Tuning Optical Properties of BODIPY Dyes by Pyrrole Conjugation for Photoacoustic Imaging

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Photoacoustic imaging (PAI) is increasingly employed in (pre-) clinical research, thus, development of suitable contrast agents, in particular fluorescence-quenched chromophores, for PAI is of high importance. Small molecule dyes are appropriate due to favorable circulation, excretion properties, and ease of conjugation to targeting moieties. The BODIPY chromophores have been widely used in bioimaging, yet they are not ideal for PAI due to high fluorescence. Hence, here nonfluorescent BODIPY are designed by 1H-pyrrole conjugation (PyBODIPY) to apply as probes for PAI. The PyBODIPYs exhibit absorption maxima up to 800 nm, and PA signal could be detected in concentrations of 1 nmol mL\(^{-1}\) and 35 pmol mm\(^{-1}\), by tube and tissue phantom, respectively. In addition to nonfluorescent, PyBODIPYs are non-phototoxic, photostable, and show high molar extinction coefficients, as well as inertness toward nucleophilic addition. PyBODIPYs are modified with PEG-400, to improve aqueous solubility and to enable in vivo imaging. Thus, PyBODIPY is an attractive small molecule to use as PA contrast agent, which could be coupled to targeting ligands for in vivo use. In addition, 1H-pyrrole conjugation might be applied to the design of novel near-infrared ranged quenchers suitable for PAI, and promote the development of probes for clinical translation.

Photocoustic imaging (PAI) is currently in the progress of translation as a viable clinical deep tissue imaging modality for the detection of breast, prostate, melanoma, cervical and ovarian cancers, as well as sentinel lymph nodes. In addition, PA imaging has been evaluated for the characterization of inflammatory bowel disease, metabolism of brown fat, Duchenne muscular dystrophy disease, and the depth analysis of periodontal pockets. Further applications in diagnostic investigation are ongoing.

The PA signal is generated by thermoelastic expansion and contraction of tissue, caused by nonradiative relaxation processes of optical absorbers and thereby induced change in temperature (few mK), when irradiated with a pulsed laser. The produced ultrasound waves have low attenuation in tissue, and thus allow for detection at high depths (device dependent, up to 7 cm). Apart from endogenous contrast (e.g., hemoglobin, melanin), exogenous contrast agents based on noble metal nanoparticles (Au, Ag), semiconducting nanoparticles, synthetic melanin, and small molecule dyes have been explored. The latter class is particularly promising for clinical diagnostic PAI, as they can be renally cleared, pharmacokinetic properties can considerably be tuned, and can be used as imaging tags of larger biomolecules without substantially changing their properties. To date, FDA-approved indocyanine green (ICG) is widely studied as (off-label) PA probe, despite its low PA-signal and short blood half-life. Other small organic chromophores, e.g., azo-dyes, (nap)thalocyanines, IR-dye fluorophores (aza)BODIPYs, porphyrins, and other dyes were also explored for PAI applications. Several of the above named probes could also be used in photothermal therapy (PTT), as both applications share a similar underlying principle, optical absorption to thermal conversion via nonradiative process. However, there is still a strong need for novel PA contrast agents as many of the above probes are either hydrophobic (e.g., (nap)thalocyanines, “black” porphyrins, or prone to nucleophilic addition (e.g., cyanine dyes for thiols) or potentially carcinogenic (e.g., azo-dyes).

Here, we aim to optimize highly biocompatible and photo-stable BODIPY dyes for PAI applications. The BODIPY probes were already explored in PAI including PTT, and to generate photodynamic effect. The 3,5-bis-p-methoxystyryl BODIPY was the first example studied in PAI, but required laser energies to generate a PA signal outside of the clinically acceptable range. In addition, they produce only a weak PA signal due to high fluorescence emission. Thus, we
hypothesized that PA efficiency of BODIPYs can be increased by quenching fluorescence, similar to the reported fluorescence-quenched naphtalocyanines. In addition, we wanted to tune the absorption maximum ($\lambda_{\text{Max}}$) of BODIPYs, which is typically for aryl conjugated BODIPYs $< 700$ nm range, into the near-infrared (NIR). The electron-rich thiophene, or polyaromatics like anthracene and pyrene-conjugated BODIPY also exhibited $\lambda_{\text{Max}} < 700$ nm (see Figure 1A). The 8-aza substituted tetraaryl azaBODIPYs also exhibit $\lambda_{\text{Max}} < 700$ nm, which have been explored in PA imaging and for analyte-induced change by ratiometric detection. Hence, in the currently known conjugated BODIPY approaches, ideally required $\lambda_{\text{Max}} \geq 800$ nm is very challenging.

Therefore, in this work we explored hitherto unknown 1H-pyrrole conjugation to BODIPYs (PyBODIPY). The 1H-pyrrole is an electron-rich moiety that expected to induce redshift in absorption as well as fluorescence quenching via photoinduced electron transfer (PeT) process. PeT is a broadly studied concept, guided by the excited state properties of the donor and acceptor moieties. In PeT, in either intramolecular or intermolecular, an electron-rich donor (D) synthon pumps an electron into the acceptor (A) orbital in the excited state, leading to the formation of a charge separated state. Thereby formed radical ions decrease both the lifetime and emission quantum yield of molecule. Hence, an electron donor in close proximity to the fluorophore can be used to quench the emission by photoinduced electron transfer (Figure 1B). Here, the excited states of molecular systems may as well undergo, competing with the PeT, other radiative and nonradiative processes.

Here, by the conjugation of 3,4,5-trimethyl-1H-pyrrole to BODIPY, we obtained Me$_3$-pyrrole-conjugated BODIPY dye that shows excellent NIR absorption ($\lambda_{\text{Max}} = 799$ nm), with molar absorption coefficients $> 70 000$ m$^{-1}$ cm$^{-1}$, complete fluorescence quenching, and a strong PA signal. Furthermore, the

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**Scheme 1.** Synthesis of BODIPY dyes and their 1H-pyrrole conjugated BODIPYs (PyBODIPY) via Knoevenagel condensation.
meso-aryl group of the PyBODIPY was used to fine-tune the aqueous solubility by addition of three PEG-400 arms, without disturbing the optical properties, which improved blood circulation time, and suitability for in vivo imaging. Here we present synthesis, characterization of 1H-pyrrole-conjugated BODIPYs and their application in PA imaging.

The precursors for PyBODIPYs, 1,3,5,7-tetramethyl BODIPY (1–3), and 3,5-dimethyl BODIPY (4–6) were prepared by procedures described in the literature using catalytic TFA, oxidation with chloranil, and followed by BF₃·OEt₂ reaction (see the Supporting Information). Then, 1H-pyrrole tethering to BODIPYs 1–6 was achieved by Knoevenagel condensation, in the presence of piperidine and methanesulfonic acid in toluene at 100 °C, to obtain PyBODIPYs 7–15 in up to 53% yields (see Scheme 1 and Table 1). In order to evaluate the electronic effects, the meso-position was substituted with “electron-neutral” phenyl (7, 8), “electron-rich” 3,5-dimethoxyphenyl (9, 10), and “electron-poor” dimethyl-3,5-isophthalate (11, 14) groups. Usage of “methyl-free” 1H-pyrrole-2-carbaldehyde resulted in an unstable dye 12, which rapidly polymerized on air. Only after introducing a methyl group at position R₃ on 1H-pyrrole-2-carbaldehyde, stable products were isolated. On average, 3,5-dimethyl BODIPYs (4–6) yielded relatively higher yields (up to 53%) in comparison to the 1,3,5,7-tetramethyl BODIPYs (1-3, up to 27%). Apart from the lower isolated yields from tetramethyl BODIPY, additional size-exclusion chromatography (using BioBeads SX-1) was required in purification.

We also prepared a PEGylated PyBODIPY 16 (Scheme 2), as the PEGylation improves aqueous solubility and increase circulation time in vivo. Hence, we introduced three polyethylene glycol (PEG400) arms at the meso-aryl group of BODIPY 17 (by “click” chemistry, see the Supporting Information), without affecting the NIR absorption, and could be dissolved in 5% DMSO/H₂O in 100 × 10⁻⁶ m concentration. The resulting (PEGylated triazolyl) BODIPY was reacted with 1H-3,5-dimethylpyrrole-2-carbaldehyde by Knoevenagel condensation, as in synthesis of 14.

The obtained PyBODIPYs exhibited a strongly redshifted absorption (λ_max > 734 nm). Increasing the degree of methylation (12 to 15) showed a further redshift of the absorption maxima with simultaneous decrease in the molar extinction coefficients (e. Figure 2B). In contrast, decreasing the degree of methylation resulted in a blueshift and higher ε value. The PyBODIPY 16 exhibited a similar absorption maximum (λ_max: 750 nm, Figure 3A) as in 14. The highest redshift was thereby obtained for PyBODIPY 15 (λ_max = 799 nm) (Figure 2B) and to the best of our knowledge, such a large redshift was not observed, for any, by Knoevenagel condensation prepared, (hetero)aryl conjugated BODIPY dyes before.

We subsequently evaluated the dyes photostability using red LED light irradiation (100 mJ cm⁻² s⁻¹) for 15–30 min (total radiant flux: 90–180 W), finding that 95% of the dyes remained intact (Figure 2C), except the 15 (Figure 2E). Since a typical PA imaging device works at an energy range of 20–100 mJ cm⁻² s⁻¹, and imaging is performed within 30 s, no decomposition of PyBODIPYs should occur during an imaging sequence. In addition, for the red light irradiation, no formation of singlet oxygen (¹O₂) was observed, as quantified by no loss of ¹O₂ indicator, 1,3-diphenylisobenzofuran (DPBF) (Figures 2D,F and 3B).

Table 1. Isolated yields of PyBODIPYs and their optical characterization.

| Compd. no.a) | R¹ | R² | R³ | R⁴ | Yield [%] | λ_maxb) [nm] | ε⁰ [× 10⁴ M⁻¹ cm⁻¹] | λ_embc) [nm] |
|--------------|----|----|----|----|-----------|---------------|-----------------|-------------|
| 15           | H  | CO₂Me | Me | Me | 23         | 778           | 6.93            | b.d.l.       |
| 14           | H  | CO₂Me | Me | H  | 56         | 740           | 6.32            | b.d.l.       |
| 13           | H  | CO₂Me | Me | H  | 10         | 748           | 5.49            | b.d.l.       |
| 12           | Me | CO₂Me | H  | H  | n.d.       | 700           | n.d.            | b.d.l.       |
| 11           | Me | OMe  | Me | H  | 52         | 762           | 7.14            | b.d.l.       |
| 10           | H  | OMe  | Me | H  | 46         | 764           | 6.67            | b.d.l.       |
| 9            | Me | OMe  | Me | H  | 27         | 734           | 6.31            | b.d.l.       |
| 8            | H  | H    | Me | H  | 53         | 762           | 7.51            | b.d.l.       |
| 7            | Me | H    | Me | H  | 17         | 734           | 5.58            | b.d.l.       |

[a]Prepared by Knoevenagel condensation as in Scheme 1; [b] All the measurements were in DMSO unless specified; [c] UV-vis maximum of reaction mixture in DCM; n.d.: not determined, b.d.l.: below the device detection limit, hence it was not possible to determine the fluorescence emission and quantum yields.

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Scheme 2. Synthesis of water-soluble PyBODIPY: a) 4.1 eq. α-azido-PEG-400, 0.1 eq. sodium ascorbic acid, 0.1 eq. CuSO₄, THF, water, 40 °C, 94%; b) 6.3 eq. 1H-3,5-dimethylpyrrole-carbaldehyde, piperidine, cat. MeSO₃H, toluene, 100 °C, 23%.
Figure 2. Optical properties of PyBODIPY dyes: A) Absorption and emission spectra of BODIPY 6 (in DMSO); B) quantitative absorption spectra of PyBODIPY 13, 14, and 15, showing redshift in absorption maxima, with increasing number of methyl groups; C,E) photostability of PyBODIPY (in DMSO) under red light irradiation; D,F) change in DPBF concentration during the irradiation.
This concludes that the possibility for phototoxic side effects is low and also confirms that the absence of fluorescence is not due to intersystem crossing. In addition, reactions of 14 with variety of reactive oxygen species (ROS; H$_2$O$_2$, HO$^\bullet$, O$_2$$^{\cdot-}$, and tBuOOH) were monitored by UV–vis, which showed no change in absorption spectra; however, with tBuO$^\bullet$, ONOO$^-$ species a blueshifted absorption peak was observed, indicating an oxidative reaction (Figure S11 and Table S2, Supporting Information).

As initially assumed, all PyBODIPYs show no fluorescence emission. We postulate that this quenching is due to PeT effect. To investigate the dynamics of PeT, we used transient absorption spectroscopy (TAS). Experimental details can be found elsewhere. Figure 4A,B shows the picosecond-nanosecond (ps-ns) TAS of 14 in DMSO upon excitation with 404 nm. The TA spectrum exhibits a blueshift of the photobleach band (477–468 nm) with over 1000 ps time. This is a clear indication of the generation of another excited state species, which we tentatively assign to photoelectron transferred state. We also measured the ps-ns TA spectra of 14 in different solvents (DCM and THF) to check the solvent polarity dependence of the PeT (Figure S12, Supporting Information). The blueshift of the photobleach in DMSO occurred faster than that of DCM and THF, indicating the PeT is significantly impacted by polarity of the solvent.

To evaluate photoacoustic characteristics, in vitro tube-phantom spectroscopic PA experiments were performed with PyBODIPY (in DMSO) using ICG as a reference standard. Surprisingly, the main absorption band (highly populated S1 excited state) did not give rise to a corresponding PA signal. Instead, the PA maximum overlapped with the left shoulder peak of the absorption band (i.e., S2 excited state, see Figure 5A). Nevertheless, the PA signal intensity of PyBODIPYs at $10 \times 10^{-6}$ M concentration (in DMSO) was up to two times higher than that of ICG at their respective maxima (Figure 5A and Figure S13, Supporting Information). Furthermore, a dilution series indicated that the PyBODIPYs could be detected at $< 2.5 \times 10^{-6}$ M concentration which is only 30% of the concentration of ICG detection limit in our device settings. In addition, PA experiments of PyBODIPYs were performed in aqueous media, by formulating them with cremophore EL (CrEL), a clinically used excipient for the formulation of hydrophobic drugs. The phosphate buffered saline (PBS, pH = 7.2) dissolved PyBODIPY:CrEL exhibited a broad, redshifted absorption band compared to the solutions in DMSO (Figure 5B). The PA spectra in PBS gave a plateau between 710 and 790 nm, and overlapped well with the absorption spectra in PBS, showing a bathochromic shift in PA maxima. The detection sensitivity of dyes was also improved in PBS, at concentrations below 1 nmol mL$^{-1}$ (Figure 5C).
Subsequently, different concentrations (250, 125, and 63 pmol in 50 µL) of dye 14:CrEL solution were injected subcutaneously at the hip of a dead albino mouse (in 2–3 mm³ volume). Hereby, 63 pmol (s.c.) still provided a detectable PA signal indicating that in vivo settings could be possible in the 100 pmol range using our PA instrumentation (Figure S5, Supporting Information).

Encouraged by these results, the newly synthesized dyes were explored in vivo applications. Before we applied 16 in vivo, chemical stability toward the thiol group was explored. The reaction of 16 with 10 equivalents of N-acetyl cysteine in MeOH at room temperature (r.t.) was monitored by reverse phase high-performance liquid chromatography (HPLC) (Figure S9, Supporting Information). To our satisfaction, no reaction of 16 with cysteine could thereby be observed over 6 h (Figure 6A), in contrast to ICG, where new reaction products were observed within less than 1 h (Figure S10, Supporting Information). As PyBODIPYs in cremophore EL gave redshifted, strong PA signal in water, 16 was also formulated in CrEL (2 mg µmol⁻¹), using the same previous procedure, and dissolved in PBS (5 × 10⁻³ m). This formulation was subsequently applied in a 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) cell viability assay (A549 cell line). We found that more than 60% metabolically active cells after incubation for 24 h, with up to 50 × 10⁻⁶ m concentrations of 16:CrEL, and 100% viable cells for 10 × 10⁻⁶ m dye (Figure 6B).

Being satisfied with the observed characteristics, we applied CrEL formulated PyBODIPY 16 in vivo, to determine the blood circulation properties. All experimental procedures were approved by the Animal Welfare Board (LANUV). The blood circulation was determined by measuring the change in PA signal in the carotid artery, over the baseline, for up to 10 min, in 22–25 individual measurements. Each mouse (n = 3) was therefore injected in the tail vein with 500 nmol (in 100 µL, 4 mg kg⁻¹) of 16:CrEL. As reference, ICG (500 nmol) was used in the same setup. The gain ratio was determined at their respective absorption maxima (16: 760 nm, ICG: 800 nm). We observed a gain of 35% for 16, and 60% for ICG. But the 16 showed improved circulation in blood than the ICG (Figure 7).

Figure 5. Photoacoustic signal generation by PyBODIPY: A) in DMSO solution (PA, dotted lines, 10 × 10⁻⁶ m) with normalized absorption spectra (solid lines) of 14, 10, and 8; B) in CrEL formulation (in PBS buffer, dotted lines, 10 × 10⁻⁶ m) with normalized absorption spectra (solid lines); C) PA image snapshot at 790 nm of 14 (in CrEL/PBS) in dilution of 10 × 10⁻⁶–1 × 10⁻⁶ m; D) PA image snapshot at 790 nm of 14:CrEL, 10:CrEL, 8:CrEL at 5 and 2.5 × 10⁻⁶ m in PBS.
In summary, we describe for the first time the design, synthesis, characterization, and application of a 1H-pyrrole-conjugated BODIPY for photoacoustic imaging. The small molecule PyBODIPYs are fully fluorescence-quenched and exhibit absorption maxima in the NIR (730–800 nm). In addition, the 1H-pyrrole conjugation allows for the PAI signal detection of PyBODIPY up to 1 nmol mL⁻¹ concentration in a tube phantom using clinically relevant laser fluence. Furthermore, the PEGylation of the compound leads to long blood circulation and thus is suitable for passive targeting. In addition, attaching a targeting ligand to PyBODIPY will achieve active targeted molecular PA imaging, which is currently focused. The results encourage us to investigate the here shown PeT mode of quenching and bathochromic shift and further uses in photothermal therapy. For future experiments, it will be interesting to explore the 1H-pyrole conjugation in combination with other fluorophores, especially those with high extinction coefficients (ε > 150 000 m⁻¹ cm⁻¹). By this, one may prepare novel NIR quencher dyes, which generate a strong photoacoustic signal. Hence, intramolecular conjugation of pyrrole to create and tune the photophysical properties of cyanine, xanthene, or phthalocyanine-based dyes is an ideal approach to develop excellent probes suitable for PAI.

**Experimental Section**

**General Procedure for BODIPYS (1–6) Synthesis:**

**Step 1:** 2.1 eq. of pyrrole and 1 eq. dimethyl-5-formylisophthalate were dissolved in dry DCM (conc.: 25–30 mL mmol⁻¹) under argon atmosphere. The reaction was initiated by addition of one drop (≈10 µL) of TFA, and the mixture was stirred for 90 min at r.t. After this, 1.0 eq of p-chloranil was added, stirred for another 90 min at r.t., and quenched by addition of saturated NaHCO₃. The mixture was extracted with DCM and dried over Na₂SO₄. The crude was concentrated in vacuum and purified by column filtration, alumina (neutral), eluting the product with hexane:ethyl acetate (4:1).

**Step 2:** The above product was dissolved in dry DCM (80 mL mmol⁻¹), followed by adding 35.0 eq. of NEt₃. This mixture was stirred for 15 min at r.t., and 48.0 eq. of BF₃·OEt₂ was added. The reaction was stirred for further 60 min at r.t., and the reaction was quenched by addition of saturated NaHCO₃. The reaction mixture was extracted with DCM (3×), the combined organic phases were dried over Na₂SO₄, and the solvent was removed under reduced pressure and the obtained product purified by column chromatography (silica gel 60, hexane:ethyl acetate).

**General Procedure for PyBODIPYS (7–15) Synthesis:** The Knoevenagel condensations were carried out in 0.1 mmol scale as follows: 1.0 eq of BODIPY, 2.0 eq of pyrrole-2-carbaldehyde, one drop of methylsulfonic acid, and 0.25 mL piperidine were dissolved in 3 mL dry toluene under argon atmosphere, and the reaction mixture was placed in a preheated oil bath at 100 °C for 1 h. The reaction was removed from oil bath, cooled, and to the cold mixture additional 2.0 eq of pyrrole-2-aldehyde and 0.25 mL piperidine were added. The reaction was placed in preheated oil bath again at 100 °C, until full conversion (up to 3–4 h). The crude mixture was diluted with DCM, washed with saturated NaHCO₃, and extracted 3–4 times with DCM. Combined organic phases were dried over Na₂SO₄ and the solvent was removed under reduced pressure and the obtained product purified by column chromatography (silica gel 60, hexane:ethyl acetate).

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

**Acknowledgements**

Financial support from Excellence Initiative of the German federal and state governments through the I3TM Seed Fund Program and I3TM Step2Projects are acknowledged. The authors acknowledge Dr. Partha Maity (Catalysis Centre, KAUST) for his help in acquiring the transient absorption spectra.
Conflict of Interest

The authors declare no conflict of interest.

Keywords
dyes/pigments, PeT effect, nitrogen heterocycles, photoacoustic imaging, NIR shifted BODIPY

Received: December 19, 2019
Revised: March 1, 2020
Published online: April 7, 2020

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