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SUPPORTING INFORMATION FOR

Exploiting the nitrilotriacetic acid moiety for biolabeling with ultrastable perylene dyes

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Contents:

Page S2-S6: Experimental section

Page S7: Fluorescence Correlation spectroscopy

Page S8: Preparation of His₆-tagged *E. coli* F₁.

Page S9: References

Experimental section

Reagents

The solvents used are of commercial grade. N-(2,6-diisopropylphenyl)-N-(4-carboxyethyl)-1,6,7,12-tetrasulfophenoxy perylene-3,4:9,10-tetracarboxydiimide **1a** and N-(2,6-diisopropylphenyl)-N'-[3-(N-succinimidyl)carboxyethyl]-1,6,7,12-tetra(4-sulfo phenoxy)-peryene-3,4:9,10-tetra carboxydiimide **1** was synthesized by a method previously developed (Scheme 1).^{1, 2} N-(5-Amino-1-carboxypentyl)iminodiacetic Acid, Tri-t-butyl Ester was purchased from Toronto Research Chemicals and used as received.

Scheme 1

Materials and methods

¹H and ¹³C NMR were recorded on Bruker Avance 250, Bruker AMX 300, Bruker DRX 500 and Bruker Avance 700. FD mass spectra were performed with a VG-Instruments ZAB 2-SE-FDP instrument. MALDI-TOF mass spectra were recorded on a Bruker MALDI-TOF spectrometer. UV/Vis spectra were recorded on a Perkin-Elmer Lambda 9, fluorescence spectra on SPEX Fluorolog 3 spectrometer.

Synthetic procedures

The synthesis of perylene nitrilotriacetic acid derivatives are shown in Scheme 2.

Scheme 2. Synthesis of the NTA perylene conjugate. A) Solution phase synthesis. B) Solid phase synthesis

Solution phase synthesis (Scheme 2A)**N-(2,6-diisopropylphenyl)-N'-[3-(6-(*tert*-butyl-2,2'-(1-*tert*-butoxy-1-oxo hexan-2-ylazanediyl)diacetate)carboxyethyl]-1,6,7,12-tetra(4-sulfophenoxy)-perylene-3,4:9,10-tetracarboxydiimide (2)**

N-(2,6-diisopropylphenyl)-N'-[3-(N-succinimidyl)carboxyethyl]-1,6,7,12-tetra(4-sulfo phenoxy)-perylene-3,4:9,10-tetracarboxydiimide (50 mg, 0.035 mmol) and diisopropyl ethylamine (DIPEA) (7.4 mg, 0.057 mmol) were added to a solution of N-(5-Amino-1-carboxypentyl)iminodiacetic acid, Tri-*t*-butyl Ester (22.8 mg, 0.053 mmol) in dry DMF (3 mL). The reaction vessel was purged with argon and stirred for 16 hours at room temperature. Ethylacetate (15 mL) was added to the reaction mixture to obtain a pink precipitate, which was filtered. The precipitate was washed first with dichloromethane and then three times with ethyl acetate (3x 40 mL) and dried under vacuum to give the product as red solid in 70 % yield. ¹H-NMR (300 MHz, DMSO-d₆, 300K): δ = 7.90 (s, 2 H), 7.88 (s, 2 H), 7.63 (m, 8H), 7.39 (t, *J* = 15 Hz, 1H), 7.29 (d, *J* = 9, 2H), 6.97 (m, 8H), 4.14 (t, *J* = 15 Hz, 2H), 3.56 (m, 2H), 3.32 (m, 4H), 3.17 (m, 2H), 2.95 (m 2H), 2.77 (m, 4H), 2.72 (m, 2H), 2.64 (m, 2H), 1.35 (s, 27H), 1.00 (d, *J* = 9Hz, 12H); ¹³C-NMR (300 MHz, DMSO-d₆, 300 K, Spinecho): δ = 171.89, 170.39, 162.97, 162.55, 155.72, 155.27, 155.21, 145.46, 128.17, 123.55, 122.88, 119.37, 80.30, 40.52, 32.56, 28.07, 22.58 ppm;

N-(2,6-diisopropylphenyl)-N'-[3-(N-(5-Amino-1-carboxypentyl)iminodi acetyl)carboxyethyl]-1,6,7,12-tetra(4-sulfophenoxy)-perylene-3,4:9,10-tetracarboxy diimide (3)

To a solution of **2** (40 mg, 0.023 mmol) in dry DMSO (2 mL) were added triisopropylsilane (1 mL) and trifluoroacetic acid (TFA). After stirring the reaction mixture for 3 hours at room temperature, the volatiles were evaporated under reduced pressure. Water (5 mL) was added and the solution applied on a column fill with Biogel P30. The product was eluted with water and freeze dried to give **3** as red solid in 95% yield. ¹H-NMR (700 MHz, CD₃OD, 300K): δ = 8.55 (s, 2 H), 8.53 (s, 2 H), 8.18 (m, 8H), 7.76 (t, *J* = 14 Hz, 1H), 7.64 (d, *J* = 7, 2H), 7.43 (m, 8H), 4.54 (t, *J* =

14 Hz, 1H), 3.87 (m, 4H), 3.49 (m, 3H), 3.32 (m, 4H), 2.70 (m, 2H), 2.28 (m, 2H), 1.83 (m, 2H), 1.68 (t, $J = 14$ Hz, 2H), 1.47 (d, $J = 7, 12$ Hz); ^{13}C -NMR (700 MHz, CD_3OD , 300K): $\delta = 168.33, 166.58, 158.54, 157.94, 156.52, 156.33, 152.51, 152.47, 149.49, 149.47, 141.82, 138.27, 138.26, 129.84, 124.08, 119.69, 114.20, 113.58, 111.91, 43.04, 38.03, 25.34, 24.71, 21.13, 19.44$ ppm; UV-Vis (H_2O): λ_{max} (ϵ) = 448 nm (16 120), 534 nm (31 224), 562 nm (35 000) Fluorescence (H_2O , excitation 562 nm) $\lambda_{\text{max}} = 614$ nm ; MS (MALDI-TOF): 1599.51 (100 %) $[\text{M}+2\text{Na}]$

Solid phase synthesis of Perylene-NTA (Scheme 2B)

All amine deprotection and coupling steps were monitored with ninhydrine. In a 25 mL manual peptide synthesis reaction vessel, Tenta TGR resin (2 g, 0.64 mmol) was swollen in dichloromethane (DCM) for 1 h. Fmoc-Lys(Mtt)-OH (1.15 g, 1.84 mmol), 1-Hydroxybenzotriazole hydrate (HOBt) (248 mg, 1.84), O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) (683 mg, 1.80 mmol), diisopropylethylamine (DIPEA) (237 mg, 1.84 mmol) was dissolved in dry dimethylformamide (DMF) (6.5 mL) added and shaken at 350 rpm at 25°C. After 12 h the resin was washed extensively with DCM. Fmoc deprotection was effected by six successive 15 min treatments of the resin with 20% piperidine in DCM. Afterwards the resin was washed extensively with DCM. To the washed resin was added a 20-fold excess of *tert*-butyl-bromoacetate (1.79 g, 9.2 mmol) with an equal amount of DIPEA (1.18 g, 9.2 mmol) and DCM (2 mL). The reaction vessel was shaken at 350 rpm at 45°C for 27 h. Fresh reagents were added and the vessel was further incubated at 45°C with shaking for 18 h. The beads were washed successively with DCM, ethyl acetate, and DCM again. The Mtt group was removed by five 3-min treatments with 10 mL of 1% TFA, 5% triisopropyl silane (TIS) in DCM. The beads were again washed extensively with DCM. N-(2,6-diisopropylphenyl)-N-(4-carboxyethyl)-1,6,7,12-tetrasulfophenoxy perylene-3,4:9,10-tetracarboxydiimide **1a** (100 mg, 0.076 mmol), HOBt (10 mg, 0.076), HBTU (50 mg, 0.074) and DIPEA (10 mg, 0.076) in dry DMF (0.5 mL) were added to the beads (86 mg, 0.02 mmol) in 2 mL of DMF. The beads were allowed to react with agitation for 24 h at 25°C. After washing of the sample, an equivalent amount of fresh reagents were added and allowed to react for 12 more hours. The beads were washed successively with DCM, ethyl acetate, methanol, DCM again, and dried briefly under vacuum. The product was cleaved

from the resin with several 5-min treatments with 10 mL of 95% TFA in DCM. The material was reduced to dryness with rotary evaporation, precipitated with ice-cold diethyl ether, and dried under high vacuum.

The dried off-red material was dissolved in water and purified by size-exclusion chromatography using BioGel as stationary phase and water as eluent. The yield, relative to the amount of Fmoc-Lys(Mtt)-OH was 15%.

Ni²⁺:NTA-Perylene³

NiCl₂ (30 μ l, 2.5×10^{-4} M/l solution in 0.01 N HCl) was added to Perylene NTA (1 ml, 5×10^{-5} M/l in water), and the solution was brought to pH 7 by addition of 0.8 mL 50 mM sodium acetate (pH 7), 200 mM NaCl. Following reaction for 30 min at 25°C, the product was purified using a Sep-Pak C18 cartridge (Millipore; pre-washed with 10 mL acetonitrile and 10 mL water; washed with 20 mL water; eluted with 1 mL 60% methanol) and dried.

Fluorescence correlation spectroscopy (FCS)

FCS measurements were carried out on a confocal setup based on an Olympus IX71 inverted microscope. A solid-state laser (JIVE, Cobolt AB, Stockholm, Sweden) with emission at 561 nm was used and attenuated to 150 μ W before focusing into the buffer solution by a water immersion objective (40x, N.A. 1.15, Olympus). The solution was placed on a microscope coverslide as a droplet of 25 to 50 μ l. Scattered laser light was blocked by a dichroic beam splitter (DCXR 575, AHF, Tübingen, Germany) and out-of-focus fluorescence was rejected by an 100 μ m pinhole in the detection pathway. Fluorescence was collected in the spectral range from 575 to 648 nm using an interference filter (AHF). Single photons were detected by an avalanche photodiode (SPCM AQR-14, Perkin Elmer) and registered by a TCSPC device (PC card SPC-630, Becker & Hickl, Berlin, Germany) for software calculation of the autocorrelation functions, $g^{(2)}(t_c)$.

The fluorescence intensity autocorrelation functions, $g^{(2)}(t_c)$, were fitted with two diffusion times, t_{D1} and t_{D2} , for the un-bound and the bound perylene dye, respectively, according to

$$g^{(2)}(t_c) = \frac{1}{N_f} \left\{ \alpha \left(\frac{1}{1+t_c/t_{D1}} \right) \left(\frac{1}{1+(\omega_0/z_0)^2(t_c/t_{D1})} \right)^{1/2} + (1-\alpha) \left(\frac{1}{1+t_c/t_{D2}} \right) \left(\frac{1}{1+(\omega_0/z_0)^2(t_c/t_{D2})} \right)^{1/2} \right\} \bullet \{1 - T - T \exp(-t_c/t_T)\}$$

with N_f , average number of fluorescent molecules in the confocal detection volume, t_c , correlation time, α , fraction of molecules with the shorter diffusion time t_{D1} , ω_0/z_0 , ratio of the $1/e^2$ radii of the detection volume in radial and axial directions, T , average fraction of fluorophores in the triplet state, and t_T , lifetime of the triplet state of the fluorophore. The ω_0/z_0 ratio was measured with a Rhodamine 101 solution as the reference and was kept at this value during the subsequent fittings of the autocorrelation functions of the perylene-Ni-NTA plus F₁-ATPase solutions. The triplet state lifetime of perylene-NTA and perylene-Ni-NTA was determined to $t_T = (2 \pm 1)$ μ s. The diffusion time of F₁-ATPase was measured independently after labeling a

cysteine (residue 106 of the γ subunit) with a water-soluble perylene-maleimide. It was used as the diffusion time t_{D2} for the calculation of the binding constant of perylene-Ni-NTA to the His tags at F_1 .

Additional photophysics of PDI-NTA and PDI-Ni-NTA :

The diffusion time of Rhodamine 101 in water was $\tau_D=251$ μ s, the diffusion time of PDI-NTA was $\tau_D= 369$ μ s and of PDI-Ni-NTA was $\tau_D= 381$ μ s. The diffusion times are related to the effective hydrodynamic radii and, therefore, scale with the molecular mass $M^{1/3}$. As the molecular weight increased by a factor of 3.17 for PDI-NTA (or 3.29 for PDI-Ni-NTA, respectively), the calculated diffusion times for PDI-NTA were $\tau_{D,calc}= 368.7$ μ s and for PDI-Ni-NTA $\tau_D= 373.3$ μ s which in excellent agreement with the measured FCS data.

Preparation of His₆-tagged *E. coli* F₁.

A His₆ tag was inserted at the N-terminus of the β subunit of *E. coli* F₁ by a two-step process. First, a PCR-based method⁴ was used to create a unique NsiI restriction site (ATGCAT) at the 5'-end of the *atpD* gene for β in plasmid p3U⁵. A cassette of complementary, 5'-phosphorylated primers was then ligated into the cleaved, dephosphorylated NsiI site of p3U. Sequencing identified a clone, p3U β H₆, with the cassette in the correct orientation and no other mutations. The modified *atpD* gene encodes β with N-terminal sequence MHHHHHHMHTGK (underlined residues replace wild-type Ala). F₀F₁ was expressed from p3U β H₆ in strain LE392 Δ (*atpI-C*)⁶, and β His₆-tagged F₁ was purified by standard methods⁵. The β His₆ tags had minimal effect on the specific ATPase activity of F₁.

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