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ARTICLE TYPE

DNA Sensor's Selectivity Enhancement and Protection from Contaminating Nuclease due to Hydrated Ionic Liquid

Hisae Tateishi-Karimata,[a] Smritimoy Pramanik,[a] and Naoki Sugimoto*[a,b]

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The thermodynamic stability of certain mismatched base pairs has made the development of DNA sequence sensing systems challenging. Thus, the stability of fully matched and mismatched DNA oligonucleotides in the hydrated ionic liquid choline dihydrogen phosphate (choline dhp) was investigated. Mismatched base pairs were significantly destabilized in choline dhp relative to those in aqueous buffer. A molecular beacon that forms a triplex with a conserved HIV-1 sequence was then designed and tested in choline dhp. The molecular beacon specifically detected the target duplex via triplex formation at concentrations as low as 1 pmol/10 μL with 10,000-fold sequence selectivity. Moreover, the molecular beacon was protected from a contaminating nuclease in choline dhp, and DNAs in aqueous solutions were not sufficiently stable for practical use.

Introduction

Systems for sensing certain DNA sequences, particularly single nucleotide polymorphisms (SNPs), are important in the fields of medicine and nanobiotechnology.[1-6] Traditional methods for sensing DNA sequences using molecular beacons, DNA microarrays, or *in situ* hybridisation are based on the formation of A–T and G–C Watson–Crick base pairs (the dash indicates the Watson–Crick base pair) between the target sequence and the probe DNA.[7-10] In general, duplexes of fully matched A–T and G–C base pairs are more stable than those with mismatched base pairs; therefore, an optimally designed probe DNA should discriminate a completely complementary target sequence from the one containing mismatches. However, two issues make these sensing systems insensitive. First, the use of these probes is compromised by thermodynamically stable mismatches such as the G–T mismatch.[11-13] In fact, approximately 30% of the SNPs result in G–T mismatches between the target and probe,[14, 15] and the formation of thermodynamically stable mismatched duplexes significantly decreases probe sensitivity. Second, nucleic acids in aqueous solution are not sufficiently stable for practical use in many applications, because these molecules either spontaneously degrade or are degraded by contaminating nucleases [16].

Certain remarkable features of ionic liquids (ILs) make them attractive alternatives to water. For example, a representative IL—choline dihydrogen phosphate (choline dhp)—dissolved in a small amount of water (~20 wt% or 4 M choline dhp) ensures the long-term stability of biomolecules such as DNA and has negligible vapour pressure.[17-22] Because high concentration salt solutions act as denaturing agents for proteins, we hypothesized that choline dhp would reduce the activity of contaminating nucleases. Moreover, we previously demonstrated that ILs impact the stability of Watson–Crick base pairs.[23, 24] In addition, whereas stable Hoogsteen base pairs only form in A- and T-rich sequence motifs in aqueous buffers at neutral pH [25, 26] because cytosines in the third strand must be protonated at N3 (pK_a = 4.5) to form C–G*C$^+$ base triplets (the star indicates the Hoogsteen base pair) [27, 28] although the pK_a values shift to higher values inside triplexes depending on their sequences. [29] Hoogsteen base pairs in triplexes are stabilized in choline dhp.[30, 31] Moreover, the choline dhp also change the stability of tetraplexes.[32, 33] These

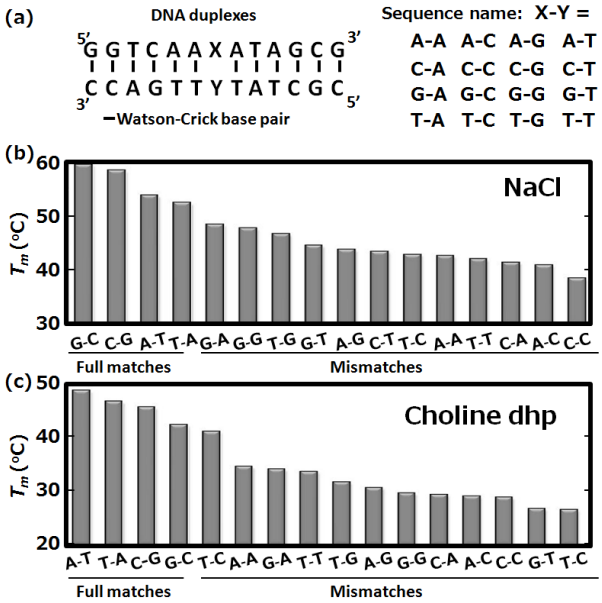

Fig. 1 (a) DNA duplex sequences and sequence names. (b) T_m values for 20 μM DNA duplexes in a solution containing 50 mM Tris (pH 7.0), 1 mM Na_2EDTA and 4 M NaCl. (c) T_m values in 50 mM Tris (pH 7.0), 1 mM Na_2EDTA and 4 M choline dhp. The T_m values for the duplexes are arranged from the highest to the lowest.

results suggest that the unique interactions between choline dhp and DNA should facilitate the development of DNA sensing systems.

Results and discussion

Herein, we describe the results of an investigation of the quantitative effects of choline dhp on the discrimination of Watson–Crick base pairs due to mismatches. The studied duplexes were formed from the 13-mer non-self-complementary oligonucleotide sequences 5′-GGTCAAXATAGCG-3′ and 5′-CGTATYTTGACC-3′, where 'X' and 'Y' denote positions at which the nucleotides were varied to investigate each possible base pair and mismatched pair (Fig. 1a). The thermal stability of the duplexes was determined in 4 M choline dhp solutions and their melting temperatures (T_m) were estimated (Fig. S1). The stability of duplexes was also evaluated in 4 M NaCl (Fig. S1, because previous quantitative analyses of DNA duplex stabilities have been performed in 1 M NaCl solutions.[12, 13] The T_m values for the duplexes are shown in Figures. 1b and 1c. In NaCl solutions, the T_m values of fully matched duplexes containing G–C and C–G pairs were higher than those for A–T and T–A pairs. Moreover, although mismatched duplexes were destabilized relative to fully matched duplexes, certain mismatched base pairs such as G–A, G–G, T–G were relatively stable, as reported previously.[12, 13] In contrast, in hydrated choline dhp, a different trend for the stabilities of the DNA duplexes was observed. The T_m values of the fully matched duplexes containing A–T and T–A base pairs were higher than those for the duplexes containing G–C and C–G base pairs. In addition, the differences in the T_m values for the mismatched and fully matched DNA duplexes were greater than those observed in aqueous solutions. Furthermore, the T_m values for the G–T and T–G base pairs were notably decreased relative to those for the A–T and T–A base pairs. Thus, choline dhp stabilized the A–T and T–A base pairs and destabilized the mismatched G–T and T–G pairs relative to their thermodynamic stabilities in aqueous buffer. Menhaj et al. reported that the choline dhp perturbed the solution pH and affected DNA duplex stability.[34] Thus, we estimated the melting curves for DNA duplexes (Fig. 1a) at different pH values (pH 6.0) with Figures 1b and 1c. As results showed, the stability of the duplexes were slightly changed; however, the differences in the T_m values for the mismatched and fully matched DNA duplexes in choline dhp were greater than those observed in aqueous solutions (Fig. S2). Thus, choline dhp can enhance the DNA mismatch discrimination.

Next, the quantitative effects of choline dhp on the discrimination of Hoogsteen base pairs in triple stranded complexes were investigated. Eight third strand sequences with the ability to bind duplex via Hoogsteen base pairs with or without mismatches were designed (Fig. 2a). The thermal stabilities of the triplexes were determined by monitoring their melting behaviour following UV irradiation at 295 nm. In solutions containing 50 mM Tris (pH 7.0), 1 mM Na_2EDTA and 4 M NaCl solution no transitions were observed at 295 nm (Fig. S3), indicating that under these conditions, the third strand did not bind to the duplex via Hoogsteen base pairing. However, in 4 M choline dhp solutions, clear melting transitions were observed at 295 nm, indicating that triplexes formed (Fig. S4). Previously we reported that choline ions facilitate triplex formation via binding to grooves in the triplex.[30] The T_m values for the T*A and C*G triplexes in the present study were 50.2 °C and 48.3 °C, respectively (Fig. 2b). Because the formation of C*G base pairs requires the protonation of C, the C*G base pairs were less stable than the T*A base pairs. In addition, although triplexes formed at pH 5.0 in NaCl, the mismatches were not discriminated effectively from Hoogsteen pairs (Fig. S5). This result is in agreement with our previous report that in choline dhp, Watson–Crick and Hoogsteen type A–T pairs are stabilized relative to G–C pairs.[24] Choline ions bind preferentially to G bases in single-stranded regions, inhibiting base pair formation with G. [24] Therefore, choline dhp enhanced the discrimination between fully matched (A–T or A*T) base pairs and stable mismatched base pairs.

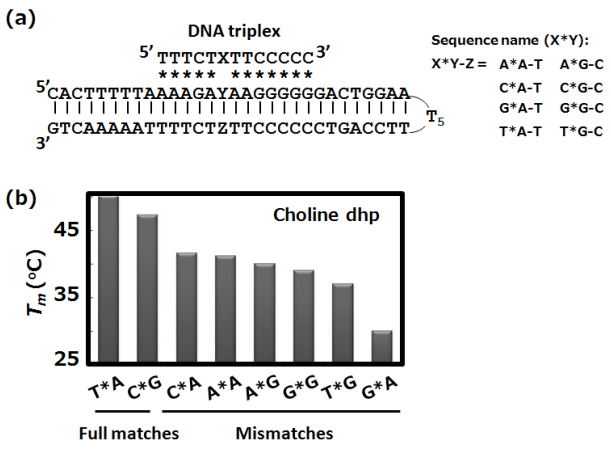

Fig. 2 (a) DNA triplex sequence and names of the third strands. (b) T_m values for 40 μM DNA triplexes in 50 mM Tris (pH 7.0), 1 mM Na_2EDTA and 4 M choline dhp.

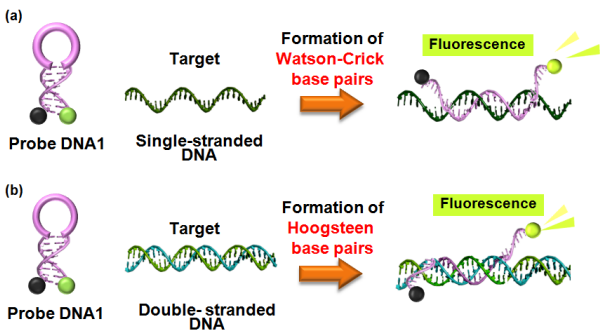

Fig. 3 Schematic of a molecular beacon detection system via the fornmation of (a) Watson–Crick and (b) Hoogsteen base pairs.

Taking advantage of this large destabilization of mismatches that are stable in water, a sensing system in choline dhp was developed. First, a 29-mer DNA molecular beacon (probe DNA1) were designed. The probe was able to form a hairpin that brings a 5′ fluorophore (6-carboxylfluorescein, 6-FAM) and a 3′ quencher (Black Hole Quencher 1) into close proximity (Figures 3a and 3b, respectively).[35] Probes DNA1 is complementary to a conserved HIV-1 sequence (binding site) via Watson–Crick and Hoogsteen base pairs (Figures 3, S6 and S7), respectively.).[28, 36] The target sequences for probe DNA1 were single-stranded DNAs (Ss1 and Ss2), and those for probe DNA1 were double-stranded DNAs (Ds1 and Ds2) (Figures 3, S6 and S7, respectively). The ability of the probe DNAs to detect the target sequence was determined using a fluorescence assay. Figure 4 shows the fluorescence spectra of 1 μM solutions of the probe DNA in the absence and presence of 1 μM target sequences. Experiments were performed at 45 °C to enhance the target selectivity. The fluorescence intensity of Alexa488 at 520 nm (excitation wavelength: 488 nm) in the absence of the target sequences was very small in NaCl and choline dhp solutions.

For probe DNA1 in the presence of Ss1, the fluorescence intensities of 6-FAM at 520 nm in 4 M NaCl and 4 M choline dhp solutions were 499 and 872 arbitrary units (AU), respectively (Figures 4a and 4b), indicating that probe DNA1 detected the single-stranded target sequence. In the presence of Ds1, the fluorescence intensities of 6-FAM at 520 nm with probe DNA1 in 4 M NaCl and 4 M choline dhp solutions were 25.1 and 802 AU, respectively (Figures 4c and 4d). Thus, the interaction between probe DNA1 and Ds1 was significantly stabilized in choline dhp solution relative to that in NaCl solution. Next, to determine whether the sequence sensing system discriminated the fully complementary target sequence from a sequence with a single nucleotide mutation (A to G) in the Watson–Crick and Hoogsteen base pairs, the two DNA sequences Ss2 and Ds2 with T–G and T*G mismatches relative to Ss1 and Ds1 (Figures S6 and S7), respectively, were designed. The fluorescence intensity of 6-FAM with probe DNA1 in the presence of Ss2 in NaCl was nearly the same as that in the presence of Ss1, indicating that the G-T mismatch was stable and the probe DNA1 was not sensitive to the mismatch (Fig. 4a). In contrast, in choline dhp, the fluorescence intensity of probe DNA1 with Ss2 was less than that with Ss1 (Fig. 4b), indicating discrimination against the G–T mismatch. For the triplex probe DNA1 in choline dhp, discrimination between Ds1 and Ds2 was very clear (Fig. 4d). The fluorescence intensity in the presence of mismatched Ds2 was one-seventh that in the presence of fully matched Ds1.

We also conducted the single mismatch detection using fluorescence change in 100 mM NaCl at pH 7.0 (physiological condition) because high NaCl concentration (4 M) is relative to physiological conditions and might show negative effects on the mismatch detection. Consequently, our results showed the NaCl concentration did not mainly affect the mismatch discrimination. For probe DNA1 in the presence of Ss1 and Ss2 the fluorescence intensities of 6-FAM at 520 nm in 100 mM NaCl were 357 and

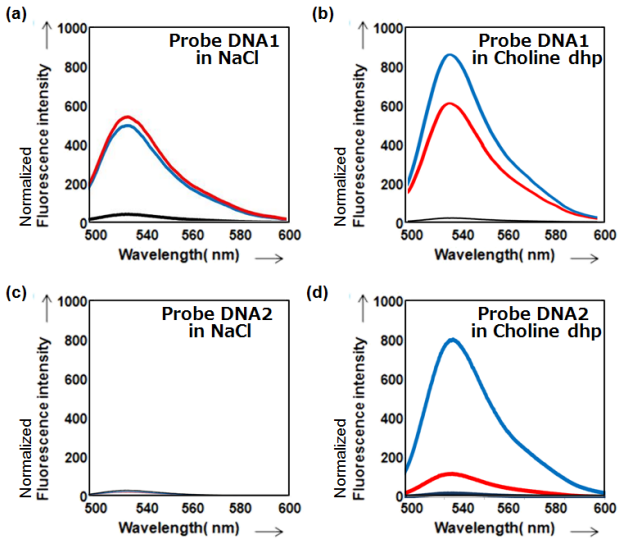

Fig. 4 Normalized emission spectra recorded at 45°C for 1 μM probe in the absence (black) and presence of 2 μM fully matched target sequence (blue) and with mismatched target (red). (a and b) Probe DNA1 with Ss 1 or Ss2 in a solution containing 50 mM Tris (pH 7.0), 1 mM Na_2EDTA, and (a) 4 M NaCl and (b) 4 M choline dhp. (c and d) Probe DNA1 with Ds1 or Ds2 in a solution containing 50 mM Tris (pH 7.0), 1 mM Na_2EDTA, and (c) 4 M NaCl and (d) 4 M choline dhp.

360 (AU), respectively (Fig. S8), indicating that probe DNA1 incorrectly detected the single-stranded target sequence with and without mismatch in 100 mM NaCl. Moreover, we estimated mismatched detection via the Hoogsteen base pairs formation. In the presence of Ds1, the fluorescence intensity of 6-FAM at 520 nm with probe DNA1 in 100 mM NaCl was less than 100, which was similar to probe DNA1 in the absence of Ds1 (Figs. 4c and 4d). Thus, the interaction between probe DNA1 and Ds1 was unstable and undetectable.

To quantify the sensitivity of the probe DNA1, the thermal denaturation of Ds1 and Ds2 in the presence of probe DNA1 was examined (Fig. S9). The value of ΔT_m (the difference between the melting temperatures of the probe with perfectly matched and mismatched targets) in the choline dhp solution was 18.3 °C. In general, detecting Watson–Crick T–G base pairs is difficult with molecular beacons, because this mismatch is more stable than other mismatches. From the parameters of the nearest neighbour model,[37] the value for $\Delta\Delta G^{o}_{25}$ (the difference in the ΔG^{o}_{25} values of the probe with a mismatched sequence with Ds1 and that of a perfectly matched probe) in the choline dhp solution was 1.4 kcal mol^{-1}, indicating that the discrimination ratio for the T–G mismatch (the ratio of the probe DNA binding to the target duplex with a T–A full match to a T–G mismatch) at 25 °C was 90:10. Interestingly, when the target sequence had the T*G mismatch, the Hoogsteen base pairs was destabilized by 6.4 $kcal^{-1}mol^{-1}$, indicating that the discrimination ratio for the T–G mismatch was 99.999:0.001. Thus, the selectivity for the target sequence was enhanced by 10,000 times in choline dhp relative to an aqueous buffer.

Next, to evaluate the utility of this system, the dependence of change in the fluorescence intensity on the concentration of Ds1 was determined. The relative fluorescence of probe DNA1 at 520 nm was calculated by subtracting the fluorescence intensity at 520 nm obtained in the absence of Ds1 from that in the presence of Ds1. Notably, the relative fluorescence intensity at 520 nm remained unchanged in 4 M NaCl after increasing the Ds1 concentration (Fig. S10), whilst in the choline dhp solution, the correlation between the relative fluorescence intensity at 520 nm and the Ds1 concentration was linear (Fig. S10). Furthermore, the relative fluorescence intensity at 30 nM Ds1 in the choline dhp solution was readily detectable at approximately a factor of 10 (3 pmol/100 μL).

To increase the selectivity of target detection and decrease the solution volume, target detection was investigated using fluorescence correlation spectroscopy (FCS). Figure S11 shows the principle of target sensing using FCS. In this method, fluorescently labelled DNA (probe DNA2) binds to the target DNA, which leads to a significant increase in the characteristic diffusion time (τ_D) of probe DNA2.[38] Thus, the FCS method can sensitively detect triplex formation. The quantitative analysis is based on the change in the τ_D as a function of the target duplex concentration. Note that, because 4 M choline dhp quenches fluorescence under the FCS experimental conditions, these FCS experiments were performed using 2 M choline dhp solutions. Figure 5 shows the normalized correlation curves for probe DNA2 and the probe DNA2-target complex. The diffusion time or the probe DNA2-target complex was greater than that for the probe DNA alone. Furthermore, the τ_D values increased as the target DNA concentration increased, which demonstrates that FCS can be used to sensitively discriminate between free and bound states of probe DNA2. Notably, even the lowest concentration of target evaluated, 10 nM (1 pmol/10 μL), was detected using this system. Ricci *et al.* reported a complicated electrochemical sensor for the detection of PCR-amplified HIV-1 targets with triplex-forming oligonucleotides with a detection limit of 10 nM.[28] The present simpler sensing system exhibited comparable sensitivity.

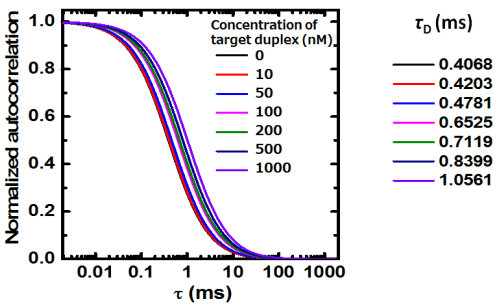

Fig. 5 Normalized autocorrelation curves for 10 nM probe DNA2 in the absence (black) and presence of the target at the concentrations indicated in the key. The solution contained 50 mM Tris (pH 7.0), 1 mM Na_2EDTA and 2 M choline dhp.

Finally, it was determined whether choline dhp can protect the probe DNA from contaminating nucleases such as those present in clinical samples. Nuclease activity was estimated by monitoring the fluorescence intensity of probe DNA1. Degradation of the probe due to cleavage results in the separation of the fluorophore from the quencher and an increase in the fluorescence intensity, which would be mistaken as a positive signal in the sensing system using probe DNA1. In an aqueous solution containing NaCl, the fluorescence intensity due to the probe at 520 nm increased approximately 80-fold after incubation for 24 h with 1 U of nuclease I (Fig. 6a, blue symbols). These observations suggest that most of probe DNA1 was degraded within 24 h after nuclease was added to the aqueous solution. On the other hand, the fluorescence intensity of the probe in 4 M choline dhp in the presence of nuclease remained unchanged after 24 h (Fig. 6a, red symbols). Probe degradation was confirmed by denaturing with PAGE. (Fig. 6b). The high salt concentration (greater than 1 M) reduced the enzyme activity, as previously observed, but the reduction in nuclease activity in the presence of choline dhp was much larger than that in NaCl. Choline ions protected the probe from degradation, indicating that choline dhp is useful not only for enhancing mismatch discrimination but also as a nuclease inhibitor.

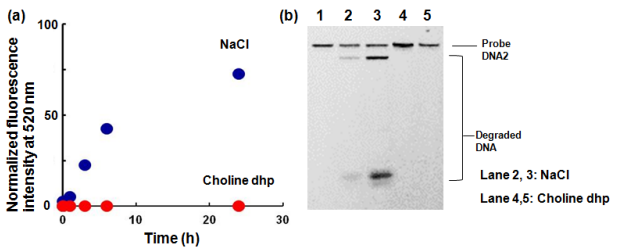

Fig. 6 (a) Fluorescence intensity at 520 nm of probe DNA1 in a choline dhp (red) or NaCl solution (blue) as a function of time after the addition of a nuclease. (b) Denaturing PAGE of probe DNA1 in the absence (lane 1) and presence of DNase I in 4 M NaCl after 6 (lane 2) and 24 h (lane 3) and in the presence of DNase I in 4 M choline dhp after 6 (lane 4) and 24 h (lane 5) at 37 °C. The concentrations of DNA1 and DNase I were 1 μM and 0.1 μM, respectively, and the solution contained 8 mM $MgCl_2$, 50 mM Tris (pH 7.0), 5 mM DTT and 4 M choline dhp or 4 M NaCl.

In this study, the hydrated IL of choline dhp was shown to enhance mismatch discrimination in DNA duplexes and triplexes relative to aqueous solution. Stable mismatches such as G–T, G–A and G–G can be detected by small ligands[39], which bind specifically to the mismatches. However, these systems require the special instrumentation or synthesis techniques. Here, the sensitive sequence-specific detection of an HIV sequence using a triplex-forming system for triplex stabilization in choline dhp was demonstrated. This new system allows the detection of a target sequence at room temperature using the naked eye (Fig. S12). Triplexes are stabilized in aqueous solution at acidic pH, but DNA strands with A- and C-rich sequences form not only triplexes but also i- and A-motif structures under these conditions[40, 41], and the nucleic acids do not exhibit long-term stability in acidic solutions. Consequently, acidic solutions are

not optimal for triplex sensing systems. On the other hand, MacFarlane *et al.* reported that DNA has long-term stability in choline dhp in the absence of nucleases.[19] In the present study, nuclease degradation of DNA was found to be significantly inhibited in a 4 M choline dhp solution. Another advantage of 4 M choline dhp is its low vapour pressure; a low-volume solution of 4 M choline dhp did not evaporate after a year at room temperature (data not shown), indicating that choline dhp can be used as a storage solution for DNAs. This system is therefore the first example of a DNA sensor with chemical stability that operates using a simple fluorescence-based method. Because hydrated ILs provide favourable environments for a wide range of chemical reactions and are green solvents, this system may be used not only for sensing applications but may also may serve as the basis for various nanotechnology devices.

Conclusions

In this manuscript, we report three important findings. First, the sequence selectivity of not only Hoogsteen base pairs but also of Watson–Crick base pairs was investigated. Choline dhp had a significant influence on the formation of Hoogsteen base pairs: there was 10,000-fold sequence selectivity for Hoogsteen pairs vs. mismatches relative to selectivity in an aqueous buffer at neutral pH. Second, to increase the selectivity for target detection and decrease the solution volume, we investigated target detection using fluorescence correlation spectroscopy and found that our system specifically detected the target duplex via triplex formation at concentrations as low as 1 pmol/10 μL. Third, we demonstrated that the DNA sensor was protected from a contaminating nuclease in choline dhp, and DNAs in aqueous solution were not sufficiently stable for practical use.

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Notes and references

[a] *Frontier Institute for Biomolecular Engineering Research (FIBER), Konan University,7-1-20 Minatojima-minamachi, Kobe 650-0047, Japan.*

[b] *Graduate School of Frontiers of Innovative Research in Science and Technology (FIRST), Konan University, 7-1-20 Minatojima-minamachi, Kobe 650-0047, Japan, Fax: +81-78-303-1495, Tel: +81-78-303-1457, E-mail: sugimoto@konan-u.ac.jp*

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