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Fluorescence enhancement of bis-acridine orange peptide, BAO, upon binding to double stranded DNA†

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Received 16th November 2004, Accepted 20th December 2004
First published as an Advance Article on the web 13th January 2005

Bis-acridine orange peptides carrying two acridine oranges at the ε-amino moieties of both terminal lysines of a tetra(lysine) chain showed a *ca.* 200-fold fluorescence enhancement upon addition of double stranded DNA.

Fluorescent dyes showing fluorescence enhancement upon binding to double stranded DNA (dsDNA) are currently increasing in importance in biotechnology and cell biology. To date, many dyes such as TOTO, YOYO and SYBR Green I have been developed, showing great fluorescence enhancement upon binding to dsDNA.[1,2] However, no dye has been reported that possesses the ability to discriminate between dsDNA and single stranded DNA (ssDNA) without showing DNA sequence specificity.[3] To develop a dsDNA-specific fluorescence dye, we designed and synthesized a series of fluorescent poly-intercalators the fluorescence of which, when quenched by intramolecular stacking of the chromophores in aqueous solution, is recovered upon dsDNA binding.[4,5] A series of poly(9-aminoacridinyl) peptides is a typical example of this approach.[4] For example, tetrakis-9-aminoacridinyl peptide (TAP) showed 1600-fold enhancement of its fluorescence upon binding to $[poly(dA\text{-}dT)]_2$. However, it did not show any fluorescence enhancement with $[poly(dG\text{-}dC)]_2$ despite its similar binding affinity for $[poly(dA\text{-}dT)]_2$ and $[poly(dG\text{-}dC)]_2$. TAP did not bind to poly(dA), nor did its fluorescence change significantly. This behaviour seemed to reflect the nature of the 9-aminoacridine unit of TAP. Therefore, if the 9-aminoacridine units were replaced with an acridine orange (AO), which is known to show fluorescence enhancement upon binding to dsDNA containing even G bases,[6] a dsDNA-specific fluorescence dye could be realized. With this consideration in mind, we developed and synthesized a bis-acridine orange peptide, BAO, which has a tetra(lysine) backbone chain carrying two AOs at the ε-amino moieties of *N*- and *C*-terminal lysine residues.

Fig. 1 shows the synthetic route for BAO. The treatment of AO (Aldrich) with sulfur afforded **1**[7] as orange crystals by the procedure of Elslager.[7] Further treatment of **1** with sodium ethoxide and methyl iodide in ethanol gave 3,6-bis(dimethylamino)-9-(methylmercapto)acridine (**2**)[7] as orange crystals, after purification by silica gel column chromatography (eluent, $CHCl_3$: MeOH : trifluoroacetic acid (TFA) = 10 : 3 : 0.1 v/v).

As a next step, the tetra(lysine) was assembled on a Model 431A peptide synthesizer (Applied Biosystems) from Fmoc-Sieber-PEG-resin (Watanabe Chemical Industries, Ltd., Japan) using the Fmoc solid-phase peptide synthesis method (SPPS) described previously[4,5] (Fig. 1). Different protection of the ε-amino moieties of the lysine residue with Boc and 4-methyltrityl (Mtt) groups allowed specific attachment of the AO to the ε-amino groups. After acetylation of the *N*-terminus of the peptide-resin, the resin was treated with 1% TFA in dichloromethane (DCM) and then with a mixed solvent (DCM : TFA : triisopropylsilane (TIPS) = 94 : 1 : 5, v/v) to detach the peptide from the resin with concomitant removal of the Mtt protecting groups. The crude peptide **5** (0.027 mmol) thus obtained was dissolved in a mixed solvent (DCM : triethylamine: trifluoroethanol = 3 : 1 : 0.1, v/v). To the solution, **2** (33.6 mg, 0.133 mmol) was added and the reaction mixture was stirred at 40 °C for 20 h. Evaporation of the solvent, followed by the addition of diethyl ether left a solid, which was dissolved in a small quantity of methanol and acetone before diethyl ether was added in an ice bath. The resulting solid was treated with a mixed solvent (TFA : thioanisole : *m*-cresol = 43 : 6 : 1, v/v) for 1 h to remove the Boc protecting groups. Crude BAO thus obtained was purified by reversed-phase HPLC using an Inertisil ODS column (4.6 × 250 mm) (GL Science Inc., Japan) and the following HPLC conditions: two solvent system; A 0.1% aqueous TFA; and B 70% acetonitrile in 0.1% aqueous TFA; a linear gradient of solvent B from 35 to 65% over 15 min at a flow rate of 1.0 ml min^{-1}. The target BAO was eluted and collected at 7 min. Its structure was confirmed by a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using α-cyano-4-hydroxycinnamic acid as the matrix: *m/z* calculated for $[C_{60}H_{87}N_{15}O_5 + H]^+$, 1099.4; found 1098.8.

Then, UV-visible (UV-vis) absorption and circular dichroism (CD) spectra of BAO were measured in 10 mM sodium 2-(*N*-morpholino)ethanesulfonate (MES) buffer (pH 6.25) containing 1 mM EDTA and 350 mM NaCl at 25 °C. The molar absorptivity of BAO at 430 nm was calculated to be 34500 cm^{-1} M^{-1} based on the absorption changes following addition of varied amounts of BAO (refer to electronic supplementary information†). The absorption maximum of BAO at 414 nm underwent a 56% hypochromic shift from that of AO (λ_{max} = 492 nm) with a blue shift of 78 nm as shown in Fig. 2. This result suggested that the AO units of BAO assumed an intramolecularly stacked conformation in the aqueous solvent. As suggested by Gardner and Mason's work on the absorption behaviour and structure of AO,[8] the two AO units of BAO seem to be disposed in a parallel orientation. The absorption spectrum of BAO in the aqueous solution underwent a hyperchromic shift with a small red shift of the absorption maximum at 420 nm upon addition of calf thymus dsDNA (Fig. 2). As shown in Fig. 3, a large exciton coupling[9] was observed in the presence of dsDNA at 350–450 nm in the CD spectrum of BAO in the same buffer solution at 25 °C. Taken together, BAO seems to bind to dsDNA with the stacked conformation of the AO units, but this conformation may be changed upon binding to dsDNA. The sign of the Cotton effect of BAO varied from negative to positive with a decrease of the wavelength in the region of 350–450 nm, but the reverse effect was observed upon binding to dsDNA (Fig. 3). Finally, fluorimetric studies on BAO were carried out in 10 mM MES buffer (pH 6.25) containing 1 mM EDTA and 350 mM NaCl at 25 °C in the presence of varied amounts of dsDNA or ssDNA. Fluorescence spectra of BAO and AO in the absence and the presence of calf thymus DNA or poly(dA) are shown in Fig. 4. The fluorescence intensity of BAO was slightly smaller than that of AO under these conditions. After addition of calf thymus DNA, the fluorescence intensity of AO was enhanced *ca.*

† Electronic supplementary information (ESI) available: Absorption change of bis-acridine orange against its concentration. See http://www.rsc.org/suppdata/ob/b4/b417391k/

DOI: 10.1039/b417391k

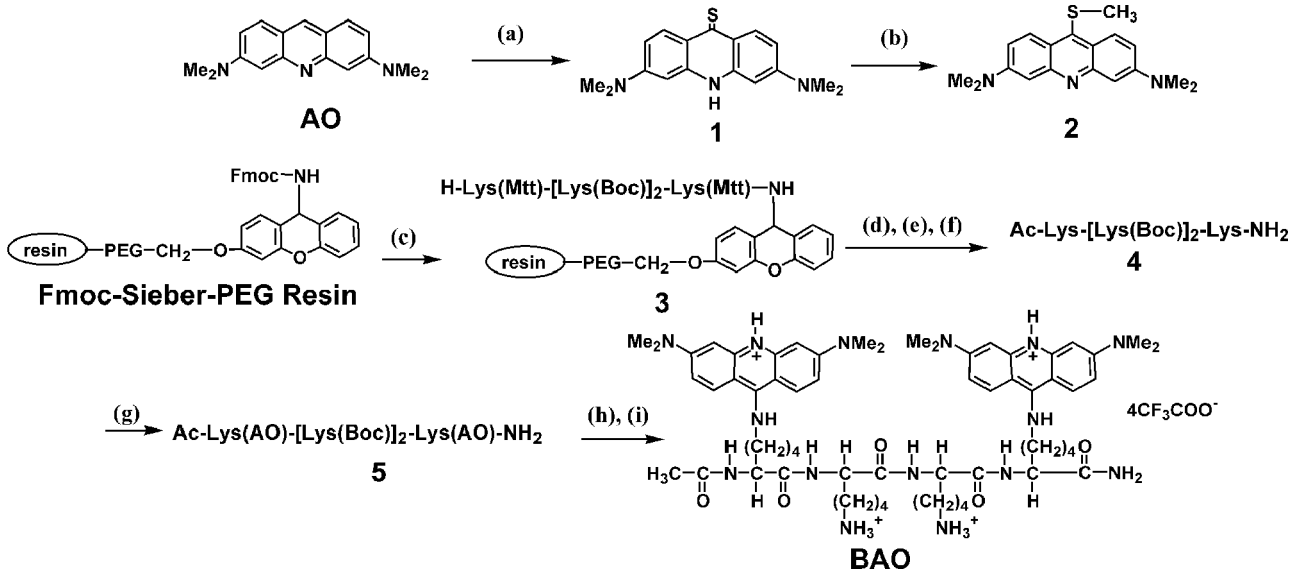

Fig. 1 Synthetic route for BAO. (a) Sulfur, 220–230 °C; (b) EtONa, CH_3I, rt; (c) SPPS with Fmoc-Lys(Boc)-OH and Fmoc-Lys(Mtt)-OH, rt; (d) acetic anhydride, DCM, rt; (e) 1% TFA–DCM, rt; (f) 0.1% TFA, TIPS, DCM, rt; (g) **2**, DCM, triethylamine, trifluoroethanol, 40 °C; (h) 86% TFA, thioanisole, *m*-cresol, rt; (i) HPLC purification.

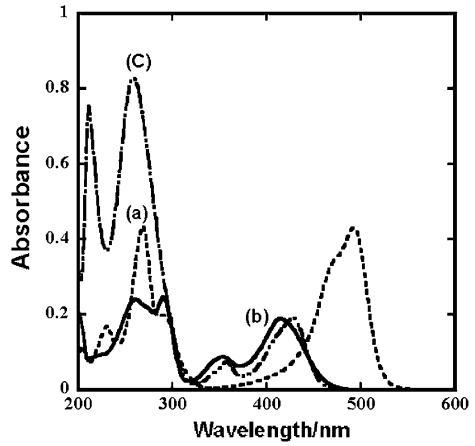

Fig. 2 UV-vis absorption spectra of 10 μM AO (a) and 5 μM BAO in the absence (b) or presence (c) of 45 μM calf thymus DNA in 10 mM MES buffer (pH 6.25) containing 1 mM EDTA and 350 mM NaCl at 25 °C.

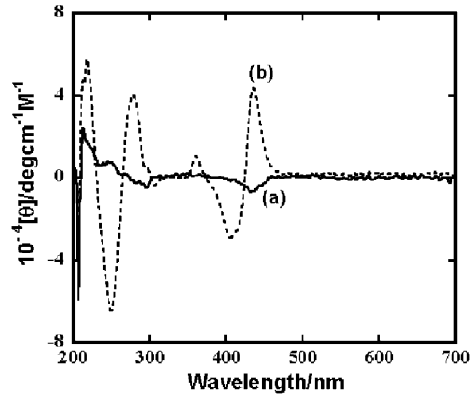

Fig. 3 CD spectra of 10 μM BAO in the absence (a) or presence (b) of 40 μM calf thymus DNA in 10 mM MES buffer (pH 6.25) containing 1 mM EDTA and 350 mM NaCl at 25 °C.

5-fold, which is in agreement with previous work[10] In contrast with AO, BAO showed *ca.* 200-fold fluorescence enhancement after the addition of calf thymus DNA, whereas no fluorescence enhancement of BAO was observed upon addition of poly(dA). The fluorescent intensity of BAO at 505 nm was plotted against

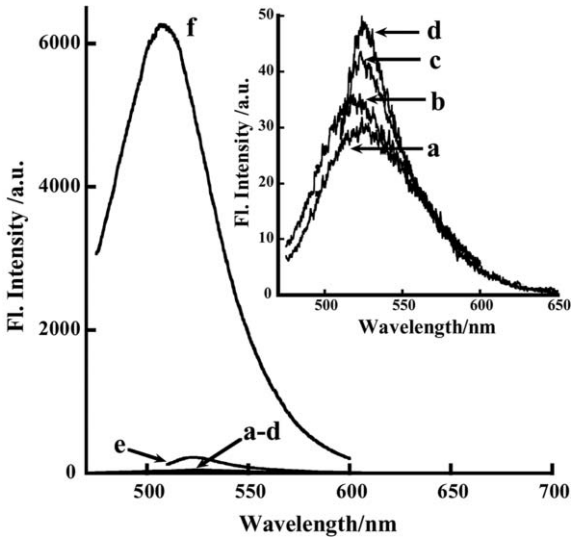

Fig. 4 Fluorescence spectra of BAO and AO in the absence and the presence of calf thymus DNA and poly(dA) in 10 mM MES buffer (pH 6.25) containing 1 mM EDTA and 350 mM NaCl; (a) 2.12 μM BAO alone; (b) 2.12 μM BAO + 23.4 μM b poly(dA); (c) 2.12 μM AO alone; (d) 2.12 μM AO + 23.4 μM b poly(dA); (e) 2.12 μM AO + 23.5 μM bp calf thymus DNA; (f) 2.12 μM BAO + 23.5 μM bp calf thymus DNA. Excitation wavelengths for BAO and AO were 429 nm and 470 nm, respectively.

the concentration of dsDNA of calf thymus DNA, $[poly(dA\text{-}dT)]_2$, or $[poly(dG\text{-}dC)]_2$, or ssDNA of poly(dA) (Fig. 5). Only in the case of dsDNA was the fluorescence enhancement of BAO clearly observed, though the magnitude of the enhancement was similar to that of calf thymus DNA with 43% GC content, but slightly smaller than that of $[poly(dA\text{-}dT)]_2$. Values of binding constant (K) and binding site size (n) for BAO–dsDNA complexes, determined by Scatchard analysis,[11] using the fluorescence change shown in Fig. 5, were as follows: for calf thymus DNA ($K = 1.1 \times 10^6$ M^{-1}, $n = 2$), for $[poly(dA\text{-}dT)]_2$ ($K = 2.5 \times 10^6$ M^{-1}, $n = 2$), and for $[poly(dG\text{-}dC)]_2$ ($K = 1.6 \times 10^6$ M^{-1}, $n = 2$). Spectrophotometric titration data showed rather small changes in absorbance with DNA addition, which may suggest that the stacked orientation of the AO units in BAO molecule is preserved upon complex formation with DNA. The detailed binding mode of BAO with dsDNA is not clear, but a

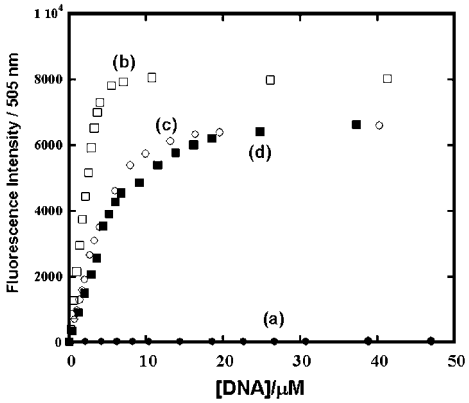

Fig. 5 Fluorescence changes of 5 μM BAO in the presence of varied amounts of poly(dA) (a), $[poly(dA\text{-}dT)]_2$ (b), $[poly(dG\text{-}dC)]_2$ (c), and calf thymus DNA (d) in 10 mM MES (pH 6.25) containing 1 mM EDTA and 350 mM NaCl at 25 °C. Excitation wavelength: 429 nm.

Type II_2 binding model[12] (*i.e.* partial insertion of one AO unit and stacking (dimerization) of a second AO unit in BAO), which is one of the suggested binding modes of AO, may be considered for BAO binding of dsDNA. This is supported by the small n value ($n = 2$) and the similarity in the CD spectra of both complexes.

In conclusion, newly synthesized BAO showed an approximate 200-fold fluorescence enhancement upon binding to dsDNA irrespective of its sequence, but not to ssDNA. This characteristic property of BAO makes itself a suitable agent for the specific analysis of dsDNA in the presence of ssDNA.

Acknowledgements

The authors are grateful to Prof. Hiroki Kondo of Kyushu Institute of Technology for helpful discussions. This work was supported by the Special Coordination Funds from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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