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ORIGINAL ARTICLE

Fluorescent Labeling of Protein Using Blue-Emitting 8-Amino-BODIPY Derivatives

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Abstract 8-Amino-BODIPY (boron-dipyrromethane) dyes show bright blue fluorescence. Disclosed here are synthesis and characterization of the photophysical properties of a series of functionalized 8-Amino-BODIPY (**BP1–4**) for protein labeling. The compact structure and solvent-insensitive absorption property of the dye are desirable features for protein labeling. For the model protein, bovine serum albumin (BSA), the labeling proceeds under mild condition via amide bond formation or thiol-ene conjugation with maintaining the bright blue fluorescence. The chromatography and mass spectroscopy analysis clearly support the labeling of the BODIPY dye on the BSA. The protein labeling with blue-emitting BODIPY would be applicable for studying protein dynamics and fluorescence resonance energy transfer (FRET) with intrinsic biomolecules.

Keywords Protein labeling · Blue-emitting BODIPY · Fluorescent labeling · Bioconjugation

Introduction

Proteins play crucial roles in a variety of cellular events, and the study of protein dynamics is critical to understanding of sophisticated living systems [1]. Particularly, visualization of protein movements has a great potential to reveal protein functions and dynamics. So far, a number of visualization methods have been developed to monitor protein behaviors [2, 3]. Among them, a protein labeling method based on fluorescence has come into spotlight due to its simplicity, high sensitivity and reliability [4, 5].

The conjugation of protein with fluorescent signaling unit have been carried out using labeling chemistries such as click-chemistry, condensation reaction, ester/amide bond formation, and thiol-ene reaction [6]. Also, various fluorescent signaling units (called dye) have been reported that in a wide range of spectrum from visible (blue, green, red) to near-infrared (NIR) [1, 7]. Fluorescein and rhodamine derivatives are representative dyes in a green and red fluorescence range, respectively. However, the development of blue-emitting dye for protein labeling have been rarely explored while it is important for studying the fluorescence resonance energy transfer (FRET) from or to intrinsic biological fluorophores such as tryptophan (Trp, $\lambda_{exi/emi} = 295/353$ nm) and flavin adenine dinucleotide (FAD, $\lambda_{exi/emi} = 450/540$ nm) [8, 9]. A few blue-emitting dyes have been reported based on coumarin backbone; Alexa Fluoro 350, AMCA, and Pacific Blue (Fig. 1) [10–13]. Coumarin is a naturally occurring benzopyran compound extracted from plants, and it have shown interesting bio-activity and photophysical properties [14]. These blue-emitting coumarin dyes are conjugated

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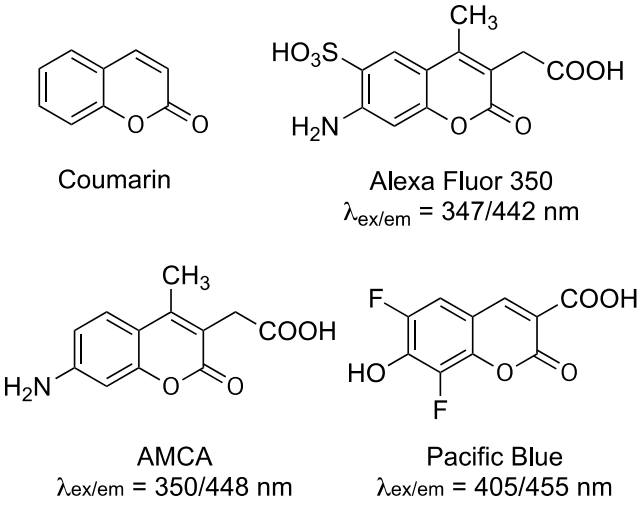


Fig. 1 Known blue-emitting dyes based on coumarin backbone

with protein via amide-bond formation, and resulting product shows strong fluorescence in biological media. However, coumarin has a drawback that comes from structure; typically electron donor–acceptor type dyes are sensitive to environment changes such as viscosity, hydrophobicity, ion concentration, pH, and polarity [15]. The changes can cause unexpected fluorescence shift/quenching or affect the protein function.

To address this issue, a new series of blue-emitting dye which shows environment-insensitive property making it a promising protein labeling is desirable. Herein, we wish to report the synthesis and characterization of novel derivatives of blue-emitting 8-Amino-BODIPY dyes for protein labeling (Fig. 2). BODIPY (boron-dipyrromethene) dyes are a class of fluorescent dyes, which have valuable properties such as environment-insensitive photophysical properties, intense absorption and emission peaks [16]. Generally, the photophysical properties of BODIPY dyes are dramatically changed depend on the electron density at 2, 3, 8-positions [16]. Among them, the substitution products with amino group at 8-position show strong blue fluorescence at 409–467 nm wavelength (absorption at 370–400 nm). In this study, we synthesized and analyzed the properties of 8-Amino-BODIPY derivative, and demonstrated the protein labeling.

Experimental

Materials

All reagents and solvents were purchased from commercial source (Sigma Aldrich; USA, TCI; Japan, Merck; USA), and used without further purification. Anhydrous solvents for organic synthesis were prepared by passing through a

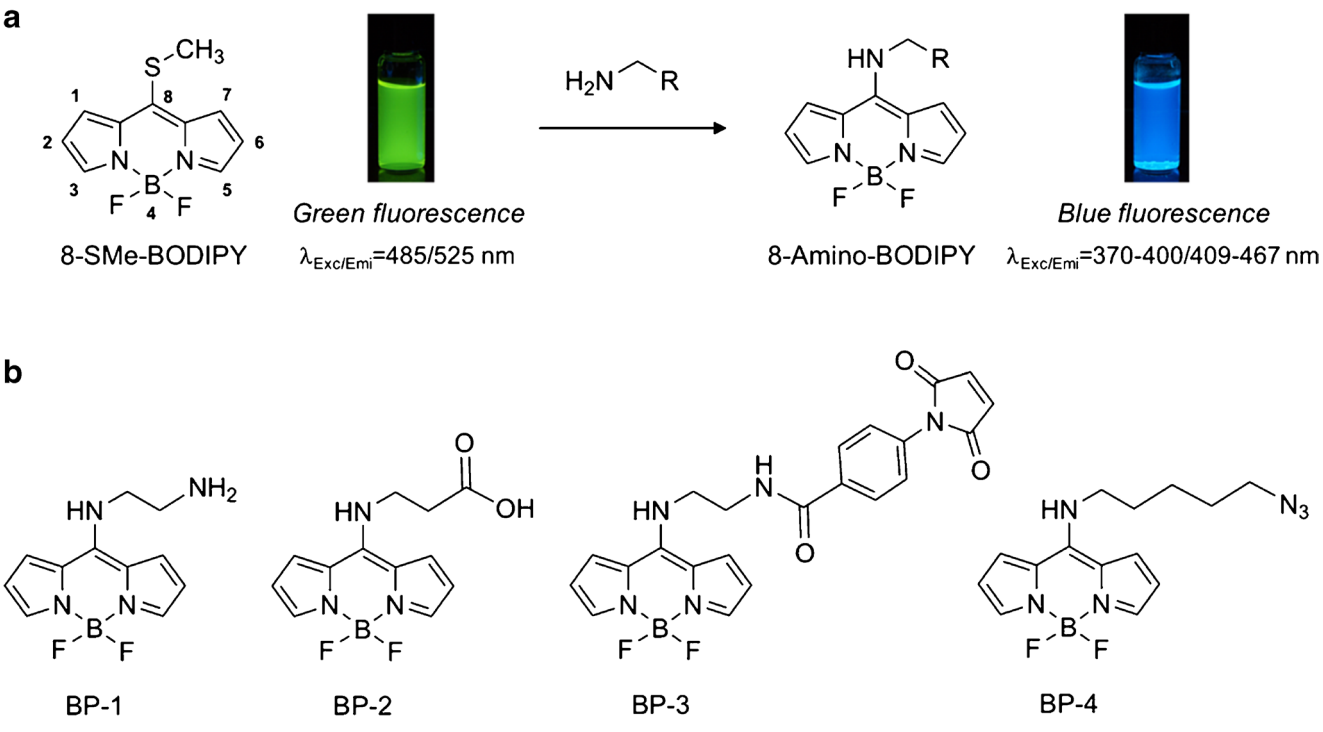


Fig. 2 **a** Structures of 8-SMe-BODIPY (inset number: numbering of BODIPY dye), and 8-Amino-BODIPY. Inset photo was taken under UV light (365 nm). **b** Structures of 8-Amino-BODIPY derivatives in this work

solvent purification tower. Silica was used silica gel 60, 230–400 mesh, Merck (USA). Thin-layer chromatography (TLC) was performed on pre-coated silica gel 60F-254 glass plates, Merck (USA).

Methods and Instruments

^{1}H and ^{13}C NMR spectra were measured with a Bruker DPX-300 and DPX-500 (Germany). Coupling constants (*J* value) are reported in Hertz. Mass spectral analysis was recorded with Jeol JMS 700 (Japan) and reported in units of mass to charge (*m*/*z*). Chemical shifts are reported as δ in parts per million (ppm). UV/Vis absorption spectra were obtained using a HP 8453 UV/Vis spectrophotometer (USA). Fluorescence spectra were recorded on a Photon Technology International Fluorescence System (USA) with a 1-cm standard quartz cell. High-resolution mass spectra of small molecules were recorded on a JEOL JMS-700 spectrometer (Japan) at the Korea Basic Science Center, Kyungpook National University and the values are reported in units of mass to charge (*m*/*z*). Mass data of protein and dye-labeled protein were measured using MALDI-TOF mass spectrometry, 4700 model proteomics analyzer with TOF/TOFTM ion Optics, ABSCIEX corp (Canada). The data were acquired with a Nd:AG laser with 200 Hz repetition rate, and up to 2000 shots were accumulated for each spectrum. LC analysis was performed using a DIONEX P580A connected to DIONEX UVD170S Detector (USA). Column: Phenomenex (USA) C18 250×4.60 mm. Wavelength: 394 nm. Eluent: DI H_2O:acetonitrile = 1:1 (*v*/*v*, 0.1% trifluoroacetic acid). Flow Rate: 1.0 mL min^{-1}.

Results and Discussion

Design

In 2010, Peña-Cabrera and co-workers reported conventional synthetic routes and unique photophysical properties of 8-Amino-BODIPY derivatives [17, 18]. Originally, 8-thiomethyl-BODIPY (8-SMe-BODIPY) has green fluorescence centered at 525 nm (the absorption maximum at 485 nm), and the amine-thiomethyl exchange reaction generate 8-Amino-BODIPY, which show bright blue fluorescence at 409–467 nm wavelength (absorption at 370–400 nm) with high quantum yield (Fig. 2a). We designed a series of 8-Amino-BODIPY derivatives that have protein conjugatable moiety; amine (**BP-1**, via amide conjugation), carboxylic acid (**BP-2**, via amide/ester conjugation), maleimide (**BP-3**, via thiol-ene conjugation), and azide (**BP-4**, via copper-catalyzed click conjugation) (Fig. 2b).

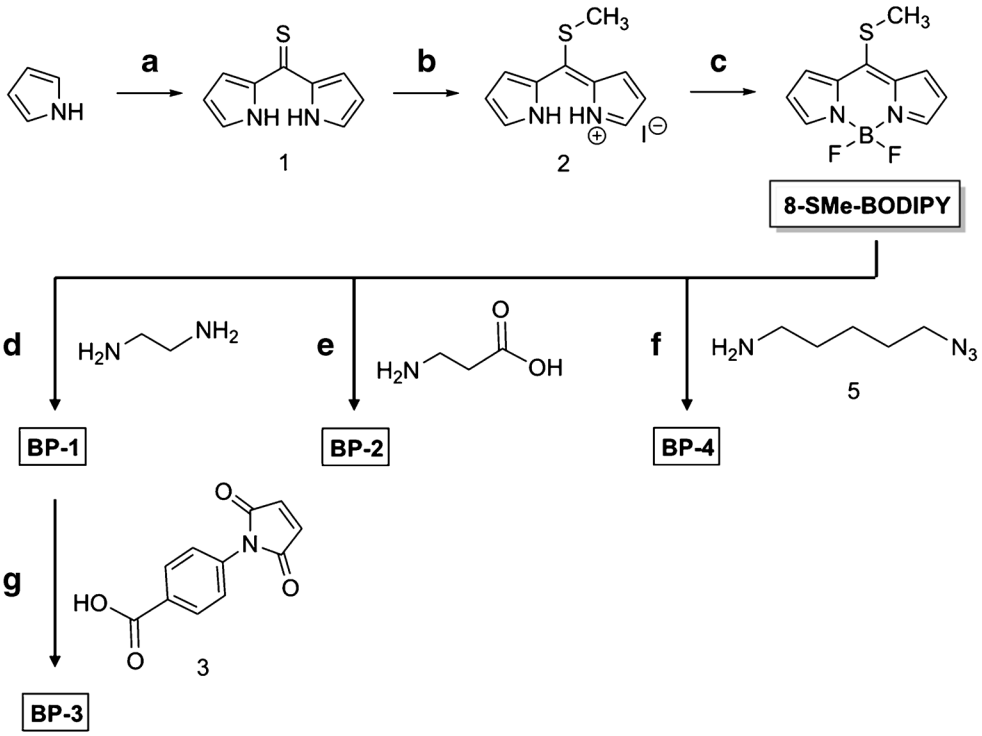
Synthesis/Method

8-thiomethyl-BODIPY (8-SMe-BODIPY) was prepared by following the literature procedure [19], and the synthetic routes to 8-Amino-BODIPY derivatives are outlined in Scheme 1 (see Scheme S1–S4 in Supplementary Material for the synthesis of linkers and protein conjugation procedure).

N^1-(4,4-Difluoro-4-bora-3a, 4a-diaza-sindacene)-ethane-1,2-diamine (BP-1) To a solution of 8-SMe-BODIPY (150 mg, 0.63 mmol) in anhydrous dichloromethane (12 mL) under argon atmosphere was added ethylenediamine (0.42 mL, 6.3 mmol). The reaction mixture was stirred for 30 min at room temperature (25 °C), and then treated with deionized water (20 mL). The two layers were separated, and the aqueous layer was extracted with dichloromethane (3×20 mL). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography (eluent: 10% methanol in dichloromethane) to afford **BP-1** as a bright yellow solid (150 mg, 95%). ^{1}H NMR ($CDCl_3$, 300 MHz, 293 K): δ 6.40 (2H, m), 7.05 (2H, m), 7.20 (2H, m), 9.77 (2H, s). ^{13}C NMR ($CDCl_3$, 75 MHz, 293 K): δ 150.7, 135.4, 132.4, 126.8, 124.8, 123.8, 117.6, 115.5, 114.3, 50.1, 40.4. HRMS (FAB) *m*/*z*: $C_{14}H_{17}BF_2N_6$ found 250.12 (M+).

3-[(4,4-Difluoro-4-bora-3a, 4a-diaza-sindacene)-amino]-propionic Acid (BP-2) To a solution of 8-SMe-BODIPY (183 mg, 0.769 mmol) in anhydrous tetrahydrofuran (13.5 mL) under argon atmosphere was added β-alanine (171 mg, 1.92 mmol) and triethylamine (0.27 mL, 1.92 mmol). The reaction mixture was stirred for 5 min at room temperature (25 °C). After 5 min, deionized water (1.5 mL) was added and the mixture was stirred for 1 h at room temperature. The solvents were removed in vacuo and the residue was extracted with ethyl acetate (3×10 mL), 1 N HCl, and deionized water. The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography (eluent: 10% methanol in dichloromethane) to afford **BP-2** as a bright yellow solid (182 mg, 85%). ^{1}H NMR (CDCl3, 300 MHz, 293 K): δ 6.40 (2H, m), 7.05 (2H, m), 7.20 (2H, m), 9.77 (2H, s). ^{13}C NMR (CDCl3, 75 MHz, 293 K): δ 177.0, 141, 140.8, 140.5, 118.3, 118.0,122.0, 121.8, 112.0, 111.0, 41.8, 38.1. HRMS (FAB) *m*/*z*: $C_{12}H_{12}BF_2N_3O_2$ found 279.16 (M+).

4-(2,5-Dioxo-2,5-dihydro-pyrrol-1-yl)-benzoic Acid (BP-3) To a solution of **BP-1** (47 mg, 0.218 mmol) in tetrahydrofuran (8 mL) were added *N*´-ethylcarbodiimide hydrochloride (50 mg, 0.262 mmol), hydroxybenzotriazole (35 mg, 0.262 mmol), *N*-methylmorpholine (72 µL, 0.654 mmol) and compound **3** (60 mg, 0.24 mmol, see the



Scheme 1 Reagent and conditions. **a** thiophosgene, diethyl ether, 0 °C, 10 min, 76%. **b** methyl iodide, dichloromethane, 25 °C, 15 h. **c** $BF_3{\cdot}Et_2O$, triethylamine, dichloromethane, 25 C, 12 h, 71% (from two steps). **d** dichloromethane, 25 °C, 30 min, 95%. **e** triethylamine, tetrahydrofuran, 25 °C, 1 h, 85%. **f** dichloromethane, 25 °C, 1 h, 91%. **g** *N*′-ethylcarbodiimide hydrochloride, hydroxybenzotriazole, N-methylmorpholine, tetrahydrofuran, 25 °C, 5 h, 61%

details in Supplementary Material). The mixture solution was stirred for 5 h under argon at room temperature (25 °C). Solvent was evaporated under vacuum, and the residue was extracted with dichloromethane (3 × 10 mL) and deionized water. The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography (eluent: 25% ethyl acetate in *n*-hexane) to afford **BP-3** as a bright yellow solid (98 mg, 61%). ^{1}H NMR ($CDCl_3$, 300 MHz, 293 K): δ 6.40 (2H, m), 7.05 (2H, m), 7.20 (2H, m), 9.77 (2H, s). ^{13}C NMR (CD_3CN, 75 MHz, 293 K): 169.70, 169.59, 148.73, 135.12, 134.60, 134.02, 132.49, 130.74, 128.05, 126.20, 123.60, 117.29, 115.82, 114.41, 113.35, 50.16, 38.72. HRMS (FAB) *m/z*: $C_{22}H_{18}BF_2N_5O_3$ found 449.15 (M+).

(5-Azido-pentyl)-(4,4-difluoro-4-bora-3a,4a-diaza-sindacene)-amine (BP-4) To a solution of 8-SMe-BODIPY (50 mg, 0.21 mmol) in anhydrous dichloromethane (4 mL) under argon atmosphere was added compound **5** (41 mg, 0.32 mmol, see the details in Supplementary Material). The reaction mixture was stirred for 1 h at room temperature (25 °C), and then treated with deionized water (10 mL). The two layers were separated, and the aqueous layer was extracted with dichloromethane (3 × 10 mL). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography (eluent: 30% ethyl acetate in *n*-hexane) to **BP-4** as a yellow solid (61 mg, 91%). ^{1}H NMR ($CDCl_3$, 300 MHz, 293 K):δ 1.47–1.79 (6H, m), 3.31–3.35 (2H, m), 3.43–3.50 (2H, m), 6.27 (1H, S). ^{13}C NMR ($CDCl_3$, 75 MHz, 293 K): 148.16, 135.78, 132.52, 124.84, 123.78, 122.27, 114.65, 113.82, 51.06, 47.07, 28.34, 24.16. HRMS (FAB) *m/z*: $C_{14}H_{17}BF_2N_6$ found 318.16 (M+).

BSA Labeling with BP3 **BP3** (10 μM) was incubated with BSA (100 μM) in PBS buffer (pH 7.4, 1% CH_3CN) for 1 h incubation at 37 °C. The labeling of **BP3** to BSA was confirmed by the fluorescence measurement and HPLC analysis.

Cellular Fluorescence Imaging of BP2-lysozyme Product 5×10^5 of TM3 mouse Leydig cell line was seeded on 6-cm cell culture dish (SPL, Pocheon, Rep. of Korea) and cultured in complete Dulbecco's modified Eagle's medium (DMEM, Hyclone, Waltham, Massachusetts, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Waltham, Massachusetts, USA) and 1 × Penicillin/Streptomycin solution (Corning, Corning, New York, USA) at 37 °C in a humidified atmosphere containing 5% CO_2 for 10 h. The cell was washed twice 1 × Phosphate buffered saline (PBS, Welgene, Gyeongsan, Rep. of Korea), followed by being applied with DMEM containing **BP2**-lysozyme product (see the preparation method in Supplementary Material, Scheme S4) for 2 h (37 °C, 5% CO_2 and Humidified condition). The dish was washed with 1 × PBS twice, and then filled with DMEM prior to the imaging. The fluorescence signal was monitored with Axioimager upright

microscope equipped at 37 °C in 5% CO_2 controlled system. (Zeiss, Jena, Germany).

Photophysical Properties

The experimental UV/Vis absorption and fluorescence emission spectra of **BP1–4** (10 μM) are measured in various solvents; protic (deionized water, methanol) and aprotic (dimethyl sulfoxide, acetonitrile, ethyl acetate, dichloromethane) (Fig. 3, Supplementary Figs. S1–S10, Table 1).

BP1–4 showed strong broad absorption bands in the range of 370–400 nm with no significant solvent dependent hypso-/bathochromic shift. Moreover, the results represent no decrement of absorbance in protic solvents while coumarin-based dyes are not. The environment non-sensitive absorption properties of BP dyes can be considered as an ideal FRET acceptor from the intrinsic biomolecules.

The strong blue fluorescence emission bands of **BP1–4** were observed in the range of 409–467 nm under excitation at the maximum absorption wavelength in the various solvents. The center of emission spectra shows a little hypsochromic shift by increasing solvent polarity (Supplementary Figs. S1–S10, Table 1). Generally, primary or secondary amine moiety reduce the fluorescence intensity of fluorophore as a quencher via photo-induced electron transfer (PeT) [21], but **BP1** has relatively strong fluorescence with high quantum yield even in the deionized water ($\Phi = 0.29$). Slightly lower fluorescent quantum yields were observed for **BP2–4** in the high polarity or protic solvents (Table 1), but the 8-Amino-BODIPY have shown sufficient fluorescence bio-imaging capability in the reported works [22].

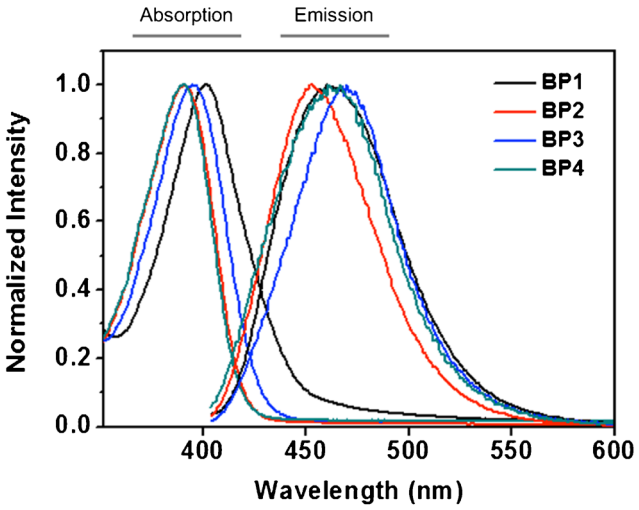


Fig. 3 Normalized absorption and fluorescence emission spectra of BP dyes (10 μM) in deionized water (DI H_2O). The emission spectra were obtained under excitation at the maximum absorption wavelength

Table 1 Photophysical properties of BP dyes (10 μM) in different solvents

Compound	Solvent	λ_{abs} (nm)	λ_{em} (nm)	Stokes shift (nm)	Φ
BP1	H_2O	402	460	58	0.29
	DMSO	396	437	41	0.09
	CH_3CN	396	445	49	0.16
	MeOH	401	454	53	0.36
	EtOAc	400	453	53	0.34
	CH_2Cl_2	404	462	58	0.46
BP2	H_2O	391	452	61	0.08
	DMSO	389	435	46	0.12
	CH_3CN	390	443	53	0.15
	MeOH	397	442	45	0.12
	EtOAc	397	451	54	0.46
	CH_2Cl_2	403	465	62	0.84
BP3	H_2O	395	470	75	0.04
	DMSO	399	442	43	0.11
	CH_3CN	394	446	52	0.19
	MeOH	400	448	48	0.17
	EtOAc	400	446	46	0.59
	CH_2Cl_2	403	449	46	0.58
BP4	H_2O	390	461	71	0.02
	DMSO	396	443	47	0.06
	CH_3CN	396	447	51	0.06
	MeOH	398	445	47	0.06
	EtOAc	400	452	52	0.36
	CH_2Cl_2	404	458	54	0.60

Φ: fluorescence quantum-yield, DMSO: dimethyl sulfoxide, CH_3CN: acetonitrile, MeOH: methanol, EtOAc: ethyl acetate, CH_2Cl_2: dichloromethane. Fluorescence quantum yield (Φ) were determined using 9,10-diphenylanthracene (DPA, 10 μM) as a standard [20]. Quantum yields were derived from the equation: $\Phi_s = \Phi_r \times (n_s^2/n_r^2) \times (I_s/I_r) \times (A_r/A_s)$, where s is the sample (BP dyes), r is the standard (DPA), n is refractive index, I is the integrated fluorescence emission, and A is the absorbance at the excitation wavelength

The time-resolved fluorescence (TRF) study of amino-BODIPY derivatives have been reported,[18] and nanosecond lifetime was characterized. We measured the time-resolved fluorescence emission of **BP1** and **BP2** in the deionized H_2O, and they showed 1.31 and 0.24 ns lifetime, respectively, which are similar to the typical decay of BODIPY (Fig. S11).

Protein Labeling with BP Dyes

As a model protein, a bovine serum albumin (BSA, Sigma, Cat. No. A2058, mol wt ~ 66 kDa) was used, which was incubated with **BP2** and **BP3** for the test of labeling reactivity.

BP2 (10 μM) was pre-treated with N-hydroxysuccinimide to form active-ester form (see details in Supplementary Material), and then BSA solution (100 μM in pH 7.4 PBS;

phosphate-buffered saline buffer containing 1% acetonitrile). The lysine amine residues in the BSA (59 lysine in the total 607 amino acid) undergo the amide bond formation with active-ester of **BP2**, and the resulting product shows bright blue fluorescent at 450 nm wavelength (Fig. 4). The fluorescence enhancement (2 times) with a little hypsochromic shift of emission maximum wavelength was observed that presumably coming from (i) environment change of **BP2** on the BSA, and (ii) reduced quenching effect of carboxylic acid to amino-BODIPY dye [23]. We conducted MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) analysis to confirm the BSA labeling with

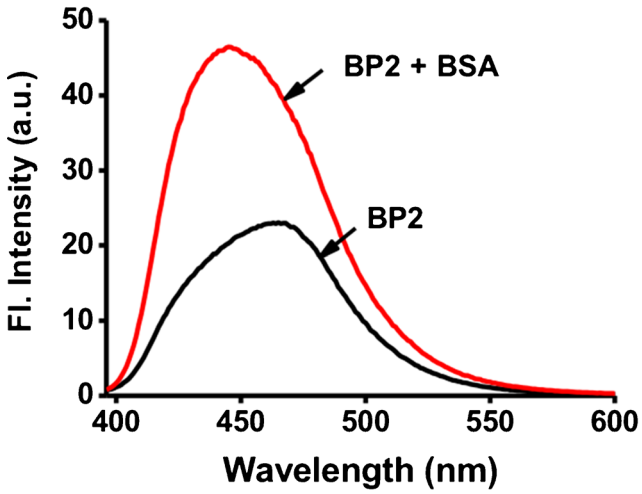


Fig. 4 Fluorescence emission spectra of **BP2** (10 μM) and BSA labeled with **BP2** [100 μM BSA, 24 h incubation at 37 °C in PBS buffer (pH 7.4, 1% acetonitrile)]. See the details in Supplementary Material (Scheme S4). The excitation wavelength was 394 nm

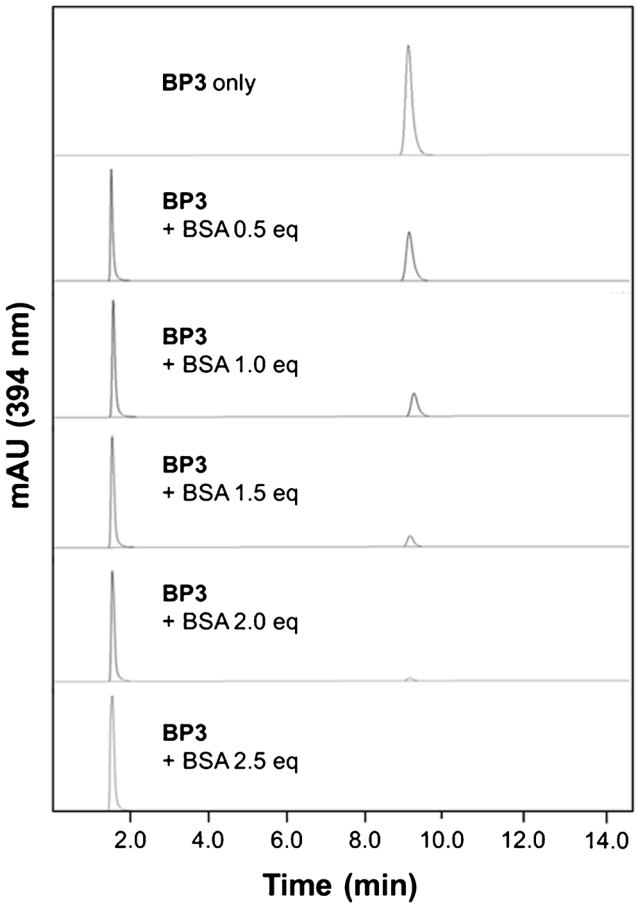


Fig. 6 High performance liquid chromatography (HPLC) spectra of **BP3** only (10 μM), and mixture of **BP3** with BSA for 1 h incubation at 37 °C in PBS buffer (pH 7.4, 1% acetonitrile). BSA concentration: 0.5 eq (5 μM)–2.5 eq (25 μM). The intensity was measured at 394 nm. Eluent was deionized H_2O:acetonitrile = 1:1 (*v*/*v*, 0.1% trifluoroacetic acid), and flow rate was 1.0 mL min^{-1}

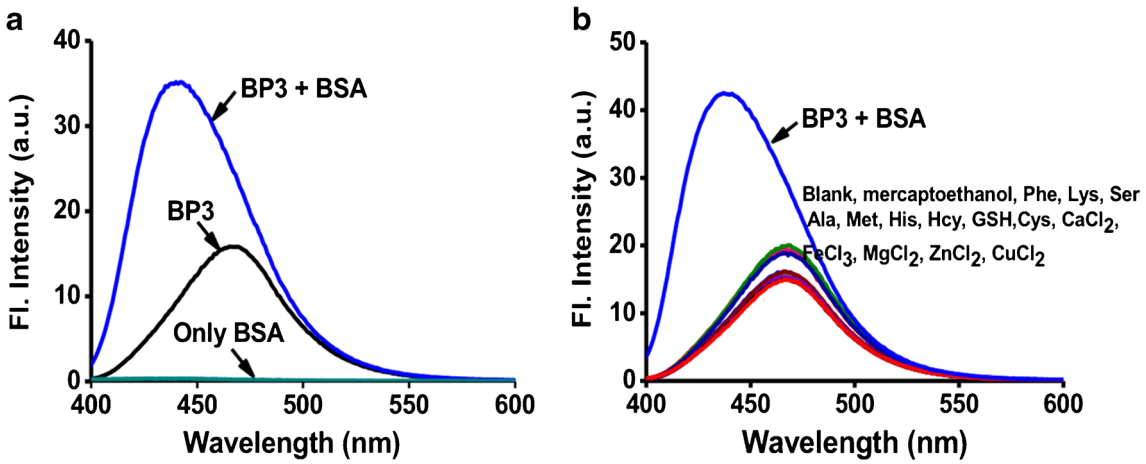


Fig. 5 **a** Fluorescence emission spectra of BSA only, **BP3** (10 μM), and BSA labeled with **BP3** [100 μM BSA, 1 h incubation at 37 °C in PBS buffer (pH 7.4, 1% acetonitrile)]. **b** Fluorescence emission spectra of **BP3** (10 μM) with BSA, amino acids, mercaptoethanol, and metal ions (100 μM, respectively), for 1 h incubation at 37 °C in PBS buffer (pH 7.4, 1% acetonitrile). The excitation wavelength was 394 nm

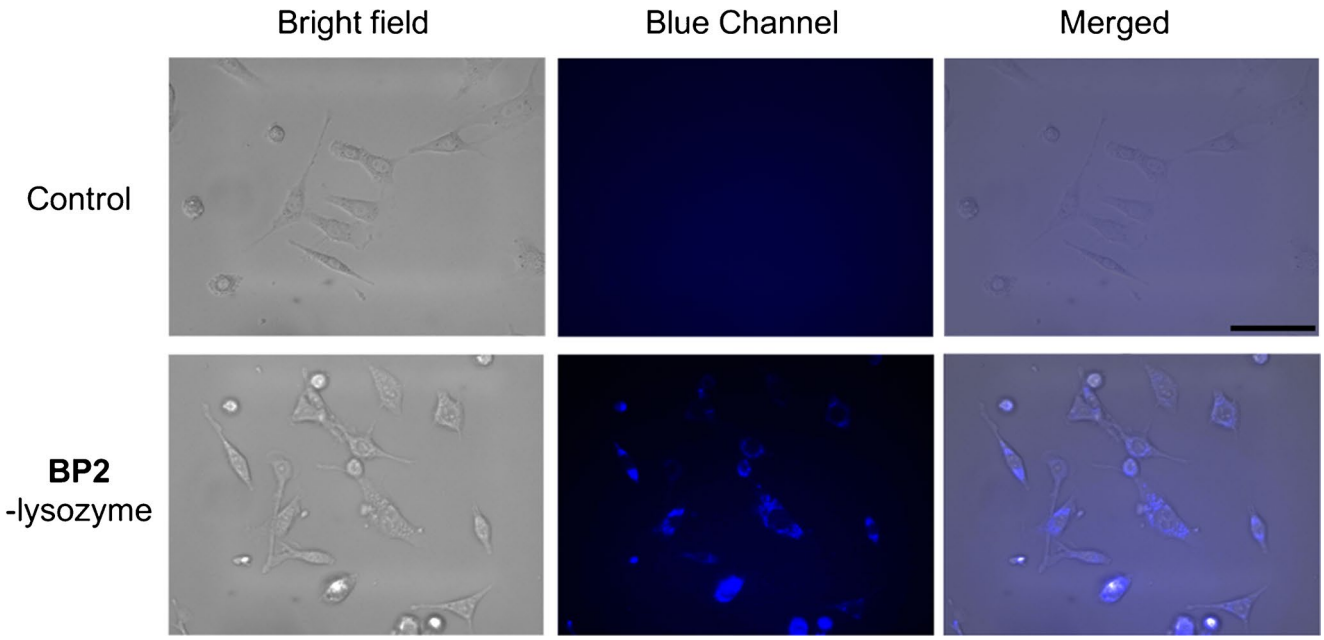


Fig. 7 Fluorescence images of TM3 mouse Leydig cells treated with the **BP2**-labeled lysozyme product. Control: without treatment. Scale bar: 250 μm. Excitation and detection wavelength were 380 nm and 420 ± 15 nm, respectively

the **BP2** (Fig. S12). The mass change of BSA with the **BP2** ($m/z = 279.05$) added was observed:$m/z = 68014.34$ (no **BP2**) ~ $m/z = 68562.52$ (+ 2 **BP2**), and the result suggests that on average 1–2 molecules of the **BP2** were labelled per each BSA.

Next, we demonstrated the BSA labeling using **BP3** which have a maleimide moiety for the thiol-ene conjugation. The primary structure of BSA has 35 cysteine residues, and the 34 cysteines are existed as disulfide form. The only one free cysteine residue, Cys34, which located in the crevice on the protein surface domain-I can react with maleimide moiety in the **BP3. BP3** (10 μM) was incubated with BSA solution (100 μM in pH 7.4 PBS buffer containing 1% acetonitrile) for 1 h at 37 °C. The fluorescence measurement of resulting product showed similar property changes likes **BP2**, emission peak shifting (15 nm) with enhancement (2.2 times) (Fig. 5a). To analyze the fluorescence change of **BP3** at various conditions, it was incubated with BSA, mercaptoethanol (HS-CH_2CH_2-OH), amino acids (Phe, Lys, Ser, Ala, Met, His, Hcy, Cys, GSH), and metal ions (Ca^{2+}, Fe^{3+}, Mg^{2+}, Zn^{2+}, Cu^{2+}), respectively (Fig. 5b). As we expected, BSA-BP3 product showed fluorescence enhancement with a peak shifting but the other thiol moiety containing substitutes such as mercaptoethanol, Hcy, Cys, GSH showed no significant changes that indicating fluorescence enhancement comes from the labeling-induced environment change of BP dye in BSA.

HPLC (high-performance liquid chromatography) was conducted to verify the bond formation of the **BP3** to the BSA (Fig. 6). In the absence of BSA, the peak of **BP3** was appeared around 9 min. Upon the addition of BSA and incubation for 1 h at 37 °C in PBS buffer (pH 7.4, 1% CH_3CN), a new peak was appeared around 2 min that corresponded with BSA-BP3 conjugated product and 2.5 equivalent of BSA gave complete conversion.

Further experiment was conducted to confirm the fluorescence imaging capability of the amino-BODIPY after the labeling of protein. The lysozyme (Sigma, Cat. No. 10,837,059,001, mol wt ~ 14 kDa) was labelled with **BP2** via amide-bond formation (6 lysine in the total 129 amino acid). The labeling was verified by measurement of fluorescence and MALDI-TOF (1–2 molecules of the **BP2** labelled per each lysozyme on average). The resulting product was lyophilized, and then incubated with TM3 mouse Leydig cells for 2 h at 37 °C under 5% CO_2. Then, the cells were rinsed with PBS (phosphate buffered saline) buffer prior to fluorescence imaging. The bright blue fluorescence was observed inside the cells, which indicates that **BP2**-labeled lysozyme penetrates the cells and maintain the fluorescence in biological media (Fig. 7).

Conclusion

Disclosed here are the synthesis and photophysical properties of a series of 8-Amino-BODIPY (**BP1**–**4**) dyes as protein labeling reagents. The primary amine moiety reacts with a thiomethyl group in 8-SMe-BODIPY proceeds fast (within

hours) under mild condition (25 °C) with high yield, and the resulting 8-Amino-BODIPY derivatives shows bright blue fluorescence at 409–467 nm wavelength (absorption at 370–400 nm). The synthesized derivatives were applied to the labeling of bovine serum albumin (BSA), a model protein, via amide bond formation (**BP2**) or thiol-ene conjugation (**BP3**). The conjugation was successfully proceeds in mild condition, and the resulting BSA-BP conjugated product maintained the strong blue fluorescence. The HPLC and MALDI-TOF mass analysis support the conjugation of BP dyes to the amino acids of BSA. The solvent-insensitive absorption and blue-emitting property of the BP dye are desirable feature for protein labeling, therefore it would be applicable for studying the protein dynamics in living cells and fluorescence resonance energy transfer (FRET) with intrinsic biomolecules.

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