Quenching of the long-lived Ru(II)bathophenanthroline luminescence for the detection of supramolecular interactions

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A feasibility study based on tailor-made peptide sequences for a new robust luminescence probe-system using the long-lived luminescence of a Ru(II)-bathophenanthroline complex in combination with an efficient anthraquinone-type quencher is presented. Due do their high chemical stability, both dyes can be introduced during solid-phase peptide synthesis avoiding post-synthetic labelling. Photophysical measurements revealed an intense quenching of the luminescence of the Ru-complex (65–68%) which was also confirmed by calculations resulting from decay time measurements. The long-lived luminescence allows for a time-gated detection scheme, which can reduce any luminescence contribution from matrix components.

Introduction

Luminescence probes as entities of fluorescence resonance energy transfer (FRET) systems are important tools to study supramolecular interactions with a special emphasis in the realm of biomolecules like DNA, RNA and proteins.[1] Furthermore, they allow structural changes in such molecules to be monitored in a real time mode and in homogeneous formats. Meanwhile, a myriad of applications has been reported. These involve binding of ligands to their pertinent protein receptors,[2] DNA–protein complexation,[3] RNA-folding and catalysis.[4] Other applications are enzyme assays based, *e.g.*, on the Förster resonance energy transfer principle,[5] and monitoring of PCR-reactions.[6]

The basis for FRET is an efficient transfer of energy from the donor after its electronic excitation to the acceptor. The intensity of the FRET depends largely on the spectral overlap between donor and acceptor as well as on the orientation of their respective dipole transition moments relative to each other. FRET is highly distance-dependent and decreases according to the Förster equation with r^{-6}, r being the distance between the donor and the acceptor. A number of different donor–acceptor pairs have been reported but despite the multitude of available systems, sensitivity—especially in the presence of background luminescence from matrix constituents—still remains an issue. A further concern is robustness of the applied dyes as well as the possibility of employing them in a modular way as broadly as possible *via* stable covalent bonds and without interference of the spectral properties of the labelled molecules.

One way to solve the sensitivity issue is the application of multivalent fluorophores.[7] Another solution is the use of FRET-systems of which one partner possesses a long excited state decay time and hence, allows for measurements in a time-resolved mode in order to reduce background luminescence. The most prominent candidates for this purpose are lanthanide ions with lifetimes up to milliseconds. One of the drawbacks is their low extinction coefficient. Due to that, they are commonly employed as chelate-complexes such as cryptates. The chromophores of the ligands act as a kind of light collector and sensitize the lanthanide luminescence, which is commonly known as "antenna effect". Leakage of the lanthanide out of cryptates is an issue of concern. Furthermore, the very long decay time might be a disadvantage in monitoring events which occur in the μs range (*e.g.* rotations of proteins).

Recently, we have reported a new FRET-system based on a carbostyril donor **1** and a Ru–bathophenanthroline-complex **2** (Fig. 1).[8]

The system has a number of interesting properties. It is very robust, *e.g.*, the dyes are not sensitive towards basic or acidic conditions. Thus, both building blocks can be inserted during solid-phase peptide synthesis, the donor directly as Fmoc-building block and the Ru-complex either at the N-terminus or at orthogonally-protected Lys-side chains. Deprotection and removal from the support after synthesis by trifluoroacetic acid (TFA) caused no harm to either dye. The spectral overlap of the emission of the carbostyril donor and the absorption of the Ru-complex is very strong and resulted in a FRET with high efficiency. The long luminescence decay time of the Ru-complex makes the system well-suited for time-resolved measurements.[9] As an application of the new FRET system, a peptide with the recognition sequence for the serine protease thrombin flanked by the two dyes was synthesised and successfully cleaved by the enzyme. The change in the ratio of the fluorescence intensity before and after cleavage could be used to monitor the protease reaction in realtime.

Here we report on a different new FRET-system in which the Ru(II)-bathophenathroline-complex itself serves as the energy donor unit and a non-fluorescent quencher is employed as acceptor. Generally, such systems have the advantage, that no background fluorescence due to the direct excitation of the acceptor can occur and in biological systems the contribution

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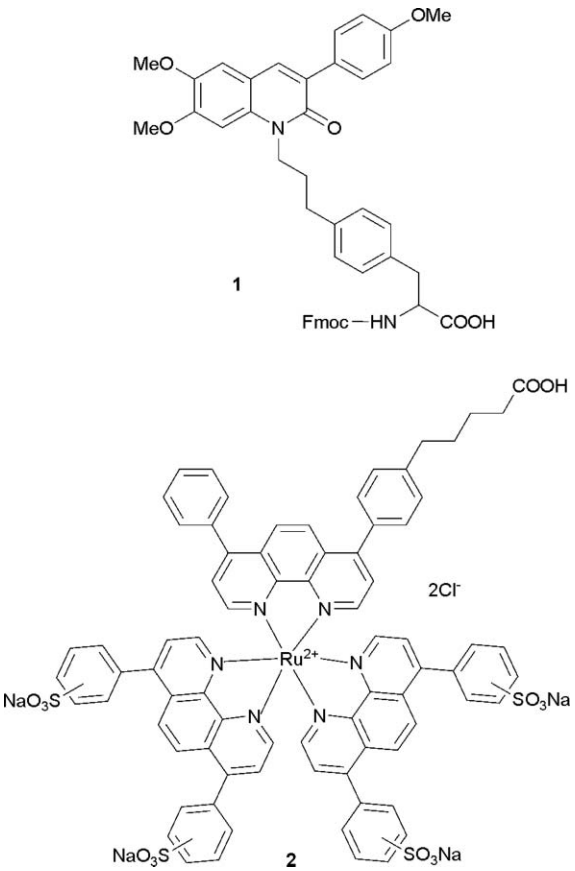


Fig. 1 Carbostyril (**1**) and Ru-complex (**2**) building blocks.

of matrix luminescence is greatly reduced due to the excitation at wavelengths $\lambda_{ex} > 400$ nm. In general, the quenching can proceed *via* an energy or an electron transfer but there exists also the possibility for static quenching caused by intramolecular dimer formation of the chromophores.[10] Static quenching is especially suited to applications in DNA, *e.g.*, in so called "molecular beacons".[11] For usage in peptides, luminescence probes present the danger of changing the conformation of the pertinent peptides and hence can have an influence, *e.g.*, on enzyme activities acting on these sequences.

In the new system, the anthraquinone derivative **3**, dubbed as Disperse Blue 3 (Fig. 2), is used to quench the emission of the Ru(II)–bathophenanthroline complex.

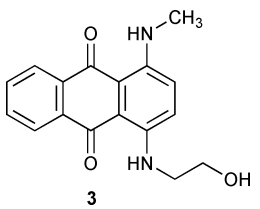


Fig. 2 Structure of Disperse Blue 3 (**3**).

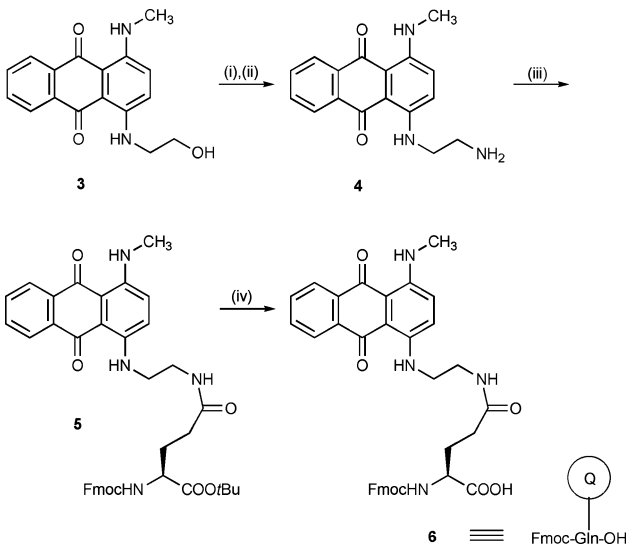
In a first report, the anthraquinone quencher **3** was used in combination with Disperse Red 1 in an enantioselectivity screening.[12] Later, it was applied to oligonucleotides in combination with a number of commonly employed dyes.[13]

Chromophore **3** has an absorption minimum at 450 nm, which matches very well with the excitation wavelength of the Ru complex **2**. Furthermore, the spectroscopic data of **3** and of the Ru(II)–bathophenathroline complex revealed a good overlap of the emission wavelength of the Ru-complex with the absorption spectrum of **3**.

Results and discussion

Our first aim was to incorporate the Ru-complex **2** and the anthraquinone quencher **3** into peptide sequences followed by an evaluation of the spectroscopic properties in combination with these biomolecules.

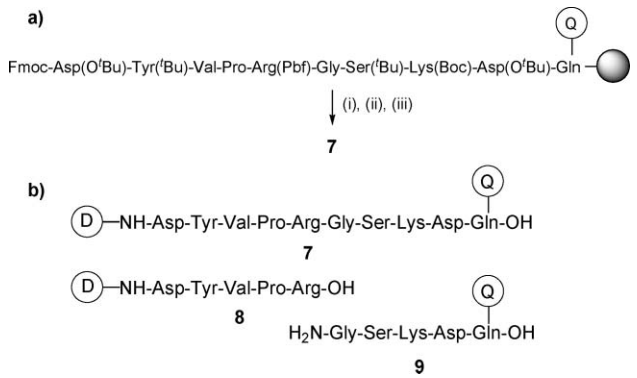
For this reason, compound **3** was transformed into a suitable Fmoc-amino acid building block amenable to peptide synthesis on solid support by the Fmoc/*t*Bu strategy (Scheme 1). Preliminary experiments had shown that the quencher molecule is stable towards 95% TFA, which means that it is stable under the conditions for removal of the side-chain protecting groups of peptides after their assembly on a solid support. Hence, the quencher amino acid can be introduced into peptides at will during their synthesis on solid support.



Scheme 1 Synthesis of the quencher-building block **6**. *Reagents and conditions*: (i) PPh_3, DEAD, phthalimide, THF; (ii) $N_2H_4 \cdot xH_2O$, CH_2Cl_2–MeOH (1 : 2) (72%, two steps); (iii) Fmoc-Glu-O*t*Bu, TBTU, DIEA, DMF (73%); (iv) TFA–CH_2Cl_2–TIS 95 : 3 : 2 (80%).

Disperse Blue 3 (**3**), obtained in pure form by column chromatography from a crude material (Aldrich) which contained about 25% of the dye, was transformed into **4** by Mitsunobu reaction with phthalimide followed by hydrazine deprotection. Reaction of **4** with side chain-unprotected Fmoc-Glu-O*t*Bu using TBTU[14] as coupling reagent led to **5** of which the *t*-butylester was cleaved with TFA yielding the desired building block **6**. All steps proceeded with good yields. It was demonstrated that building block **6** could be introduced with high efficiency during solid-phase synthesis by applying 1.5 equivalents as demonstrated in the course of a synthesis of a small peptide. The only problem was the blue colour of the resin after introduction of **6** which hampered the monitoring of the coupling efficiency by the Kaiser-test.[15]

In order to investigate the photophysical properties of the system we have synthesized the peptide sequences **7–9** (Scheme 2). In peptide **7**, the Ru-complex as donor (D) and the anthraquinone entity as quencher (Q) are interspaced by 9 amino acids which should result in a relatively intense FRET due to the close proximity of D and Q. Apart from the dyes the peptide contains also a cleavage site for the serine protease thrombin which cleaves selectively after Pro-Arg and leads to fragments **8** and **9**, respectively. Hence, cleavage of **7** will increase the distance of the dyes which should result in a diminished FRET.



Scheme 2 a) Representative synthesis of **7**. *Reagents and conditions*: i) 20% piperidine in DMF, 15 min. ii) Ru-Complex **2**, TBTU, DIEA, DMF, 1 d. iii) CF_3COOH–CH_2Cl_2–TIS 95 : 3 : 2, 2 h. b) *Sequences of Peptides* **7–9**. The FRET donor and quencher are represented by D and Q.

In Fig. 3 the absorption spectra of compounds **2**, and **7–9** are depicted together with the substrate **7** after cleavage with thrombin (**8** + **9**). From the comparison of the absorption spectra it can be concluded that the spectra of the donor and quencher are not altered in **7** when compared to the single-labeled compounds (**8** and **9**) or the pure Ru-complex-donor **2**. Hence, the electronic systems of donor and acceptor in their ground state are basically unaffected by the proximity of the two moieties in **7**. A closer look reveals that in the donor absorption the vibrational structure around $\lambda = 470$ nm is slightly less resolved and for the quencher in **7** a slight red shift ($\Delta\lambda \approx 5$ nm) is observed.

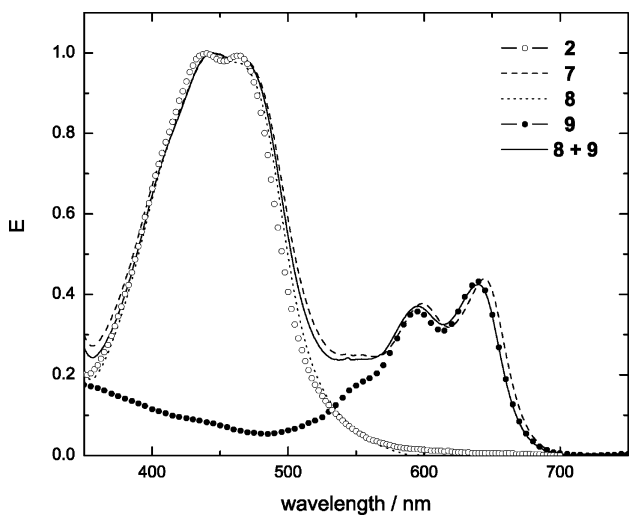


Fig. 3 Absorption spectra of compounds investigated.

In Fig. 4 the normalized fluorescence spectra of **8** and **2** are compared. Here, also the shoulder around $\lambda_{em} = 665$ nm is slightly less resolved in **8**, which could be attributed to the presence of the polypeptide. In summary, it can be concluded that due to the presence of the polypeptide in the absorption and in the fluorescence spectra of **7** only minor changes are observed and hence, no strong ground-state complex formation is present. The changes in the absorption spectrum might be indicative of a weak electronic coupling of donor and quencher. From the absorption spectra cleaved and uncleaved peptide **7** cannot be distinguished since in both samples the experimental absorption spectrum is basically the sum of the two individual spectra of **8** + **9** (see Fig. 3).

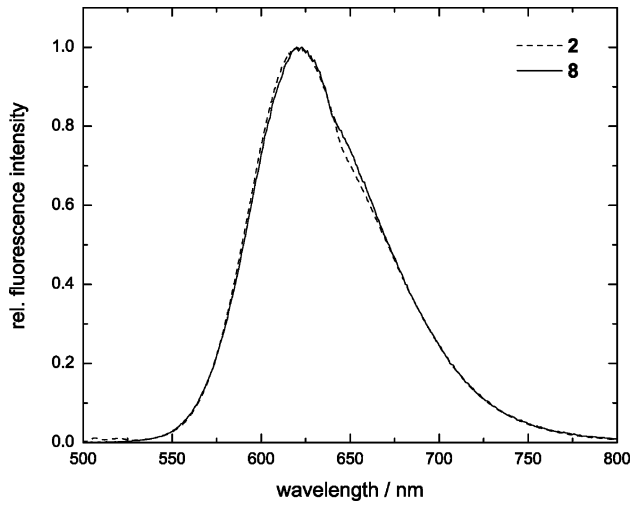


Fig. 4 Comparison of fluorescence spectra of **2** (dashed) and **8** (solid) ($\lambda_{ex} = 450$ nm).

As far as the decay times for compounds **8** and **2** are concerned, a clear influence of the peptide on the fluorescence decay time was observed. While **2** shows a fluorescence decay time of 400 ns under ambient conditions (*e.g.*, in the presence of oxygen), in **8** the fluorescence decay time is increased by a factor of about five (2150 ns!). This value is close to the decay time found for **2** after oxygen has been removed from the solution by a pump, freeze and thaw technique. The effective shielding of **2** against external quenching, when bound to macromolecules, has been observed for other luminescence probes by others as well and is attributed to a kind of protection effect by the peptide chain.[16,17]

A comparison of the absorption spectrum of the quencher peptide (**9**) and the fluorescence spectrum of the donor peptide (**8**) demonstrates the large spectral overlap. In Fig. 5 the area-normalized (area = 1) fluorescence spectrum $F_D(\nu)$ and the absorption spectrum $\varepsilon_A(\nu)$ are shown. Although the extinction coefficient of the quencher is not very large (*e.g.*, compared to fluorescein or rhodamine-based systems), the spectral overlap is very intense. Based on the experimental data a spectral overlap integral $J = 1.25 \times 10^{-16}$ cm^6 mol^{-1} was calculated. Using the following equation, the critical Förster distance R_0 can be calculated.

$$R_0^6 = \frac{(9000 \ln 10)\kappa^2 \Phi_D}{128\pi^5 n^4 N_A \nu} \int_0^\infty \frac{F_D(\nu)\varepsilon_A(\nu)}{\nu^4} d\nu$$

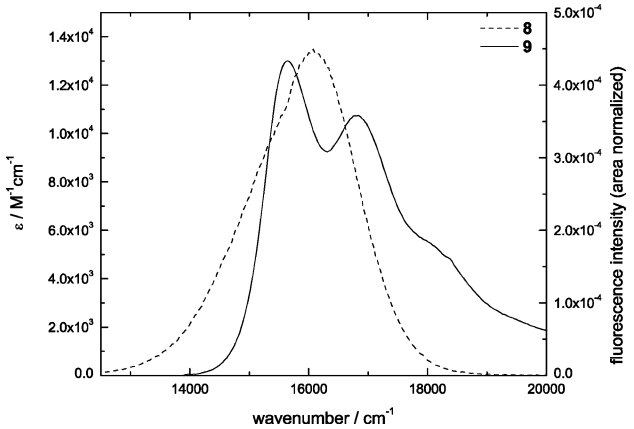


Fig. 5 Spectral overlap between fluorescence emission of **8** (dashed line, $\lambda_{ex} = 450$ nm) and absorption of **9** (solid line).

R_0 describes the distance between D and Q, at which the probability of energy transfer occurring is 50%. Based on the experimental data an $R_0 = (1.5 \pm 0.2)$ nm was calculated, which corresponds well to distances recently reported for similar D/Q systems.[18] In the calculation a fluorescence quantum yield of the donor $\Phi_D = 0.2$ was taken into account and the orientation factor κ was set to 2/3. The assumption of $\kappa = 2/3$ can be questioned since D and Q are bound to a peptide. However, D and Q are free to rotate, which makes it probable to assume that no preferential orientation between D and Q is present.

In Fig. 6 the fluorescence spectra of compound **7** before and after cleavage with thrombin are depicted. HPLC investigations had revealed that the cleavage rate was >90%. The fluorescence measurements were performed under identical experimental conditions. Upon cleavage of **7** the fluorescence intensity is drastically increased, which indicates that in **7** effective quenching of the donor fluorescence is present. According to the equation below from the steady-state measurements a FRET efficiency $E = 65\%$ was calculated. I_F^0 and I_F are the fluorescence intensities in the absence and presence of acceptor. For the present samples I_F^0 corresponds to the cleaved sample.

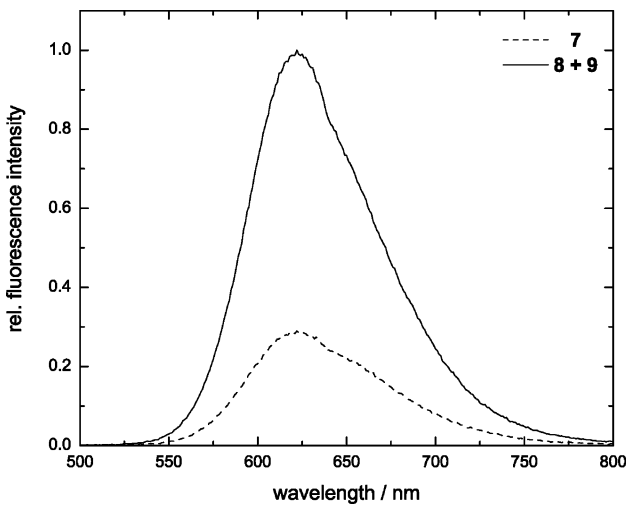


Fig. 6 Fluorescence spectra of **7** (dashed line) and **8** + **9** (after cleavage, solid line). Both samples were excited at $\lambda_{ex} = 450$ nm.

$$E = 1 - \frac{I_F}{I_F^0} = 1 - \frac{\tau_F}{\tau_F^0}$$

From the TCSPC measurements the fluorescence decay times were calculated. In Fig. 7 the fluorescence decays of compound **7** before and after cleavage are shown. For **7** a complex fluorescence decay was found. Due to an efficient FRET, the experimentally measured fluorescence decay appears non-monoexponential and was fitted with a bi-exponential decay law.[18,19]

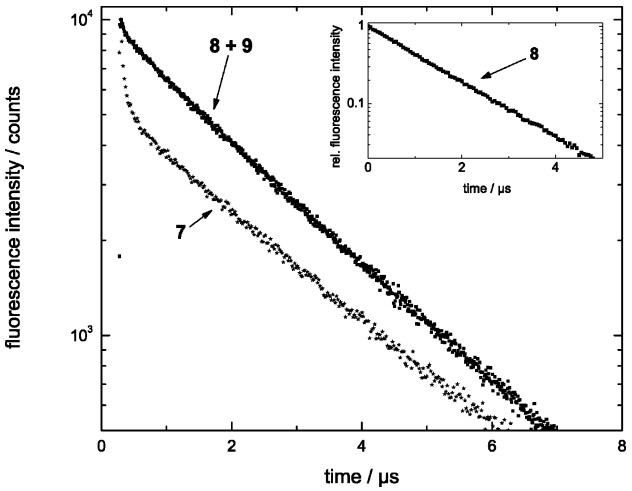


Fig. 7 Luminescence decays of **7**, **8** + **9** and **8** (inset). $\lambda_{ex} = 430$ nm, $\lambda_{em} = 620$ nm; measured in TCSPC mode.

The average fluorescence decay time $\langle\tau\rangle$ was calculated according to

$$\langle\tau\rangle = \frac{A_1\tau_1 + A_2\tau_2}{A_1 + A_2}$$

The complex fluorescence decay in **7** is attributed to a distribution of distances between D and Q due to molecular motion either of the peptide chain itself or of the linker that attaches D and Q to the peptide. The remaining fluorescence at long times (t > 500 ns) is a consequence of conformers with low quenching efficiency either due to larger distance and/or due to unfavourable angles between D and Q (influence of κ^2!). The explanation of unquenched fluorescence due to single labelled compounds can be ruled out. This was checked by HPLC comparison of the labelled peptide sequences.

Upon enzymatic cleavage the fluorescence decay became monoexponential with a fluorescence decay time identical to reference compound **8** (see inset in Fig. 7). The fluorescence decay of the cleaved sample **7** shows a trace of a remaining FRET (at short measurement time: first few nanoseconds) which indicated that the cleavage of **7** was not quantitative. The fluorescence decay curves were evaluated with respect to the FRET efficiency E i) based on the total number of counts and ii) based on the averaged fluorescence decay time τ_F. The evaluation of the fluorescence decays has the advantage that they are concentration independent. The analysis of the time-resolved data yielded FRET efficiencies E of 68% and 66%, respectively. These results are in excellent agreement with the efficiency calculated from the steady-state measurements.

From the average efficiency E of about 66% and the Förster radius $R_0 = (1.5 \pm 0.2)$ nm an average distance between D and Q in compound **7** of $R = (1.3 \pm 0.2)$ nm was determined.

Conclusions

In summary, we have developed a new robust FRET system composed of a Ru(II)–bathophenanthroline complex and a highly efficient anthraquinone-type quencher. The feasibility of the system was demonstrated in taylor-made peptide sequences (**7**–**9**) which illustrated at the same time that both building blocks (**2** and **6**) can be inserted during solid phase peptide synthesis and are stable during coupling and the final deprotection with TFA. Hence, post-synthetic labelling procedures could be avoided.

Photophysical studies revealed an intense fluorescence quenching in peptide **7** which diminished after cleavage with thrombin. The quenching efficiency was as high as 65–68% determined by steady state measurements and confirmed by calculations resulting from the fluorescence decay times. The observed fluorescence quenching was attributed to an efficient FRET, which is in excellent agreement with comparable systems reported in literature for which a Förster radius R_0 of 1.5 ± 0.2 nm was reported.[18]

One advantage of the new system is the excitation wavelength ($\lambda_{ex} = 450$ nm) which greatly reduces the direct excitation of matrix background fluorescence. Another advantage is the long decay time of the Ru-complex allowing for time-gated spectroscopy, in which any short-lived luminescence contribution of matrix constituents is eliminated. In order to perform measurements at quasi zero background conditions, further improvement with respect to the rotational and vibrational freedom of the fluorophores is necessary. Nevertheless, the system should be suitable for investigations of supramolecular interactions involving peptides/proteins. Experiments toward these goals and an extension to applications in DNA are currently under way.

Experimental

General

All reagents were purchased from commercial sources and used without further purification. Amine-free DMF (Roth) was employed throughout peptide synthesis, water for the protease assays was purified *via* a Direct-Q system (Millipore), and THF was dried over Na–benzophenone before use. Column chromatography (CC): silica gel 60 (Merck). Peptide synthesis: semi automatic SP-4000 synthesizer (Labortec AG). Semi-prep. HPLC: Agilent-1100 system, with Nucleosil 100–5 C18 PPN columns (Machery Nagel) for peptides or a Source 5RPC ST 4.6/150 column (Amersham Pharmacia Boitech) for Ru-complexes. NMR spectra: at 300 or 400 MHz (^{1}H), and at 75.5, 100.6 or 125.7 MHz (^{13}C); chemical shifts δ in ppm relative to the respective solvent signals, J in Hz. MS: Finnigan MAT-8200 (EI), TSQ-7000 (ESI); in m/z. The absorption spectra were recorded on a Lambda 750 UV/Vis Spectrometer (Perkin Elmer). The steady state luminescence measurements were carried out using a Fluoromax3 spectrometer (Jobin Yvon). The luminescence decay times were measured on a FL920 fluorescence spectrometer (Edinburgh Instruments) operated in the time-correlated single photon counting (TCSPC) mode. For the excitation the frequency double output (second harmonic generation, SHG) of a Titan sapphire laser ($\lambda = 860$ nm, $\lambda_{SHG} = 430$ nm) was directed into the sample and the luminescence was measured at 90 degrees using a multichannel plate (Europhoton) for the detection. The decay curves were measured at different emission wavelengths 550 nm $< \lambda_{em} <$ 700 nm. The detection window was set to 20 μs. In the data analysis least-square fitting based on the Levenberg-Marquardt algorithm was used.

1-[(2-Aminoethyl)amino]-4-(methylamino)anthraquinone (4). 1-[(2-Hydroxyethyl)amino]-4-(methylamino)anthraquinone (Disperse Blue 3, Aldrich) **3** was purified from crude material by silica gel column chromatography with ethylacetate–cyclohexane (50 °C; 1 : 1). The primary alcohol of pure Disperse Blue 3 (0.10 g, 0.33 mmol), was then transformed first to the phthalimide protected amine in a Mitsunobu reaction with DEAD (57 μl, 0.37 mmol), phthalimide (0.06 g, 0.37 mmol) and PPh_3 (0.10 g, 0.37 mmol) according to literature.[13] Cleavage of the phthalimide protecting group with hydrazine monohydrate (0.08 ml, 1.69 mmol) according to literature[13] yielded product **4** in 73% (0.07 g). The obtained spectroscopic data are in accordance with those reported in literature.[13]

(*S*)-*tert*-Butyl α-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)-δ-(2-(4-(methylamino)-9,10-dioxo-9,10-dihydroanthracen-1-ylamino)-ethylamino)-glutamic acid (5). A mixture consisting of **4** (0.23 g, 0.78 mmol, 1.0 eq.), Fmoc-Glu-O*t*Bu (0.40 g, 0.94 mmol, 1.2 eq.), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium-tetrafluoroborate (TBTU)[14] (0.38 g, 1.18 mmol, 1.5 eq.), and DIEA (0.80 mL, 4.68 mmol, 6.0 eq.) was suspended in dry DMF (25 mL) under argon and stirred for 12 h. The solvent was removed under reduced pressure. The blue residue was dissolved in CH_2Cl_2 (200 ml) and extracted with HCl (2 M, 2 × 90 mL) and H_2O (3 × 100 mL). After evaporation of the solvent the product was dried by azeotropic coevaporation with CH_3CN. Recrystallisation from MeOH yielded **5** as a blue solid (0.40 g, 0.57 mmol, 73%). ^{1}H-NMR ($CDCl_3$, 400 MHz): $\delta = 1.45$ (9H, s, -C(CH_3)$_3$), 1.92–2.03 (1H, m, -CH-CH_2-CH$_2$-), 2.21–2.32 (1H, m, -CH-CH_2-CH$_2$-), 2.32–2.40 (2H, m, -CO-CH_2-CH$_2$-), 2.99 (3H, d, $^3J = 5.2$ Hz, -NH-CH_3), 3.49 (2H, t, $J = 5.0$ Hz, -CH_2NH-CO-), 3.54 (2H, t, $^3J = 5.1$ Hz, -CH_2NH-Ar), 4.10 (1H, t, $^3J = 6.7$ Hz, Fmoc-C*H*-), 4.23–4.35 (3H, m, -NH-C*H*-CH$_2$, Fmoc-CH_2-), 5.74 (1H, d, $^3J = 5.7$ Hz, -CO-N*H*-CH-), 6.80 (1H, t, $^3J = 4.9$ Hz, -CO-N*H*-CH$_2$-), 6.96 (1H, d, $^3J = 9.8$ Hz, C*H*Ar^2), 7.08 (1H, d, $^3J = 9.9$ Hz, C*H*Ar^3), 7.18–7.25 (2H, m, arom. Fmoc), 7.29–7.36 (2H, m, arom. Fmoc), 7.45 (2H, d, $^3J_{app} = 7.5$ Hz, arom. Fmoc), 7.61 (2H, m, C*H*Ar^{5,8}), 7.67 (2H, d, $^3J_{app.} = 7.5$ Hz, arom. Fmoc), 8.16–8.25 (2H, m, C*H*Ar^{6,7}), 10.49 (1H, q, $^3J = 5.1$ Hz, -N*H*-CH$_3$), 10.69 (1H, t, $^3J = 5.2$ Hz, -CH$_2$-N*H*-Ar); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ = 27.99, 28.94, 29.41, 32.51, 39.51, 41.89, 47.12, 54.03, 67.00, 82.50, 109.63, 110.07, 119.89, 119.92, 122.93, 123.10, 125.04, 125.92, 125.94, 126.99, 127.64, 131.86, 132.01, 134.22, 134.44, 141.21, 143.69, 143.82, 145.70, 146.96, 156.41, 171.14, 172.70, 181.93, 182.44; MS (ESI-pos.): m/z (%) = 725 (100, [M + Na]$^+$), 703 (11, [M + H]$^+$); elemental analysis found C 69.8, H 6.0, N 7.9; $C_{41}H_{42}N_4O_7$ (702.79) requires C 70.1, H 6.1, N 8.0%.

(*S*)-α-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)-δ-(2-(4-(methylamino)-9,10-dioxo-9,10-dihydroanthracen-1-ylamino)ethylamino)-glutamic acid (6). The protected amino acid **5** (0.31 g,

0.44 mmol, 1.0 eq.) and TIS (0.24 mL, 1.17 mmol, 2.7 eq.) were dissolved in TFA–CH_2Cl_2 (1 : 1, 10 mL) and stirred for 3 h. The solvent was removed *in vacuo*. Precipitation with Et_2O gave **6** as a blue solid (0.23 g, 0.36 mmol, 82%). ^{1}H-NMR ($CDCl_3$, 500 MHz): $\delta = 1.80$–1.88 (1H, m, -CH-CH_2-CH$_2$-), 2.00–2.09 (1H, m, -CH-CH_2-CH$_2$-), 2.23 (2H, t, $^3J = 7.7$ Hz, -CO-CH_2-CH$_2$-), 3.06 (3H, d, $^3J = 5.4$ Hz, -NH-CH_3), 3.28–3.36 (2H, t, $^3J = 5.0$ Hz, -CH_2NH-CO-), 3.49–3.56 (2H, m, -CH_2NH-Ar), 4.10 (1H, m, -NH-CH-CH$_2$-), 4.18–4.31 (3H, m, Fmoc-CH-), 7.29–7.33 (2H, m, arom. Fmoc), 7.36–7.43 (2H, m, arom. Fmoc), 7.55 (1H, d, $^3J = 9.8$ Hz, CHAr^2), 7.68 (1H, d, $^3J = 9.7$ Hz, CHAr^3), 7.71 (2H, d, $^3J_{app} = 7.3$ Hz, arom. Fmoc), 7.75–7.78 (2H, m, CHAr^{5,8}), 7.87 (2H, d, $^3J = 7.5$ Hz, arom. Fmoc), 8.14 (1H, d, $^3J = 5.5$ Hz, -NH-CH-CH$_2$), 8.20–8.24 (2H, m, CHAr^{6,7}), 10.60 (1H, q, $^3J = 5.2$ Hz, -NH-CH$_3$), 10.79 (1H, t, $^3J = 6.0$ Hz, -CH$_2$-NH-Ar); ^{13}C-NMR (DMSO-d$_6$, 100 MHz): $\delta = 26.69$, 29.08, 29.21, 31.76, 41.32. 46.61, 53.47, 65.64, 103.50, 105.85, 107.41, 108.43, 108.63, 110.46, 111.80, 120.07, 124.10, 124.42, 125.60, 125.69, 127.04, 127.60, 132.20, 132.25, 133.80, 133.83, 140.67, 143.78, 145.84, 146.76, 156.10, 171.90, 173.63, 180.56, 180.80; MS (ESI-pos.): m/z (%) = 669 (42, [M + Na]$^+$), 647 (100, [M + H]$^+$), 527 (16), 381 (14), 289 (27); elemental analysis found C 68.5, H 5.4, N 8.5; $C_{37}H_{34}N_4O_7$ (646.69) requires C 68.7, H 5.3, N 8.7%.

Peptide synthesis

The peptide synthesis was carried out on a 0.02 mmol scale using the Fmoc/*t*Bu-protocol and Wang resin (loading 0.75 mmol g^{-1}) with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) as a coupling reagent.[14] Standard Boc and *t*-Bu side-chain-protected amino acids were employed. As building block for the incorporation of the quencher, compound **6** (20 mg, 0.03 mmol, 1.5 eq.), was used. For the incorporation of the Ru-complex to the solid phase-bound peptide at the N-terminus, a solution of **2** (37 mg, 0.022 mmol, 1.1 eq.), DIEA (30 µL, 0.18 mmol, 9.0 eq.) and TBTU (7 mg, 0.022 mmol, 1.1 eq.) in DMF (1.5 mL) was added to the resin, which was agitated for 24 hours. The peptides were deprotected and cleaved from the solid support by exposure to CF_3COOH–CH_2Cl_2–i-Pr$_3$SiH 95 : 3 : 2, and then purified by RP-HPLC and analyzed by LC-MS.

Data for 7. ESI-MS: 1471 (100, [M − 4Na + 4H − 2Cl]$^{2+}$), 981 (36, [M − 4Na + 5H − 2Cl]$^{3+}$).

*Data for **8***. ESI-MS: 1074 (100, [M − 4Na + 4H − 2Cl]$^{2+}$).

*Data for **9***. ESI-MS: 812.2 (100, [M + 1H]$^+$).

Thrombin assay

To a solution of peptide **7** (0.125 µmol) in a mixture of 330 µl of H_2O and 50 µl of buffer (0.2 M Tris·HCl (pH 8), 0.8M NaCl), 20 µl of human thrombin (20 U) were added. Before and after incubation of the solution at 30 °C for 24 h, aliquots were taken, deactivated at 100 °C for 30 s, and diluted with the same volume of MeCN. The samples were analyzed by RP-HPLC to determine the cleavage fragments. For the measurement of the fluorescence-emission spectra, the samples were further diluted with H_2O–MeCN 1 : 1 to result in a final peptide concentration of about 0.4 µM.

Notes and references

1 K. E. Sapsford, L. Berti and I. L. Medintz, *Angew. Chem., Int. Ed.*, 2006, **45**, 4562–4589.
2 M. Parsons, B. Vojnovic and S. Ameer-Beg, *Biochem. Soc. Trans.*, 2004, **32**, 431–433; Y. Sako, S. Minoguchi and T. Yanagida, *Nat. Cell Biol.*, 2000, **2**, 168–172; S. Brasselet, E. J. G. Peterman, A. Miyawaki and W. E. Moerner, *J. Phys. Chem.*, 2000, **104**, 3676–3682; A. Cha, G. E. Snyder, P. R. Selvin and F. Bezanilla, *Nature*, 1999, **402**, 809–813; Y. Suzuki, T. Yasunaga, R. Ohkra, T. Wakabayashi and K. Sutoh, *Nature*, 1998, **396**, 380–383.
3 I. Rasnik, S. Myong, W. Cheng, T. M. Lohman and T. Ha, *J. Mol. Biol.*, 2004, **336**, 395–408; W. Shen, M. F. Bruist, S. D. Goodman and N. C. Seeman, *Angew. Chem., Int. Ed.*, 2004, **43**, 4750–4752; D. A. Hiller, J. M. Fogg, A. M. Martin, J. M. Beechem, N. O. Reich and J. J. Perona, *Biochemistry*, 2003, **42**, 14375–14385; A. I. Dragan, J. Klass, C. Read, M. E. A. Churchill, C. Crane-Robinson and P. L. Privalov, *J. Mol. Biol.*, 2003, **331**, 795–813; P. J. Rothwell, S. Berger, O. Kensch, S. Felekyan, M. Antonik, B. M. Wöhrl, T. Restle, R. S. Goody and C. A. M. Seidel, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 1655–1660.
4 M. K. Nahas, T. J. Wilson, S. Hohng, K. Jarvie, D. M. J. Lilley and T. Ha, *Nat. Struct. Mol. Biol.*, 2004, **11**, 1107–1113; Z. Xie, N. Srividya, T. R. Sosnick, T. Pan and N. F. Scherer, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 534–539; H. D. Kim, G. U. Nienhau, T. Ha, J. W. Orr, J. R. Williamson and S. Chu, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 4284–4289.
5 O. Wichmann, J. Wittbrodt and C. Schultz, *Angew. Chem., Int. Ed.*, 2006, **45**, 508–512; J.-J. Li and T. D. H. Bugg, *Chem. Commun.*, 2004, 182–183; J. Hirata, C. F. d. Jong, M. M. v. Dongen, J. Buijs, F. Ariese, H. Irth and C. Gooijer, *Anal. Chem.*, 2004, **76**, 4292–4298; H. Takakusa, K. Kikuchi, Y. Urano, H. Kojima and T. Nagano, *Chem. Eur. J.*, 2003, **9**, 1479–1485; R. G. Kruger, P. Dostal and D. G. McCafferty, *Chem. Commun.*, 2002, 2092–2093; D. Summerer and A. Marx, *Angew. Chem., Int. Ed.*, 2002, **41**, 3620–3622.
6 P. M. Holland, R. D. Abramson, R. Watson and D. H. Gelfand, *Proc. Natl. Acad. Sci. U. S. A.*, 1991, **88**, 7276–7280; S. Tyagi and F. R. Kramer, *Nat. Biotechnol.*, 1996, **14**, 303–308.
7 M. Ternon and M. Bradley, *Chem. Commun.*, 2003, 2402–2403.
8 E. K. Kainmüller, E. Pinyol Ollé and W. Bannwarth, *Chem. Commun.*, 2005, 5459–5461; E. K. Kainmüller and W. Bannwarth, *Helv. Chim. Acta*, 2006, **89**, 3056–3070.
9 W. Bannwarth, D. Schmidt, R. L. Stallard, C. Hornung, R. Knorr and F. Müller, *Helv. Chim. Acta*, 1988, **71**, 2085–2099.
10 M. K. Johansson and R. M. Cook, *Chem. Eur. J.*, 2003, **9**, 3466–3471.
11 V. V. Didenko, *Biotechniques*, 2001, **31**, 1106–1121; L. Tan, Y. Li, T. J. Drake, L. Moroz, K. M. Wang, J. Li, A. Monteanu, C. Y. J. Yang, K. Martinez and W. H. Tan, *Analyst*, 2005, **130**, 1002–1005; W. H. Tan, K. M. Wang and T. J. Drake, *Curr. Opin. Chem. Biol.*, 2004, **8**, 547–553.
12 M. D. Weingarten, K. Sekanina and W. C. Still, *J. Am. Chem. Soc.*, 1998, **120**, 9112–9113.
13 J. P. May, L. J. Brown, I. Rudloff and T. Brown, *Chem. Commun.*, 2003, 970–971; J. P. May, L. J. Brown, I. Van Delft, N. Thelwell, K. Harley and T. Brown, *Org. Biomol. Chem.*, 2005, **3**, 2534–2542.
14 R. Knorr, A. Trzeciak, W. Bannwarth and D. Gillessen, *Tetrahedron Lett.*, 1989, **30**, 1927–1930.
15 E. Kaiser, R. L. Colescott, C. D. Bossinger and P. I. Cook, *Anal. Biochem.*, 1970, **34**, 595–598.
16 B. Geisser, A. Ponce and R. Alsfasser, *Inorg. Chem.*, 1999, **38**, 2030–2037.
17 A. A. Marti, C. A. Puckett and N. J. Turro, *J. Am. Chem. Soc.*, 2007, **129**, 8680–8681.
18 H. J. Youn, E. Terpetschnig, H. Szmacinski and J. R. Lakowicz, *Anal. Biochem.*, 1995, **232**, 24–30.
19 J. S. Kang, G. Piszczek and J. R. Lakowicz, *J. Fluoresc.*, 2002, **12**, 97–103.

