Fluorescence and electrochemical detection of pyrimidine/purine transversion by a ferrocenyl aminonaphthyridine derivative†

Kotaro Morita,[a,b] Yusuke Sato,[a] Takehiro Seino,[a] Seiichi Nishizawa[a,b] and Norio Teramae*[a,b]

Received 29th October 2007, Accepted 23rd November 2007
First published as an Advance Article on the web 4th December 2007
DOI: 10.1039/b716682f

A novel hydrogen bond-forming ligand for pyrimidine/purine transversion, which contains both a fluorescent naphthyridine moiety and a ferrocenyl group as an electrochemical indicator, is described. Hydrogen bond-mediated recognition for a target nucleobase at an abasic site in a DNA duplex is confirmed by both fluorescence and electrochemical measurements. The analysis by fluorescence titration reveals that the ligand shows significant fluorescent quenching upon formation of a 1 : 1 complex with the target nucleobase opposite the abasic site, and the selectivity is in the order of cytosine > thymine > adenine, guanine, reflecting the stability of the hydrogen bond formation.

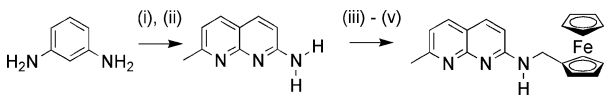
Detection of single nucleotide polymorphisms (SNPs) is important from the viewpoint of developing tailor-made diagnoses, because they can be in correlation with a disease or the side effects of a given drug. Although lots of detection methods for SNPs have been developed in recent years,[1] most adopt a discrimination principle based on the difference in the thermodynamic stabilities between a probe strand and a complementary target strand over a mismatched one in order to differentiate fully matched DNAs from mismatched DNAs or SNPs. However, these methods can only detect the presence of a mismatch in the base pairing and can not distinguish the type of nucleobase involved in the mismatch[2] since they are based on the nature of DNA duplex formation.

We have recently demonstrated a simple SNP detection method utilizing hydrogen bond formation between a ligand and a target nucleobase, which can provide target base-selective SNP discrimination.[3] In contrast to the current methods, our approach is based on an abasic (AP) site-containing DNA duplex, which allows the use of a small ligand to selectively recognize target nucleobases, irrespective of the difference in the thermodynamic stabilities of fully matched and mismatched base pairs. While naturally occurring AP sites are one of the most common forms of DNA damage,[4] we intentionally constructed an AP site in a DNA duplex so as to orient the AP site toward a target nucleobase. Moreover, the selectivity of the ligand–target base binding is accomplished by using the AP site as a hydrophobic microenvironment suitable for hydrogen bond formation in an aqueous solution.

[a]*Department of Chemistry, Graduate School of Science, Tohoku University, Aoba-ku, Sendai 980-8578, Japan. E-mail: teramae@mail.tains.tohoku.ac.jp; Fax: +81-22-795-6552; Tel: +81-22-795-6549*
[b]*CREST, Japan Science and Technology Agency (JST)*

† Electronic supplementary information (ESI) available: Characterization data for FcAMND synthesis, detailed experimental procedures for photochemical and electrochemical measurements, and comments on the fluorescence intensity of FcAMND and AMND. See DOI: 10.1039/b716682f

Naphthyridine derivatives have been found to form very stable complexes with cytosine opposite the AP site.[3a–c] When a ferrocenyl group is attached to naphthyridine, the resulting compound is expected to serve as an electrochemically active cytosine-selective ligand. In this report, we synthesized a novel naphthyridine derivative (*N*-ferrocenylmethyl-7-methyl-1,8-naphthyridin-2-amine; FcAMND) and used it as a novel hydrogen bond-forming ligand with fluorescence and electrochemical activities. The synthesis procedure and structure are shown in Scheme 1.



Scheme 1 Synthesis of *N*-ferrocenylmethyl-7-methyl-1,8-naphthyridin-2-amine. (i) H_3PO_4, 24 h, 90 °C. (ii) NaOH. (iii) Ferrocenecarboxaldehyde, CH_2Cl_2, 6 h, reflux. (iv) $NaBH_4$, 1 h. (v) HCl, NaOH.

FcAMND was prepared according to the standard procedures given in the literature.[5] Briefly, ferrocenecarboxaldehyde was condensed with 2-amino-1,8-naphthyridine, followed by sodium borohydride reduction to give FcAMND. The structure of FcAMND was fully confirmed by elemental analysis, ^{1}H-NMR and mass spectroscopy (detailed synthetic procedure is described in the ESI†). Fluorescence spectra and electrochemical measurements of FcAMND both in the presence and absence of an AP site-containing DNA duplex were done in an aqueous buffer solution (pH 7) containing 0.1 M NaCl, 0.01 M sodium cacodylate, and 1 mM EDTA. Binding behavior of FcAMND was examined for adenine (A), cytosine (C), guanine (G), and thymine (T), *i.e.*, target bases opposite the AP site in 23-meric DNA duplexes (5′-GTG TGC GTT GCN CTG GAC GCA GA-3′/3′-CAC ACG CAA CGX GAC CTG CGT CT-5′, N = target base, X = spacer-C3, *i.e.*, a propylene residue‡).

Fig. 1 shows absorption and fluorescence spectra of FcAMND and AMND in an aqueous buffer solution. For comparison, the absorption spectrum of *N*,*N*-dimethylaminomethylferrocene (Me_2NMeFc) is also shown. The FcAMND has its absorption maximum at 363 nm ($\log\varepsilon = 4.1$) accompanied by a small absorption band at 420 nm ($\log\varepsilon = 2.3$), whereas AMND has its maximum at 343 nm ($\log\varepsilon = 4.1$). The FcAMND absorption spectrum shows the characteristic feature of a ferrocene derivative (for Me_2NMeFc, $\lambda_{max} \approx 420$ nm) and has a slight red-shift peak compared to the spectrum of AMND (see ESI†). By contrast, the fluorescence spectrum of FcAMND is almost the same as that of AMND regarding the shape and position of the emission band. This result indicates that there is a small interaction between the AMND moiety and the ferrocenyl group in the ground state. On

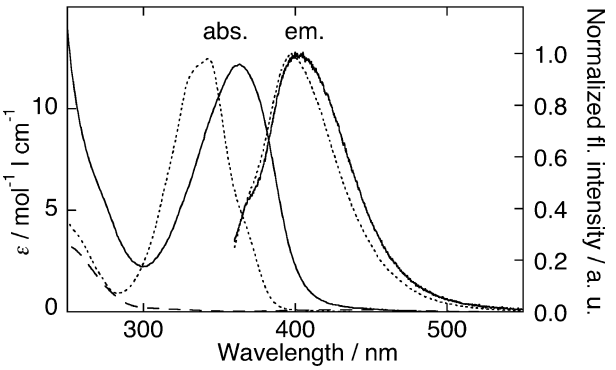


Fig. 1 Absorption and normalized fluorescence spectra of FcAMND (solid line) and AMND (dotted line) in a buffer solution. The absorption spectrum of Me_2NMeFc is given by a broken line. The vertical axis for the absorption spectra plots the molar absorption coefficient. Detailed measurement conditions are described in the ESI.†

the other hand, the singlet excited state of the AMND moiety is not significantly altered by the ferrocenyl group.

Next, using fluorescence measurements we examined the complexation between FcAMND and target bases in the 23-meric model AP site-containing DNA duplexes. The fluorescence spectra of FcAMND in the presence of the DNA duplexes containing different bases opposite an AP site were measured at an excitation wavelength of 356.5 nm. In the spectrum of the FcAMND without a DNA duplex, the fluorescence peak is observed at 405 nm, as shown in Fig. 2. In contrast, the fluorescence spectra of FcAMND with the duplex formed by an AP site-containing DNA and a target DNA (N = A, C, G, T) shows quenching of the fluorescence intensity. The fluorescence intensity at 405 nm is quenched by as much as 85% in the presence of equimolar AP site-containing DNA duplex when the target base is pyrimidine (N = C, T). In the binding to purine bases (N = A, G), the fluorescence quenching is moderate (15% and 30% for adenine and guanine, respectively). Apparently, the fluorescence intensity of FcAMND is strongly influenced by the type of a base opposite the AP site, and particularly when cytosine is the base opposite the AP site,

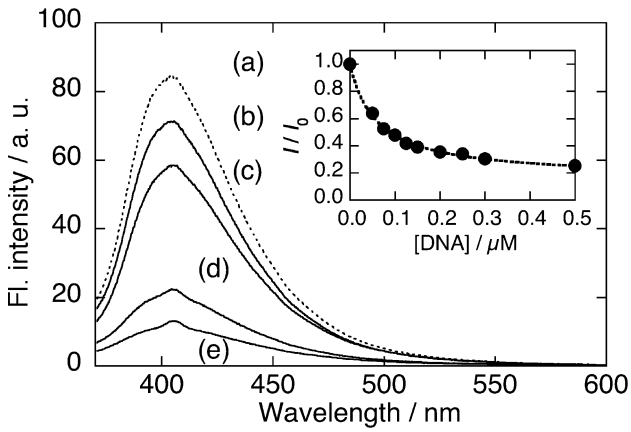


Fig. 2 Fluorescence spectra of FcAMND (5 μM) in the absence (a) and presence (b–e) of AP site-containing DNA duplexes (5 μM) in a buffer solution at 5 °C; N = (b) A, (c) G, (d) T, and (e) C. The inset shows the changes in fluorescence intensity of FcAMND as a function of the AP site-containing DNA duplex (N = C); [FcAMND] = 0.1 μM, [DNA] = 0.0–0.5 μM.

the fluorescence of FcAMND is greatly weakened. It seems likely that FcAMND binds to cytosine *via* a three-point hydrogen-bonding motif as reported in our previous NMR study.[3c] Thus, the change in the fluorescence behavior of FcAMND depending on the base opposite the AP site facilitates identification of the type of bases located at a specific site on a target DNA strand. In addition, the binding constants between FcAMND and target nucleobases opposite the AP site are estimated as 9.7×10^7 M^{-1} for cytosine and 1.4×10^7 M^{-1} for thymine, by the titration curve shown in the inset of Fig. 2 which used a non-linear fitting method described previously.[3a] By contrast, probably due to a steric effect, such effective binding is not obtained for purine target bases, where binding constants are less than 10^6 M^{-1} (6×10^4 M^{-1} for adenine and 2×10^5 M^{-1} for guanine). We note that FcAMND shows a larger binding constant with cytosine than AMND. The binding constant of AMND with cytosine opposite the AP site was previously calculated as on the order of 10^6 M^{-1}.[3b] The enhancement in the binding constant of FcAMND is promoted by introduction of the ferrocenyl group to the AMND moiety. Although FcAMND has the same hydrogen bond-forming moiety as AMND, the ferrocenyl group of FcAMND can give an electrostatic interaction with a DNA backbone,[6] which is expected to contribute to the decrease in the dissociation rate. Moreover, based on preliminary molecular modeling, we think that the ferrocenyl group is located in a minor groove when the target base is cytosine. To clarify this point, molecular mechanics calculations were conducted for optimization of binding structure.

A possible computer-generated molecular model of the complex of an AP site-containing DNA duplex (N = C) with FcAMND is shown in Fig. 3, which was optimized using AMBER* force fields. The AMND ring of FcAMND is located at the AP site of the DNA duplex where the formation of three hydrogen bonds is evident between AMND and cytosine, and the ferrocenyl group is located in the minor groove of the DNA duplex. A similar molecular modeling result was reported by Sato *et al.*[6b] for the binding of naphthalenediimide bearing two ferrocenyl groups as a threading intercalator for DNA duplexes. In their result, two ferrocenyl groups served as an anchor, where one of the substituents lay in the major groove and the other in the minor groove in the complex with a DNA duplex, to prevent the intercalator from dissociating from double stranded DNA. Therefore, it can be concluded that the ferrocenyl group of FcAMND gives rise to a stabilizing

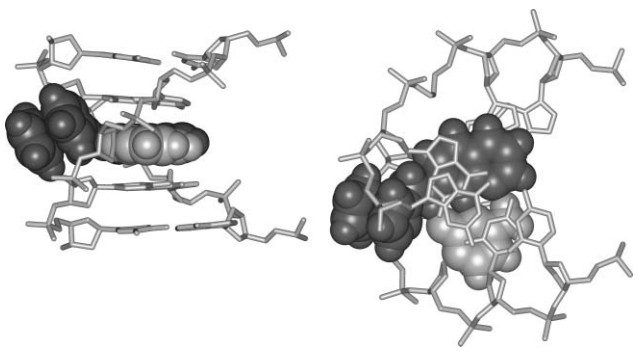


Fig. 3 Possible binding structures, obtained by MacroModel 9.0, for the complex between FcAMND and cytosine opposite the abasic site; (left) side view, (right) top view. FcAMND (dark gray) and cytosine (light gray) are shown as a space-filling model.

interaction between FcAMND and an AP site-containing DNA duplex, resulting in an increase in the binding constant.

Electrochemical measurements were also made to utilize the redox activity of FcAMND for SNP detection. In accordance with the results of the fluorescence measurement, selectivity for pyrimidines over other purines is clearly seen from the square wave voltammogram (SWV) as shown in Fig. 4. In the absence of a DNA duplex, FcAMND exhibits an oxidation peak at 0.25 V *vs.* Ag/AgCl (sat. KCl). The peak position was unchanged in the pH range studied (2 to 7), showing independency of the peak position upon pH change. Since naphthyridine derivatives are known to have a reduction potential at around −1.5 V *vs.* Ag/AgCl (in acetonitrile),[7] the electrochemical response is ascribed to the ferrocenyl group of FcAMND.

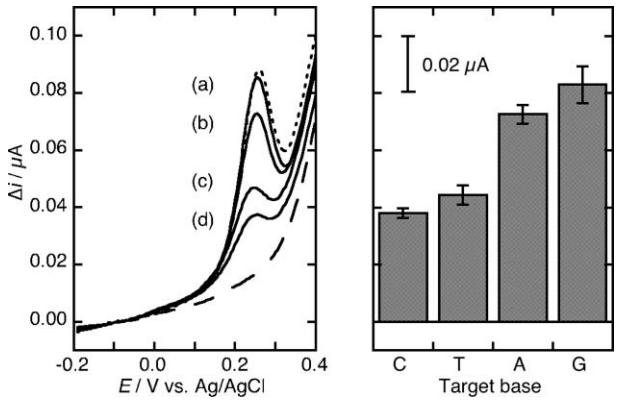


Fig. 4 (Left) Solid lines show square wave voltammograms of FcAMND (5 μM) in the presence of AP site-containing DNA duplexes (5 μM) in a buffer solution; N = (a) G, (b) A, (c) T, and (d) C, respectively. The dotted line shows FcAMND alone and the broken line shows background current. (Right) Peak current dependency of target nucleobase ($n = 3$).

Typical SWV curves for 5 μM FcAMND in the presence of 5 μM of an AP site-containing DNA duplex are shown in Fig. 4. Addition of an AP site-containing DNA duplex into the FcAMND solution results in a decrease in the peak currents for all target sequences. For the case of the cytosine target, the peak current decreases to 60% of the initial current without DNA. The current decrease upon addition of a DNA duplex can be mainly ascribed to the decrease in the diffusion coefficient of FcAMND. It is reasonably expected that the diffusion coefficient of FcAMND when bound to DNA duplex is much smaller than that of FcAMND in free solution. Similar results were reported for the interaction between DNA and electrochemically active intercalators.[8] Therefore, it can be considered that the change in current upon DNA addition is described in terms of the diffusibility between free and DNA-bound FcAMND. As described above, FcAMND shows an ability to discriminate pyrimidines from purines by a fairly large margin. We demonstrated here, in combination with AP site-containing DNA duplexes, that FcAMND selectively binds to pyrimidines with significant changes in fluorescence and electrochemical response. The detection method described in this study is expected to be applicable to a simple SNP typing procedure[9] or *in situ* gene analysis with a flow-through system for PCR amplification. Further studies are currently in progress along this line.

Notes and references

‡ There are two major types of AP site structure: a THF analog (dSpacer) and a propylene residue (spacer-C3). It is predicted by preliminary molecular modeling study that the dSpacer prevents FcAMND from facing to the target base opposite the AP site because of steric hindrance. When spacer-C3 is used in the modeling, FcAMND is able to locate at the AP site and to form favorable hydrogen bonds with target bases. Therefore, we employed spacer-C3 as the AP site in this work.

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