis of Aminonaphthyridine Derivatives[a]



[a] Reagents and conditions: (a) 3,3-diethoxypropylamine, 81%; (b) diaminopropane, 95%; (c) (i) 80% AcOH, 80 °C, 30 min, (ii) $NaBH_3CN$, Et_3N, MeOH, 5 h, 12%; (d) 3,3-iminobis(pentafluorophenyl propionate), 67%; (e) 4 N HCl/ethyl acetate, 80%.

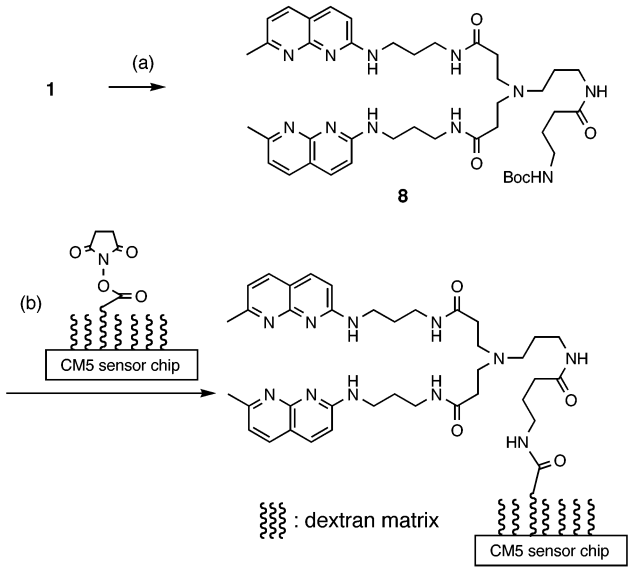
mismatches regardless of the flanking sequences as determined by the increase in melting temperatures (T_m). The **1**-immobilized sensor detected 27-mer duplex containing a C−C mismatch at the 10 nM concentration. Most genetic diseases are caused by the interaction of many genetic factors (polygenic) but not caused by a single genetic disorder (monogenic). It is anticipated that a large number of SNP sites must be determined for diagnoses of genetic diseases in the future. While G to C (or C to G) mutation producing a C−C mismatch in heteroduplex analyses is not high in frequency, under such situations, typing of G to C (or C to G) mutation would be indispensable.

Synthesis of Aminonaphthyridine Derivatives. A series of aminonaphthyridine derivatives **1**−**3** were synthesized as shown in Scheme 1. Substitution of chloride in 2-chloro-7-methyl-1,8-naphthyridine (**5**)[22] with diaminobutane and 3,3-diethoxypropylamine leads to amine **3** and diethylacetal **6**, respectively. Aminonaphthyridine dimer **2**, having the same linker length as that of acylaminonaphthyridine dimer **4**,[6,7] was obtained by a reductive coupling of **3** and **6**. Thus, the aldehyde derived from **6** by acid hydrolysis was treated with **3** under conditions of a reductive amination. Condensation of 3,3-iminobis(pentafluorophenyl propionate) with **3** afforded Boc-protected aminonaphthyridine dimer **7**. Finally, the amine-protecting Boc group of **7** was removed with 4 N HCl to give **1**.



Preparation of Aminonaphthyridine Dimer-Immobilized Sensor. A synthetic scheme of **1**-immobilized sensor surface was shown in Scheme 2. Aminoalkyl linker was attached to **1** by a reductive amination with 4-((*tert*-butoxy)carbonylamino)-*N*-(3-oxopropyl)butanamide to give **8**. Following the deprotection of the Boc group of **8** with 4 N HCl, the resulting amine was coupled to the activated carboxylic acid of the CM5 sensor chip using a standard procedure of an amine coupling method.[23] The degree of immobilization of the ligands was monitored by the increasing SPR signal and controlled by changing the reaction time. After immobilization of the ligand, the activated esters that remained intact on the surface were destroyed by being treated with ethanol amine.

Scheme 2. Preparation of Aminonaphthyridine Dimer-Immobilized Surface[a]



[a] Reagents and conditions: (a) 4-((*tert*-butoxy)carbonylamino)- *N*-(3-oxopropyl)butanamide, $NaBH_3CN$, AcOH, MeOH, 1 h, 95%; (b) (i) 4 N HCl/ethyl acetate, 30 min, (ii) amine coupling kit.

Melting Temperature of Mismatches in the Presence of Aminonaphthyridine Derivatives. Aminonaphthyridine dimer **1** consisted of two chromophores of 2-amino-7-methyl-1,8-naphthyridine and an alkyl linker connecting the exocyclic amino group of each chromophore. The molecule was discovered by a straightforward screening using increases of T_m of the mismatch-containing duplex DNA as an index. The UV melting curves of 11-mer duplexes of 5′-d(CTA AG**X** CAA TG)-3′/3′-d(GAT TC**Y** GTT AC)-5′, where **X**−**Y** are any nucleotide combinations, were measured in the presence of **1** in sodium phosphate buffer (10 mM, pH = 7.0). The difference in the melting temperature (ΔT_m) in the absence and presence of **1** (100 μM) was summarized in Table 1.

The remarkable increase of T_m by 18.0 °C was observed for the C−C mismatch, whereas the T_m values of fully matched duplexes were not increased at all under the same conditions. Increases in T_m by 11.3, 7.2, and 4.0 °C were observed for C−T, T−T, and C−A mismatches, respectively. Other mismatches, including A−A, G−T, G−A, and G−G, showed little increase of their T_m values. These results indicated that aminonaphthyridine dimer **1** strongly stabilized the C−C mismatch and the mismatches containing a cytosine or thymine with a reduced efficiency. The extraordinary high selectivity of **1** to pyrimidine-containing mismatches was remarkable.

(22) Brown, E. V. *J. Org. Chem.* **1965**, *30*, 1607−1610.
(23) Löfås, S.; Johnson, B. *J. Chem. Soc., Chem. Commun.* **1990**, *21*, 1526−1528.

Table 1. ΔT_m Values for the 11-mer Duplexes Containing a Mismatch[a]

5′-CTAAG**X**CAATG-3′
3′-GATTC**Y**GTTAC-5′

		ΔT_m		
X–Y	T_m[b]	**1**[c]	**2**[c]	**3**[d]
C−C	18.0 (0.4)	18.0 (0.4)	14.0 (0.7)	12.4 (0.8)
C−T	22.3 (0.5)	11.3 (0.3)	8.7 (0.1)	6.5 (0.8)
T−T	23.0 (0.8)	7.2 (0.6)	6.0 (0.6)	3.6 (1.2)
C−A	27.6 (0.4)	4.0 (0.1)	1.6 (0.2)	1.4 (0.6)
A−A	24.2 (0.4)	1.8 (0.8)	2.0 (0.5)	0.9 (0.3)
G−T	29.8 (0.2)	0.5 (0.7)	1.4 (0.2)	0.3 (0.0)
G−A	32.6 (0.6)	0.3 (0.8)	0.8 (0.7)	0.1 (1.1)
G−G	36.1 (0.5)	−0.1 (1.0)	0.3 (0.4)	−0.2 (1.0)
A−T	37.0 (0.3)	−0.1 (0.1)	0.0 (0.4)	0.0 (0.3)
C−G	43.1 (0.2)	−0.3 (0.5)	0.3 (0.3)	0.2 (0.4)

[a] T_m values of duplexes (4.5 μM) were measured in 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl. All measurements were taken three times, and standard deviations are shown in parentheses. [b] T_m values of oligomers. [c] The concentration of **1** and **2** was 100 μM.[d] The concentration of **3** was 200 μM.

Table 2. ΔT_m Values for the 13-mer Duplexes Containing a C−C Mismatch in Different Flanking Sequences[a]

5′-GCTAA**xCz**AATGA-3′
3′-CGATT**yCw**TTACT-5′

5′-xCz-3′/3′-yCw-5′	T_m	ΔT_m
gCc/cCg	25.4 (0.3)	17.9 (0.6)
gCt/cCa	23.9 (0.3)	15.7 (0.2)
cCc/gCg	22.3 (0.3)	15.3 (0.7)
gCa/cCt	26.2 (0.4)	15.0 (0.5)
aCt/tCa	20.7 (0.4)	14.2 (0.4)
aCa/tCt	23.2 (0.3)	13.5 (0.1)
cCt/gCa	25.3 (0.1)	13.2 (0.2)
tCa/aCt	24.8 (0.5)	12.2 (0.7)
cCa/gCt	29.0 (0.1)	11.2 (0.3)
cCg/gCc	32.6 (0.2)	10.4 (0.2)

[a] Conditions of T_m measurements were the same as those in Table 1.

Aminonaphthyridine dimer **2**, where two naphthyridine chromophores were connected with a short alkyl linker compared with that in **1**, and monomer **3** were examined as reference molecules. The ΔT_m values obtained for the C−C mismatch in the presence of **2** (100 μM) and **3** (200 μM) were 14.0 and 12.4 °C, respectively. A decreased ΔT_m by 4.0 °C showed the significance of the linker structure on the stabilization of the mismatch. A much-reduced stabilization of the C−C mismatch by **3** showed that a covalent connection of two aminonaphthyridines is in fact effective.

Effects of sequences flanking to the C−C mismatch on the **1**-binding were examined with 13-mer duplex 5′-d(GCTAA **xCz** AATGA)-3′/3′-d(CGATT **yCw** TTACT)-5′ containing a C−C mismatch in the sequence of 5′-**xCz**-3′/3′-**yCw**-5′, where **x**−**y** and **z**−**w** were any combination of Watson−Crick base pairs. The ΔT_m values obtained for all sequences were over 10 °C, indicating that **1** could stabilize the C−C mismatches regardless of the flanking sequences (Table 2). Rather low ΔT_m values of 10.4 and 11.2 °C recorded for **cCg**/**gCc** and **cCa**/**gCt** were due to the high T_m values of the duplex.[24]

Evaluation of Aminonaphthyridine-Immobilized Sensor. Having discovered that **1** strongly stabilized the C−C mismatches, we synthesized SPR chips for the C−C mismatch detection by immobilizing **1** on the sensor surface. SPR analyses of mismatch-containing 27-mer 5′-d(GTT ACA GAA TCT G**X**C AAG CCT AAT ACG)-3′/3′-d(CAA TGT TTC AGA CYG TTC GGA TTA TGC)-5′ were performed with **1**-immobilized sensor surface (for 810 response units (RU)) at pH 7.0. A marked SPR response was obtained for the duplex containing the C−C mismatch at the DNA concentration of 200 nM (Figure 1a). The response at 180 s after an injection of DNA was 399 RU, about 83 times stronger than that obtained for the fully matched duplex (4.8 RU). The duplex containing a C−T mismatch produced an intermediate response (80 RU). As shown in Figure 1b, SPR responses of the C−A and T−T mismatches were weak (19 and 9 RU, respectively) but distinguishable from that of the fully matched duplex. SPR responses of other mismatches were indistinguishable from the fully matched duplex (data not shown). These results were consistent with the ΔT_m of the mismatches in the presence of **1** (cf. Table 1). To further evaluate the novel SPR sensor, the detection limit of the C−C mismatch and the concentration dependency of SPR responses were examined. The sensor surface where **1** was immobilized for 955 RU successfully detected the C−C mismatch at the 10 nM concentration (Figure 1c). Further lowering the concentration of the C−C mismatch resulted in a loss of signals. By using aminonaphthyridine dimer **1** and the Biacore 2000 equipment, a concentration of 10 nM for the 27-mer duplex was found to be the lower limit for the detection of the C−C mismatch. Because the SPR intensity increased with increasing molecular weight of the analyte, the detection limit of longer duplexes containing the C−C mismatch would be

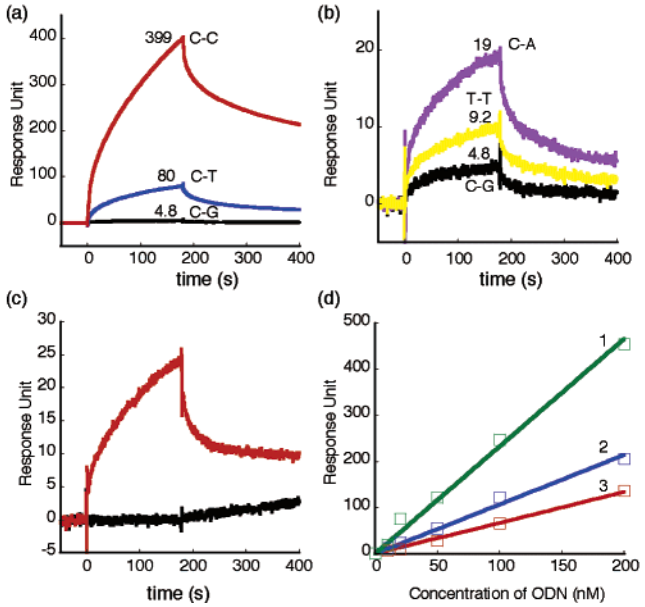


Figure 1. (a) and (b) SPR assay of 27-mer duplex (**GXC**/**CYG**) (0.2 μM) containing C−C (**GCC**/**CCG**) (red), C−T (**GCC**/**CTG**) (blue), C−A (**GCC**/**CAG**) (purple), and T−T (**GTC**/**CTG**) (yellow) mismatches and CG (**GCC**/**CGG**) match (black) with the **1**-immobilized sensor surface. Binding was measured for 180 s and dissociation for 220 s in a phosphate buffer (200 mM, pH 7.0) containing NaCl (150 mM). (c) SPR assay of C−C mismatch (red) and C−G match (black) at 10 nM. (d) Concentration dependency for the SPR response of the C−C mismatch (0−200 nM). The response after 100 s of association time was plotted against the DNA concentration. Experimental data obtained with the surface where **1** was immobilized for (1) 955 RU (green square), (2) 506 RU (blue square), and (3) 434 RU (red squares) were overlaid with a linear fitted line.

(24) For discussions on the relationship between ΔT_m and the binding affinity, see: (a) Nakatani, K.; Horie, S.; Saito, I. *Bioorg. Med. Chem.* **2003**, *19*, 51−55. (b) Peyret, N.; Seneviratne, P. A.; Allawi, H. T.; SantaLucia, J., Jr. *Biochemistry* **1999**, *38*, 3468−3477.

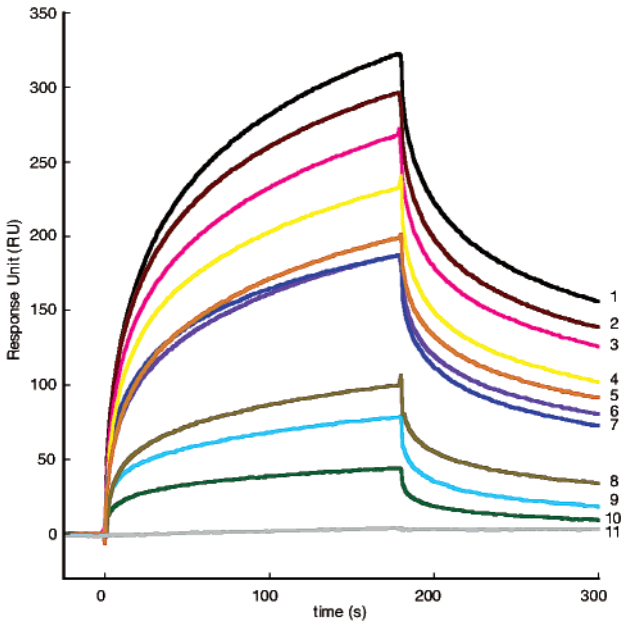


Figure 2. SPR sensorgrams were obtained by analyzing 27-mer 5′-d(GTT ACA GAA TCT **XCZ** AAG CCT AAT ACG)-3′/3′-d(CAA TGT CTT AGA **YCW** TTC GGA TTA TGC)-5′ containing all kinds of flanking sequences. Binding to **1**-immobilized sensor where **1** was immobilized for 380 RU was measured in a phosphate buffer (200 mM, pH 7.0) containing NaCl (150 mM). Key: (1) **GCC**/**CCG**, (2) **GCT**/**CCA**, (3) **CCC**/**GCG**, (4) **CCT**/**GCA**, (5) **CCG**/**GCC**, (6) **GCA**/**TCA**, (7) **ACT**/**TCA**, (8) **CCA**/**GCT**, (9) **TCT**/**ACA**, (10) **TCA**/**ACT**, and (11) **GCC**/**CGG**.

below 10 nM. SPR responses of the C−C mismatch obtained by three sensors containing different amounts of immobilized **1** are proportional to the concentration of the C−C mismatch in a range up to 200 nM (Figure 1d). These observations validate that the observed SPR responses are in fact due to the interaction between **1** on the surface and the C−C mismatched DNA.

Thermodynamics for the binding of the C−C mismatches to the aminonaphthyridine dimer-immobilized surface were investigated with regard to the effect of the flanking sequence to the mismatch. Sensorgrams of 27-mer 5′-d(GTT ACA GAA TCT **XCZ** AAG CCT AAT ACG)-3′/3′-d(CAA TGT CTT AGA **YCW** TTC GGA TTA TGC)-5′ containing all kinds of flanking sequences (5′-**XCZ**-3′/3′-**YCW**-5′) were clearly distinguishable from that of the fully matched duplex (Figure 2). The response obtained for **GCC**/**CCG** where the mismatch was flanked by two G−C base pairs was 323 RU at 180 s. A 7-fold decrease in response was observed by replacing the two G−C base pairs with two A−T base pairs in **TCA**/**ACT** (44.2 RU). However, the response to **TCA**/**ACT** was about 10-fold larger than that to the fully matched duplex. The order of magnitude in SPR response was consistent with that in ΔT_m with different flanking sequences (cf. Table 2). Association constants (K_a) of **1** to the C−C mismatches were estimated by fitting the sensorgrams to a 1:1 Langmuir model with BIAevaluation software (version 3) (Table 3). The largest K_a of 7.8×10^6 M^{-1} was obtained for **GCC**/**CCG**, whereas the smallest K_a of 1.5×10^6 M^{-1} was obtained for **TCA**/**ACT**. These data demonstrated that the binding of **1** to the C−C mismatches was stronger for those flanking G−C base pairs than those flanking A−T base pairs. This is rationalized by an improved stacking stabilization of the complex by the flanking G−C base pairs compared to that by the A−T base pairs.[25,26]

Table 3. Association Constants for **1**-Binding to C−C Mismatches[a]

5′-XCZ-3′/3′-YCW-5′	K_a (10^6 M^{-1})
GCC/CCG	7.8
GCT/CCA	6.9
CCC/GCG	7.3
CCT/GCA	6.1
CCG/GCC	5.3
GCA/CCT	5.0
ACT/TCA	4.5
CCA/GCT	3.0
TCT/ACA	1.7
TCA/ACT	1.5

[a] Association constants for the binding of **1** to 27-mer 5′-d(GTT ACA GAA TCT **XCZ** AAG CCT AAT ACG)-3′/3′-d(CAA TGT CTT AGA **YCW** TTC GGA TTA TGC)-5′ were estimated by fitting the sensorgrams (Figure 2) to a 1:1 Langmuir model with BIAevaluation software, version 3.

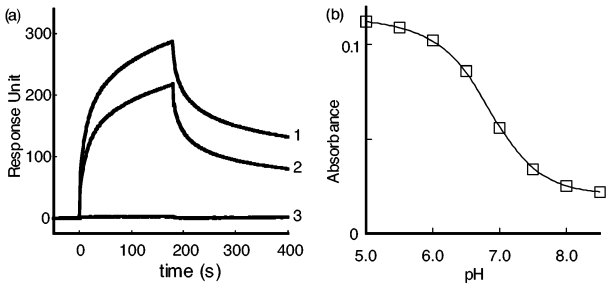


Figure 3. (a) SPR sensorgrams for the binding of 27-mer duplex (**GCC**/**CCG**) (1.0 μM) containing a C−C mismatch to the **1**-immobilized sensor surface. Binding was measured in a phosphate buffer (200 mM) containing NaCl (150 mM) at (1) pH 6.0, (2) pH 7.0, and (3) pH 8.0. (b) pH dependency of a UV absorbance of **3** at 370 nm. UV absorbance was measured in 10 mM sodium phosphate buffer in the presence of **3** (10 μM) at various pH conditions.

Model of Base Pairing between Aminonaphthyridine. We have reported that the structurally closely related compound **4**, where exocyclic amino groups in two 2-amino-7-methyl-1,8-naphthyridines were connected by an amide linkage, strongly stabilized the G−G mismatch but not the C−C mismatch at all.[6,7,27] Since both **2** and **4** have the same hydrogen-bonding surface complementary to guanine but not to cytosine, the striking differences in base selectivity between **2** and **4** need rational explanations. It was found that SPR response of the **1**-immobilized surface was sensitive to the pH of the buffer. A strong binding of 27-mer duplex (1.0 μM) containing a C−C mismatch with the **1**-immobilized sensor surface was observed at pH 6.0 and 7.0, whereas little binding was detected at pH 8.0 (Figure 3a). The responses at pH 6.0, 7.0, and 8.0 at 180 s after injection of 27-mer were 287, 216, and 2.7 RU, respectively. These results showed that the binding of aminonaphthyridine to a cytosine is dramatically suppressed at pH 8.0. The pH dependency of the UV absorbance of **3** revealed that the pK_a of the protonated **3** is about 6.8, showing that about 40% of the 2-aminonaphthyridine chromophore in **1**, **2**, and **3** in a free state would be protonated at pH 7.0 (Figure 3b). Protonation of the chromophore at the N1 position modulates from the hydrogen-bonding surface complementary to guanine to that to cytosine (Figure 4a). It is reasonable to estimate that

(25) Sponer, J.; Leszczynski, J.; Hobza, P. *J. Phys. Chem.* **1996**, *100*, 5591−5596.
(26) Alhambra, C.; Luque, F. J.; Gago, F.; Orozco, M. *J. Phys. Chem.* **1997**, *101*, 3846−3853.
(27) Murray, T. J.; Zimmerman, S. C. *J. Am. Chem. Soc.* **1992**, *114*, 4010−4011.

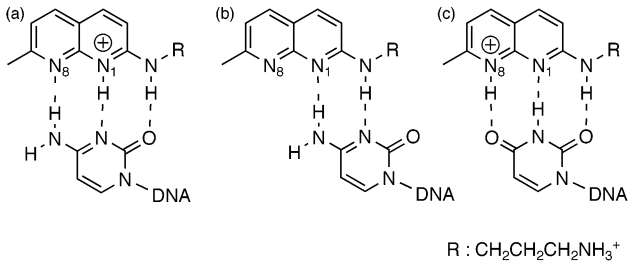


Figure 4. Proposed models of base-pairing between (a) cytosine and N1-protonated **3**, (b) cytosine and **3**, and (c) thymine and N8-protonated **3**.

the protonation of N1 in a bound state of 2-aminonaphthyridine to cytosine at neutral pH would be more facile than the protonation in a free state. While hydrogen bonding between nonprotonated aminonaphthyridine and cytosine is feasible through two hydrogen bonds (Figure 4b), energy gain by the hydrogen bonding calculated at the B3LYP/6-31G** level was 6.9 kcal/mol smaller than that between 1-methylcytosine and N1-protonated 2-methylamino-7-methylnaphthyridine.[28,29] On the basis of these arguments, the remarkably high selectivity of **1** to the C−C mismatch is most likely due to a facile protonation of the chromophore under neutral pH.

The presence of a carbonyl group next to the exocyclic amino group in 2-aminonaphthyridine is responsible for the remarkable difference in binding selectivity to the mismatch. The strength of a hydrogen bond is related to the hydrogen-bonding acidity of the donor group involved.[30,31] Beijer et al. have reported that the association constant of the complex of 2,6-diaminopyridine with *N*-propylthymine increases approximately 10-fold upon acylation of the amino groups.[32,33] It is highly likely that an acylation of the exocyclic amino group of 2-aminonaphthyridine significantly improves the stability of the hydrogen-bonding pair to a guanine. Since the acylated aminonaphthyridine is less susceptible to the protonation at N1 and N8, the dimer of acylated naphthyridine showed negligible binding to the C−C mismatch.

Protonation of 2-amino-1,8-naphthyridine is feasible both at N1 and N8 positions. While N1 protonation produces the hydrogen-bonding surface that is complementary to that of cytosine as discussed above, N8 protonation produces a surface complementary to that of thymine (Figure 4c). This is most likely the molecular basis for the binding of **1** to the C−T mismatch. The decreased binding to the C−T mismatch compared to the binding to the C−C mismatch was rationalized by an unfavorable secondary interaction in the hydrogen-bonded complex with alternating donor (D) and acceptor (A) groups. Studies on secondary interaction in a triple hydrogen-bonded complex by Jorgensen et al. predicted that the base pair of ADA/DAD (e.g., T/N8-protonated **3**) is 11.3 kcal/mol less stable than that of DDA/AAD (e.g., C/N1-protonated **3**).[34−37]

Conclusion

A SPR sensor where a dimeric form of 2-amino-1,8-naphthyridine was immobilized was found to detect the C−C mismatch. The SPR intensity was affected by the sequence flanking to the mismatch, but clearly distinguished from that of the fully matched duplex, indicating that the sensor is potentially useful for the SNP typing in heteroduplex analyses. A modest SPR intensity and T_m increase observed for the C−T mismatch by **1** are important clues for the molecular design of drugs selectively binding to the thymine-containing mismatch.

Experimental Section

Thermal Denaturation Profiles. All UV melting experiments were carried out with a duplex containing an X−Y mismatch (4.5 μM strand) in a sodium phosphate buffer (10 mM, pH 7.0) containing NaCl (100 mM) using a SHIMADZU UV-2550 UV−vis spectrometer linked to a Peltier temperature controller. The absorbance of the sample was monitored at 260 nm from 4 to 70 °C with a heating rate of 1 °C/min in the absence and presence of a ligand.

***N*-(7-Methyl-[1,8]naphthyridin-2-yl)-propane-1,3-diamine (3).** 2-Chloro-7-methyl-1,8-naphthyridine **5** (168 mg, 0.94 mmol) was added to 1,3-diaminopropane (1.5 mL, 9.4 mmol) and stirred for 5 h at 80 °C. The solvent was evaporated, and the residue was suspended in $CHCl_3$. The organic layer was washed with 1 M NaOH, dried over $MgSO_4$, and evaporated to dryness to give **3** (196 mg, 95%) as yellow gum. ^{1}H NMR (CD_3OD, 400 MHz): δ = 7.83 (d, 1H, J = 8.0 Hz), 7.71 (d, 1H, J = 9.2 Hz), 7.01 (d, 1H, J = 8.0 Hz), 6.71 (d, 1H, J = 9.2 Hz), 3.61 (t, 2H, J = 6.8 Hz), 2.78 (t, 2H, J = 6.8 Hz), 2.55 (s, 3H), 1.84 (tt, 2H, J = 6.8 Hz J = 6.8 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 161.8, 161.5, 157.4, 138.3, 137.9, 116.6, 114.4, 39.1, 38.6, 32.5, 24.6. ESI-TOFMS calcd for $C_{12}H_{17}N_4$ [(M + H)$^+$], 217.1453; found, 217.1487.

(3,3-Diethoxy-propyl)-(7-methyl-[1,8]naphthyridin-2-yl)-amine (6). 2-Chloro-7-methyl-1,8-naphthyridine **5** (673 mg, 3.8 mmol) was added to 3,3-diethoxypropylamine (6.0 mL, 37.6 mmol) and stirred for 5 h at 80 °C. The solvent was evaporated, and the residue was suspended in $CHCl_3$. The organic layer was washed with $NaHCO_3$, dried over $MgSO_4$, and evaporated to dryness. The residue was purified by column chromatography on silica gel to give **6** (882 mg, 81%) as pale as yellow gum: ^{1}H NMR (CD_3OD, 400 MHz): δ = 7.90 (d, 1H, J = 9.0 Hz), 7.78 (d, 1H, J = 8.4 Hz), 7.06 (d, 1H, J = 7.6 Hz), 6.72 (d, 2H, J = 9.0 Hz), 4.68 (t, 1H, J = 5.6 Hz), 3.69 (q, 4H, J = 8.8 Hz), 3.59 (t, 4H, J = 6.9 Hz), 3.52 (q, 4H, J = 8.8 Hz), 2.59 (s, 3H), 1.97 (dt, 2H, J = 5.6 Hz, J = 5.6 Hz), 1.18 (t, 6H, J = 6.9 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 161.8, 161.3, 157.7, 138.3, 137.8, 118.9, 116.6, 114.3, 103.2, 62.9, 38.2, 34.5, 24.5, 15.7. HR−FABMS calcd for $C_{16}H_{24}N_3O_2$ [(M + H)$^+$], 290.1869; found, 290.1872.

***N*-(7-Methyl-[1,8]naphthyridin-2-yl)-*N*′-[3-(7-methyl-[1,8]naphthyridin-2-ylamino)-propyl]-propane-1,3-diamine (2).** To a solution of 80% AcOH−H_2O (0.5 mL) was added **5** (10 mg, 34.6 μmol). After being stirred at 60 °C for 0.5 h, triethylamine (0.9 mL), **3** (10 mg, 46.3 μmol), and sodium cyanotrihydroborate (2.2 mg, 35 μmol) were added. After being stirred for 1 h, the reaction mixture was diluted with $CHCl_3$, washed with saturated $NaHCO_3$, and dried over $MgSO_4$. The solvent was evaporated to dryness, and the residue was purified by column

(28) Calculations at the B3LYP/6-31G** level revealed that the energy gain for the formation of a hydrogen-bonding pair between 1-methylcytosine and N1-protonated 2-methylamino-7-methyl-1,8-naphthyridine is 10.9 kcal/mol, whereas that between 1-methylcytosine and nonprotonated 2-methylamino-7-methyl-1,8-naphthyridine is 4.0 kcal/mol.

(29) Binding of 2-amino-7-methyl-1,8-naphthyridine selective to the cytosine opposite the abasic site in duplex was recently reported: Yoshimoto, K.; Nishizawa, S.; Minagawa, M.; Teramae, N. *J. Am. Chem. Soc.* **2003**, *125*, 8982−8983. These authors prefer the rationalization involving two hydrogen bonds between the ligand and the cytosine. Since the N-acylated naphthyridine showed striking preference to guanine (ref 6), they assumed an additional stacking interaction that modulates the binding selectivity from G to C.

(30) Abraham, M. H. *J. Phys. Org. Chem.* **1993**, *6*, 660−684.

(31) Abraham, M. H. *Chem. Soc. Rev.* **1993**, *22*, 73−83.

(32) Beijer, F. H.; Sijbesma, R. P.; Vekemans, J. J. M.; Meijer, E. W.; Kooijman, H.; Spek, A. L. *J. Org. Chem.* **1996**, *61*, 6371−6380.

(33) Beijer, F. H.; Kooijman, H.; Spek, A. L.; Sijbesma, R. P.; Meijer, E. W. *Angew. Chem., Int. Ed. Engl.* **1988**, *37*, 75−78.

(34) Jorgensen, W. L.; Pranata, J. *J. Am. Chem. Soc.* **1990**, *112*, 2008−2010.

(35) Pranata, J. Wierschke, S. G.; Jorgensen, W. L. *J. Am. Chem. Soc.* **1991**, *113*, 2810−2819.

(36) Sartorius, J.; Schneider, H. J. *Chem.−Eur. J.* **1996**, *2*, 1446−1452.

(37) Murray, T. J.; Zimmerman, S. C. *J. Am. Chem. Soc.* **1992**, *114*, 4010−4011.

chromatography on silica gel to give **2** (14.3 mg, 12%) as pale yellow gum. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.73 (d, 2H, J = 8.8 Hz), 7.63 (d, 2H, J = 8.8 Hz), 6.98 (d, 2H, J = 8.0 Hz), 6.61 (d, 2H, J = 8.8 Hz), 3.72 (dt, 4H, J = 5.6 Hz, J = 5.6 Hz), 2.78 (t, 4H, J = 6.4 Hz), 2.65 (s, 6H), 1.91 (tt, 4H, J = 6.4 Hz, J = 6.4 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 161.5, 159.3, 156.7, 136.8, 136.1, 118.0, 115.0, 112.2, 40.1, 39.7, 32.4, 25.3. ESI-TOFMS calcd for $C_{24}H_{30}N_7$ [$(M + H)^+$], 416.2563; found, 416.2546.

Bis-{2-[3-(7-methyl-[1,8]naphthyridin-2-ylamino)-propylcarbamoyl]-ethyl}-carbamic Acid *tert*-Butyl Ester (7). To a solution of *N*-(*tert*-butoxycarbonyl)imino-3,3′-bis(pentafluorophenyl propionate) (198 mg, 0.33 mmol) in dry $CHCl_3$ (2 mL) were added **3** (144 mg, 0.67 mmol) and triethylamine (139 μL, 1.0 mmol). The reaction mixture was stirred at room temperature for 15 h. The solvent was evaporated to dryness, and the residue was purified by column chromatography on silica gel to give **7** (148 mg, 67%) as pale yellow solids. ^{1}H NMR (CD_3OD, 300 MHz): δ = 7.88 (d, 2H, J = 7.8 Hz), 7.76 (d, 2H, J = 8.7 Hz), 7.05 (d, 2H, J = 7.8 Hz), 6.71 (d, 2H, J = 9.0 Hz), 3.55 (t, 4H, J = 6.9 Hz), 3.51 (t, 4H, J = 6.9 Hz), 3.26 (t, 4H, J = 6.9 Hz), 2.59 (s, 6H), 2.47 (t, 4H, J = 6.9 Hz), 1.83 (tt, 4H, J = 6.9 Hz, J = 6.9 Hz), 1.40 (s, 9H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 174.3, 162.3, 161.8, 158.1, 157.4, 138.7, 138.3, 119.4, 117.0, 114.9, 81.7, 77.9, 48.6, 46.0, 39.6, 38.4, 36.9, 36.4. ESI-TOFMS calcd for $C_{35}H_{48}N_9O_4$ [$(M + H)^+$], 658.3829; found, 658.3813.

***N*-[3-(7-Methyl-[1,8]naphthyridin-2-ylamino)-propyl]-3-{2-[3-(7-methyl-[1,8]naphthyridin-2-ylamino)-propylcarbamoyl]-ethylamino}-propionamide (1).** To a solution of **7** (76 mg, 0.133 mmol) in $CHCl_3$ (1 mL) was added ethyl acetate containing 4 M HCl (2 mL), and the reaction mixture was stirred at room temperature for 0.5 h. The solvent was evaporated to dryness to give **1** (51 mg, 80%) as white solids. ^{1}H NMR (CD_3OD, 300 MHz): δ = 7.88 (d, 2H, J = 7.8 Hz), 7.75 (d, 2H, J = 8.7 Hz), 7.05 (d, 2H, J = 7.8 Hz), 6.71 (d, 2H, J = 9.0 Hz), 3.51 (t, 4H, J = 6.9 Hz), 3.27 (t, 4H, J = 6.9 Hz), 2.86 (t, 4H, J = 6.9 Hz), 2.47 (s, 6H), 2.43 (t, 4H, J = 6.9 Hz), 1.82 (tt, 4H, J = 6.9 Hz, J = 6.9 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 175.1, 162.3, 161.8, 158.1, 138.7, 138.3, 119.4, 117.0, 114.9, 79.9, 46.8, 39.6, 38.3, 36.9, 30.5, 25.0. ESI-TOFMS calcd for $C_{30}H_{40}N_9O_2$ [$(M + H)^+$], 558.3305; found, 558.3253.

{3-[3-(Bis-{2-[3-(7-methyl-[1,8]naphthyridin-2-ylamino)-propylcarbamoyl]-ethyl}-amino)-propylcarbamoyl]-propyl}-carbamic Acid *tert*-Butyl Ester (8). To a solution of **1** (11.7 mg, 0.021 mmol) and 4-((*tert*-butoxy)carbonylamino)-*N*-(3-oxopropyl)butanamide (11 mg, 0.042 mmol) in CH_3OH (1 mL) was added sodium cyanotrihydroborate (3.3 mg, 0.05 mmol) at room temperature. The reaction mixture was kept at pH 6 with acetic acid for 1 h. The reaction mixture was diluted with $CHCl_3$, washed with saturated $NaHCO_3$, dried over $MgSO_4$, and evaporated to dryness. The residue was purified by column chromatography on silica gel to give **8** (14.3 mg, 95%) as pale yellow solids. ^{1}H NMR (CD_3OD, 400 MHz): δ = 7.85 (d, 2H, J = 8.0 Hz), 7.73 (d, 2H, J = 8.4 Hz), 7.04 (d, 2H, J = 7.6 Hz), 6.70 (d, 2H, J = 8.8 Hz), 3.54 (t, 4H, J = 6.0 Hz), 3.26 (t, 4H, J = 6.4 Hz), 3.11 (t, 2H, J = 6.8 Hz), 3.01 (t, 2H, J = 6.8 Hz), 2.74 (t, 4H, J = 6.0 Hz), 2.58 (s, 6H), 2.45 (t, 2H, J = 6.4 Hz), 2.37 (t, 2H, J = 6.4 Hz), 2.14 (t, 2H, J = 7.6 Hz), 1.81 (m, 4H), 1.70 (m, 2H), 1.60 (m, 2H), 1.40 (s, 6H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 175.1, 169.3, 161.9, 161.3, 157.6, 138.3, 137.9, 133.6, 132.4, 129.9, 118.9, 116.6, 114.5, 79.9, 69.1, 52.0, 51.1, 40.3, 40.2, 39.3, 38.6, 34.7, 34.4, 31.6, 30.2, 28.8, 27.4, 24.9, 24.0, 14.4. ESI-TOFMS calcd for $C_{42}H_{62}N_{11}O_5$ [$(M + H)^+$], 800.4935; found, 800.4884.

1-Immobilized Sensor Surface. To a solution of **8** (1.0 mg, 1.25 μmol) in $CHCl_3$ (0.5 mL) was added ethyl acetate containing 4 M HCl (1 mL), and the reaction mixture was stirred at room temperature for 0.5 h. After evaporation of the solvent, the residue was dissolved in borate buffer (1 mL, pH 9.2) and immobilized on a sensor chip CM5 (carboxymethylated dextran surface) using amine coupling kit with a BIAcore 2000 system (BIAcore, Uppsala, Sweden). The amount of **1** immobilized on the surface was monitored as an increase of SPR signal. 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 on the surface could be calculated by the difference in SPR response before and after the immobilization.

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