Turn-on fluorescence switch involving aggregation and elimination processes for β-lactamase-tag†

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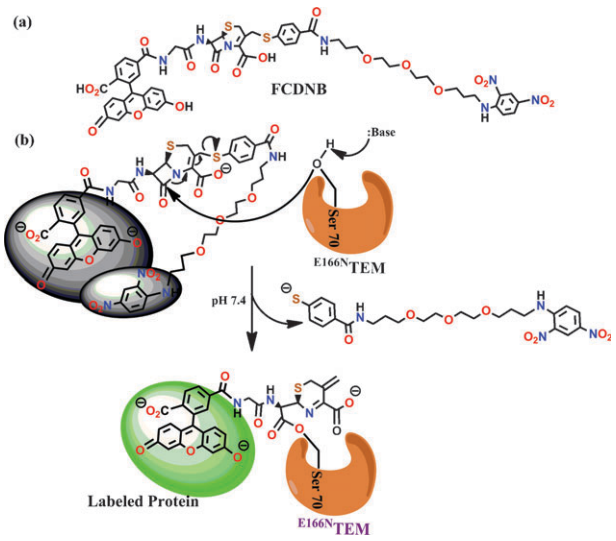
The targeted protein of interest is fused with genetically modified β-lactamase enzyme, which reacts with the probe in physiological conditions to break the aggregated interaction between the fluorophore and quencher. This alliance–separation technique is new for protein labeling and is probed *in vitro* and in live cell imaging studies.

Molecular imaging with the aid of fluorescence microscopy has become an indispensable means to study the proceedings in living cells, which would be extensively valuable for the future of medical science. The practical approach involving chemistry to control modification and monitoring specific proteins has proved useful.[1] The direct imaging of protein interactions in single living cells is possible due to the site-specific labeling of proteins with molecular tags, which is an authoritative means for studying the structure–function relationships of proteins.[2] The rationale of fluorescence microscopy in cell biology has been thoroughly tailored with fluorescence proteins.[3] To conquer the shortcomings intrinsic to proteins due to the lack of robust modification for target phenomena and nonfluorogenicity, bright and photostable small fluorophores can be advantageous over their fluorescent protein counterparts.[4] Tetracysteine-tag and its modified version provided a route for using small molecules in fluorogenic labeling and were found to be effective for activation of G protein-coupled receptors in living cells.[5] Specificity towards labeling was observed with SNAP-tag and its modified version with semisynthetic fluorescent sensor proteins.[6] In our protein labeling method our aim was to combine fluorogenicity and specificity of the probe. Several other protein-[7] and enzyme-mediated[8] labeling methods have enriched the recent literature. Among the small fluorogenic molecules, fluorescein is approved by the U.S. Food and Drug Administration (FDA) for medical use.[9] Herein, we have chosen fluorescein for our protein labeling experiment.

Recently we have shown[10] a protein labeling system that coalesces genetically modified β-lactamase (BL-tag) with low molecular weight fluorogenic β-lactam probes. The study concerned FRET to optimize the emission of the probe. A shortcoming of the strategy involved the suitable choice of donor fluorophore, acceptor quencher and their application in physiological conditions. To surmount the restraint in the FRET process, our new approach is based on the switching mechanism involving aggregation followed by elimination processes. In the modified version, the quenching ability of the fluorescence quencher does not depend upon the emission wavelength of the fluorophore, as is desired for an effective FRET process. In this case the quenching phenomenon is the intrinsic property of the quencher part.

We have continued our study with the same BL-tag protein. The reaction of TEM-1 (class A β-lactamases) with β-lactam rings involves acylation and deacylation steps. Glu166 is indispensable for the deacylation step,[11] and the mutant version of TEM-1 (^{E166N}TEM or BL) restricts the deacylation step.[12] Our aim was to exploit the properties of BL for covalent attachment with a fluorescent substrate with a better versatile technique. Here, we present a rationally designed fluorogenic probe that takes advantage of the aggregation of fluorophore and quencher. In the aggregated form, the interaction between the fluorogenic part and the quencher restricts the emission of the fluorophore. The viability of the fluorescently labeled BL-tag under physiological situations has been explored with the newly designed and synthesized a cephalosporin-based fluorescent probe (**FCDNB**, Scheme 1a). We have used the *m*-dinitrobenzene (DNB) group as quencher, whose quenching efficiency is well established.[13] The probe contains 6-carboxyfluorescein as fluorophore and DNB as quencher and they are connected to different sides of the β-lactam ring of the central cephalosporin part. A flexible spacer can control the emission[14] and enzyme activity prompts



Scheme 1 Labeling strategy with (a) structure and (b) labeling state of the fluorescent probe **FCDNB**.

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the fluorescence recovery. This inspired us to introduce polyethylene glycol in the newly developed system for better aggregation interaction and solubility in physiological conditions. Incubation with BL-tag protein leads to the subsequent elimination of quencher to label the covalent modification of the tag protein with the desired fluorophore. Using this modification, we can achieve a new and better route to a turn-on fluorescence protein labeling system compared to our previous FRET analogue under the same physiological conditions. The advantage of this design principle is robustness towards fluorescein, whose fluorescence intensity was not easily modified by the previous design,[10] since the quenching mechanism is due to simple molecular interactions.

The labeling mechanism is illustrated in Scheme 1b. Cleavage of the β-lactam group of **FCDNB** by Ser70 in BL produced covalent labeling of the fluorescein to the protein with concomitant release of DNB. The labeled protein was detected by irradiating the gel with visible light (λ = 470 nm) in an SDS-PAGE study. A protein band of ~29 kDa was observed, exhibiting green fluorescence (Fig. 1a). To confirm that the labeling activity is due to the BL-tag, we carried out a similar experiment with wild-type (WT) TEM-1. In contrast to BL, incubation of WT TEM with **FCDNB** ended with no labeled fluorescence. Labeling in a biological medium with HEK293T cell lysate was also checked. SDS-PAGE analysis confirmed competent and selective labeling of **FCDNB** (Fig. 1b).

Compound **8** (Fig. S2†) with only DNB quencher showed maximum absorption at 364 nm and negligible absorption after 475 nm in HEPES buffer, whereas there is no substantial emission of fluorescein in this region (Fig. S3†). The probability of FRET from fluorescein to the DNB group can be overruled due to the insignificant overlap of fluorescein emission and DNB absorbance. The absorption peaks of fluorescein and the DNB group in **FCDNB** were obtained at 507 nm and 375 nm, respectively, in HEPES buffer. In methanol, the absorption peaks for both fluorescein and DNB were shifted by 10–15 nm (Fig. S4†). These positional shifts suggest a different nature of interaction between the fluorophore and quencher in the studied media.

The fluorescence quantum yield of **FCDNB** was found to be 0.05 in 100 mM HEPES buffer. This result confirmed that the

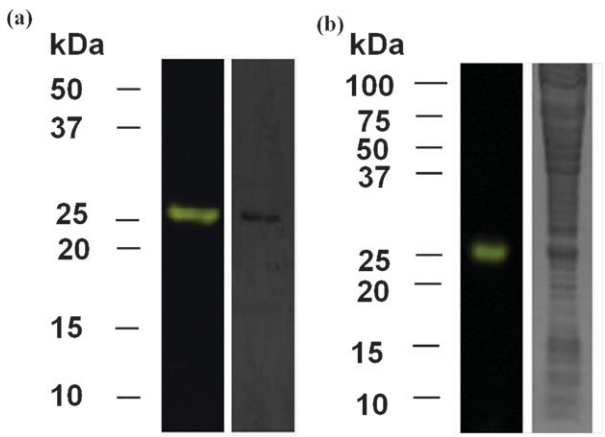

Fig. 1 **FCDNB** incubated fluorescence (left) and CBB-stained (right) gel images of (a) BL and (b) BL mixed with HEK293T cell lysate.

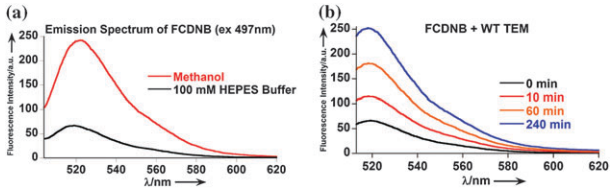

Fig. 2 (a) Emission spectra of **FCDNB** in 100 mM HEPES buffer (pH 7.4) and methanol (conc. of **FCDNB** 0.5 μM). (b) Time-dependent emission spectra (λ_{ex} = 507 nm) of **FCDNB** in the presence of WT TEM in 100 mM HEPES buffer (pH 7.4) containing 0.05% DMSO at 25 °C.

fluorescein emission was sufficiently quenched because of intramolecular interaction between the fluorophore and DNB group. 2,4-Dinitroaniline is an efficient intramolecular fluorescence quencher for fluorescein labeled oligonucleotides.[15] The mechanism of fluorescence attenuation can be attributed to electron-rich aromatic rings that can π-stack with the electron-poor nitroaromatics.[16] The inherent quenching phenomenon of DNB does not depend upon the nature of fluorophores, rather it has been found effective in the case of several fluorogenic probes.[17] The quantum yield of **FCDNB** in methanol is 0.42. This significant difference of emissions in these two solutions (Fig. 2a) implies that the aggregation phenomenon is favorable in the physiological conditions only. Buffer comparison studies of **FCDNB** and the DNB-free version of the probe (**FA**, Fig. S5†) showed that **FCDNB** is sufficiently quenched in each buffer medium (Table S1†). A pH probe fluorescence assay of **FCDNB** (Fig. S6†) suggests that emission from fluorescein is more effective at physiological conditions compared to in acidic medium.

The effects of BL-tag and WT TEM on the emission properties of the probe were studied. The fluorescence intensity at 520 nm due to fluorescein was monitored after the incubation of BL with **FCDNB**. Slow enhancement of the fluorescence intensity was observed with time (Fig. S7†). This indicates that the cleavage of the β-lactam of **FCDNB** was performed by BL but the disaggregation followed by elimination of the DNB group is very slow. The process is significantly different in case of the incubation study with WT TEM. The DNB group was eliminated by WT TEM with a much faster rate and the fluorescence signal increased a considerable amount (Fig. 2b) as expected due to the favorable deacylation step of the catalytic activity. The change in fluorescence enhancement was found to be slow after 4 h. The initial rate of fluorescence enhancement in the case of WT TEM was found to be ~20 times that of the BL-tag. This noticeable difference in the rate of reaction is also due to the controlled acylation path as a result of mutation at Glu166.[10] In comparison, emission intensities of fluorescein in **FA** remain the same with time for both WT TEM and BL-tag.

The site-specific labeling of target proteins has been probed by the demonstration of **FCDNB** on the surface of living cells (Fig. 3a). For this purpose we have chosen the N-terminus of epidermal growth factor receptor (EGFR). The target fluorophore recognizes the transfected protein as a result of covalent bond formation. The BL-tag fused to the EGFR, and then it was expressed with HEK293T cells. After treatment with **FCDNB**, fluorescence images of the HEK293T cells were

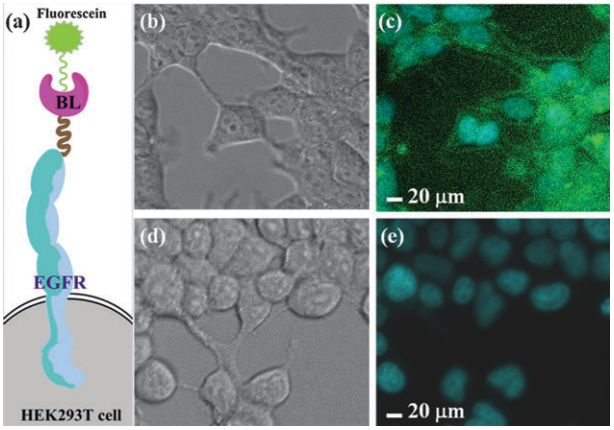

Fig. 3 (a) Labeling of protein with the probe through the BL-tag. (b)–(e) Optical microscopic images of **FCDNB**-labeled HEK293T cells expressing (b,c) BL-EGFR and (d,e) EGFR. (b,d) DIC images, (c,e) fluorescence microscopic images. The cell nuclei were stained with Hoechst 33342. For fluorescence microscope images, the cells were excited at 330–385 nm for Hoechst 33342, and 460–490 nm for **FCDNB**.

taken under an inverted fluorescence microscope. The cell nuclei were stained with Hoechst 33342. Only the cells expressing the BL-EGFR fusion protein emitted green fluorescence as a consequence of specific labeling by the probe (Fig. 3c). In the case of HEK293T cells expressing the EGFR protein without any BL-tag, the cell nuclei show only cyan fluorescence due to Hoechst 33342. In this case no fluorescein-labeled cell was observed (Fig. 3e).

In conclusion, we have modified our protein labeling method with an improved and straightforward technique that merges a BL-tag with a low molecular weight fluorogenic β-lactam probe. Through appropriate modification of our probe design, we succeeded in labeling targeted proteins with a familiar and useful fluorophore *in vitro* and also in living cells. Despite the similarity in molecular weight of the BL and Green Fluorescent Protein (GFP), fluorogenicity can be introduced through BL-tag technology, which is rather impossible with GFP. In this tailored version, the system can be considered as preliminary proof of a newly developed principle. By introducing a more efficiently quenched probe, this system can solve the problem of washing procedures after the labeling method. We are presently engaged in the aspect of the versatile use of this customized *modus operandi* with a range of fluorophores.

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