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Diazaoxatriangulenium: synthesis of reactive derivatives and conjugation to bovine serum albumin†

Ilkay Bora, Sidsel A. Bogh, Martin Rosenberg, Marco Santella, Thomas Just Sørensen* and Bo W. Laursen*

The azaoxa-triangulenium dyes are characterised by emission in the red and a long fluorescence lifetime (up to 25 ns). These properties have been widely explored for the azadioxatriangulenium (ADOTA) dye. Here, the syntheses of reactive maleimide and NHS-ester forms of the diazaoxatriangulenium (DAOTA) system are reported. The DAOTA fluorophore was conjugated to bovine serum albumin (BSA) and investigated in comparison to the corresponding ADOTA-BSA conjugate. It was found that the fluorescence of DAOTA experienced a significantly higher degree of solvent quenching if compared to ADOTA as non-conjugated dyes in aqueous solution, while the fluorescence quenching observed upon conjugation to BSA was significantly reduced for DAOTA when compared to ADOTA. The differences in observed quenching for the conjugates can be explained by the different electronic structures of the dyes, which renders DAOTA significantly less prone to reductive photoinduced electron transfer (PET) quenching from e.g. tryptophan. We conclude that DAOTA, with emission in the red and inherent resistance to PET quenching, is an ideal platform for the development of long fluorescence lifetime probes for time-resolved imaging and fluorescence polarisation assay.

Introduction

Fluorescence technology is prevalent in high-tech applications from diagnostics,1,2 point-of-care devices,3,4 and display technology to DNA sequencing,5 drug-discovery,6 and imaging.7,8 Each application relies on dye development, be it bioengineered fluorescent proteins,9 emissive nanoparticles,10 inorganic luminophores,11,12 or organic dyes.13–16 Organic dye development is limited by fluorophore design, where many of the existing molecular frameworks have been known for more than a century.17 Many dye systems have been synthesised using these well-known scaffolds,18–21 and have been optimised to show significantly enhanced performance.22–31 In particular, photostability, absorption cross-section, fluorescence quantum yield, and emission wavelength have been enhanced to the extent possible for the available fluorophores. In the group of organic dyes, the triangulenium dyes are different.12 In these molecules, a small absorption cross-section, and the resulting low fluorescence rate constant, does not infer low photostability and low quantum yield. While donor-substituted triangulenium dyes are bright emitters similar to rhodamine and fluorescein dyes,33–36 the azaoxatriangulenium dyes are highly photostable, highly emissive, long fluorescence lifetime dyes.37–41 This group of triangulenium dyes includes azadioxatriangulenium (ADOTA) and diazaoxatriangulenium (DAOTA) shown in Chart 1 (for details on the triangulenium nomenclature see ESI†). The aza-bridges are readily functionalised with groups compatible, with the reaction conditions used to form the aromatic core.

The triangulenium dyes are synthesised from common precursors using sequences of highly selective nucleophilic
aromatic substitution reactions \( \left( S_{\text{Ar}} \right) \).\(^{33,35,38,42}\) For the azaoxa-
triangulenium dyes, each substitution step occurs in a cascade of
two \( S_{\text{Ar}} \)-reactions forming a heteroatom bridge.\(^{37,38}\) A similar
approach has recently been used to form the aza-bridge in
carbazoles.\(^{43}\) Alternatively, ether-cleaving reaction conditions
can be used to initiate an intramolecular \( S_{\text{Ar}} \)-reaction with
the formation of an oxygen bridge.\(^{44}\) Here, we elaborate on the
synthesis of diazaoxatriangulenium (DAOTA) in an effort to
make DAOTA derivatives, with reactive linkers for conjugation
of the DAOTA fluorophore to biomolecules.

We chose to use bovine serum albumin (BSA) as a demonstra-
tor for bioconjugation, although native BSA is not an ideal
model system,\(^{26,29,45}\) the results allow for a direct comparison
between DAOTA and ADOTA BSA-conjugates.\(^{46}\) We have pre-
viously investigated bioconjugates of ADOTA to BSA and IgG,
and used both of the azaoxa-triangulenium dyes in
bioconjugates of ADOTA to BSA and IgG,\(^{37,38}\) a similar
two SNAr-reactions forming a heteroatom bridge.\(^{37,38}\) A similar
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synthesis of diazaoxatriangulenium (DAOTA) in an effort to
make DAOTA derivatives, with reactive linkers for conjugation
of the DAOTA fluorophore to biomolecules.

Experimental

Materials and methods

Absorption spectroscopy was recorded with a double-beam
spectrophotometer using the pure solvent as baseline. Steady
state fluorescence spectra were recorded with a standard L-con-
figuration fluorimeter equipped with single grating monochro-
mators. All solvents used for spectroscopic experiments were
of HPLC grade and used as received. Phosphate buffered saline
(PBS) was prepared from slabs according to common protocols.
The pH value of the buffer was determined and subsequently
adjusted to 7.4. Molar absorption coefficients were determined
for each of the dyes using Lambert–Beer’s law by measuring the
absorption spectrum of three stock solutions with di-

\[ I(t) = \alpha \exp(-t/\tau) \]

In eqn (1) \( \alpha \) is the amplitude and \( \tau \) is the fluorescence life-
time. All time-resolved emission decay profiles and fits are
shown in the ESI.\(^{\dagger}\)

Synthetic procedures

Unless otherwise stated, all starting materials were obtained
from commercial suppliers and used as received. Solvents were
of HPLC grade for reactions and recrystallisations and techni-
gal grade for column chromatography and were used as
received.\(^{1}\) H NMR and \( 1^C \) NMR spectra were recorded on a
500 MHz or a 300 MHz instrument (500/300 MHz for \( 1^H \) NMR
and 126 MHz for \( 1^C \) NMR). Proton chemical shifts are
reported in ppm downfield from tetramethylsilane (TMS) and
carbon chemical shifts in ppm downfield of TMS, using
the resonance of the residual solvent peak as internal standard.
High-resolution mass spectra (HRMS) were recorded with an
ESP-MALDI-FT-ICR spectrometer equipped with a 7 T magnet
(prior to experiments, the instrument was calibrated using
NaTFA cluster ions) using dithanol as matrix. Column
chromatographic purifications were performed on Kieselgel 60
(0.040–0.063 mm particle size). Dry column vacuum chromato-
graphy was performed on Kieselgel 60 (0.015–0.040 mm
particle size). Thin layer chromatography was carried out using
aluminum sheets pre-coated with silica gel 60F.

Synthesis of 2 and 4. Compound 1 was prepared according
to the published procedure, see ref. 37. The compounds \( 2a–d \)
and \( 4a–d \) were prepared as reported in ref. 47.

N-(3-Carboxypropyl)-N'-methyl-1,13-dimethoxy-quin[2,3,4-k]-
acridinium tetrafluoroborate \( 3a \). 1,8-Dimethoxy-10-(2,6-
dimethoxyphenyl)-9-(3-carboxypropyl)-acridinium methyl
ester tetrafluoroborate \( 2a \) (0.5 g, 0.9 mmol) was placed in a sealable
tube and dissolved in acetonitrile (5 mL) and excess methyl-
amine (15 mL, 33 wt% in ethanol) was added. The solution
was stirred at 60 °C for five days. After it had cooled to ambient temperature it was poured into diethyl ether (500 mL)
to precipitate the product. The crude material was dissolved in
potassium hydroxide solution (1 M, 0.1 L) and stirred at refluxing
conditions for 5 h. After cooling aqueous tetrafluoroboric acid
(50 wt% in water) was used to acidify the solution when a dark
precipitate formed, which was filtered off. The crude
compound was dissolved in warm acetonitrile, filtered through a
paper filter, and precipitated twice from a solution of aceto-
nitrile with diethyl ether. Recrystallisation from \( i \)-propanol/
acetonitrile and yielded dark green crystals, which are washed
with dichloromethane and dried in vacuum (0.315 g, 69%).

\( 1^H \) NMR (500 MHz, Acetonitrile-\( d_3 \)) \( \delta \) 8.10 (\( t, J = 8.4 \) Hz, \( 1H \),
7.91 (\( t, J = 8.5 \) Hz, \( 1H \)), 7.86 (\( t, J = 8.4 \) Hz, \( 1H \)), 7.72 (\( d, J = 8.6 \) Hz,
1H), 7.67 (d, J = 8.9 Hz, 1H), 7.43–7.34 (m, 2H), 6.93–6.84 (m, 2H), 4.78–4.68 (m, 1H), 4.53–4.43 (m, 1H), 4.02 (s, 3H), 3.72 (s, 3H), 3.71 (s, 3H), 2.68 (t, J = 6.3 Hz, 2H), 2.34–2.19 (m, 2H). 13C NMR (126 MHz, Acetonitrile-d3) δ 175.2, 160.5, 160.2, 143.7, 143.3, 143.1, 140.6, 139.7, 138.1, 137.8, 137.3, 120.1, 113.9, 113.9, 108.6, 108.5, 106.0, 105.8, 104.1, 104.0, 56.5, 38.2, 31.2, 21.9. HRMS (MALDI-TOF): m/z calculated for C32H25N2O4+: 429.1809; found, 429.1811.

N-(4-Aminophenyl)-N′-(4-carboxyphenyl)benzimidazole tetrafluoroborate 3a. 1H NMR (500 MHz, Chloroform-d) δ 8.18 (s, 1H), 7.94–7.92 (m, 1H), 7.70–7.67 (m, 1H), 7.53 (d, J = 8.5 Hz, 1H), 7.23 (t, J = 7.2 Hz, 2H), 7.08 (d, J = 8.1 Hz, 1H), 6.96 (d, J = 8.0 Hz, 1H), 5.20 (d, J = 4.9 Hz, 1H), 4.43 (m, 1H), 4.02 (s, 3H), 3.34 (m, 2H). 13C NMR (126 MHz, Acetonitrile-d3) δ 144.9, 144.6, 144.6, 142.0, 141.2, 141.0, 138.7, 138.3, 138.0, 137.4, 120.0, 115.0, 113.9, 108.9, 107.3, 106.7, 104.5, 101.6, 56.4, 56.3, 38.0, 27.1. HRMS (MALDI-TOF): m/z calculated for C29H27N3O4+: 476.1969; found, 476.1972.

N-(4-Aminophenyl)-N′-(3,4,4′-trifluorobenzylidene)-1,13-dimethoxy-quin[2,3,4-kl]acridinium tetrafluoroborate 3b. 1H NMR (500 MHz, Acetonitrile-d3) δ 8.28 (d, J = 8.5 Hz, 1H), 7.94–7.92 (m, 1H), 7.70–7.67 (m, 1H), 7.55 (d, J = 4.0 Hz, 1H), 7.28 (t, J = 8.1 Hz, 1H), 6.96 (d, J = 8.0 Hz, 1H), 5.20 (d, J = 4.9 Hz, 1H), 4.43 (m, 1H), 4.02 (s, 3H), 3.34 (m, 2H). 13C NMR (126 MHz, Acetonitrile-d3) δ 144.6, 144.6, 144.6, 142.0, 141.2, 141.0, 138.7, 138.3, 138.0, 137.4, 120.0, 115.0, 113.9, 108.9, 107.3, 106.7, 104.5, 101.6, 56.4, 56.3, 38.0, 27.1. HRMS (MALDI-TOF): m/z calculated for C32H25N2O4+: 429.1809; found, 429.1811.

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ether gave a fine powder which was recristallized from i-propanol/methanol to yield the pure compound as dark crystals which are washed with cold acetonitrile and methanol (0.15 g, 17.5%).

$^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 8.17–8.09 (m, 2H), 7.96 (t, $J = 8.4$ Hz, 1H), 7.77 (d, $J = 8.9$ Hz, 1H), 7.59 (d, $J = 8.6$ Hz, 1H), 7.44–7.37 (m, 2H), 7.15 (d, $J = 8.6$ Hz, 2H), 6.93 (d, $J = 8.6$ Hz, 2H), 6.73 (d, $J = 8.7$ Hz, 1H), 6.67 (d, $J = 8.5$ Hz, 1H), 5.78 (s, 2H), 4.03 (s, 3H).

$^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 152.1, 151.8, 150.6, 142.8, 141.6, 141.0, 140.3, 139.7, 139.2, 138.6, 138.1, 128.5, 124.5, 115.8, 111.0, 110.5, 109.9, 108.3, 108.2, 107.3, 107.1, 107.0, 106.1, 35.6. Anal. Calcd for C$_{23}$H$_{18}$BF$_4$N$_3$O: C, 65.71; H, 3.82; N, 9.10; Found: C, 65.94; H, 3.54; N, 9.10. HRMS (MALDI-TOF): m/z calcd for C$_{23}$H$_{18}$BF$_4$N$_3$O$^+$, 388.1444; found, 388.1455.

N-(3-Carboxypropyl)-N'-methyl-diazaoxatriangulenium NHS ester tetrafluoroborate 6a. N-(3-Carboxypropyl)-N'-methyl-diazaoxatriangulenium tetrafluoroborate 5a (0.20 g, 0.42 mmol), N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (0.17 g, 0.55 mmol) and diisopropylethylamine (0.12 mL, 0.68 mmol) were dissolved in DMSO (20 mL) and stirred at ambient temperature overnight. The product was precipitated by addition of sodium tetrafluoroborate solution (0.2 M, 0.25 L) and filtered off. Then the crude material was redissolved in dichloromethane and a minor volume acetonitrile and dried over sodium sulfate, filtered, and the solvent was removed in vacuum. Precipitation from a solution of acetonitrile with diethyl ether gave a fine powder, which was purified by column chromatography (dichloromethane/methanol 19:1) to yield the compound as a red powder, which is washed with dichloromethane and dried in vacuum (0.11 g, 43%).

$^1$H NMR (500 MHz, Acetonitrile-$d_3$) $\delta$ 8.20 (t, $J = 8.6$ Hz, 1H), 8.04–8.01 (m, 1H), 8.01–7.97 (m, 1H), 7.57 (d, $J = 8.7$ Hz, 1H), 7.50 (d, $J = 8.6$ Hz, 1H), 7.44 (d, $J = 8.8$ Hz, 1H), 7.36 (d, $J = 8.6$ Hz, 1H), 7.19 (d, $J = 7.7$ Hz, 1H), 7.15 (d, $J = 7.8$ Hz, 1H), 4.43–4.35 (m, 2H), 3.84 (s, 4H), 3.73 (s, 3H), 2.66 (t, $J = 6.6$ Hz, 2H), 2.12–2.09 (m, 2H).

$^{13}$C NMR (126 MHz, Acetonitrile-$d_3$) $\delta$ 174.4, 153.3, 153.2, 142.4, 141.6, 141.2, 140.7, 140.6, 140.3, 139.6, 139.5, 118.0, 112.1, 110.3, 110.1, 109.5, 109.4, 108.3, 108.1, 107.0, 106.8, 52.5, 48.1, 36.4, 30.8, 21.2. HRMS (MALDI-TOF): m/z calcd for C$_{23}$H$_{18}$N$_3$O$^+$, 417.1234; found, 417.1236.

N-(4-Aminophenyl)-N'-methyl-diazaoxatriangulenium NHS ester tetrafluoroborate 6b. N-[2-(4-Aminophenyl)ethyl]-N'-methyl-diazaoxatriangulenium tetrafluoroborate 5b (0.10 g, 0.19 mmol) and N,N,N',N'-tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (0.085 g, 0.28 mmol) were dissolved in acetonitrile (8 mL) and triethylamine (0.06 mL, 0.34 mmol). The solution was stirred at ambient temperature for 90 min. Then the product was precipitated with diethyl ether and filtered off. Washing with water (10 mL) and threefold precipitation from a solution of acetonitrile with diethyl ether gave the pure product as red powder (0.108 g, 91%).

$^1$H NMR (500 MHz, Acetonitrile-$d_3$) $\delta$ 8.17 (t, $J = 8.6$ Hz, 1H), 8.05–8.00 (m, 2H), 7.97 (t, $J = 8.5$ Hz, 1H), 7.53 (d, $J = 8.2$ Hz, 2H), 7.48 (d, $J = 8.8$ Hz, 1H), 7.42 (d, $J = 8.8$ Hz, 1H), 7.38 (dd, $J = 8.6$, 5.9 Hz, 2H), 7.21 (d, $J = 3.7$ Hz, 1H), 7.19 (d, $J = 3.8$ Hz, 1H), 7.12 (d, $J = 3.5$ Hz, 2H).
Laubing procedure. Labelling of bovine serum albumin (BSA) was achieved either by activating the free carboxylic acid substituted dyes with O-\((N\text{-succinimidyl})\)-N,N,N',N'-tetramethyltrifluoroborurate (TSTU) in the presence of diisopropylethylamine (DIPEA), resulting in in situ formation of the N-hydroxysuccinimide (NHS) ester, which was subsequently reacted with BSA. Alternatively, NHS esters of the dyes were used directly. The BSA conjugates were subsequently purified by dialysis. Consult ref. 46 for the full labelling protocols and procedures. Despite the tendency of BSA to bind small molecules electrostatically, we did not observe unbound dye in the optical experiments.

Results and discussion

Synthesis

The synthesis of azaoxa-triangulenium dyes was developed in our lab\(^{37,38}\) based on the work of Martin and Smith\(^{41}\) who first synthesised the trioxatriangulenium (TOTA) system. In our early work, azadioxa-, diazaoxa-, and triaza-triangulenium (ADOTA, DAOTA, and TATA) was reported.\(^{37,38}\) Lacour and co-workers have later expanded the series of triangulenium salts to also include derivatives with a single sulfur bridge.\(^{60}\) The incorporation of more than one sulfur atom in the triangulenium core seems not to be possible due to the distortion enforced by the significantly different C=S bond length as compared to C-N and C-O bonds.\(^{61}\) The azaoxa-triangulenium dyes are all made from a common precursor; tris(2,6-dimethoxyphenyl)-carbeneium tetrafluoroborate (1) which, can be reacted stepwise with primary amines to form between one and three aza-bridges. 1 or each intermediate may be reacted under ether cleaving conditions at elevated temperatures to form the fully ring closed triangulenium core with one, two, or three oxa-bridges. The oxa-bridges are themselves reactive towards primary amines. However, this substitution reaction is slow compared to attack on the methoxy groups of the open precursors, but it is still highly selective.\(^{35}\)

Post-functionalisation of the substituents on the aza-bridges have previously been reported\(^{62,63}\) and we have synthesised and explored reactive derivatives of ADOTA for bioconjugation.\(^{46,48}\)

The syntheses of ADOTA with reactive NHS esters and maleimide groups (Scheme 1) are straightforward, and proceed by a \(S\text{NAr}\) reaction between 1 and a suitable amino acid or diamine. The primary amine attack one of the methoxy substituted carbon atoms in 1 followed by elimination of methanol upon formation of a transient intermediate set up for an intramolecular \(S\text{NAr}\) reaction, eliminating methanol and forming an aza-bridge. The product is an \(N\)-substituted tetramethoxy-acridinium salt (2). Reacting 2 in molten pyridinium chloride yields the fully ring closed azadioxa-triangulenium (4, ADOTA).\(^{37,38}\) Alternatively, a second aza-bridge can be introduced by reacting 2 with a second primary amine forming a \(4\) helicenium ion. The product is an \(N\text{,N'\text{-substituted 1,13-dimethoxyquinacridinium (DMQA) salt}}\) (3, for details on this nomenclature see...
ESI†), which in molten pyridinium chloride yields the fully ring closed diaza-oxa-triangulenium core (5, DAOTA).

The steps to ADOTA-derivatives suitable for bioconjugation as active esters and maleimides follow the direct path from 1 over 2 to 4. Similarly, the 3-carboxypropyl derivative of DAOTA (2a) could be synthesised by introducing first one (2a), and then a second aza-bridge to give the DMQA derivative (3c). Subsequent reaction of the DMQA compounds in molten pyridinium chloride followed by basic hydrolysis of the intermediate amide yielded the desired N-(3-carboxypropyl)-N'-methyl-DAOTA tetrafluoroborate (5a, Scheme 1) as a red powder in a good yield.49 The synthesis of the 4-aminophenyl DAOTA derivative (5d) was also performed via the route through the DMQA derivative (3d, Scheme 1). This yielded 3d in a high yield, while the ring closure reaction for conversion of 3d into 5d yielded 5d in a low yield. In attempts to prepare the 2-(4-carboxyphenyl)-ethyl and 4-carboxyphenyl DAOTA derivatives (5b and 5c, respectively) via the same synthetic route as described for 5a and 5d, we found that this was associated with difficulties. The compound 3b was isolated in a high yield. However, the following ring-closure reaction in molten pyridinium chloride yielded the desired product (5b) in combination with impurities, which were inseparable from 5b. Similarly, the DMQA derivative 3c could not be prepared as the basic hydrolysis of the intermediate amide derivative (3c’, Scheme 2) was not successful.
Thus, 5b and 5c were synthesised via an alternative route, which to our knowledge has only been reported for the N,N'-dipropyl-DAOTA salt by Laursen and Krebs. The compounds 5b and 5c were obtained in good yields via reaction of the ADOTA derivatives (4b and 4c) with methylvamine in acetonitrile/ethanol or NMP/ethanol mixtures. The acid derivatives 5a–c were reacted with N,N,N',N'-Tetramethyl-O-(N-succinimidy)luronium tetrafluoroborate (TSTU) in acetonitrile or DMSO solution to form the reactive NHS esters (6a–c) in good yields. The NHS ester functional group is used to conjugate fluorescent dyes to biomacromolecules via coupling to a primary amine residue of the biomacromolecule.

Compound 5d was reacted with maleic anhydride to form the corresponding maleimide 7 in a two-step/one pot reaction (Scheme 1). The maleimide group is used to selectively label thiol groups in biomolecules.

Optical spectroscopy

In the following N-(4-carboxyphenyl)-N'-methyl-DAOTA tetrafluoroborate 5c is used to demonstrate the spectroscopic properties of the reactive ADOTA derivatives. The properties of 5c are compared to those of the corresponding N-(4-carboxyphenyl)-ADOTA tetrafluoroborate 4c. The spectra displayed in Fig. 1 show that DAOTA (5c) has more desirable absorption and emission properties. These are in the red region of spectrum, which is better for measurements on biological systems as compared to ADOTA (4c). Table 1 summarises the photophysical properties of 4c and 5c in acetonitrile, dimethyl sulfoxide (DMSO), and phosphate buffered saline at pH = 7.4 (PBS). The differences between 4c and 5c in organic solvents are closely related to the oscillator strengths of the lowest energy transition of the two parent chromophores. The higher molar absorption coefficient at the lowest energy transition of DAOTA as compared to that of ADOTA (ε = 16 000 vs. 10 000 M⁻¹ cm⁻¹ in acetonitrile solution) results in a shorter fluorescence lifetime (τ = 19 vs. 21 ns in MeCN). In PBS solution, DAOTA 5c exhibits a significant change in fluorescence lifetime (τ) and fluorescence quantum yield (φ), where both are reduced; φ from 55% to 35% and τ from 19 ns to 14 ns. We have found that chloride is not a specific quencher of DAOTA, and suggest that the observed 30% reduction in φ must be due to unspecific solvent quenching, likely due to the high hydrophobicity of the DAOTA core of 5c. ADOTA 4c is less hydrophobic and does not show more than 18% reduction in φ in PBS solution as compared to the organic solvents.

The fluorescence lifetime (τ) for DAOTA 5c is exceptionally long for a red emitting organic dye, even when reduced to 14 ns by unspecific solvent quenching in PBS solution. The combination of a long fluorescence lifetime (>10 ns) and emission in the red (>600 nm) makes DAOTA ideally suited to monitor rotational correlation times of biomolecules and as a fluorescent probe for fluorescence polarisation based assays.

DAOTA-BSA conjugates

To test the ability of DAOTA as a probe for measuring the rotational motion of proteins the reactive ester of DAOTA 5c, N-(4-carboxyphenyl)-N'-methyl-diazaoxatriangulenium NHS ester tetrafluoroborate 6c, was conjugated to BSA. This reaction is expected to predominantly result in conjugation of the triangulenium based probe to the N-terminus of the protein. The labelling protocol was optimised to give a low degree of labelling (DOL) to ensure that complications arising from multiple labels, such as energy transfer between labels, was minimal. When developing a fluorescence polarisation assay, these effects can be probed by looking for Weber's red-edge effect. A DOL of 0.9 DAOTA dyes per BSA was used to obtain the results presented below.

The Perrin equation (eqn (2)) describes the ideal relationship between: the observed fluorescence anisotropy (r), the fundamental anisotropy of the dye (r0), the rotational correlation time of the rotating volume (θ), and the fluorescence lifetime (τ).

$$ r = \frac{r_0}{1 + \frac{3}{2} r_0 \tau / \theta} $$

The rotational correlation time (θ) is directly related (eqn (3)) to the rotational volume (V) and the viscosity of the surrounding

| Table 1 Photophysical properties of N-(4-carboxyphenyl)-az diaoxatriangulenium tetrafluoroborate (ADOTA, 4c) and N-(4-carboxyphenyl)-N'-methyl-diazaoxatriangulenium tetrafluoroborate (DAOTA, 5c) in acetonitrile (MeCN), dimethyl sulfoxide (DMSO), and phosphate buffered saline at pH = 7.4 (PBS) solutions |
|---|---|---|---|---|
| Solvent | ADOTA 4c | DAOTA 5c |
| λabs / nm | 536 | 539 | 539 | 557 | 561 | 556 |
| λem / nm | 562 | 570 | 560 | 590 | 600 | 592 |
| ΔStokes / nm | 26 | 31 | 21 | 33 | 39 | 36 |
| τfl / ns | 21.0 | 17.1 | 17.9 | 18.7 | 15.1 | 14.0 |
| φ | 0.64 | 0.64 | 0.57 | 0.55 | 0.45 | 0.35 |
| τ / ns | 32.8 | 26.7 | 31.4 | 34.0 | 33.6 | 40.0 |

λabs is the wavelength of the lowest energy absorption maximum given in nm. λem is the wavelength at the emission maximum given in nm. ΔStokes is the Stokes’ shift given in nm, τfl is the fluorescence lifetime given in ns, and φ is the measured fluorescence quantum yield using rhodamine 6G as a reference, τ0 is the radiative lifetime: τ0/φ0 given in ns.
medium (\(\eta\)). For proteins, the rotational correlation time can be related (eqn (3)) to the molecular mass (\(M\)), the specific density (\(\nu\)), and the average hydration (\(h\)) of the protein.\(^{66}\)

\[
\frac{r_0}{r} = 1 + \frac{\tau_\eta}{\theta}
\]  
\(\theta = \eta V / RT = \eta M / RT \cdot (\nu + h)
\] (3)

Two factors related to the fluorescent probe used are found in the equations: the fluorescence lifetime (\(\tau_\eta\)) and the fundamental fluorescence anisotropy (\(r_0\)). The fluorescence lifetime determines the range of rotational correlation times that may be probed, in other words the range of molecular weights that can be investigated. The fundamental anisotropy, ranging from 0.4 to −0.2, determines the dynamic range of the experiments. Dyes with a low \(r_0\) value are poor probes for fluorescence polarisation-based methods. DAOTA has \(r_0 = 0.38\),\(^{65}\) which is very close to the maximal value (0.4), while the fluorescence lifetime allows for probing biomolecules with a molecular weight up towards 1000 kDa.\(^{56}\)

Fig. 2 shows a Perrin plot of 5c conjugated to BSA (5c-BSA), where the steady-state fluorescence anisotropy \(r\) measured at four different temperatures \(T\) are plotted as \(1/r\) against \(T/\eta\) and used to determine the \(\theta/\nu\), and the apparent anisotropy \(r_0^{app}\) by extrapolation of \(T/\eta\) to zero. The latter is a measure for the flexibility of the dye label, when conjugated to the biomolecule. Ideally, if the label upon conjugation loses all degrees of freedoms, except co-rotation with the biomolecule, the apparent and fundamental anisotropy will be identical. Any local flexibility of the dye label will induce a pathway for fast scrambling of the photoselection not related to the motion of the biomolecule. The result is a lowering of the apparent anisotropy \(r_0^{app}\) in a Perrin plot, and a loss of dynamic range in any fluorescence polarisation based experiment. For 5c-BSA the apparent anisotropy is at \(r_0^{app} = 0.36\) surprisingly high, clearly indicating that the DAOTA label is immobilised on the surface of BSA.

While the effect of the long fluorescence lifetime of 5c may be hard to identify in the steady state spectra, it is directly visible in time-resolved experiments. Fig. 3 (top) shows a time-resolved emission decay profile for 5c and 5c-BSA measured in PBS solution, obtained using time-correlated single photon counting (TCSPC). Cursory inspection of the fluorescence decays profiles in Fig. 3 (top) shows that the 5c-BSA has a longer fluorescence lifetime than 5c in PBS solution (\(r_{f,5c} = 14.0\) ns vs. \(r_{f,5c\text{-BSA}} = 21.2\) ns, see ESI†), and that photons can be detected well beyond 150 ns when using the standard settings of TCSPC with a maximum count of 10,000. By using longer acquisition times (higher maximum number of counts), photons arising from emission of the 5c-BSA conjugates may be detected up towards 250 ns after excitation, this is without equal when considering organic dyes with emission in the red.

Fig. 3 (bottom) shows the time-resolved anisotropy decay for 5c-BSA. The data allow for direct determination of the rotational correlation time of the rotating volume \(\theta\). The long fluorescence lifetime is important in obtaining these data, as photons must be emitted in a time interval long enough to describe the rotational motion of the biomolecule. Fig. 3b

Fig. 2 Perrin plot of 5c-BSA in phosphate buffered saline at pH = 7.4 solution. The experiment was performed on a sample with a DOL = 0.9, where the steady state fluorescence anisotropy \(r\) was determined at four different temperatures \(T\).

Fig. 3 Top: Time-resolved emission decay profile for \(N\)-(4-carboxyphenyl)-\(N\')-methyl-diazaoxatriangulenium tetrafluoroborate (DAOTA, 5c) and the corresponding BSA conjugate (5c-BSA) measured in PBS solution. Bottom: Time-resolved anisotropy decay profile for 5c-BSA measured in PBS solution. For details on fits and the resulting parameters see the ESI†.

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[Image: Fig. 2 Perrin plot of 5c-BSA in phosphate buffered saline at pH = 7.4 solution. The experiment was performed on a sample with a DOL = 0.9, where the steady state fluorescence anisotropy \(r\) was determined at four different temperatures \(T\).]

[Image: Fig. 3 Top: Time-resolved emission decay profile for \(N\)-(4-carboxyphenyl)-\(N\')-methyl-diazaoxatriangulenium tetrafluoroborate (DAOTA, 5c) and the corresponding BSA conjugate (5c-BSA) measured in PBS solution. Bottom: Time-resolved anisotropy decay profile for 5c-BSA measured in PBS solution. For details on fits and the resulting parameters see the ESI†.]

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shows that in the case of 5c-BSA the photoselection is fully scrambled by rotational motion in ~100 ns. That is, the anisotropy has decayed to zero. The long rotational correlation time determined for BSA from these data is θ_{BSA} = 40 ns (see ESI for details†), a number identical to the average literature value of θ_{BSA} = 40 ns. Note that BSA is not a perfect spherical rotor, and the value determined will be influenced by the position and relative orientation of the dye in the bioconjugate.

While emission from either of the azaoxa-triangulenium dyes can be used to follow the rotational motion of large biomolecules for more than 100 ns,27 there is a clear difference in the behaviour of 4a-BSA and 5c-BSA. Where ADOTA fluorescence is quenched in the conjugates (4a-BSA) when compared to the non-bounded dyes (4a), the DAOTA fluorescence appears to be enhanced upon conjugation, as seen by the significantly increased fluorescence lifetime (Fig. 3). As the fluorescence lifetime only report on the emitting population of dyes, the actual emission intensity of each dye was determined. Fig. 4 shows the results for 4a-BSA and 5c-BSA. The total emission intensity of either dye is decreased upon conjugation to BSA, but the effect is much less pronounced for the DAOTA derivative. We rationalised the quenching of the ADOTA fluorescence as a result of reductive PET quenching by tryptophan,50 a process the increased cation stability of DAOTA makes less favoured (see ESI for details†).51 Thus, we see less quenching of the DAOTA fluorescence in the 5c-BSA conjugates. The significant increase in lifetime upon conjugation of 5c to BSA must be due to a reduction in the non-specific solvent induced quenching of a population of DAOTA dyes that is partially shielded by the protein surface, while the reduction in overall intensity must be due to an almost fully quenched population of 5c-BSA. The quenched population does not contribute to the time-resolved emission decay profile, and will not influence the fluorescence anisotropy. The net result is that the fluorescence quantum yield of the 5c-BSA conjugate at ϕ_{q} = 0.34 is very close to that of the free dye 5c in PBS at ϕ_{q} = 0.35, although with a more complicated time-resolved fluorescence decay profile (see ESI†).

**Conclusions**

The syntheses of six new derivatives of diazaoxatriangulenium (DAOTA) salts were reported, and it was shown that these dyes can be accessed via two synthetic routes. One set of substituents may favour one route over the other.

The photophysical properties of the DAOTA fluorophore were investigated in view of using the red emitting, long fluorescence lifetime dye in fluorescence polarisation assays. We showed that the DAOTA fluorophore undergoes unspecific solvent fluorescence quenching in aqueous buffer, reducing the fluorescence lifetime from 19 ns in acetonitrile solution to 14 ns in PBS solution. 5c was conjugated to BSA, and we found that 5c-BSA had a significantly increased longest fluorescence lifetime component at 21.2 ns (as well as intensity weighted average fluorescence lifetime of 19.2 ns). Furthermore, the overall emission intensity of the conjugates (5c-BSA) was found to be equal to that of the free dye 5c measured in PBS solution, as 5c is less quenched by tryptophan. Thus the DAOTA fluorophore, with emission further in the red and a longer fluorescence lifetime in biomolecule conjugates, was found to be superior to the ADOTA fluorophore as a probe for developing fluorescence polarisation assays for large biomolecules.

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