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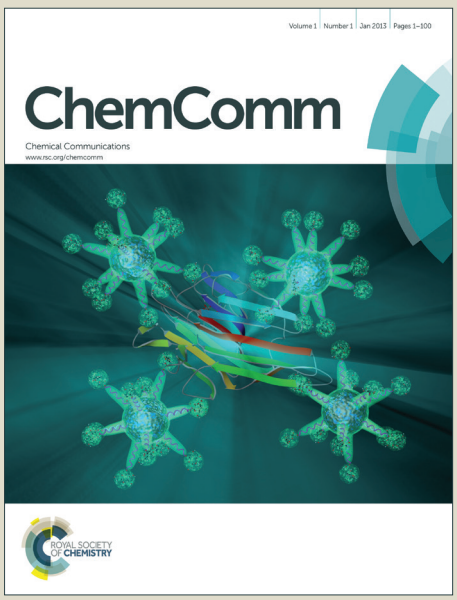

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Rational design of a photo-crosslinking BODIPY for *in situ* protein labeling

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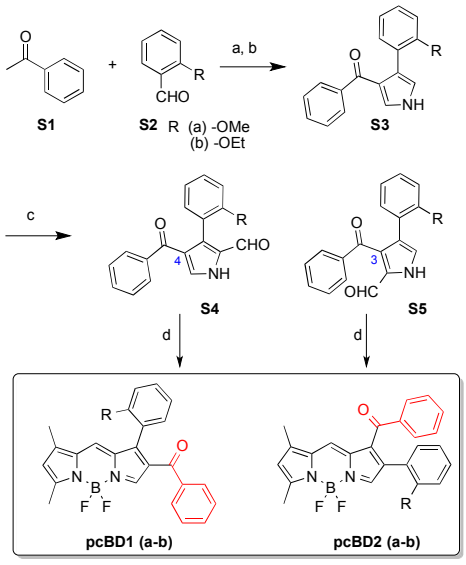
Photo-crosslinking agents have emerged as critical tools to investigate protein-protein interactions in complex proteome, but there are few photocrosslinkers available at the moment. Here, we report the first rational design of a photo-crosslinking BODIPY fluorophore (pcBD) and its biological application for biomolecule labeling. As a photosensitizing functional motif, aryl ketone group was incorporated into BODIPY fluorophore, and series of proteins were labeled by pcBD compounds upon UV irradiations. In order to investigate protein-protein interactions in whole protein mixture, amino functionalized pcBD was prepared and covalently attached to ubiquitin ligase binding peptide. Upon UV irradiation, we could successfully visualize the substrates out of total lysates. These results provided a proof of concept of spatially controllable tagging via photo-activations of pcBD scaffold, and demonstrated its potential usage for *in situ* labeling applications.

Protein-protein interactions (PPI) underlie varieties of biological signaling pathways, and detail understandings of the interactome networks are crucial for biomedical research and drug discovery.[1, 2] Traditionally, reductionist strategy was applied to investigate PPI using purified proteins *in vitro,* however, many of these interactions are difficult to study *in vitro* condition since their functions are tightly controlled in complex cellular context.[3] Although several techniques have been developed to study PPI, such as tandem affinity purification (TAP) or yeast 2 hybrid (Y2H), these methods provides indirect evidence of PPI with relatively high false positive rate.[4] A powerful alternative strategy is utilizing *in situ* photo-affinity labeling (PAL). Upon photo-irradiation, photo-crosslinking functional group generates highly reactive species that react to adjacent molecules resulting direct covalent modification. Since PAL can capture non-covalent interaction partner spatio-selectively, photo-crosslinking agents have emerged as critical tool for PPI study.[5] Despite of the significance, there are only few photo-crosslinkers available at the moment, mainly benzophenone, aryl azides, and diazirines.[6] Further conjugation of these agents via bio-orthogonal reactions (i.e. click chemistry) can be used for in situ fluorescence labeling of proteins. We envisioned that photo-crosslinking fluorophores would enable PPI and fluorescence labeling in one single step. Here we report a design of a novel photo-crosslinking fluorophore, and its application for ubiquitin ligase substrate labeling.



Scheme 1. Synthesis of photo-crosslinking BODIPY (**pcBD**) compounds. See supporting information for detail synthetic procedures (a) LiOH, EtOH, overnight at rt; (b) LiOH, *p*-toluenesulfonylmethyl isocyanide, EtOH; (c) $POCl_3$, DMF/CH_2Cl_2; (d) 2,4-dimethyl pyrrole, $POCl_3$, 3h at rt; BF_3OEt_2, DIEA, rt for 3h.

We initiated our studies by determining fluorescent scaffold and photosensitizing motif. Boron dipyrromethane (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene, BODIPY) was chosen as fluorophore due to its ease of synthesis and exceptional photophysical properties, such as high photostability, high extinction coefficient and fluorescence quantum yield, and narrow emission band width.[7, 8] Along with desirable BODIPY fluorophore, we selected a photo-crosslinking motif based on following criteria. First, non-activated form should be a bio-orthogonal and stable form until photo-irradiation. Second, activated intermediates must not disrupt the core BODIPY fluorophore structure. Third, reactivity of activated species has to be moderate to minimize nonspecific crosslinking. Despite all three popular photo-crosslinkers (benzophenone, aryl azides, and diazirines) are basically bioorthogonal, aryl azides produce ketenimide intermediate that can break the dipyrrolemethene conjugation system in BODIPY, and carbene species produced by photo-activation of diazirine have extreme reactivity that reduce photo-crosslinking efficiency against targets due to the nonspecific crosslinking with waters.[6] Aryl ketones, including benzophenone, are activated by UV light, and generate diradical that can react with any adjacent C-H bonds to form covalent modification. Since this diradical can persist upto 120 μs and relaxed back to original form, aryl ketone has high efficiency of photo-crosslinking.[9] Consequently, we decided to attach aryl ketone groups to BODIPY core.

In order to incorporate aryl ketone group to BODIPY, we designed synthetic path utilizing benzoyl pyrrole. Benzoyl pyrroles were synthesized by Van Leusen methods.[10, 11] α,β-unsaturated ketones were prepared by Knoevenagel condensation reaction between benzaldehydes and acetophenone, and they were reacted with *p*-toluenesulfonylmethyl isocyanide to produce benzoyl pyrroles (Scheme 1). After formylation of benzoyl pyrroles, we obtained two regioisomers of benzoyl pyrrole carbaldehydes. 4-benzoyl-3-phenyl-1*H*-pyrrole-2-carbaldehyde (**S4**) was obtained as a major product, and 3-benzoyl-4-phenyl-1*H*-pyrrole-2-carbaldehyde (**S5**) was a minor product. After purification of individual regioisomers, final BODIPY compounds were prepared by condensation of benzoyl pyrrole carbaldehydes and 2,4-dimethyl pyrrole followed by the addition of BF_3OEt_2. We dubbed these new BODIPY scaffolds synthesized from **S4** and **S5** pyrroles as **pcBD1** and **pcBD2**, respectively.

Photophysical properties of final 4 **pcBD** compounds were measured in various organic solvents, and summarized in Table S1. Interestingly, regioisomers of BODIPY compounds showed remarkably distinct fluorescence property. **pcBD1** series showed green fluorescence emission (520 nm ~ 531 nm, Fig S1-2), but the other regioisomers, **pcBD2** series, exhibited a red shifted emission (576 nm ~ 613 nm, Fig S3-4) with higher solvent polarity dependency. Intrigued by this observation, we further investigated the cause of red shifted emission. One of the notable distinctions between **pcBD1** and **pcBD2** compounds was chemical shift value of BODIPY core's *meso*-position proton. Typically, BODIPY core's *meso*-proton exhibits singlet peak with chemical shift value around 7.2 ppm.[12, 13] Likewise, ^{1}H-NMR spectra from all **pcBD1** compounds showed singlet peak at the region. However, we could not find singlet proton around 7.2 ppm for all **pcBD2** compounds. Instead, there was downfield shifted singlet peak around 7.6 ppm (Fig 1). This observation suggested that intramolecular hydrogen bonding between *meso*-proton and

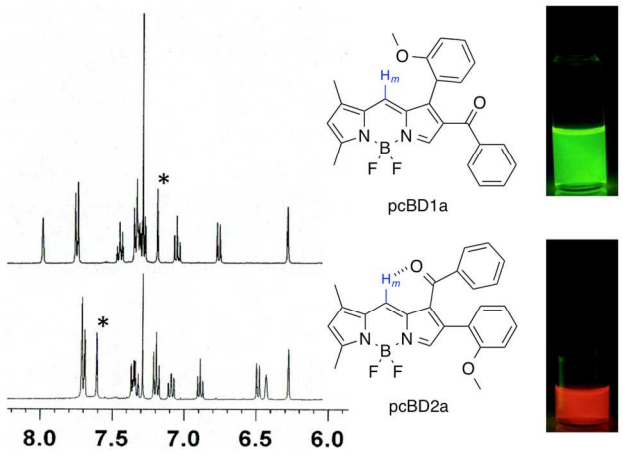


Figure 1. Intramolecular hydrogen bonding mediated fluorescence red shift. Chemical shift of *meso*-position proton was shifted to downfield compared to the corresponding regioisomer.

ketone group might be the origin of red shifted fluorescence emission. It was further validated by reducing the ketone to hydroxyl group. We observed green color emission after treatment of sodium borohydride to **pcBD2** compounds, which supported that ketone group play crucial role for red shifted emission (Fig S5).

Next, photo-crosslinking properties of **pcBD** compounds were examined using amino acid monomers and purified proteins. Amino acids containing both aliphatic and aromatic side chains were tested, and their photo-crosslinking efficiencies were measured using LC-MS traces. Each amino acid (2.75 mM) was mixed with **pcBD2a** (0.5 mM) and irradiated under UV light (100W, Mercury lamp). After 3 hour irradiation, we could observe photo-crosslinking reaction products from LC-MS traces, and calculated their efficiency based on total ion counts values (Table 1). Both aliphatic and aromatic amino acids can be labeled by **pcBD** scaffold, and photo-crosslinking efficiency varies depending on the amino acids (Table S2). In general, **pcBD** exhibited higher efficiency of photo-crosslinking compared with benzophenone (averaged crosslinking efficiency of **pcBD** for amino acids: 0.45 %, water: 0.72 %; averaged crosslinking efficiency of benzophenone for amino acids: 0.06 %, water: 0.04 %). It is noteworthy that tyrosine exhibited high efficiency for photo-crosslinking, which is consistent with the previous radical intermediate based protein tagging study.[14]

Table 1. Photo-crosslinking efficiency of **pcBD2a** compounds toward 4 representative amino acids (Detail LC-MS traces Fig S6-S9, and Table S2).

	Aliphatic amino acids		Aromatic amino acids	
	Ala (A)	Gly (G)	Phe (F)	Tyr (Y)
Photo-crosslinking efficiency(%)	0.59	0.36	0.13	3.56

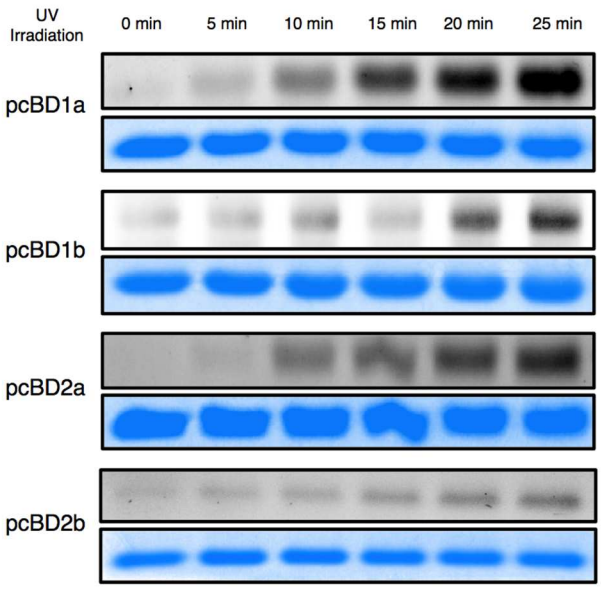


Figure 2. *In vitro* photo-crosslinking for lysozyme using **pcBD** compounds. 10 μM **pcBD** compounds were mixed with 100 μg/ml lysozyme, and irradiated 254 nm UV light (6W) for given time. For each compound, photo-labeled proteins were visualized by fluorescence emission signal (gray; excitation: blue LED, emission: 530 nm/bandwidth 28 nm), and loading controls were visualized by coomassie staining (blue).

To validate photo-labeling of proteins *in vitro*, we incubated **pcBD** compounds (10 μM) with 3 purified proteins (100 μg/ml), and the mixtures were exposed to UV lights from 0 to 25 min. After photo-reaction, the mixture was separated by SDS-PAGE, and visualized **pcBD** labeled proteins by fluorescence imager (Fig 2: lysozyme, Fig S10-S11: human serum albumin (HSA) and bovine serum albumin (BSA)). All three proteins were nicely labeled with **pcBD** compounds using conventional bench top UV lamp upon photo-activation in time dependent manner, which demonstrated the potential of this scaffold for protein labeling applications. Particularly, **pcBD** could crosslink proteins under both 254 nm and 365 nm UV lights (Fig S12). These results suggest that **pcBD** can be easily employed to label protein *in situ* without any special light source.

The main advantage of **pcBD** for photo-activated protein labeling is the capability to attach BODIPY fluorophore at any kinds of target proteins depending on the spatial vicinity. Encouraged by the successful photo-crosslinking of purified proteins, we next attempted to visualize protein-protein interaction in complex proteome using **pcBD** compound. We chose ubiquitin ligase complex as model system since several enzymes tightly cooperate to tag substrate with ubiquitin during ubiquitination process. Ubiquitination is one of the major post-translational modification, and ubiquitin-proteasome pathway play critical role in cellular functions, including nonlysosomal protein turnover, membrane receptor endocytosis, and cell cycle regulation.[15, 16] There are three enzymes mediating ubiquitination; ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3).

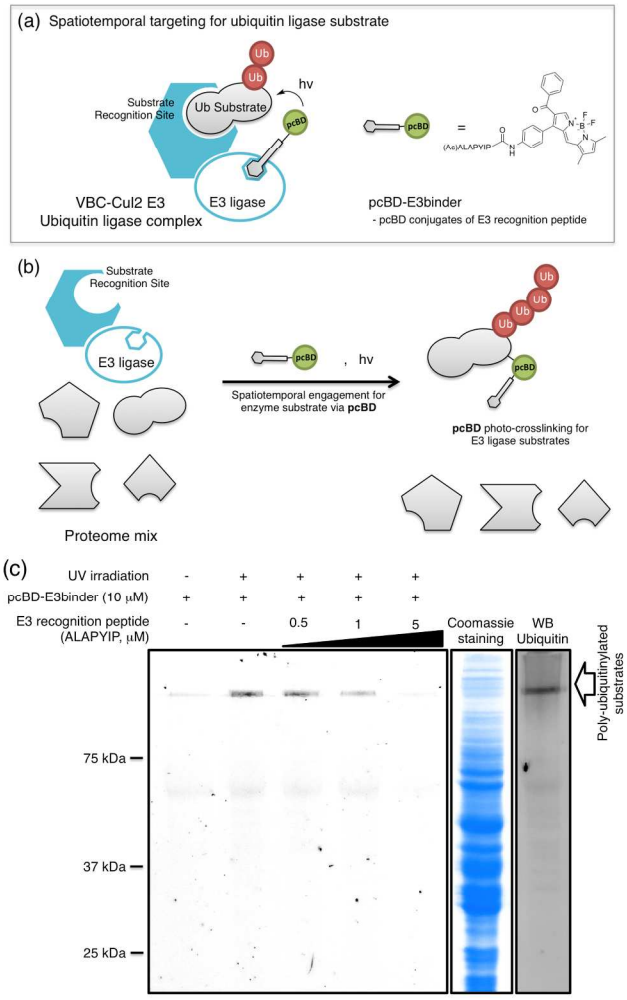


Figure 3. Spatial targeting for ubiquitin ligase substrate using **pcBD**. (a) Schematic presentation of photo-crosslinking strategy to tag enzymatic substrates of VBC-Cul2 E3 ubiquitin ligase complex. (b) Selective fluorescent tagging for ubiquitin ligase substrates using **pcBD-E3binder** (pcBD conjugate of E3 recognition peptides, ALAPYIP sequence) in complex proteome. (c) Fluorescence SDS gel image of spatiotemporal tagging for ubiquitin ligase substrates out of total HEK293 lysate. Photo-labeled proteins were visualized by fluorescence emission signal (gray; excitation: blue LED, emission: 530 nm/bandwidth 28 nm), and total proteins were visualized in coomassie staining (blue, loading controls in Fig S13). Western blot analysis using ubiquitin antibody showed that photo-labeled proteins were substrates of ubiquitin ligase enzyme.

Briefly, ubiquitin activation is performed by E1 enzyme in an ATP-dependent manner, and E1 transfer ubiquitin to E2 enzyme. Ubiquitin loaded E2 enzyme form complex with E3 ubiquitin ligase that recruits substrates, and this complex mediates ubiquitination of substrate.

In this ubiquitin ligation process, we envisioned that fluorescent photo-crosslinker could be utilized to examine enzyme-substrate interaction. To visualize substrates of E3 ubiquitin ligase out of total cellular proteome mixture, we synthesized a photo-affinity probe, **pcBD-E3binder**, by conjugating E3

ligase binding peptide (ALAPYIP sequence) and amine functionalized **pcBD** (Fig 3-a, b). This short peptide was discovered as minimum recognition domain for the von Hippel-Lindau tumor suppressor protein (VHL),[17] which is a part of the VNC-Cul2 E3 ubiquitin ligase complex.[18] Therefore, spatially adjacent substrates can be tagged by **pcBD-E3binder** only under photo-activation. Total protein lysates (2.7 mg/ml) of HEK293 cell line was mixed with **pcBD-E3binder** (10 μM), and the mixture was either stored in the dark or irradiated 365 nm UV light for 30 min (Fig 2-c, first and second lane). As we expected, strong fluorescence signal was observed only the irradiated sample. It was further confirmed that the labeling event was E3 ligase binding dependent by the following competition assay with free E3 ligase binding peptide (Fig 3-c, three to five line). It should be noted that **pcBD-E3binder** labeling was clearly decreased dose dependent manner in the competition assay. Lastly, western blot analysis exhibited the target of photo-labeled proteins were highly ubiquitinylated protein, in other words substrates of ubiquitin ligase. This result proved the utility of photo-crosslinking fluorophore for *in situ* protein labeling in complex mixture.

In summary, we rationally designed the first photo-affinity BODIPY fluorophore, and demonstrated usage for spatially controlled bio-molecule tagging. Compared with a conventional dual tagging approach, **pcBD** tagging provides a major advantage in terms of a small versatile tag. In this study, we conjugated **pcBD** to ubiquitin ligase binding peptide to probe their substrates out of total lysate, but its application was not limited in specific enzyme. Any enzyme ligand could be attached into **pcBD**, and visualized their substrates. Such efforts could significantly contribute to enhance protein-protein interaction in complex biological system. In addition to photo-affinity application, **pcBD** scaffold also provided interesting photophysical property of BODIPY scaffold. Regioisomers of **pcBD** compounds exhibited unique emission shift depending on the position of benzoyl group in BODIPY core, and red shifted emission was originated by intramolecular hydrogen bonding mediated extension of conjugation system. This feature can be applied in various fluorophore designs to tether fluorescence property.

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Author Contribution

D.P. Murale and S.C. Hong contributed equally for chemical synthesis experiments. J.-S.Lee designed overall experiment for photo-affinity BODIPY compound design and spatio-temporally controlled in situ protein labeling. J.Yun. contributed for protein labeling experiment. D.P. Murale and J.-S.Lee wrote the main manuscript text and prepared all figures. C.N.Yoon and J.-S.Lee contributed reagent/material/analysis tools. All authors reviewed the manuscript.

Notes and references

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