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Effect of LNA nucleobases as an enhancer for the binding of amiloride to an abasic site in DNA/DNA and DNA/RNA duplexes

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Introduction

Much attention has been paid to the development of small nucleic acid-binding molecules, i.e. ligands due to their promising abilities as therapeutical agents to modulate or inhibit various functions of DNAs and RNAs.[1] These ligands also function as the staining agents for nucleic acids,[2] in which the binding-induced fluorescence response can be utilized to detect nucleic acids in solutions, gels and microarrays. Recent efforts have been devoted to designing site-selective ligands that can recognize intrahelical nucleobases.[3] Useful binding selectivity and affinity can be achieved by the combination of hydrogen bonding, π-π stacking, electrostatic interactions, and molecular shape-complementarity. It has been demonstrated that such ligands are applicable to gene diagnostic analysis and therapy for targeting the specific DNAs and RNAs.[4] In addition, they can be utilized as site-selective staining agents for designing label-free nucleic acid-based assays for detecting target analytes including metal ions,[5a-5c] small molecules,[5a-5h] and nucleic acids.[5i]

In this context, we have developed a series of abasic (apyrimidinic or apurinic; AP) site-binding fluorescent ligands[6] in DNA duplexes for genotyping of single-nuleotide polymorphisms (SNPs) as well as for designing label-free DNA aptamers and molecular beacon. This class of ligands are flat aromatic molecules having hydrogen bonding sufaces to form pseudo-base pairing with the intrahelical nucleobase opposite an AP site. The binding events are further promoted by stacking interactions with the nucleobases flanking the AP site. In the genotyping assay, an AP site-containing DNA probe is hybridized with a target DNA so as to place the AP site toward a target nucleobase, and subsequently, the ligands can discriminate the target nucleobase in the preformed AP site-containing DNA duplexes (Fig. 1A). In the case of aptamers and molecular beacons, our ligands function as not only affinity-labeling agents but also fluorescence reporters to transduce the recognition events of the target analytes to the fluorescence signaling.[5] One of the key issues for these applications is to attain strong binding of the ligands for the target nucleobases in working toward the goal of sensitive bioanalytical assays suitable for practical use.

For this purpose, chemical modification of AP site-binding ligands has been demonstrated as an effective approach, as is the case for usual DNA-binding ligands.[7] 2-Amino-1,8-naphthyridine can function as a cytosine-selective ligand in the DNA duplexes and its binding affinity significantly was improved by methyl group substitution into the parent naphthyridine ring due to the favorable hydrophobic effect on the binding reaction.[6a] The introduction of the cationic guanidinium moiety into thymine-selective 2,6-diamino-pyrazine derivative resulted in increased binding affinity.[6b]

In this work, we describe another approach for improving the binding abilities of the AP site-binding ligands, for which LNA (locked nucleic acid)-DNA probes are utilized as an alternative to DNA ones (cf. Fig. 1A). LNAs are conformationally restricted nucleic acid analogues[8] and their monomers contain a ribose moiety in which the 2'-O and 4'-C are linked by a methylene bridge (Fig.

1A), rendering the sugar locked in the C3'-endo conformation. DNA probes containing LNA nucleobases are one of the probes widely used in various hybridization-based assays[9] because they can more strongly and specifically recognize the complementary sequences compared to DNA and RNA probes. Here, we focus on such LNA nucleobases as an enhancer of the binding of amiloride (Fig. 1A).[6b,10] Amiloride can selectively bind to the target thymine opposite an AP site in the DNA duplexes whereas the binding to a fully-matched DNA duplex was negligible.[10c] We adopted DNA probes with LNA nucleobases flanking the AP site (Fig. 1B) due to the significant effects of the flanking nucleobases on amiloride binding.[10b,10d] Despite the usefulness of LNA in a wide variety of applications,[9] there are few reports on the interactions between small ligands and LNA-containing duplexes. From the binding study of various intercalators and minor groove binders, the binding affinity of most ligands was found to be significantly reduced for the LNA-containing DNA duplexes relative to the corresponding DNA duplexes.[11] While the reason for this is not clear, the results indicate that the LNA nucleobases in the DNA duplexes lead to the decrease in the binding affinity of intercalators and groove binders. By contrast, the present work reveals a significant favorable effect of LNA nucleobases flanking an AP site on the binding affinity of amiloride. Amiloride showed remarkable enhancement of the binding affinity for the target T opposite an AP site in the LNA-DNA/DNA duplexes compared to the corresponding normal DNA duplexes (Fig. 1B). Such a favorable effect of LNA nucleobases on the amiloride binding was discussed based on the examination of thermodynamic parameters. Moreover, we also demonstrate that amiloride showed enhanced affinity for the LNA-DNA/RNA hybrid duplexes compared to DNA/RNA hybrid duplexes.

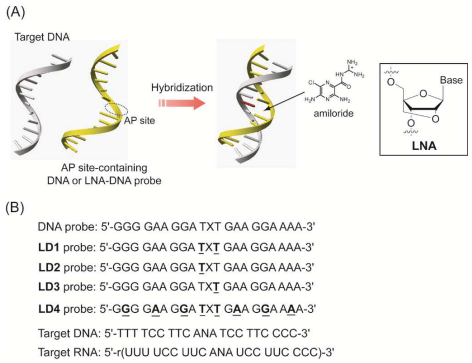

Fig. 1 (A) Schematic illustration of abasic site (AP site)-binding ligand (amiloride)-based assay for the genotyping of SNPs in combination with an AP site-containing DNA or LNA-DNA probe. Chemical structure of LNA is also shown. (B) DNA, LNA-DNA, and RNA sequences examined in this study (X = AP site (spacer C3; a propyl residue), N = target nucleobases (G, C, A, or T (U)). Bold and underlined nucleobases denote the LNA nucleobases.

Results and discussion

For the systematic investigation of the effect of LNA nucleobases, we prepared a series of DNA probes that possess LNA nucleobases flanking the AP site (Fig. 1B). **LD1**, **LD2**, and **LD3** probes contain LNA nucleobase on both 5' and 3'-sides, only the 5'-side, and only the 3'-side of the AP site, respectively. Besides the LNA nucleobases flanking the AP site, the additional LNA nucleobases were involved in the **LD4** probe so as to promote the effect of LNA nucleobases on the structural property of the duplexes.[12] The hybridization between these probes and the target DNAs (Fig. 1B) allowed the formation of the LNA-DNA/DNA duplexes containing target G, C, A, or T opposite the AP site. First, the conformation of these duplexes containing target T was examined by CD experiments (Fig. 2). In the case of the **LD1**-containing duplex, the CD spectrum exhibits a positive band at 277 nm and a negative band at 248 nm, with these two bands being indicative of the B-formed conformation. Similar spectra of the typical of B-form are observed when **LD2** and **LD3** probes were used. These spectra profiles of LNA-DNA/DNA duplexes are similar to those of the corresponding DNA duplexes, which indicates that LNA nucleobases flanking the AP site in the probes have little influence on the conformation of the duplexes. By contrast, the CD spectrum of the **LD4**-containing duplex is distinct from that of the DNA duplex and the spectrum is characteristic of the A-form. The observed change in the global conformation can be ascribed to the large content of LNA nucleobases in the **LD4** probe, as observed for the normal duplexes having no AP sites.[13] The LNA contents also affect the thermal stabilities of the AP site-containing LNA-DNA/DNA duplexes (Fig. S1).[14] The melting temperature (T_m) of **LD4**-containing duplexes (T_m = 62.7 ± 0.1°C; n = 3) is much higher compared to DNA duplexes (T_m = 46.2 ± 0.1°C; n = 3) while the T_m values of **LD1**, **LD2**, or **LD3**-containing duplexes are slightly smaller or comparable (T_m/°C: **LD1**, 44.3 ± 0.1; **LD2**, 46.3 ± 0.3; **LD3**, 45.8 ± 0.2; n = 3).

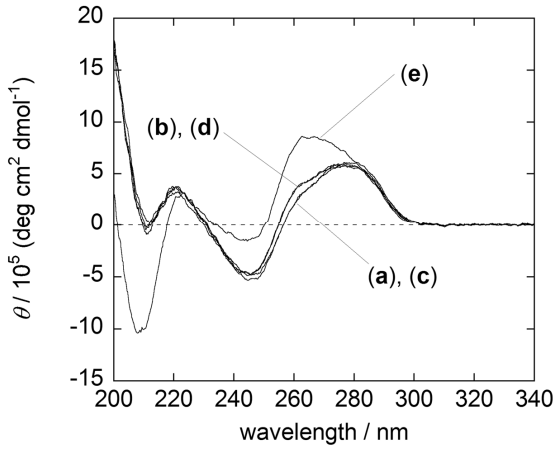

Fig. 2 CD spectra of (a) DNA/DNA, (b) **LD1**/DNA, (c) **LD2**/DNA, (d) **LD3**/DNA, (e) **LD4**/DNA duplexes (10 μM) containing the target T. Sample solutions were buffered to pH 7.0 with 10 mM sodium cacodylate containing 100 mM NaCl and 1.0 mM EDTA. Temperature, 20°C.

Next, we examined the fluorescence response of amiloride to AP site-containing LNA-DNA/DNA duplexes. Fig. 3A shows the fluorescence spectra of amiloride in the absence and presence of **LD1**/DNA duplexes. In the absence of the duplexes, amiloride has an emission with a maximum at 415 nm (curve **a**). The addition of the **LD1**/DNA duplexes causes the fluorescence enhancement responses of amiloride and the response strongly depends on the target nucleobase opposite the AP site. Amiloride shows a significant response for the target T (curve **e**) while almost no or only moderate responses are observed for other target nucleobases, suggesting the binding selectivity of amiloride for the target T in the **LD1**/DNA duplexes. This is consistent with the results for the UV-visible absorption spectral changes (Fig. S2), where amiloride shows a strong decrease in the absorption intensity (32.8%) and a large red shift (11.4 nm) upon binding to the target T. Likewise, the most significant fluorescence responses are observed for the target T in the **LD2** and **LD3**-containing duplexes (Fig. S3). Considering the T-selectivity for the DNA duplexes (Fig. 3B),[9b, 9c] the selectivity of amiloride remains unchanged by using LNA flanking nucleobases in the AP site-containing duplexes. Furthermore, amiloride shows the most significant response for the target T in the A-formed **LD4**/DNA duplexes (Fig. S3). Then, the global conformation of the AP site-containing duplexes does not alter the selectivity of amiloride, which is in good agreement with our previous result.[10a]

The fluorescence titration experiments reveal the significant influence of LNA flanking nucleobases as the enhancer for amiloride binding. The binding-induced fluorescence response of amiloride was monitored when increasing the concentration of duplexes (Fig. S4A). For all duplexes, the resulting titration curves are well analyzed by nonlinear least-squares regression based on a 1:1 binding isotherm (Inset of Fig. 3),[6] giving the binding affinity with the dissociation constants (K_d). The K_d value for the target T in the **LD1**/DNA duplex is determined as 57.6 ± 2.8 nM (n = 3). This affinity is significantly larger than that for other target nucleobases (K_d > 3500 nM). This result shows the strongest binding affinity for the target T in the **LD1**/DNA duplex. Notably, the binding affinity for **LD1**/DNA duplexes is found to be much larger than that for normal DNA duplexes, in which the affinity for the target T in the DNA duplex (K_d = 890 ± 2.4 nM (n = 3); cf. Inset of Fig. 3B and Fig. S4B) is 15-times weaker compared to the **LD1**/DNA duplex. Hence, the LNA flanking nucleobases of the AP site result in the significant enhancement of the binding affinity of amiloride for the target T.

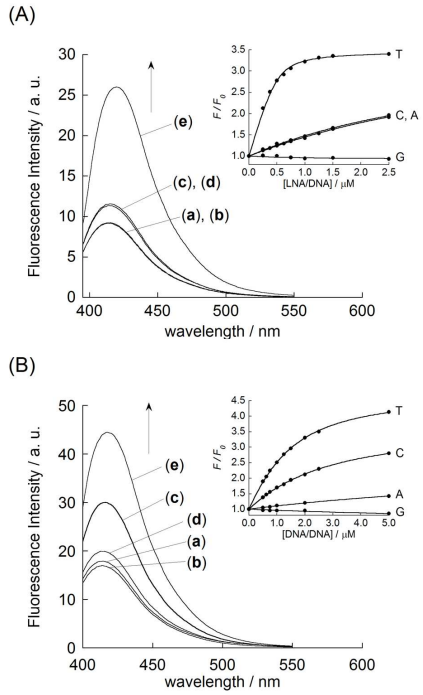

Fig. 3 Fluorescence response of amiloride to the target nucleobase in (A) **LD1**/DNA and (B) DNA duplexes: (a) duplex free, target nucleobase = (b) G, (c) C, (d) A, or (e) T. (A) [amiloride], [duplex] = 0.5 μM, (B) [amiloride], [duplex] = 1.0 μM. Other solution conditions are the same as those given in Fig. 2. Excitation, 380.5 nm. Temperature, 20°C. Inset: Nonlinear regression analysis of the changes in the fluorescence intensity ratio at 415 nm based on a 1:1 binding isotherm model. F and F_0 denote the fluorescence intensities of amiloride in the presence and absence of duplexes, respectively.

Although we observed a similar effect of the LNA flanking nucleobases on the binding affinity for the **LD2**/DNA and **LD3**/DNA duplexes, the enhancement degree of the binding affinity is moderate (Fig. S3). The affinities for the target T are enhanced 3.0 and 7.4-times in the **LD2** and **LD3**-containing duplexes, respectively compared to DNA duplexes (K_d / nM (n = 3): **LD2**, 301 ± 23; **LD3**, 121 ± 6.3). Meanwhile, the enhancement is slight for the duplex containing the LNA nucleobase that does not flank the AP site (K_d = 382 ± 6.4 nM (n = 3); 5'-GGG GAA GG**A** TXT GAA GGA AAA-3'/3'-TTT TCC TTC ATA TCC TTC CCC-5'; **A**, LNA nucleobase). From these observations, we consider that LNA flanking nucleobases on both 5'- and 3'-sides of the AP site can effectively improve the binding affinity of amiloride. We note that such a favorable effect from the

LNA flanking nucleobases on both 5'- and 3'-sides is observed in the cases of other flanking nucleobases (GXG, CXC, and AXA) while the degree of the enhancement depends on the kind of flanking nucleobases (Table S1). Additional LNA nucleobases besides the flanking nucleobases (**LD4**) are found to result in further enhanced affinity of amiloride (K_d = 46.2 ± 2.0 nM (n = 3)), but its enhancement of the affinity over **LD1**-containing duplex is small.

In order to clarify the favorable effect of the LNA flanking nucleobases on the binding affinity of amiloride, we examined the thermodynamic parameters based on the ITC experiments, with focusing on the binding to the target T in the **LD1**- or **LD4**-containing DNA duplex because of its remarkable enhancement of the affinity relative to the normal DNA duplexes. Fig. 4 shows typical ITC data for amiloride binding to the target T in the **LD1**/DNA duplexes. For all duplexes, a large exothermic heat of reaction is observed upon addition of amiloride aliquots into the solution containing the DNA duplexes (Fig. S5). As is seen in Table 1, the binding reaction of amiloride is driven by the enthalpy (ΔH_{obs}) for all duplexes. Significantly, the loss in the binding entropy ($T\Delta S_{obs}$) for **LD1-** or **LD4-**containing duplex is lower than that for the DNA duplex whereas the gain in ΔH_{obs} becomes small for these LNA-containing duplexes. Accordingly, the LNA nucleobases in the duplexes can reduce the loss in the binding entropy, which is indeed responsible for the enhanced binding affinity of amiloride for target T in the LNA-containing duplexes compared to the DNA duplexes. On the other hand, amiloride binding to target T in LNA-containing duplexes causes little change in CD spectra of the duplexes (Fig. S6), indicating no drastic conformational changes of the duplexes. Thus, the observed decrease in the entropic loss is highly likely to arise from the increased rigidity of the AP site by using the LNA flanking nucleobases due to the restricted ribose moiety that allows the preorganized conformation.[15] On the other hand, we note that the thermodynamic paramters for **LD1** and **LD4**-containing duplexes are almost comparable considering the standard deviations while larger enthalpy gain is observed for **LD4**-containing duplex than **LD1**-containing duplex.

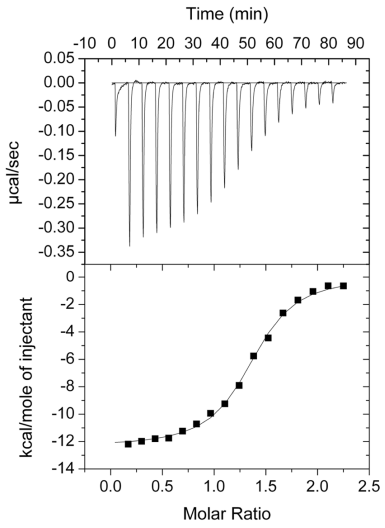

Fig. 4 ITC data for the addition of amiloride aliquots (60 μM) into the solution containing **LD1**/DNA duplex having the target T (5.0 μM). Other solution conditions are the same as those given in Fig. 2. Temperature, 20°C. The data were best-fitted to a model that assumes a single set of identical binding sites. The analysis gives the binding enthalpy (ΔH_{obs}) and the binding stoichiometry (n).

Table 1. Thermodynamic parameters for amiloride binding to the target T in the AP site-containing LNA-DNA/DNA and DNA duplexes[a]

	n	ΔG_{obs} (kcal/mol)	ΔH_{obs} (kcal/mol)	$-T\Delta S_{obs}$ (kcal/mol)
LD1	1.3	–9.7 ± 0.057	–12.4 ± 0.40	2.7 ± 0.41
LD4	1.3	–9.8 ± 0.025	–13.1 ± 0.23	3.3 ± 0.23
DNA	1.2	–8.1 ± 0.015	–14.5 ± 0.46	6.4 ± 0.46

[a] n is the stoichiometry of the binding of amiloride for the duplexes. ΔG_{obs} is the observed binding free energy calculated from $\Delta G_{obs} = -RT \ln K_{11}$ (K_{11} (the binding constant) = $1/K_d$ (cf. Figs 3A and S3C in the Supplementary Data)). ΔH_{obs} was directly determined by isothermal titration calorimetry (ITC; Figs 4 and S5). $T\Delta S_{obs}$ was calculated from $T\Delta S_{obs} = \Delta H_{obs} - \Delta G_{obs}$. [b] Errors are the standard deviations obtained from three independent experiments.

Finally, we examined whether the LNA flanking nucleobase can function as the enhancer of amiloride binding to DNA/RNA hybrid duplexes similar to DNA duplexes as described above, where the hybrid duplexes consisting of **LD1** or **LD4** probe and the target RNAs (Fig. 1B) were utilized. It is clearly seen that amiloride shows selectivity for the target U opposite an AP site in the

LNA-containing hybrid duplexes (Fig. 5). From the fluorescence titration experiments (Fig. S7), we note there is significant binding affinity of amiloride for U in these hybrid duplexes (K_d / nM (n = 3): **LD1**, 110 ± 11; **LD4**, 48 ± 2.0). These values are 16.4 and 37.5 times superior to the corresponding hybrid duplexes (Fig. S8; K_d = 1800 ± 99 nM (n = 3)). Therefore, amiloride shows significant enhanced affinity for the AP site-containing LNA-DNA/RNA hybrid duplexes compared to the DNA/RNA hybrid duplexes. Such an improved affinity of amiloride for the LNA-containing hybrid duplexes is useful for RNA analysis such as detection of the single base mutation in microRNA[16] as has been demonstrated in our recent study.[10a,17]

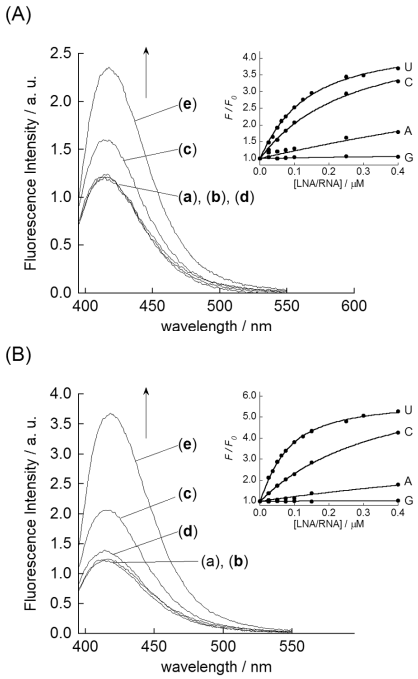

Fig. 5 Fluorescence response of amiloride to the target nucleobase in (A) **LD1**/RNA and (B) **LD4**/RNA: (a) duplex free, target nucleobase = (b) G, (c) C, (d) A, or (e) U. [amiloride], [duplex] = 0.05 μM. Other solution conditions were the same as those given in Fig. 2. Excitation, 380.5 nm. Temperature, 20°C. Inset: Nonlinear regression analysis of the changes in the fluorescence intensity ratio at 415 nm based on a 1:1 binding isotherm model. F and F_0 denote the fluorescence intensities of amiloride in the presence and absence of duplexes, respectively.

While we focused on the enhancement of the binding affinity of amiloride for AP site-containing DNA duplexes and DNA/RNA hybrid duplexes, it should be noted that the fluorescence properties of the ligands are another important issues for the practical application of our ligand-based assays from the analytical point of view. In order to achieve large binding-induced fluorescence responses of the ligands, the use of quencher such as iodide should be effective because

it can suppress the background emission of the ligands in the absence of DNAs. On the other hand, the conjugation of AP site-binding ligands with another fluorophores can be useful for monitoring the long-wavelength fluorescence signaling, which should be advantageous for the bioanalytical applications. Therefore, we expect that these approaches are effective for further enhancement of the practicability of the present approach that uses LNA nucleobase as the enhancer of the binding of amiloride.

Conclusion

In summary, we described the significant influence by LNA nucleobases on the binding of amiloride to AP site-containing DNA duplexes. The binding affinity of amiloride for the target T was remarkably improved for the DNA duplexes possessing LNA nucleobases that flank the AP site, compared to the corresponding DNA duplex. Moreover, such an enhancement of the binding affinity of amiloride was also achieved for the AP site-containing DNA/RNA hybrid duplexes. These results clearly showed the approach using LNA flanking nucleobase-containing probes is quite effective for improving the binding affinity of AP site-binding ligands, enabling to enhance the practicability of our ligand-based gene analysis[6] as well as bioanalytical applications.[5] Use of the duplexes containing LNA flanking nucleobases (cf. **LD1**-containing duplex) is expected to allow an effective affinity-labeling of the ligands without reducing the sensing ability of the aptamers[5a-5h] and molecular beacons[5i] since this kind of LNA modification shows little influence on the thermal stability and the conformation of the duplexes (Figs 2 and S1), which can improve the sensitivity for detecting the target analytes. On the other hand, the use of many LNA-modified duplexes (cf. **LD4**-containing duplex) is expected to improve the hybridization selectivity for the target DNAs and RNAs considering the observation of the increased thermal stability as well as the global conformational change of the AP site-containing duplex (Figs 2 and S1) like the normal duplexes containing no AP sites,[9,13] leading to accurate gene analysis in our assay.[6,10a,17] Moreover, this work revealed new aspects of LNA nucleobases regarding interactions between nucleic acids and small ligands, which will lead to the development of various LNA-containing nucleic acid-based assays for therapeutic and diagnostic applications. We are now undertaking further studies in this direction.

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Experimental Section

Materials

LNA-DNA, DNA, and RNA oligonucleotides were custom synthesized and HPLC-purified by Gene Design Inc. (Ibaraki, Japan), Nihon Gene Reserach Laboratories Inc. (Sendai, Japan), and

Sigma-Genosys (Ishikari, Japan), respectively. Amiloride and other reagents were commercially available with analytical grade and were used without further purification. Water was deionized (≥18.0 MΩ cm specific resistance) by an Elix 5 UV Water Purification System and a Milli-Q Synthesis A10 system (Millipore Corp., Bedford, MA), followed by filtration through a BioPak filter (Millipore Corp.) in order to remove RNase.

The concentrations of LNA-DNAs were determined according to the manufacture's instruction and those of RNAs and DNAs were determined from the molar extinction coefficients at 260 nm, according to the literature.[18]

Unless otherwise mentioned, all measurements were performed in 10 mM sodium cacodylate buffer solutions (pH 7.0) containing 100 mM NaCl and 1.0 mM EDTA. Before measurements, the sample solutions were annealed as follows: heating at 75°C for 10 min, gradually cooling to 5°C (3°C/min), and raising the solution temperature to 20°C (1°C /min).

UV-visible and fluorescence spectra measurements

Absorption and fluorescence spectra were measured at 20°C with a JASCO model V-570 UV–vis spectrophotometer and FP-6500 spectrofluorophotometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan), respectively. Both instruments were equipped with thermoelectrically temperature-controlled cell holders. Measurements of absorption and fluorescence spectra were done using a 2 × 10 mm quartz cell (optical path length: 10 mm) and a 3 × 3 mm quartz cell, respectively. In the absorbance measurements, bandwidth of 2 nm and scan rate of 40 nm/min were used. Typically, in the fluorescence measurements, excitation and emission band width were set to 3 nm and 5 nm, respectively, and a scan rate was 100 nm/min. In the fluorescence measurements, excitation wavelengths for amiloride was set at the isosbetic point (380.5 nm for all duplexes), determined from UV-vis spectral changes for amiloride binding to the duplexes. Fluorescence titration experiments for the determination of the binding affinity of amiloride were performed done as described in our previous study.[10a]

Determination of the binding affinity from fluorescence titration

The changes in fluorescence intensity of amiloride at 415 nm were monitored as a function of the concentration of the duplex. The resulting titration curve was analyzed by nonlinear least-squares regression based on a 1:1 binding isotherm:[19]

$$F/F_0 = \{1 + kK_{11}[D]/1 + K_{11}[D]\} \quad (1)$$

where F and F_0 are the observed fluorescence intensities of amiloride in the presence and absence of duplexes, respectively, and k (= k_{11}/k_L) represents the ratio of proportionality constants connecting the fluorescence intensities and concentrations of the molecules (1:1 complex: k_{11}, free ligand: k_L). K_{11} is the 1:1 binding constant. The concentration of the free duplex, [D], can be related to known

total concentrations of the duplex (D_0) and ligand (L_0), by the following equation:

$$[D] = K_{11}(D_0 - L_0) - 1 + \sqrt{\{K_{11}(D_0 - L_0) - 1\}^2 + 4K_{11}D_0} \,/\, 2K_{11} \qquad (2)$$

Together, eqs. (1) and (2) describe the system, which allows to determine the binding affinity with the dissociation constant ($K_d = 1/K_{11}$) by the fitting using KaleidaGraph software.

CD measurements

Circular dichroism (CD) spectra were measured with a JASCO model J-800 spectropolarimeter (scan rate, 5 nm/min; band width, 1 nm; response, 16 sec) that was equipped with a thermoelectrically temperature-controlled cell holder (Japan Spectroscopic Co. Ltd.) using a 2 × 10 mm quartz cell (optical path length: 10 mm).

Melting temperature (T_m) measurements

Absorbance of LNA-DNA and DNA duplexes was measured at 260 nm as a function of temperature using an UV-vis spectrophotometer Model UV-2450 (Shimadzu Corp., Kyoto, Japan) equipped with a thermoelectrically temperature-controlled micro-multicell holder (8 cells; optical path length = 1 mm). The temperature ranged from 2°C to 92°C with a heating rate of 1.0°C /min. The resulting absorbance versus temperature curve was analyzed by a differential method to determine T_m values.

Isothermal titration calorimetry

ITC experiments were carried out in order to examine the thermodynamic parameters for amiloride binding using a Microcal VP-ITC calorimeter (Microcal Inc., Northampton, MA). ITC experimental details are given in our previous report. [10a]