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Synthesis of nitric oxide probes with fluorescence lifetime sensitivity

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Abstract

We present the rationale, synthesis and evaluation of the first activatable fluorescent probe that utilizes fluorescence lifetime change for detection of nitric oxide. The new probe DAP-LT1 features a near-infrared polymethine skeleton with diaminobenzene functionality incorporated into the meso-position. The probe is partially quenched, and upon reaction with a nitric oxide shows an increase in the fluorescence lifetime from 1.08 ns to 1.24 ns.

INTRODUCTION

Reactive nitrogen species (RNS) are known biomarkers of inflammation and tumour proliferation. They participate in a great number of physiological functions from cell signalling to cell damage oxidation. The current methods for assessing NO include: direct measurements with NO sensitive electrodes,¹ chemiluminescence through NO reactions with ozone,² staining tissues for nitrosylated proteins with antinitrotyrosine specific antibodies, measuring the expression of NOS via qPCR³ and analysis of nitrite with absorbance Griess reagent.⁴ With the rise of fluorescent techniques in the last two decades a number of activatable fluorescence probes for measuring nitric oxides and its metabolites in cells and thin tissues have been developed. Today, a variety of activatable “OFF-ON” probes that include NBD methylhydrazine, fluorescein based DAF-FM, rhodamine DAR-2 for nitric oxide are available commercially and new Cu-based,⁵ NK-green-2,⁶ HK-green-3,⁷ PyBor⁸ for peroxynitrite detection has been recently published.

The sensitivity of all-to-date published and commercially available “OFF-ON” fluorescent probes for RNS research rely on the increase of the fluorescence intensity. However, the fluorescence intensities are difficult to quantify in heterogeneous systems such as thick tissues and biofluids where the recorded intensity changes may be due to a concentration gradient instead of differences in RNS expression. In contrast to intensity, the fluorescence lifetime is an intrinsic property of the fluorophore⁹ and has been recently introduced as complementary modality to overcome the problem of concentration artifacts.^{10, 11} Additional benefits of fluorescence lifetime includes contrast enhancement through alleviation of some of the typical problems associated with intensities including scattering,¹² sample turbidity, and autofluorescence.¹³⁻¹⁵ Recently, several papers describing lifetime sensitive probes based on cyanine dyes,^{16, 17} quantum dots^{18, 19} and fluorescent proteins²⁰ have been published.

Herein, we present a first example of the fluorescence lifetime probe for NO detection. As with other lifetime probes, two criteria have to be met. First, the change in the lifetime upon reaction with NO should be substantial enough to overcome the sensitivity of current fluorescence lifetime instrumentation (50-100 ps). Second, the fluorescence intensity of the probe before and after reaction with the substrate should be strong, otherwise an impractically long scanning time will be needed to measure the lifetime. So far, all published probes according to the corresponding references exhibit high level of quenching (>100x) which is ideal for intensity imaging, however, detrimental to fluorescence lifetime imaging.

RESULTS AND DISCUSSION

Probe design and synthesis

The design of the NO lifetime probes was motivated by our recently published work where amine-containing pyrazoles were inserted into the meso-position to form fluorescence lifetime pH sensitive dyes.¹⁶ Fluorescence lifetime of polymethine probes is, in general, sensitive to the environment and structural modifiers.⁹⁻¹¹ Placing electron rich amines in close proximity to the fluorophore decreases the emission due to photoinduced electron transfer (PET)²¹ also known as excited state electron transfer (ESET) effect that results in the lower fluorescence lifetime. This approach was implemented in the design of NO sensitive lifetime probes where the NO reactive diaminopolymethine sensor was placed in the meso-position of the heptamethine chain to form diaminopolymethine polymethine lifetime probes (DAP-LTs) shown in Fig. 1.

Upon reacting with NO in the presence of molecular oxygen, the diaminebenzene is converted into a benzotriazole, leading to a restoration of fluorescence.²² This approach has been successfully used for preparation of a number of NO sensitive fluorescent probes based on fluoresceins,²³ rhodamines,²⁴ spirobifluorenes,²⁵ and polymethines.²⁶ All these probes were designed to achieve the highest quenching to maximize the sensitivity of the probe. However, none of them have been reported to show any fluorescence lifetime sensitivity.

The synthesis of DAP-LTs was based on methodology developed for selective substitution of meso-positions in polymethine dyes via Suzuki coupling.²⁷ Briefly, arylbromide **1** or a methylated form of 4-bromo-2-nitroaniline **2** was converted to the boronic esters **4-5** using bis-pinacolato diboron **3** under Suzuki coupling conditions (Scheme 1). The introduction of the methyl group was motivated by the structure of commercial NO sensor DAF-FM (see below). Subsequent coupling of the **4-5** with a commercially available dye-precursor IR783 led to a formation of C-C bond in dyes **6-7**. A standard method for reduction of nitro group with tin chloride^{26, 28} was successful; however, we were unable to purify the products from tin. This was apparently due to a strong complexation between the tin chloride and *o*-diamino group. The presence of tin after several rounds of purifications including orthogonal column chromatography (reverse and normal phase), trituration, gel filtration and dialysis was confirmed by ICP-MS analysis and NMR.

To avoid potential complexation with metals on the reduction step, we utilized a catalytic hydrogen transfer approach. In this mild and selective reduction method, hydrogen is transferred from a hydrogen donor ammonium formate to the substrate in the presences of catalysts such as Pt or Pd.^{29, 30} The reduction of the nitro group in **6-7** with excess of ammonium formate in the presence of 10% Pd/C in air-free methanol at room temperature led to the desired product diamino-compounds DAP-LT1 **8** and DAP-LT2 **9**. The reaction was clean and complete within 20 min. To the best of our knowledge, this is the first time catalytic hydrogen transfer has been used in the chemistry of polymethine dyes.

Since the reaction between NO and diaminebenzene leads to the formation of a benzotriazole product, a representative dye **11** was synthesized (Scheme 2). The dye was prepared in a manner similar to DAP-LT1 via Suzuki coupling of IR783 and a corresponding commercially available benzotriazole boronic ester **10**. Optical properties of the dyes are tabulated in Table 1.

Fluorescence intensity of the probe in the presence of NO donor

Having the starting materials and the products in hand, we tested DAP-LT1 and DAP-LT2 in the presence of a known NO donor, DEA NONOate (Scheme 2) in 10% serum/PBS at room temperature. This compound is relatively stable under basic condition but decomposes to produce NO under neutral and acidic conditions with the half-life of 16 min at room temperature and pH 7.4.³⁴ The choice of serum was dictated by potential applications of the probe for clinically relevant measurements of NO in biological fluids.^{35, 36} The visible probe DAF-FM with almost complete quenching was utilized as a positive control.

Both DAP-LT1 and DAP-LT2 showed reasonably good sensitivity to NO as judged by the fluorescence intensity measurements but were not completely quenched with the QY in the initial state at ~2%. Within 15 min after addition of the NO donor, fluorescence increased ~5.3 times for DAP-LT1 without the change of the absorption spectra (Fig. 2). Such behaviour is typical for PeT-type compounds^{37, 38} and confirms the suggested mechanism of fluorescence quenching.

DAP-LT2, having a methylated amine like in DAF-FM, was even more sensitive with ~9 times change of fluorescence intensity also without the change of the absorption spectra (Fig. S2, ESI[†]).

For biological application it is important for a probe to react fast to monitor the NO burst in real time. The reaction rates of DAP-LTs with NO calculated from fluorescence intensity data were similar between the studied DAP-LT probes but lower than that of DAF-FM (Table 1, Fig. 3). Although the overall reaction kinetics between the probes and NO are complex because of the non-stationary conditions (change in NO concentration with time, Fig. 3), the difference in reaction rates can be, in part, explained by steric effects induced by serum proteins. The measured binding constant of DAP-LT1 to bovine serum albumin (BSA), the major serum protein, was found to be 92,400 M⁻¹ (Fig. S4, ESI[†]), more than three times higher than that of fluorescein (~28,100 M⁻¹).³¹ This comparison suggests that probes with lower affinity to plasma proteins, such as more hydrophilic dyes (PEGylated or conjugated to nanoparticles), will respond to NO faster.

Fluorescence lifetime of the probe in the presence of NO donor

Out of two synthesized probes, only DAP-LT1 showed appreciable fluorescence lifetime sensitivity to NO growing from 1.03 ns to 1.24 ns within 30 min (Table 1, Fig. 4A, the corresponding fluorescence decays are shown in Fig. S3, ESI). This increase in the lifetime was in the agreement with the lifetime of the dye **11** – the final product of the reaction between DAP-LT1 and NO. This product of the reaction (entry DAP-LT1+NO) and benzotriazole **11** with the lifetime of 1.25 ns, showed almost identical fluorescence decays of the reaction (Fig. 4B).

Similar sensitivity was observed under physiological temperature (37 °C). The compounds retain their fluorescence lifetime difference although the absolute values of the lifetimes were lower due to the higher rate of non-radiative deactivation at higher temperature⁹

[†]Electronic Supplementary Information (ESI) available: optical spectra, fluorescence decays and NMR spectra.

(decays at 37 °C are shown in Fig. S4, ^{ESI†}). Despite the larger fluorescence intensity sensitivity of DAP-LT2 to NO, the compound showed weak lifetime response with visually indistinguishable fluorescence decays before and after the reactions (Table 1, Fig. S5, ^{ESI†}). Similarly, no fluorescence lifetime sensitivity was observed for DAF-FM during the reaction (Fig. 5) despite of the very strong change in the fluorescence emission. The absence of fluorescence lifetime sensitivity for DAF-FM can be explained by almost complete quenching of the original fluorophore which as mentioned violates the second criterion for the lifetime sensitivity.

Activatable probes are a broad class of probes with a non- or minimally fluorescent initial state that only “turned on” under certain conditions.³⁹ These conditions might include enzymatic activity,⁴⁰ change in the pH^{41, 42} or the presence of metals⁴³ and metabolites such as reactive oxygen species^{44, 45} and nitric oxide (see ref above). Several photochemical mechanisms can be utilized in the activatable probe design, including homo- and hetero-fluorescence resonance energy transfer (FRET), aggregate formation, and PET. In these cases, the excited state energy of the fluorophore is deactivated resulting in an initially low fluorescence intensity of the probe. Upon removal the quenching effects, the significant rise of the fluorescence intensity and in certain cases, the fluorescence lifetime increase such as described throughout this work, can be observed.

The lack of direct correlation between strong enhancement in fluorescence intensity and the change in the fluorescence lifetime demonstrates the complexity of the photophysical processes in the excited state. Understanding such behaviour requires a larger set of examples and model compounds. However, the number of publications reporting fluorescence lifetime changes in activatable probes is still limited to a few reports such as describing calcium probes,⁴³ fluorescent proteins^{46, 47} and cyanine dyes.^{11, 40} We optimistic that more research will be conducted to better understand the underlying principles of fluorescence lifetime probe design.

Conclusions

We synthesized an activatable fluorescent probe for detecting nitric oxide that is based on the fluorescence lifetime change. The new probe DAP-LT1 is built on a heptamethine scaffold with incorporated diaminobenzene functionality in the meso-position. The probe is only partially quenched, but upon reaction with a nitric oxide donor shows the increase of the fluorescence lifetime from 1.08 ns to 1.24 ns. A control benzotriazole derivative that corresponds to the product of the reaction showed the lifetime of 1.25 ns confirming the suggested PeT mechanism of quenching. The probe could find applications in monitoring nitric oxide in a biological relevant media such as blood serum and other biological fluids. The observed fluorescence lifetime change is sufficient for NO monitoring in vitro, however such conclusion for in vivo yet has to be demonstrated. We envision that the probe performance could be further improved by modification the skeleton of the cyanine molecule and/or by placing the diamino functionality in a different location of the dye.

MATERIALS AND METHODS

Materials

Solvents, 10% Pd/C, tin(II) chloride, ammonium formate, methyl iodide, bovine serum albumin (BSA, grade agarose gel electrophoresis, 99% purity) 6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-benzo[d][1,2,3]triazole (Sigma-Aldrich), 1X PBS buffer (Mediatech Inc), Pd(PPh₃)₄ (Strem Chemicals, Inc.), dye IR783 (American Dye Source Inc.), sodium hydride (Alfa Aesar), bis-pinacolate diboron (Oakwood Products Inc), NMR solvents (Cambridge Isotope Lab). DEA NONoate (Cayman Chemical Company), fetal bovine serum

heat inactivated (Life Technologies) were used without purification, Millipore water was used throughout this work. All chemicals were used without further purification.

Chemical analysis methods

^1H and ^{13}C NMR data were recorded on a 400 MHz Varian spectrometer at ambient temperature in either chloroform-*d* or methanol-*d*₄ and referenced to tetramethylsilane (TMS) as an internal standard unless otherwise stated. Chromatographic purification was performed on C18 silica gel column. LC-MS spectra were recorded on a Shimadzu 2010A (ESI, positive mode) equipped with UV/Vis detector with up to 900 nm detection limit. Thin layer chromatography (TLC) was performed on either alumina-backed plates coated with 0.20 mm silica gel Xtra SIL G/UV₂₅₄ or 0.15 mm RP-18W/UV₂₅₄ silica gel. ICP-MS for measurements of Sn were conducted using Elan DRC II ICP-MS (PerkinElmer).

Optical measurements

General spectroscopy methods—UV/Vis spectra of samples were recorded on a Beckman Coulter DU640 UV-visible spectrophotometer. The molar absorptivities were obtained using Beer's law at 0.1-0.6 μM concentration of the dye. Steady state fluorescence spectra and fluorescence lifetime were recorded on a Nanolog spectrofluorometer (Horiba Jobin Yvon). Fluorescence quantum yield of the samples were measured using ICG as a standard.⁴⁸ The fluorescence lifetime of dyes was determined using time-correlated single photon counting (TCSPC) technique with excitation sources NanoLed-740 nm, emission 790 nm for NIR dyes or Nanoled-460 nm, emission 520 nm for fluorescein as described previously.¹⁰ Temperature was adjusted using a cuvette holder connected to a thermostat.

Measuring the binding constants to BSA—The details of the method were described previously.⁴⁹ Briefly, BSA was dissolved in 1× PBS buffer to obtain a concentration of 0.08 mg/mL and kept as a stock solution. DAP-LT1 concentration was varied from 2.5×10^{-7} to 4.0×10^{-6} M. For binding measurements, a solution of albumin (2 mL) was placed in a quartz cuvette and titrated with dye in microliter scale increments. After each addition, the solution was stirred and allowed to equilibrate for a few minutes, and its emission spectra of tryptophan residue were recorded (ex/em. 279/294-450 nm). The binding constant was obtained from Eq. 1.

$$(F_0 - F) / F = K [\text{dye}] \quad \text{Eq. 1}$$

Where F_0 is initial fluorescence, F is fluorescence after addition of the dye, $[\text{dye}]$ is dye concentration, K is a binding constant.

Sensitivity of the probes to NO—The probe was predissolved in water and added to a quartz cuvette with 10% serum in PBS buffer. Serum solution was chosen to maintain conditions close to in vivo. The NO donor DEA NONoate (10 μM) was added to the solution of the probe in 10% serum at room temperature or at 37 °C in order to mimic the NO level found in biological tissues.⁵⁰ Direct measurement of the detection limit was difficult due to a non-steady concentration of the NO donor in the solution. Based on the indirect estimate using the rate of DEA NONoate decomposition and kinetics of the lifetime changes shown in Fig. 4A the lowest measurable sensitivity was 4 μM of NO. DAF-FM was pre-incubated in 10% serum for 5 min prior the addition of the NO donor to ensure complete deacetylation of the probe by serum proteases. The reactions were monitored by measuring fluorescence intensity or fluorescence lifetime. Kinetics of DEA NONoate decomposition was modelled according to Eq. 2 assuming half-life time of DEA NONoate 16 min at 22-25 °C.⁵¹

$$N(t) = N_0(1/2)^{t/t_{1/2}} \quad \text{Eq. 2}$$

where N_0 - 100%, t - reaction time, $t_{1/2}$ =16 min. All in 10% serum/PBS buffer.

Synthesis

General procedure for synthesis of boronic esters (I)—To a solution of a corresponding aryl-bromide in oxygen-free THF, $\text{Pd(PPh}_3)_4$ (0.06 mol %) was added under argon atmosphere followed by bis-pinacolato diboron (2.0 eq.) and potassium acetate (2.5 eq.) The reaction mixture was left for overnight reflux, cooled down, and filtered through a small layer of silica gel using ethyl acetate/hexanes mixture as eluent. Solvents were evaporated under reduced pressure; the residue was subjected to the normal phase column chromatography on silica gel using gradient ethyl acetate/hexanes as eluent. Fractions containing the desired product were collected, combined and solvent evaporated to afford a pure product.

General procedure for Suzuki coupling of IR783 with boronic esters (II)—Dye IR783 was dissolved in oxygen-free water purged with argon. $\text{Pd(PPh}_3)_4$ (0.06 mol. %) was added to the stirred solution of IR783 under argon atmosphere followed by addition of solution of an appropriate boronic ester (2.0 eq.) in small amount of DMF (water:DMF ratio 5:1 by vol.). The reaction mixture was sealed and left for overnight reflux, analysed by TLC and/or LC-MS. The reaction mixture was cooled down and solvents were evaporated under reduced pressure. The residue was subjected to reverse phase column chromatography using water/methanol gradient as eluent. Fractions containing desired product (by LS-MS) were collected, combined, and methanol was evaporated under the reduced pressure. The remaining solution was lyophilized.

General procedure for reduction of nitro-group with SnCl_2 (III)—To a solution of the corresponding dye in methanol, SnCl_2 (6.0 eq.) was added, the reaction mixture was left with stirring at 50 °C. The reaction was monitored by LC-MS until the complete conversion of the starting material was confirmed. The reaction mixture was filtered through a layer of Celite and the solvent was evaporated. A cascade of purification techniques was used to remove SnCl_2 and Sn-products from the reaction mixture. Trituration of the dye with diethyl ether from methanol solution, dialysis, and column chromatography on reverse phase silica gel led to the single compound which contained SnCl_2 apparently complexed with the diamino group. Presence of tin complex was confirmed by broader ^1H NMR signals and by ICP-MS analysis.

General procedure for reduction of nitro-group via catalytic hydrogen transfer (IV)—To a solution of the corresponding dye in methanol thoroughly purged with argon, 10% Pd/C was added followed by the addition of ammonium formate (20 eq.) The reaction mixture was sealed and stirred at room temperature. After ca. 40 min, TLC indicated a complete conversion of the starting nitro-derivative into a desired product. The reaction mixture was filtered through a layer of Celite, the solvent was evaporated and the residue was subjected to the column chromatography on reverse phase silica gel using water/methanol gradient as eluent. Fractions containing desired product were collected, combined and lyophilized.

Synthesis of 4-bromo-N-methyl-2-nitroaniline (2)—To a solution of 4-bromo-2-nitroaniline (500 mg, 2.29 mmol) in dry THF (2.0 mL), sodium hydride (92 mg of 60% suspension in mineral oil, 2.29 mmol) was added at 0 °C under argon atmosphere and stirred for 10 min. Methyl iodide (325 mg, 2.29 mmol) was added to the reaction mixture, the

temperature of the reaction mixture was raised to ambient and the mixture was stirred for two hours. TLC showed complete conversion of the starting material. The reaction mixture was washed with 5% HCl(aq), extracted with ethyl acetate twice, washed with brine, and dried over sodium sulfate. Solvents were evaporated under reduced pressure; the residue was subjected to the column chromatography on silica gel using mixtures of hexanes/ethyl acetate as eluent affording 530 mg (99%) of pure 4-bromo-N-methyl-2-nitroaniline as reddish solid. ^1H NMR (400 MHz, CDCl_3) δ 8.32, 8.32, 8.02, 7.54, 7.53, 7.52, 7.51, 7.26, 6.77, 6.75, 3.03, 3.02. ^{13}C NMR (400 MHz, CDCl_3) δ 145.23, 138.98, 128.89, 115.11, 110.03, 106.37, 29.85.

Synthesis of 2-nitro-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline (4)—

The compound **4** was obtained according to the standard procedure (**I**) as colorless solid. ^1H NMR (400 MHz, CDCl_3) δ 8.59 (s, 1H), 7.73-7.71 (d, 1H), 6.78-6.76 (d, 1H), 6.20 (br. s, 2H), 1.33 (s, 12H). ^{13}C NMR (400 MHz, CDCl_3) δ 146.46, 141.06, 133.72, 132.14, 117.95, 83.98, 24.84.

Synthesis of N-methyl-2-nitro-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline (5)—

The compound **5** was obtained according to the standard procedure (**I**) as colourless solid. ^1H NMR (400 MHz, CDCl_3) δ 8.64 (s, 1H), 8.19 (br. s, 1H), 7.83-7.81 (d, 1H), 6.82-6.79 (d, 1H), 3.05-3.03 (d, 3H), 1.33 (s, 12H). ^{13}C NMR (400 MHz, CDCl_3) δ 147.87, 141.72, 134.29, 131.96, 112.54, 83.90, 29.74, 25.03, 24.85.

Synthesis of intermediate dye (6)—

The dye **6** was obtained according to the standard procedure (**II**) as green powder. Purity 95% area LC-MS. ^1H NMR (400 MHz, CD_3OD) δ 7.81 (s, 1H), 7.61-7.52 (m, 3H), 7.47-7.45 (m, 1H), 7.33-7.30 (m, 2H), 7.25-7.22 (m, 3H), 7.17-7.14 (m, 3H), 7.08-7.04 (t, 2H), 6.16-6.13 (d, 2H), 4.03-4.00 (t, 4H), 2.79-2.76 (t, 4H), 2.63 (m, 3H), 2.59 (s, 2H), 1.93 (br s, 2H), 1.83 (m, 8H), 1.20-1.16 (d, 12H). ^{13}C NMR (400 MHz, CD_3OD) δ 171.76, 147.43, 145.88, 142.23, 140.65, 136.98, 134.36, 134.22, 133.11, 132.32, 131.68, 131.58, 130.50, 129.88, 129.83, 129.45, 128.59, 128.47, 128.33, 126.49, 125.85, 125.53, 124.51, 121.89, 121.77, 119.14, 113.66, 110.47, 99.89, 50.40, 48.52, 43.33, 33.95, 26.70, 26.33, 25.78, 25.07, 24.36, 22.17, 21.08.

Synthesis of intermediate dye (7)—

The dye **7** was obtained according to the standard procedure (**II**) as green powder. Purity >95% area LC-MS. ^1H NMR (400 MHz, CD_3OD) δ 7.91 (d, 1H), 7.32-7.27 (m, 3H), 7.25-7.23 (d, 4H), 7.18-7.16 (m, 3H), 7.08-7.05 (t, 2H), 6.18-6.14 (d, 2H), 4.04-4.01 (t, 4H), 3.05 (s, 3H), 2.79-2.76 (t, 4H), 2.66-2.63 (t, 4H), 2.59 (s, 1H), 1.95 (m, 2H), 1.83 (m, 8H), 1.19-1.15 (d, 12H). ^{13}C NMR (400 MHz, CD_3OD) δ 171.79, 160.11, 147.41, 145.82, 142.23, 140.65, 137.82, 131.82, 131.05, 128.33, 127.24, 124.74, 124.49, 121.88, 114.05, 110.48, 99.92, 50.39, 48.51, 43.31, 28.76, 26.71, 26.32, 25.77, 24.43, 24.39, 22.17, 21.08.

Synthesis of DAP-LT1 (8)—

DAP-LT1 was obtained according to the standard procedure (**IV**) as pure green powder with 55% yield. Purity >95% area LC-MS. ^1H NMR (400 MHz, CD_3OD) δ 8.30 (s, 1H), 7.51-7.47 (m, 2H), 7.25-7.20 (m, 4H), 7.14-7.12 (d, 2H), 7.07-7.03 (t, 2H), 6.81-6.79 (d, 1H), 6.51-6.50 (d, 1H), 6.34-6.31 (dd, 1H), 6.07-6.04 (d, 1H), 4.00-3.96 (t, 4H), 2.79-2.76 (m, 5H), 2.58-2.56 (m, 5H), 1.91-1.88 (t, 2H), 1.81 (m, 8H), 1.21-1.18 (d, 12H). ^{13}C NMR (400 MHz, CD_3OD) δ 171.46, 164.98, 153.86, 148.83, 142.38, 140.69, 139.02, 135.08, 133.89, 131.46, 128.92, 128.12, 123.88, 123.15, 121.72, 120.28, 117.48, 115.24, 109.95, 107.12, 105.14, 99.01, 50.47, 48.46, 43.23, 33.85, 28.88, 26.70, 25.75, 24.29, 22.17, 21.24.

Synthesis of DAP-LT2 (9)—DAP-LT2 was obtained according to the standard procedure (**IV**) as green powder with 50% yield. Purity >95% area LC-MS. ¹H NMR (400 MHz, CD₃OD) δ 7.48–7.44 (d, 2H), 7.22–7.21 (d, 4H), 7.14–7.12 (d, 2H), 7.06–7.02 (t, 2H), 6.71–6.69 (d, 1H), 6.57 (d, 1H), 6.51–6.48 (dd, 1H), 6.08–6.05 (d, 2H), 3.97 (m, 4H), 2.87 (s, 3H), 2.78–2.75 (t, 4H), 2.59–2.56 (m, 4H), 1.91–1.88 (t, 2H), 1.81 (m, 9H), 1.20–1.17 (d, 12H). ¹³C NMR (400 MHz, CD₃OD) δ 171.46, 161.07, 148.82, 142.37, 140.64, 132.84, 128.21, 125.42, 124.15, 122.11, 121.81, 116.52, 110.17, 109.99, 99.01, 50.46, 48.46, 43.23, 30.01, 26.78, 26.72, 25.75, 24.35, 22.16, 21.29.

Synthesis of benzotriazole dye (11)—The dye 11 was obtained from commercially available 6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-benzo[d][1,2,3]triazole **10** and IR783 according to the standard procedure (**II**) as pure green powder with 57% (31 mg) yield.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig.1.
Design and principle of DAP-LT fluorescence lifetime NO probes.

Scheme 1.

Synthesis of diamino- polymethine lifetime probes DAP-LT1 and DAP-LT2

Scheme 2.

Synthesis of benzotriazole polymethine dye from IR783 via Suzuki coupling and via the reaction between DAP-LT1 and the NO

Fig. 2. DAP-LT1 reaction with DEA NONOate. A: Absorption change. B: Fluorescence intensity change, (ex. 720 nm)

Fig. 3.
Kinetics of DAP-LT1 fluorescence increase after treatment with DEA NONOate and DEA NONOate decomposition. Half-life time of DEA NONOate 16 min at 22-25 °C

Fig. 4.

A: Fluorescence lifetimes of DAP-LT1 increase after reaction with DEA NONOate at different time points. B: Fluorescence decays of DAP-LT1 after reaction with DEA NONOate and benzotriazole dye **11** (ex/em 740/790 nm, 10% serum/PBS buffer).

Fig. 5.
Fluorescence lifetime of DAF-FM in the presence of DEA-NONOate 10% serum/PBS buffer. Lifetime: ex/em: 460/520 nm

Table 1

Spectral properties of the probe before and after reaction with NO donor in 10% of serum in PBS

Probe	Rate constant, K (min^{-1})	Binding constant to BSA (M^{-1})	$\lambda_{\text{abs}}/\lambda_{\text{fl}}$ (nm)	τ (ns)	ε ($\text{M}^{-1}\text{cm}^{-1}$)	Φ_{fl}^c
DAP-LT1	-	92,400	760/780	1.08 ^a	247,000	0.037
DAP-LT1 +NO	0.24 $\pm$ 0.03	see 11	767/782	1.24 ^a	220,000 ^d	0.28 ^d
DAP-LT2	-	n/d	762/788	0.92 ^a	240,000	0.040
DAP-LT2+NO	0.33 $\pm$ 0.09	-	767/788	0.96 ^a	212,000	0.40 ^d
Entry 11	no reaction	142,000	778/795	1.25 ^a	214,000	0.367
DAF-FM	-	28,100 ³¹	558/515	3.75 ^b	n/d	0.005 ^e
DAF-FM+NO	1.82 $\pm$ 0.53	n/d	488/515	3.72 ^b	n/d	0.81 ^e

^a lifetime, ex/em 740/790 nm^b lifetime, ex/em 460/510 nm^c Quantum yield (QY) measured in DMSO, relative to ICG (QY=0.22 in DMSO^{32, 33})^d estimated based on the change in absorption and fluorescence spectra^e relative to QY of fluorescein