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Rapid and Selective Labeling of Endogenous Transmembrane Proteins in Living Cells with a Difluorophenyl Ester Affinity-Based Probe

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Abstract: The long-term stability of affinity-based protein labeling probes is crucial to obtain reproducible protein labeling results. However, highly stable probes generally suffer from low protein labeling efficiency and pose significant challenges when labeling low abundance native proteins in living cells. In this paper, we report that protein labeling probes based on an ortho-difluorophenyl ester reactive module exhibit long-term stability in DMSO stock solution and aqueous buffer, yet they can undergo rapid and selective labeling of native proteins. This novel electrophile can be customized with a wide range of different protein ligands and is particularly well-suited for the labeling and imaging of transmembrane proteins. With this probe design, the identity and relative levels of basal and hypoxia-induced transmembrane carbonic anhydrases were revealed by live cell imaging and in-gel fluorescence analysis. We believe that the extension of this difluorophenyl ester reactive module would allow for the specific labeling of various endogenous membrane proteins, facilitating in-depth studies of their distribution and functions in biological processes.

Membrane proteins are involved in a variety of vital biological processes including selective transport of molecules, cell adhesion, intracellular signal transduction and cell-cell communication. Due to their requisite roles in the survival of organisms, many membrane proteins are central targets for therapeutics and medical diagnosis.[1] Therefore, the analysis of the structure, function and expression level of membrane proteins is highly significant in medical and pharmaceutical applications as well as for fundamental research.

Among the modern methods to study membrane proteins in live cells, several methods based on the selective protein labeling are widely popular and highly effective.[2] As compared with the fluorescent protein fusion technology, protein labeling with small molecule fluorophores enables the incorporation of a wide range of fluorophores with superior photophysical properties to the protein of interest (POI), coupled with the precise control of the location and timing of labeling by the appropriate regulation of probe delivery. Furthermore, protein labeling also exhibits a strong advantage over fluorescent protein fusion technology for the imaging of membrane proteins where significant fluorescence from the fluorescent proteins can be observed in intracellular secretory pathways.[3] Currently, most protein labeling strategies rely on protein/peptide tags and bioorthogonal chemistry-based methods. Although these genetic approaches are robust and versatile, the major drawback is that they are applicable only to genetically engineered proteins, thus cannot be directly employed in the study of endogenous proteins in live cells.

As a complementary strategy to the labeling tags, affinity-based protein labeling is a tag-free approach that is applicable to study endogenous membrane protein of live cells.[4] In a typical design, the small molecule probe binds to the POI by a specific protein–ligand interaction, inducing a chemical reaction between the reactive group and an amino acid near the active pocket of the protein via proximity effect. To date, a variety of reactive modules have been developed and used in affinity-based protein labeling, including activated esters, sulfonyl chlorides, epoxides, maleimides, α-halocarbonyls, tosylate, and acyl imidazole.[5] Although these reactive moieties have been applied for proteomics analysis, protein imaging, biosensor construction and irreversible inhibition of protein activity, the reactive nature of the electrophiles on the probe is also the cause of many selectivity and stability problems.[4c,6]

Ideally, biocompatible electrophile exhibiting large reaction rate constants is highly desirable for efficient labeling of endogenous proteins. However, high reactivity may also result in elevated levels of nonspecific reactions. For example, derivatives of N-hydroxysuccinimide (NHS, $pK_a = 6.0$) and pentafluorophenol ($pK_a = 5.5$) activated esters exhibit fast reaction with proteins, but they are not stable in an aqueous buffer and decompose slowly in DMSO, a popular organic solvent used for preparing stock solution.[7] The main reason for the low stability and high reactivity of these two ester groups is due to the low pK_a values of the electrophile. Therefore, it is important to modulate the pK_a value of electrophile in order to create an affinity-based protein labeling probe with optimal reactivity and stability.

In this paper, we report that an ortho-difluorophenyl ester, which shows high stability in DMSO stock solution and aqueous buffer, can be incorporated in affinity-based protein labeling

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probes for the efficient and selective labeling of target protein in complex biological environments (Figure 1a). The reported experimental pKa value for ortho-difluorophenol is 7.51.[8] As compared with the previous ortho-dibromophenol electrophile which has two bulky bromo atoms and a pKa value of 5.59, protein labeling probes based on ortho-difluorophenol should display faster labeling rates and higher stability.[9] Therefore we believe that this difluorophenyl ester reactive module can overcome the shortcomings of many previously reported protein labeling probes. To generate the affinity-based labeling probes for selective, sensitive and rapid protein labeling, a cell-impermeable Cy5 dye was incorporated with 2,6-difluorophenol and the corresponding protein ligand (Figure 1b). Based on this modular design, probe **1** and **2** were synthesized which consist of a binding ligand, arylsulfonamide and biotin, for the specific labeling of human carbonic anhydrase II (hCAII) and streptavidin proteins.[10]

We first examined the selectivity of protein labeling by incubating **1** and recombinant hCAII (0.5 μM each) for 15 minutes in *E.coli* lysate at 37 °C and analyzed by using in-gel fluorescent imaging (Figure 2a). The result showed that a clear single fluorescent band corresponding to hCAII (MW ~32 KDa) appeared when the cell lysate was incubated with **1**. On the other hand, no detectable fluorescence was observed in the presence of 100 μM hCAII inhibitor, ethoxzolamide (EZA), indicating the specific ligand controlled labeling of the difluorophenyl ester probe in complex biological medium. To further prove that the protein labeling occurs solely based on the protein-ligand affinity binding, we also synthesized a controlled reagent **Cy5-DF** which consists of only the reactive

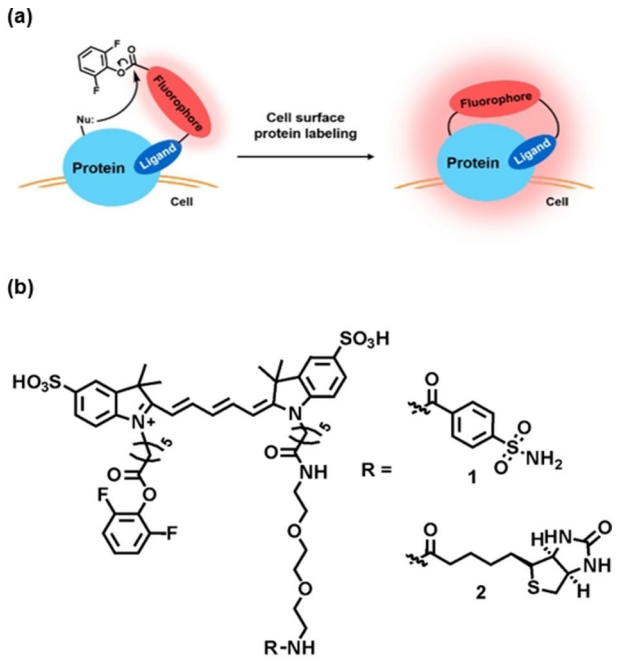

Figure 1. (a) Schematic illustration of an affinity-based protein labeling probe design based on the difluorophenyl ester as a reactive electrophile. (b) Chemical structures of difluorophenyl ester protein labeling probes. **1** and **2** are for human carbonic anhydrase (hCA) and streptavidin proteins, respectively.

difluorophenyl ester linked with a Cy5 dye. No obvious fluorescent band was observed by treating **Cy5-DF** with hCAII protein (Figure S1). Furthermore, we also studied the labeling sensitivity of **1** and found that the probe can label as low as 50 nM of hCAII in just 15 minutes (Figure S2).

Similar to the results from **1**, the labeling of streptavidin (MW ~53 KDa, tetramer) with **2** in *E.coli* lysates displayed a single band which was not observed in the presence of 100 μM biotin (Figure 2b). The labeling selectivity of **1** and **2** were also tested with different non-target proteins (BSA, lactoferrin, RNase A, HSA, and lysozyme) and only in the presence of hCAII and streptavidin showed one distinctive band (Figure S3). For **2**, we also performed the labeling reaction with streptavidin in fetal bovine serum (FBS) which has about 56 mg/mL of total proteins as determined by BCA assay (Figure S4). The results showed that as little as 5 ng of streptavidin can be labeled in FBS. By using MALDI-TOF, we also identified the labeling sites for the recombinant hCAII to be at Lys190 and Lys192, respectively (Figure S5). Overall, these results indicate that our labeling probes based on the difluorophenyl ester electrophile can modify the target protein selectively and rapidly in complex environments containing various non-target biomolecules.

The stability of labeling probes in stock solutions upon long-term storage and freeze/thaw cycles is a fundamental concern for the reproducibility of the results. It is known that many chemical compounds decompose over time when stored in DMSO, which is the most common solvent employed for preparing stock solutions.[11] To examine the chemical integrity of difluorophenyl ester protein labeling probes, we conducted HPLC analysis of **1** stored at −80 °C in DMSO stock solution (Figure 2c and Figure S7). The results from the HPLC chromatogram showed that 97% of **1** remained after 8 months of storage. In comparison, Cy5 derivatized with NHS ester degraded to 76% purity after 1-month storage in the same condition (Figure S8). Furthermore, we also studied the stability of **1** in pH 7.4 PBS buffer (Figure S9). Approximately 80% of **1** remained after 24 hours of incubation. Evidently, the half-life of **1** in aqueous buffer is much longer as compared with the highly reactive NHS and pentafluorophenyl esters reported in the literature.[7b] We believe that the long term stability of the probe in the DMSO stock solution and aqueous buffer can be attributed to the moderate pKa value of difluorophenol.

Next, we analyzed the labeling kinetics of hCAII with **1** by monitoring the increase in fluorescence intensity over time at SDS-PAGE gel (Figure 2d and Figure S10). Full labeling can be achieved after 1 hour of incubation and the second-order rate constant (k_2) was calculated to be about 404 $M^{-1}s^{-1}$. With the given k_2 values, the reaction rate of difluorophenyl ester is comparable to the thiol-maleimide Michael addition labeling.[5b,11] Although the two electrophiles share similar reaction rates, protein labeling using difluorophenyl ester has many advantages over the maleimide electrophile; there are numerous lysine residues on the protein surface to facilitate reactions with the phenyl ester, the formation of a highly stable peptide bond in contrast to the thio-succinimide adduct which often undergo a site-dependent retro-Michael reaction leading to deconjugation, and there is less interference from the

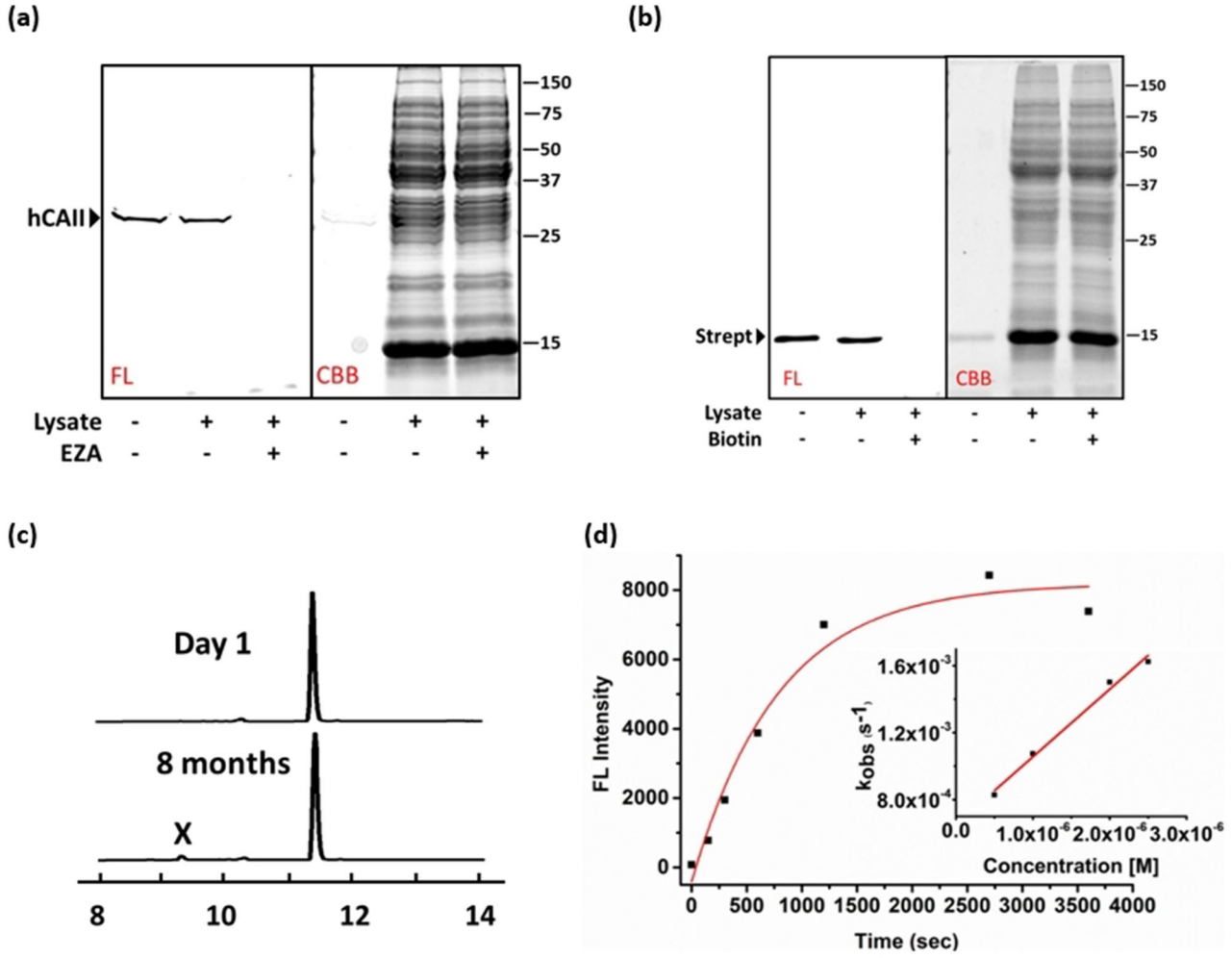

Figure 2. SDS-PAGE in-gel analysis of the labeling of (a) **1** with recombinant hCAII and (b) **2** with streptavidin for 15 minutes in PBS buffer and 6 mg/mL *E. coli* lysates at 37 °C. The concentrations of the probes and proteins are 0.5 μM. The gel was analyzed by in-gel fluorescence imaging (FL) and stained with Coomassie Brilliant Blue (CBB). (c) HPLC trace of probe **1** on day 1 and after storage in DMSO at −80 °C for 8 months. **X** denotes decomposed byproduct of probe **1**. (d) Time course of fluorescence increase for the reaction of hCAII with **1** at 37 °C in pH 7.4 PBS buffer. The inset shows the kinetic plot of the apparent reaction rate constant k_{obs} (s^{-1}) versus various concentrations of **1** ($R^2 = 0.99$).

biological medium which often contains high level of thio derivatives. Moreover, the preparation of protein conjugates using maleimide chemistry can be a cumbersome process. The reducing agents such as dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) used to activate cysteines, can also inactivate maleimide and so must be removed before conjugation. Thus, by introducing the ortho-difluorophenol moiety with moderate electrophilicity as a leaving group, we have successfully created a general affinity-based protein labeling probe approach with rapid protein labeling rate without compromising the stability of the probe.

To demonstrate the application of our protein labeling probes for live cell imaging, we first applied **1** to label HeLa cells with overexpressed recombinant CFP-hCAII-PDGFR protein on the extracellular surface. The transfected HeLa cells were incubated with 0.5 μM **1** for 15 minutes and fluorescence images were taken after removing the excess probe. The fluorescence from the Cy5 channel overlaid very well with the CFP-hCAII-PDFGR protein expressed on the cell surface (Figure 3a and Figure S11). In contrast, addition of EZA reduced the Cy5 fluorescence while the emission from the CFP channel was not affected (Figure 3a, ii). These results indicate that **1** can specifically label and image extracellular hCAII with high specificity.

Carbonic anhydrases (CAs) form a family of enzymes that catalyze the inter-conversion between carbon dioxide and carbonic acid. It has been reported that transmembrane-type human carbonic anhydrases hCAIX (MW ~50 KDa) and hCAXII (MW ~44 KDa) are substantially expressed under the condition of hypoxia in many tumor cell lines which makes hCAs a valuable biomarker for preclinical and diagnostic imaging.[13] To show that our difluorophenyl ester probes are sensitive and selective enough to detect native proteins, **1** was further applied for the imaging and identification of basal transmembrane CA isozyme of MCF7 under normoxia condition (Figure 3a, iii and iv). The imaging results showed that clear Cy5

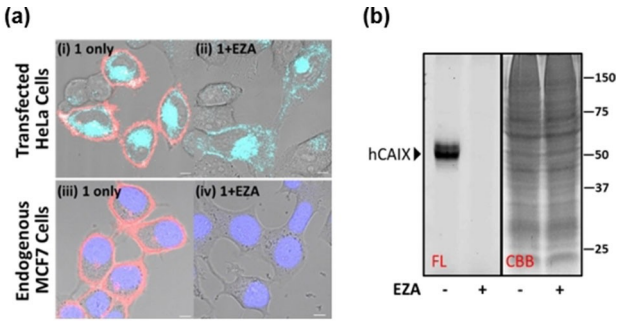

Figure 3. Live cell labeling of overexpressed and endogenous transmembrane carbonic anhydrases with **1**. (a) Live-cell imaging of HeLa cells transfected with CFP-hCAII-PDGFR plasmid and MCF7 cells expressing native transmembrane carbonic anhydrase. CFP and PDGFR encode for cyan fluorescent protein and platelet-derived growth factor receptor, respectively. (i) and (iii) were treated with **1** only, while (ii) and (iv) were with **1** and 100 μM EZA. Images were taken after incubating the cells with 0.5 μM **1** for 15 minutes at 37 °C in DMEM medium. Scale bar: 20 μm. (b) SDS-PAGE in-gel fluorescence analyses of the labeling of endogenous hCAIX with 0.5 μM **1** in living MCF7 cells in the absence or presence of 100 μM EZA.

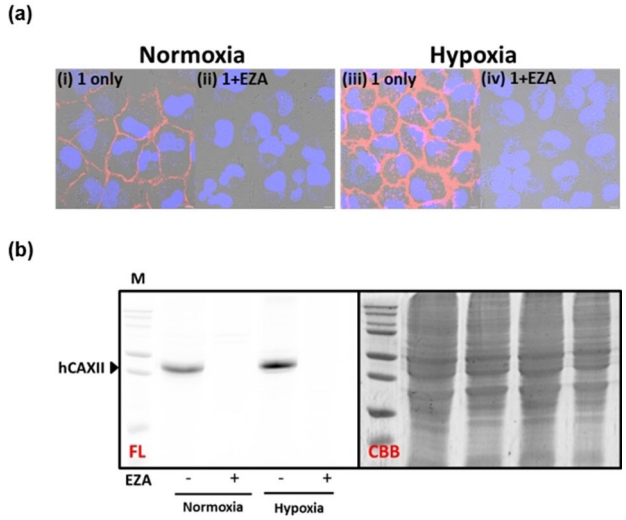

Figure 4. Live cell labeling of normoxia and hypoxia-induced carbonic anhydrase isozymes of A549 cells with **1**. (a) Images of normoxia and hypoxia-mimetic A549 cells labeled with 0.5 μM **1** for 15 minutes in DMEM medium. (i) and (iii) were treated with **1** only, while (ii) and (iv) were with **1** and 100 μM EZA. Scale bar: 20 μm. (b) In-gel fluorescence analyses of the labeling of living A549 cells with 0.5 μM **1** for 15 minutes in the absence or presence of EZA inhibitor. The cells were cultured under normoxia and hypoxia-mimetic conditions, respectively. M = Molecular weight marker.

fluorescence along the plasma membrane were observed for the MCF7 cells treated with **1** and were not detected in the presence of EZA inhibitor (Figure S12). To characterize the extracellular CA isozymes, the MCF7 cells labeled with **1** were lysed and identified by in-gel fluorescence and Western blot analysis. The fluorescent gel showed that distinct fluorescent bands at around 50 KDa can be observed clearly and was not visible in the presence of EZA (Figure 3b). It is important to note that hCAIX contains a unique N-linked glycosylation site that bears high mannose-type glycan structures. As a result, multiple fluorescent bands can be observed with in-gel fluorescent analysis. In line with the in-gel fluorescence results, Western-blot bands were observed for MCF7 cells with anti-hCAIX antibody and not with anti-hCAXII antibody (Figure S13). These results are consistent with many previous reports which indicate that the major extracellular CA isozyme for MCF7 cells is hCAIX.[13b,14]

It has been reported that A549 cells express higher level of hCAXII proteins when cultured under hypoxia conditions.[13a,15] Since the sulfonamide ligand has similar binding affinities to hCAIX and hCAXII, the relative abundance of hCAXII under normoxia and hypoxia conditions can be determined by measuring the fluorescence intensity of the **1**-labeled hCAXII bands. Thus, we applied **1** to label A549 cells cultured under normoxia and hypoxia-mimetic conditions to determine the relative level of hCAXII. To induce the overexpression of hCAXII, A549 cells were cultured in the presence of 200 μM $CoCl_2$ for 24 hours to mimic the hypoxia condition.[16] As shown in Figure 4a, stronger fluorescence signals were observed on the cell membrane of the hypoxia-mimetic A549 cells (Figure S14). To estimate the relative levels of hCAXII, the **1**-labeled cells were lysed and analyzed by in-gel fluorescence imaging and quantified using ImageJ software (Figure 4b). In contrast to MCF7 cells in which hCAIX is the major isozyme, a fluorescent band at around 44 KDa was observed which corresponds to the molecular weight of hCAXII. Under the hypoxia conditions, the expression level of hCAXII was increased by about 35%. For comparison, western-blot analysis showed that hypoxia-A549 cells expressed about 30% higher level of hCAXII (Figure S15). These results are in accordance with many previous findings which indicate that higher level of hCAXII was found in A549 cells under hypoxia condition.[13d,14,17] Thus, cell-impermeable **1** can be harnessed as a new sensitive and selective protein labeling probe to study the expression of different transmembrane hCAs as demonstrated by the quantitative analysis of the hCAXII level under basal and stimulated conditions.

In summary, we have developed a stable yet reactive affinity-based protein labeling probe approach based on the ortho-difluorophenyl ester for the selective and rapid labeling of target proteins in the complex biological environments. The moderate pK_a value of difluorophenol imparts long-term stability of the probe in DMSO stock solution and aqueous buffer, while maintaining high labeling reaction efficiency with its target protein. Owing to the modular design and the versatile synthetic scheme, different ligands can be customized easily on the probes to label various natural (or engineered) proteins with nucleophilic amino acids located near the ligand binding site, as shown in the cases of streptavidin and hCAs. With the cell-impermeable sulfonamide probes, we have also successfully shown that the major CA isozymes expressed on the cell surface of MCF7 and A549 cells to be hCAIX and hCAXII respectively. Furthermore, the sensitivity of proteins labeling was comparable to the standard protein detection methods, such as the Western blot technique. We envision that the extension of this difluorophenyl ester reactive module should allow for multi-color imaging of various endogenous membrane

proteins in living cells, thereby enhancing its applications in drug discovery in the future.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Protein labeling · Membrane proteins · Hypoxia · Fluorescent Imaging · Difluorophenyl ester

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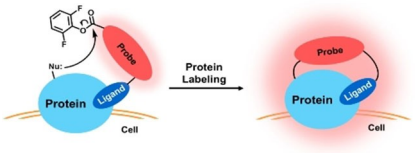

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