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A Rapid and Fluorogenic TMP-AcBOPDIPY Probe for Covalent Labeling of Proteins in Live Cells

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Supporting Information

ABSTRACT: Protein labeling is enormously useful for characterizing protein function in cells and organisms. Chemical tagging methods have emerged as a new generation protein labeling strategy in live cells. Here we have developed a novel and versatile TMP-AcBOPDIPY probe for selective and turn-on labeling of proteins in live cells. A small monomeric tag, *E. coli* dihydrofolate reductase (eDHFR), was rationally designed to introduce a cysteine in the vicinity of the ligand binding site. Trimethoprim (TMP) that specifically binds to eDHFR was linked to the BOPDIPY fluorophore containing a mildly thiol-reactive acrylamide group. TMP-AcBOPDIPY rapidly labeled engineered eDHFR tags via a reaction termed affinity conjugation (a half-life of ca. 2 min), which is one of the top fast chemical probes for protein labeling. The probe displays 2-fold fluorescence enhancement upon labeling of proteins. We showed that the probe specifically labeled intracellular proteins in live cells without and with washing out the dye. We demonstrated its utility in visualizing intracellular processes by fluorescence-lifetime imaging microscopy (FLIM) measurements.

Studying protein function *in vitro* or in the context of live cells and organisms is of vital importance in biological research. Genetic tags such as fluorescent proteins (FPs) are widely used to detect proteins. However, compared to chemical tags, FPs have several limitations, including environmental sensitivity, limited flexibility of modification, and less amenability for temporal control.[1] In contrast, chemical probes are able to achieve properties that are not readily possible when using fluorescent proteins, such as fluorophore-assisted light inactivation, real-time detection of protein synthesis, and multicolor pulse-chase labeling.[1b] Many organic dyes are superior to fluorescent proteins in terms of brightness, photostability, far red emission, environmental sensitivity, and potential for modifications to their spectral and biochemical properties. Altogether, chemical labeling has become an important strategy for the study of protein function in live cells and organisms.[1]

Recent advancements in *in vivo* chemical labeling techniques involve the combination of the specificity of genetically encoded tags and the flexibility of small molecule probes. Such amino acid sequences include tetracysteine/tetraserine motifs,[2] metal chelation motifs,[3] peptide tags for enzymatic modification,[4] ligand binding domains,[5] and self-labeling enzymatic domains.[6] These methods have profoundly enriched the spectrum of tools used to investigate biological events. However, intrinsic limitations include cytotoxicity, off-target labeling, high background staining, and a slow labeling rate. Many of them rely on either reversible noncovalent labeling or enzymatic reactions that have limitations of low reactivity in some organelles and steric hindrance. These limitations represent a great challenge for the development of chemical labeling tags *in vivo*. There remains a high demand for intracellular labeling reagents with low cytotoxicity, good cell permeability, fast reaction rates, and low background staining.

Fluorogenicity is a desirable feature in protein labeling particularly in a complex biological context, as it allows a dye to "turn-on" after labeling and hence helps to reduce labeling background and substantially enhances the signal-to-noise ratio. Therefore, strategies for fluorogenic protein labeling are gaining enormous interest from the perspective of protein chemistry and cell biology.[7] Affinity probes are an attractive approach, because selectivity can be conferred by specific ligand binding. Recently, affinity probes and baits have been specifically incorporated into proteins *via* selective chemical conjugations, which are mediated by inhibitor binding or metal chelating.[8] Covalent probes, as opposed to noncovalent ones, resulted in the permanent labeling of target proteins and are therefore often more superior in many applications.[4,6] Furthermore, protein labeling kinetics is another essential consideration in live cell labeling. A fast labeling rate confers better temporal control, which is a fundamental feature of chemical labeling. A combination of high selectivity, fast covalent reaction, and fluorogenicity in an individual chemical probe is profoundly beneficial for chemical labeling in cells. However, only limited progress has been achieved in this development.[8,9] In this study, we aimed to develop a rapid and fluorogenic chemical probe for the covalent labeling of intracellular proteins in live cells.

The trimethoprim (TMP) tag has been introduced by Cornish's lab.[5b] TMP has high affinity (K_I = 1nM) for *Escherichia coli* dihydrofolate reductase (eDHFR), but a much lower affinity (K_I = 4−8 μM) for a mammalian form of DHFR.[10] eDHFR is small (18 kD, two-thirds of GFP) and monomeric and marginally perturbs the function of proteins that it fuses with. Moreover, TMP can be derivatized without significantly disrupting its binding to eDHFR.[5b] Here we

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develop a novel probe for chemical protein labeling in live cells, termed affinity conjugation, using the TMP tag. The general scheme of this approach is shown in Figure 1.

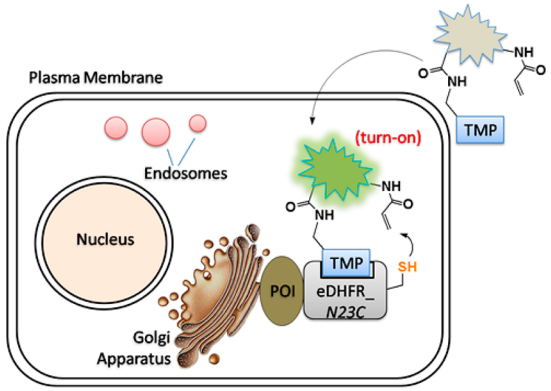
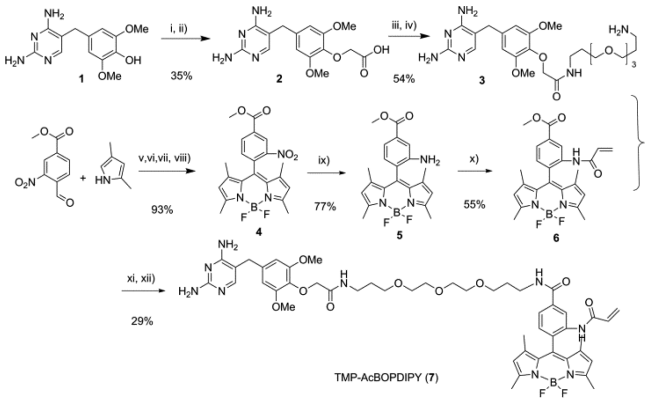


Figure 1. Principle of labeling of protein *via* affinity conjugation in live cells using the TMP-AcBOPDIPY probe.

We first designed a TMP-probe, TMP-AcBOPDIPY, featuring four modules including a ligand (TMP), a spacer [TEG (tetraethyleneglycol)-like linker (diethylene glycol bis-aminopropyl terminated)], a fluorophore (BOPDIPY), and a mildly thiol-reactive acrylamide group (compound **7**) (Scheme 1). To make a fluorogenic probe, we use an environment-

Scheme 1. Synthesis of TMP-AcBOPDIPY[a]



[a](i) $BrCH_2COOMe$, *t*-BuOK, DMSO, rt; (ii) NaOH, MeOH, rt, 1 h; (iii) Boc-TEG-NH_2, EDC, HOBt, DIEA, DMF, rt, 5 h; (iv) TFA, DCM, 3 h; (v) neat TFA, rt, 4 h; (vi) DDQ, MePh, rt, 5 min; (vii) Et_3N; (viii) $BF_3{\cdot}OEt_2$, 10 min; (ix) 10% Pd/C, MeOH, rt, overnight; (x) acryloyl chloride, Et_3N, 1,4-dioxane; (xi) LiOH, H_2O, THF, *i*PrOH; (xii) **3**, EDC, HOBt, DIEA, DMF, rt.

sensitive boron phenyldipyrromethene (BOPDIPY) dye. BOPDIPY can freely rotate around the single bond between the BODIPY moiety and the phenyl group. The fluorescence intensity of BOPDIPY increases substantially with increasing solvent viscosity because of the restricted rotation of the phenyl group.[11] We speculate that BOPDIPY would display dramatic fluorescence enhancement when it binds to proteins. TMP-AcBOPDIPY was synthesized *via* a convergent synthetic route. First, the BOPDIPY intermediate **4** was prepared through the condensation reaction between the benzaldehyde derivative and dimethylpyrrole followed by oxidation.[12] The nitro group of **4** was reduced to an amino group *via* hydrogenation, and the resulting **5** was acrylated yielding the key intermediate **6**. Second, TMP was demethylated to produce a 4′-phenol derivative of TMP (**1**) that was introduced with a carboxylate group (**2**), and subsequently coupled with a TEG-like linker, yielding the intermediate TMP-TEG-NH_2 (**3**). Finally, AcBOPDIPY (**6**) was coupled with TMP-TEG-NH_2 (**3**), affording the target molecule, TMP-AcBOPDIPY (**7**). The quantum yield of the TMP-AcBOPDIPY probe was determined to be Φ 0.31 in 50 mM PBS buffer (pH 7.4) using Fluorescein as the reference (Φ_R = 0.87 at pH 7.4). In PBS buffer (pH 7.4), TMP-AcBOPDIPY displays maximum absorption at 503 nm (ε = 4.9 × 10^3 M^{-1} cm^{-1}) and maximum emission at 519 nm (Figure S1).

On the other hand, we designed eDHFR mutants with one or two cysteine mutations in the vicinity of the TMP binding site to enable Michael addition with the acrylamide group on the TMP-AcBOPDIPY probe. The key issue is to identify mutations that do not influence the tight and specific binding of TMP to eDHFR. Analysis of the crystal structure identified several amino acid residues (A19, N23, G51, and R52) located in two loop regions in the vicinity of the binding site (Figure 2A). These amino acid side chains do not appear to be involved

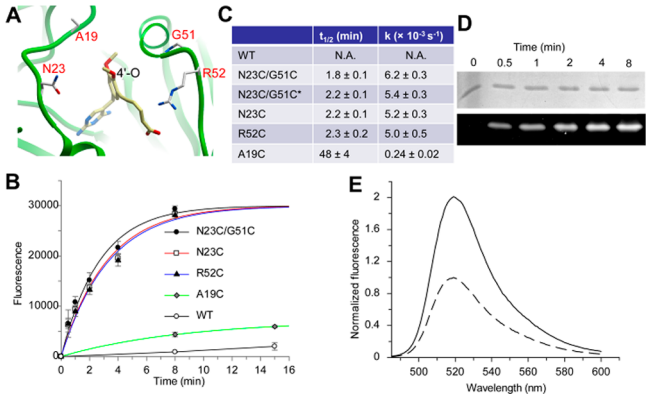


Figure 2. Labeling rate and fluorescence enhancement. (A) The TMP-binding site of eDHFR. The G51, R52, N23, and A19 residues in close proximity to the ligand binding pocket are highlighted. (B) Kinetic study of the labeling of eDHFR mutants by TMP-AcBOPDIPY. Recombinant eDHFR wild type or eDHFR mutant (5 μM) was labeled with 10 μM probes at 37 °C. At time points, reaction was quenched by denaturing SDS-sample buffer with 9 M Urea and resolved by SDS-PAGE. (C) Summary of the first-order reaction kinetic constants and half-lives. * Reaction was performed in the presence of 50 μM NADPH. (D) SDS-PAGE analysis of eDHFR_N23C/G51C reaction with TMP-AcBOPDIPY (upper: Coomassie stain, lower: fluorescent scan). (E) Emission spectra of TMP-AcBOPDIPY in the absence (dashed line) and the presence of eDHFR_N23C/G51C (solid line).

in essential interactions and are well exposed on the surface. Moreover, their side chains are close (ca. 8−9 Å) to the 4′-O of the trimethoxybenzyl group. We generated four eDHFR mutants (A19C, N23C, N23C/G51C, and R52C) and purified recombinant proteins from *E. coli*.

We evaluated the *in vitro* reactivity of the TMP-AcBOPDIPY probe with these eDHFR mutants. The reaction was assayed by denaturing SDS-PAGE. As can be seen from the fluorescent gel, time-dependent incorporation of TMP-AcBOPDIPY into eDHFR mutants was observed, but not on the wide type eDFHR (Figure 2B). The reaction traces can be fitted to a single exponential function, giving the first-order reaction kinetic constant of each eDHFR mutant (Figure 2C).

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Apparently, eDHFR N23C and R52C display a very fast labeling rate with a half time of ca. 2 min. It is worthy to note that the double mutant, eDHFR N23C/G51C, reacts even faster with an unprecedented labeling half time of only 1.8 min. The reaction rate is 10−57-fold faster than those of previous A-TMP probes[8d,e] and is also superior to that of most affinity labeling reactions.[13]

Next, we investigated fluorescence enhancement upon labeling. As can be seen from Figure 2E, over 2-fold of fluorescence enhancement was observed after reaction with eDHFR_N23C/G51C. The "turn-on" effect seems to solely originate from the binding of BOPDIPY to eDHFR rather than covalent conjugation because the similar fluorescence enhancement was also observed when using the wild type eDHFR (Figure S2). Binding of BOPDIPY to a presumably hydrophobic site of eDHFR may also facilitate positioning the reactive acrylic group to the close proximity of the mutated cysteine residue and hence substantially enhance the reaction efficacy. Therefore, the TMP-AcBOPDIPY probe confers the fast and "turn-on" labeling of proteins.

Thereafter, we proceeded to investigate the labeling of intracellular proteins in live cells using TMP-AcBOPDIPY. We fused eDHFR_N23C with the K-Ras C-terminal sequence (CAAX) which targets the plasma membrane (PM), the nucleus localization peptide (NUC) which targets the nucleus, Rab1 protein residing at the Golgi apparatus, and Rab5 protein which localizes at the endosomes. We added 1 μM TMP-AcBOPDIPY to the culture medium of Hela cells expressing one of these fusion proteins. The confocal fluorescence microscopic images before and after washing out the dye were both recorded (Figure 3A). TMP-AcBOPDIPY is cell-permeable and clearly labeled the target proteins in live cells. As shown in Figure 3A, after washing only the cells expressing the protein tag are highly fluorescent. Because of the fluorogenic properties of the probe, images taken before washing out the probe are analogous to those taken after washing in the expressed cells (Figure 3A), suggesting that specific labeling and imaging of intracellular proteins in live cells can be accomplished under nonwashing conditions. To compare the covalent labeling with the noncovalent labeling, we labeled the cells expressing eDHFR wild type (eDHFRwt)-CAAX with TMP-AcBOPDIPY. As shown in Figure S3, the staining of the plasma membrane showed a significant decrease after washing, suggesting that the covalent labeling is more stable than the noncovalent labeling.

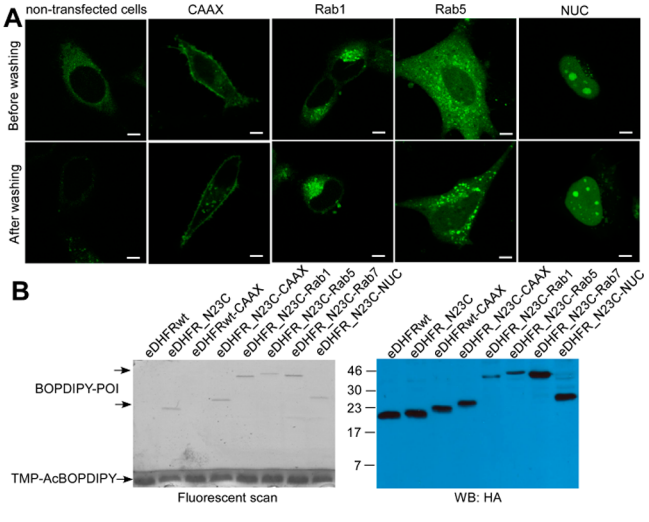

Figure 3. Labeling of intracellular proteins in live cells. (A) Confocal microscopy of live HeLa cells expressing eDHFR_N23C fused with K-Ras C-terminal sequence (CAAX), Rab1, Rab5, and nucleus localizing sequence (NUC) locating at the plasma membrane, the Golgi body, the endosomes, and the nucleus, respectively. Scale bar 10 μm. (B) Selective and covalent labeling of proteins in cells. HeLa cells expressing the indicated fusion proteins were lysed and subjected to denaturing SDS-PAGE analysis.

In order to further confirm the specific and covalent labeling reactions occurred inside live cells, we lysed the labeled cells and subjected the cell lysate to denaturing SDS-PAGE. The expression levels of eDHFR wild type (eDHFRwt) or various eDHFR mutant (eDHFRmt) fusion proteins were confirmed by Western blot (WB) analysis of the Hemagglutinin (HA) tag that is fused with each protein. While cell lysates showed a range of proteins based on Coomassie staining, only a single fluorescent band was observed in the cells expressing eDHFRmt fusion proteins, but not in the cells expressing eDHFRwt or eDHFRwt-CAAX (Figures 3B and S4). This suggests that TMP-AcBOPDIPY selectively and covalently labels the proteins of interest inside cells.

In order to demonstrate the utility of the TMP-AcBOPDIPY probe for visualization of intracellular processes, we measured the Föster resonance energy transfer (FRET) by fluorescence-lifetime imaging microscopy (FLIM) in live cells. FRET is highly useful and can be employed to elucidate many intracellular processes, such as protein−protein interactions (PPIs) and substrate−enzyme binding. As a proof of principle, a red fluorescent mCherry protein was fused N-terminally to eDHFR_N23C-CAAX to constitute an "interacted" protein pair. After labeling with TMP-AcBOPDIPY, the FRET between the donor BOPDIPY and the acceptor mCherry can be determined by the FLIM measurement (Figure 4). FLIM is an imaging technique based on measurement of the lifetime of a fluorophore. Energy transfer from the donor molecule to the acceptor molecule will lead to a decrease in the lifetime of the donor, which can be recorded by FLIM. Since the FLIM

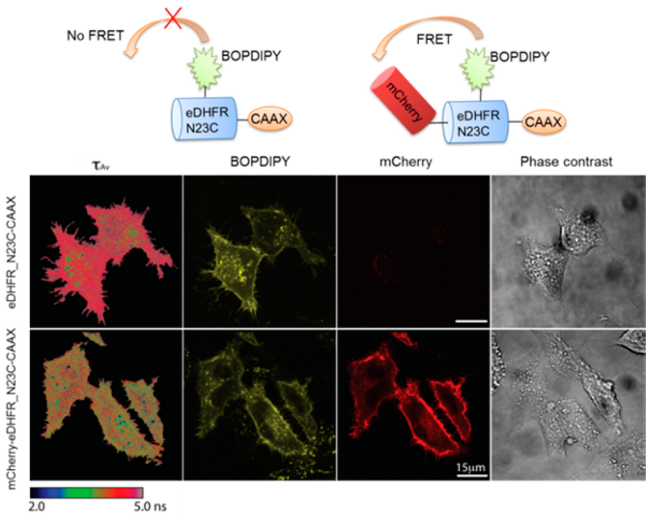

Figure 4. FLIM measurements in live cells. Fluorescence lifetime images of BOPDIPY (left panel), fluorescence confocal images of BOPDIPY and mCherry (middle panels), and phase contrast images (right panel) of HeLa cells expressing eDHFR_N23C-CAAX (upper panels) or mCherry-eDHFR_N23C-CAAX (lower panels).

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measurements are insensitive to the concentration of fluorophores and can thus filter out artifacts resulted from changes in the concentration and emission intensity, FLIM has been a very useful technique for monitoring protein interactions in cells.[14] Hela cells expressing eDHFR_N23C-CAAX or mCherry-eDHFR_N23C-CAAX were treated with TMP-AcBOPDIPY. In labeled Hela cells expressing eDHFR_N23C-CAAX, BOPDIPY displayed an average fluorescence lifetime of 4.3 ns. In contrast, in Hela cells expressing mCherry-eDHFR_N23C-CAAX, the fluorescence lifetime of BOPDIPY substantially reduced to 3.6 ns, suggesting a significant energy transfer from BIDIPY to mCherry (Figure 4). Noteworthy, the BOPDIPY fluorescence lifetime is much higher than that of fluorescent protein donors (ca. 2.5 ns for EGFP and ca. 3.0 ns for Citrine) and displays a superior dynamic range in FLIM measurements. This study demonstrates that the TMP-AcBOPDIPY probe is well suited for intracellular FRET studies.

In summary, we have developed a rapid (reaction half-lives of ca. 2 min) and fluorogenic TMP-AcBOPDIPY probe for the intracellular labeling of proteins fused with an engineered eDHFR tag. To our knowledge, this is the first affinity probe which combines all three features (high selectivity, fast covalent reaction, and fluorogenicity). We expect that the novel probe can be widely used for the labeling of proteins in cells and for the elucidation of various cellular processes.

■ ASSOCIATED CONTENT

Supporting Information

General procedures and materials, detailed preparation of TMP-AcBOPDIPY, fluorescence enhancement of TMP-AcBOPDIPY upon binding to wild type eDHFR, Coomassie staining of the gel shown in Figure 3B, etc. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

‖W.L., F.L., X.C., and J.H. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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