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A simple method for monitoring protein–DNA interactions

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ABSTRACT

A simple, efficient and cheap method is reported for monitoring interactions between single stranded desoxyribonucleic acids and proteins, using fluorescence spectroscopy and complexes of 5′-dye–DNA conjugates with bovine serum albumin as probes. In the presence of a single stranded DNA-binding protein the complexes with bovine serum albumin are disrupted, which results in a reduction of fluorescence intensity.

Nucleic acids and proteins are ubiquitous in all forms of life. Interactions between these molecules play a crucial role in many steps of gene expression. Therefore, understanding of the kinetics and thermodynamics of this process is of fundamental importance for biological sciences.

Proteins are intrinsically fluorescent due to presence of tryptophan and tyrosine amino acid residues. Fluorescence of these chromophores is affected (usually quenched) when a protein binds a nucleic acid. This effect has been utilized to monitor protein–nucleic acid interactions in real time.[1] The method is quick and does not require any special instrumentation. However, its sensitivity is not sufficient in many cases since the brightness ($\varepsilon \times \Phi_f$: extinction coefficient × fluorescence quantum yield) of natural fluorophores is low. The sensitivity can be improved by using molecular beacons (**MB**s). A **MB** is a nucleic acid probe, whose termini are modified with a fluorophore and a fluorescence quencher. The terminal sequences in these probes are usually complementary to each other. As a consequence, in solution they are folded into the hairpin structure, where the fluorophore is positioned in close proximity to the quencher; in this state the probe is not fluorescent.[2] When a protein binds to the loop part of the beacon the hairpin is opened and the fluorescence intensity is increased. **MB**s have already been applied to monitor the interactions of nucleic acids with single stranded DNA (ssDNA)-binding protein (**SSP**)[3] and different lactate dehydrogenase (**LDH**) enzymes.[4]

The price of **MB**s is high because their synthesis includes modification of both termini of nucleic acids with expensive building blocks.[5] Moreover, their hydrophobicity can make the purification of **MB**s difficult. In contrast, probes that carry only one modification are substantially cheaper and their synthesis and purification are trivial. A method that relies on such simple compounds, rather than **MB**s, therefore has potential to become a valuable and broadly accessible biochemical tool for studying nucleic acid–protein interactions.

Herein, we report on the development of such a method. Its concept is illustrated in Figure 1. In particular, we use a 5′-dye–DNA conjugate, which binds bovine serum albumin (**BSA**) due to a non-covalent interaction (dye–**BSA**). In the resulting complex the fluorophore is located in the hydrophobic pocket of the protein, so its Φ_f is high. In the presence of a ssDNA-binding protein, for example, **SSP** or **LDH**, the **BSA**–dye–DNA complex is replaced by a dye–DNA–ssDNA-binding protein complex. In the latter state the dye is exposed to water and its Φ_f is low. Thus, a decrease of fluorescence intensity correlates with concentration of ssDNA-binding proteins.

Our first task was to obtain a dye whose Φ_f is low in water and high in the presence of **BSA**. We have selected 4-substituted 1,8-naphthaleneimides, since they are highly photostable, cheap and their chemical modification is straightforward. Moreover, these

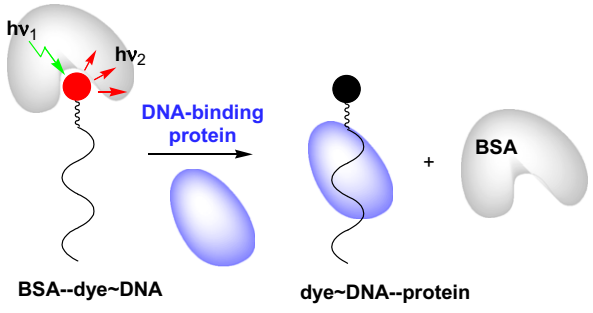

Figure 1. A method for monitoring of single stranded nucleic acid–protein interactions: bovine serum albumin (**BSA**) is shown in grey color, a single stranded DNA–binding protein—in blue color; the red sphere is a dye in the hydrophobic pocket of **BSA** (fluorescent); the black sphere is the same dye in aqueous solution (non-fluorescent).

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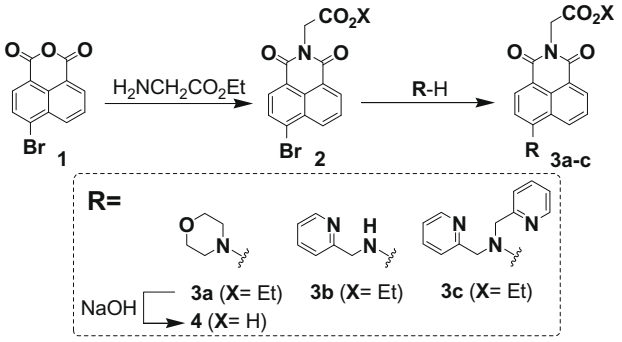


Scheme 1. Synthesis of dyes **3a–c** and **4**.

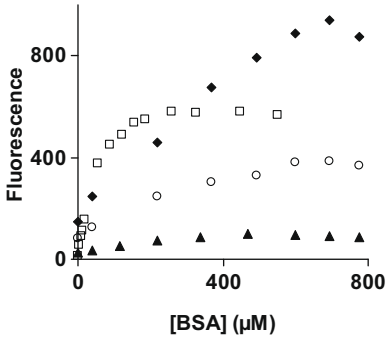


Figure 2. Fluorescence intensity (arbitrary units) as a function of **BSA** concentration; tris-(hydroxylmethyl)-aminomethane (TRIS) 20 mM (pH 7.5), NaCl 50 mM, $MgCl_2$ 10 mM: open squares—dye **4**, 10 μM; filled diamonds—**F1**–DNA1, 10 μM; open circles—**F1**–DNA2, 10 μM; filled triangles—**F2**–DNA1, 2 μM.

Table 1
Properties of 4-substituted 1,8-naphthaleneimide dyes

Dye	Absorption: λ_{max}, nm	Emission: λ_{max}, nm −**BSA**/+**BSA**[a]	F(+**BSA**)/F(−**BSA**) (λ, nm)[b]
3a	400	555/525	19.5 (525)
3b	430	544/525	1.9 (524)
3c	420	540/520	8.9 (518)

[a] Emission maxima of **3a–c** with and without **BSA** (Sigma/Aldrich: PN A9647, Lot. Nr. 10GH1395) 600 μM; λ_{ex} = 400 nm; phosphate buffer 20 mM (pH 7), NaCl 50 mM.

[b] Emission intensity at a wavelength given in the brackets without (−**BSA**) and with **BSA** 600 μM (+**BSA**).

dyes exhibit large Stoke's shifts due to formation of an intramolecular charge transfer (ICT) state upon absorption of light.[6] It is known that the fluorescence of ICT-dyes is highly sensitive to their environment.[7]

Synthesis of the dyes was conducted in accordance with Scheme 1. In the first step a reaction between the ethyl ester of glycine and 4-bromo-1,8-naphthalenedicarboxylic anhydride (**1**) has resulted in imide **2**. Then, dyes **3a–c** have been obtained by nucleophilic substitution of 4-Br with different alkylamines.[8]

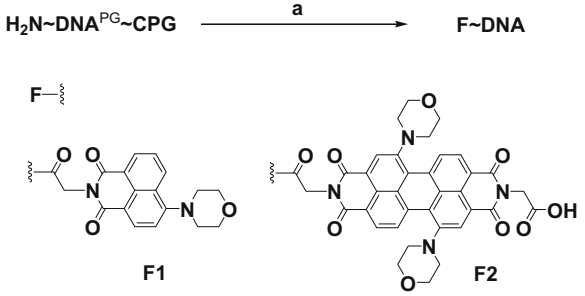
All of the prepared compounds show fluorescence that is sensitive to their environment (Table 1). The effect is large for **3a** and **3c**, but is dramatically smaller for **3b**. This tendency does not follow the basicity order of the 4-substituents (**R**): morpholine > 2-picolylamine > bis-(2-picolyl)amine.[9] It can rather be explained by the theory of twisted ICT states,[10] which indicates that the ICT-state is destabilized in the case of 4-mono-alkylamino substituted dyes, for example, **3b**.

We have selected a morpholino-derivative for further studies. **3a** was converted to carboxylic acid **4** (Scheme 1). The acid was coupled to a 5′-amino linker modified DNA, which was otherwise fully protected and bound to a controlled pore glass (CPG) support. The conjugate obtained was cleaved from the support and deprotected with aqueous ammonia solution and, finally, purified by HPLC (Scheme 2).[11] The purity of the conjugates prepared by this method was higher than 90%.

Compared to dye **4**, the affinity of conjugate **F1**–DNA1 to **BSA** is substantially lower (Fig. 2). This is apparent from a comparison of the **BSA** concentrations required to saturate the fluorescence of the dye ($[\mathbf{BSA}]_{sat}$): 250 μM for **4** and 600 μM for **F1**–DNA1 (Table 2). The affinity is not further reduced in the presence of 1 equiv of complementary **DNA3**.[12] This effect may be explained by repulsive interactions between negatively charged DNA strands and hydrophobic sites within **BSA**.

Upon the addition of **SSP** to **BSA**–**F1**–DNA1 complex, its fluorescence intensity is reduced by 4.2-fold (Table 2). The resulting fluorescence intensity matches that of the **F1**–DNA1 conjugate, which is not complexed to **BSA**. **SSP** alone does not affect substantially fluorescence of the **F1**–DNA1: F(−**SSP**)/F(+**SSP**) = 1.2. These data indicate that the initial complex is dissociated in the presence of **SSP**. The driving force for this process is binding of **SSP** to the DNA part of the **F1**–DNA1 conjugate.

In some cases it may be useful to monitor protein–DNA interactions directly in biological mixtures, for example, cell lysates and



5'-->3':
H_2N~DNA1: $H_2N(CH_2)_6OPO_2$-CGCACCATAAACCTT
H_2N~DNA2: $H_2N(CH_2)_6OPO_2$-GTTCATCACGCGCTCCCACTTTTT
DNA3: 3'-GCGTGGTGTTTGGAA
DNA4: 3'-CAAGTAGTGCGCGAGGGTGAAAAA

Scheme 2. Synthesis of the 5′-modified DNAs: **PG**s—standard protecting groups for nucleobases; a) (1) **F**-OH, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate (HBTU), *N*-hydroxy-benzotriazole (HOBT) and diisopropylethylamine (DIEA); (2) aqueous NH_3 (20%).

Table 2
Fluorescence of **F**–**DNA** conjugates in the presence of **BSA** and single stranded DNA binding proteins: **SSP** and **LDH-5**

DNA[a]	F(+**BSA**)/ F(−**BSA**)[b]	$[\mathbf{BSA}]_{sat}$ mM[c]	F(+**BSA**)/ F(+**BSA**+**SSP**)[d]	F(+**BSA**)/ F(+**BSA**+**LDH-5**)
F1–DNA1	9.9	0.6	4.2	3.9
F1–DNA1/DNA3	9.0	0.7	2.4	3.2
F2–DNA1	5.5	0.5	2.6	3.1
F2–DNA1/DNA3	2.2	0.5	2.6	2.2
TAMRA–DNA1	1.0	—	—	1.2
F1–DNA2	7.4	—	—	4.9
F1–DNA2/DNA4	5.6	—	—	2.5

[a] Probes: [**F1**–DNA1] = [**F1**–DNA2] = [**TAMRA**–DNA1] = 10 μM, [**F2**–DNA1] = 2 μM; 1 equiv of DNA3 and DNA4 were used relative to the corresponding labeled DNAs; buffer: TRIS 20 mM (pH 7.5), NaCl 50 mM, $MgCl_2$ 10 mM.

[b] Emission intensity at 525 nm (λ_{ex} = 400 nm for the derivatives of **F1**), at 765 nm (λ_{ex} = 650 nm for the derivatives of **F2**) and at 585 nm (λ_{ex} = 540 nm for **TAMRA**–DNA1) without (−**BSA**) and with **BSA** (+**BSA**); [**BSA**] = 0.6 mM.

[c] $[\mathbf{BSA}]_{sat}$: saturating concentrations of **BSA**.

[d] [**SSP**] = 2.3 μM; [**LDH-5**] = 23 units/mL.

serum. Since autofluorescence of the components of these mixtures will contribute to the background emission at <650 nm,[13] the sensitivity of the assay based on the **BSA**–**F1**–DNA probe (λ_{em} = 525 nm) is expected to be low. We solved this problem by replacing **F1** with a fluorescent dye, which emits at >650 nm. We selected another ICT-dye, a derivative of perylene: **F2**-OH, λ_{em} = 765 nm, λ_{ex} = 650 nm. This compound was prepared by using the known protocol.[14] It has been reported that the natural perylene, hypericine binds efficiently to IIA subdomain of human serum albumin.[15] Since this drug is larger than **F2**, one could expect that the binding site of serum albumin will be able to accommodate **F2** as well. **F2** was attached to the DNA according to the approach outlined in Scheme 2. We were pleased to observe that the fluorescence of **F2**–DNA (2 μM) is also enhanced in the presence of **BSA**; $[\mathbf{BSA}]_{sat}$ = 0.6 mM. Further, we have tested whether the **BSA**–**F2**–DNA complex can sense presence of **SSP** in solution. We have observed that **SSP** reduces fluorescence of the **BSA**–**F2**–DNA complex by 2.6-fold (Table 2). Analogously to the **F1**–DNA1 conjugate, **SSP** alone does not affect fluorescence of the **F2**–DNA1: F(−**SSP**)/F(+**SSP**) = 1.3. Thus, the **BSA**–**F2**–DNA as well as **BSA**–**F1**–DNA can be applied for monitoring protein–DNA interactions. Another ssDNA-binding protein, **LDH-5** has a similar effect on both **BSA**–**F2**–DNA and **BSA**–**F1**–DNA complexes (Table 2).

Next, we have prepared an analogue of **F1**–DNA1 (15-mer) with a longer DNA strand: **F1**–DNA2 (24-mer, Scheme 2) to show that the effect observed is not restricted to one particular DNA sequence. The fluorescence of **F1**–DNA2 is weakly modulated by **LDH-5** alone: F(−**LDH-5**)/F(+**LDH-5**) = 1.5, whereas the fluorescence of **BSA**–**F1**–DNA2 is strongly affected by **LDH-5**: F(−**LDH-5**)/F(+**LDH-5**) = 4.9.

One could expect that **BSA**–**F**–DNA associates would bind ssDNA-binding proteins stronger than **BSA**–**F**–DNA/complementary DNA associates would do. This was not the case for the 15-mer **F**–DNA probes (Table 2) that indicates that the corresponding duplexes are less stable than ssDNA–protein (**LDH-5** or **SSP**) complexes. In agreement with this assumption, the inhibitory effect of the complementary DNA on the probes with the longer DNA strand (24-mer, **F1**–DNA2) was significant (Table 2).

In contrast to the ICT-dyes **F1** and **F2**, **TAMRA** fluoresces from an S_1 excited state. As expected, fluorescence of a control conjugate **TAMRA**–DNA1 is affected neither by **BSA** nor the ssDNA-binding proteins (Table 2).

In summary, we have developed a simple, efficient and cheap method for monitoring interactions between single stranded DNAs and proteins by using fluorescence spectroscopy. As probes, we have selected fluorescent complexes of 5′-dye–DNA conjugates with physiological concentrations of serum albumin. In the presence of a single stranded DNA–binding protein (**SSP** or **LDH**) these complexes are destabilized, and the fluorescence intensity is reduced. We have used this signal change for monitoring ssDNA-binding protein–DNA interactions in real time. We have also demonstrated that two different dyes (λ_{em} = 525 and 765 nm) may be applied in this assay, confirming that it can be tuned for application-specific needs. For example, the assay could potentially be used for monitoring protein–DNA interactions directly in cell lysates.

Acknowledgments

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8. *Synthesis of **2***: 4-Bromo-1,8-naphthalenedicarboxylic anhydride (5.54 g, 20 mmol) was dissolved in DMF (60 mL) and glycine ethyl ester hydrochloride (3.09 g, 22 mmol) in pyridine (19 mL) was added at once. The mixture was heated to 100 °C and left at this temperature for 25 h. Energetic stirring was conducted all this time. Then water was added in 50 mL-portions until no more yellowish precipitate was released. The precipitate was filtered, washed with water and re-crystallized from ethanol. Yield 3.2 g (44%). TLC in CH_2Cl_2/EtOH (95/5): R_f = 0.34. ^{1}H NMR in $CDCl_3$, δ (ppm): 1.20 (triplet, 3H), 4.23 (quartet, 2H), 4.82 (singlet, 2H), 7.8–8.70 (multiplet, 5H).*Synthesis of **3a***: Imide **2** (0.98 g, 2.7 mmol) was dissolved in *N*-methylpyrrolidone (NMP, 15 mL) and morpholine (1.2 mL, 13.5 mmol) together with triethylamine (TEA, 3.8 mL, 27 mmol) were added. The mixture was stirred for 2 h at 85 °C. Then water (80 mL) was added, which induced formation of yellow precipitate. The precipitate was filtered, washed with water (100 mL) and dried. Yield 0.67 g (67%). TLC in EtOAc/TEA (98/2): R_f = 0.7. ^{1}H NMR in $CDCl_3$, δ (ppm): 1.29 (triplet, 3H), 3.20 (triplet, 4H), 3.92 (triplet, 4H), 4.22 (quartet, 2H), 4.92 (singlet, 2H), 7.5 (multiplet, 1H), 7.65 (triplet, 1H), 8.34–8.58 (multiplet, 3H). ESI-MS, positive mode, *m/z*: observed 369.07 ([M+H]$^+$), calcd for $[C_{20}H_{21}N_2O_5]^+$ 369.15. CHN-analysis: calcd for $C_{20}H_{20}N_2O_5$ (%): C, 65.22; H, 5.43; N, 7.61. Found: C, 64.90; H, 5.43; N, 7.58.*Synthesis of **3b***: Imide **2** (2.25 g, 6.2 mmol) was dissolved in NMP (25 mL) and 2-picolylamine (3.2 mL, 31.1 mmol) together with TEA (8.6 mL, 62.2 mmol) were added. The mixture was stirred for 3 h at 110 °C. Then NMP was removed under reduced pressure and hexane was added to the oil left. Black precipitate was formed. It was filtered, washed with hexane and purified by column chromatography on silica. Yield 0.86 g (35%). TLC in EtOAc/TEA (96/4): R_f = 0.42. ^{1}H NMR in $CDCl_3$, δ (ppm): 1.29 (triplet, 3H), 4.23 (quartet, 2H), 4.69 (singlet, 2H), 4.92 (singlet, 2H), 6.70 (duplet, 1H), 7.25–7.95 (multiplet, 4H), 8.34–8.70 (multiplet, 4H). ESI-MS, positive mode, *m/z*: observed 390.14 ([M+H]$^+$), calcd for $[C_{22}H_{20}N_3O_4]^+$ 390.15. CHN-analysis: calcd for $C_{22}H_{20}N_3O_4$ (%): C, 67.86; H, 4.88; N, 10.79. Found: C, 67.44; H, 5.07; N, 10.82.*Synthesis of **3c***: A mixture of imide **2** (350 mg, 0.97 mmol) and bis(2-picolyl)amine (385 mg, 1.93 mmol) was flushed with argon and heated up to 150 °C. It was at this temperature for 5 h. Then the mixture was cooled to 22 °C, all volatiles were removed and the residue was purified by column chromatography on silica. The oily residue obtained was recrystallized from CH_2Cl_2–hexane mixture. Yield 230 mg (50%). ^{1}H NMR in DMSO-d_6, δ (ppm): 1.22 (triplet, 3H), 4.17 (quartet, 2H), 4.77 and 4.75 (2 unresolved singlets, total—6H), 7.21–7.35 (multiplet, 3H), 7.46 (duplet, 2H), 7.68–7.9 (multiplet, 3H), 8.26 (duplet, 1H), 8.45–8.60 (multiplet, 3H), 8.97 (duplet, 1H). ESI-MS, positive mode, *m/z*: observed 381.13 ([M+H]$^+$), calcd for $[C_{28}H_{25}N_4O_4]^+$ 381.19.
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11. *Synthesis of dye **4***: Ester **3a** (40 mg, 0.11 mmol) was dissolved in MeOH (10 mL) and aqueous NaOH solution (4 M, 10 mL) was added. The emulsion was stirred at 22 °C for 24 h, then neutralized with HCl (1 M) and extracted with ethylacetate until aqueous phase was practically colorless. The organic phases were combined, dried under Na_2SO_4 and evaporated. The product was purified by column chromatography on silica. The product was purified by flash chromatography on silica. Yield 19.6 mg (58%). TLC in EtOAc/AcOH (99/1): R_f = 0.65. ^{1}H NMR in DMSO-d_6, δ (ppm): 3.15 (triplet, 4H), 3.81 (triplet, 4H), 4.31 (singlet, 2H), 7.36 (duplet, 1H), 7.81 (triplet, 1H), 8.36–8.55 (multiplet, 3H). ESI-MS, positive mode, *m/z*: observed 341.11 ([M+H]$^+$), calcd for $[C_{18}H_{17}N_2O_5]^+$ 341.14.*Synthesis of **F1**–DNA1*: A mixture of **F1**-OH (34 mg, 100 μmol), HBTU (34 mg, 90 μmol) and HOBT (14 mg, 100 μmol) was dried at 0.01 mbar for 30 min, dissolved in DMF (1 mL), and mixed with DIEA (38 μL, 200 μmol). The darkened solution was immediately added to 5′-amino linker modified DNA (1 μmol DNA, 33 mg CPG), which was fully protected and bound to CPG. The resulting suspension was mixed for 40 min at 22 °C. After this the solution was filtered, the CPG was washed with DMF (3 × 2 mL) and CH_3CN (3 × 2 mL) and dried at 0.01 mbar. Finally, the sample was treated with aqueous ammonia solution at 22 °C for 24 h. The solution was filtered, diluted with water and lyophilized. The rest was re-dissolved in water (250 μL) and purified by HPLC. Yield 40 nmol. MALDI-TOF MS: calcd for $C_{168}H_{212}N_{57}O_{93}P_{15}$ [M−H]$^-$: 4980.3, found: 4979.9.**F1**–DNA2 and **F2**–DNA1 were prepared

analogously.***F1***–*DNA2*: Yield 120 nmol; MALDI-TOF MS: calcd for $C_{255}H_{327}N_{80}O_{155}P_{24}$ $[M-H]^-$: 7703.8, found 7695.6.***F2***–*DNA1*: Yield 50 nmol; MALDI-TOF MS: calcd for $C_{186}H_{223}N_{59}O_{98}P_{15}$ $[M-H]^-$: 5315.1, found 5311.7.

12. UV-melting points (T_m) were measured in phosphate buffer (10 mM, pH 7) in the presence of NaCl (150 mM); DNA strand concentration was 1 μM: T_m(**F1**–DNA1/DNA3) = 60.1 ± 0.1 °C, T_m(**F1**–DNA2/DNA4) = 74.2 ± 0.9 °C, T_m(**F2**–DNA1/DNA3) = 61.8 ± 0.7 °C. All protein titrations have been conducted at lower NaCl concentration—50 mM and at 22 °C. According to Howley, P. M.; Israel, M. F.; Law, M.-F., and Martin, M. A. *J. Biol. Chem.* **1979**, 254, 4876–4883, T_m(at 150 mM NaCl) − T_m(at 50 mM NaCl) will be less than 10 °C. Thus, all duplexes are expected to be stable at our experimental conditions.
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