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Targeted pH-dependent fluorescent activity-based cathepsin probes†‡

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Bifunctional, pH-activatable BODIPY dyes were developed and incorporated in mannose cluster-containing activity-based probes for cysteine proteases. Mannose receptor-dependent uptake of the probes in dendritic cells, followed by trafficking to acidic cellular compartments resulted in fluorescence as seen by live-cell imaging, and subsequent cathepsin inhibition.

Fluorescent dyes are applied in many different areas of chemical biology. Among these dyes, boron-dipyrromethene (BODIPY) derivatives are frequently used due to their excellent photochemical properties and relative stability under physiological conditions.[1,2] For example, covalent attachment of a BODIPY dye to an activity-based probe (ABP) facilitates the study of its target enzyme, by means of fluorescence scanning and microscopy.[3–7] Urano *et al*. recently developed a series of acidic pH-activatable BODIPY dyes.[8] Due to photoinduced electron transfer (PeT) of the *meso* aniline substituent toward the BODIPY fluorophore, fluorescence is quenched at neutral or basic pH.[9–11] Upon protonation of the aniline nitrogen, fluorescence is restored.

Depending on the choice of alkyl substituents on the aniline nitrogen, the pK_a and thus the pH-dependency of the fluorophore can be tuned.[8] A great advantage of these kind of fluorophores is that they do not fluoresce when unprotonated, making them convenient tools to study in-cell processes with fluorescence microscopy. Since a (slightly) acidic pH is a prerequisite for fluorescence, these fluorophores are ideally suited for incorporation in probes that are transported to acidic cellular compartments like lysosomes. In a previous study, we have shown that lysosomal targeting of the epoxysuccinate activity-based probe DCG-04[12] can be accomplished by attachment of a synthetic mannose cluster.[3]

This mannose cluster binds to the mannose receptor, which is predominantly present on professional antigen-presenting cells such as dendritic cells and macrophages. Mannose receptor-mediated internalization of the construct is followed by trafficking in the endocytic pathway towards the lysosomes. Subsequently, multiple lysosomal cysteine proteases of the cathepsin[13] family are covalently and irreversibly addressed by the ABP. We reasoned that incorporation of a pH-activatable fluorophore in our construct would result in less background because of selective fluorescence in the cellular compartments of interest. Hence, we designed a set of bifunctional pH-sensitive dyes that allowed orthogonal ligation to the mannose cluster and DCG-04 amine (Fig. 1). We here report the development of three different pH-activatable fluorescent activity-based cathepsin probes that are selectively taken up by professional antigen-presenting cells *via* the mannose receptor.

We followed a modular synthetic approach, in which the constructs are assembled from three building blocks in the two final steps (Fig. 1A). The mannose cluster and cathepsin inhibitor DCG-04 amine were synthesized as previously reported.[3,12] To obtain bifunctional pH-dependent BODIPY dyes, the synthetic route of Urano and coworkers[8] was modified and extended, yielding azido-BODIPY-OSu intermediates **3a–d** (Scheme S1, ESI‡). Condensation of BODIPYs **3a–d** and DCG-04 amine followed by ligation to the mannose cluster by copper(I) catalysed Huisgen 1,3-cycloaddition[14,15] gave constructs **5a–d** (Fig. 1A).

To assess the fluorescence properties of the dyes, fluorescence-pH curves of probes **5a–d** in a citric acid/phosphate buffer system were measured (Fig. 2A and B). The resulting curves for **5a–c** (Fig. 2B) were shifted to the left as compared to the curves reported by Urano *et al*.,[8] indicative of a shift in pK_a values. Upon addition of a low amount (0.2%) of the detergent sodium dodecyl sulphate (SDS) the values resembled the expected pK_a's. Since the pH of the lysosomes in antigen presenting cells such as macrophages is estimated to be 4.5–4.9[16] we expected that probe **5c**, with pK_a $\sim$ 5.1 would be the best candidate for imaging in living cells.

The ability of probes **5a–d** to label lysosomal cysteine proteases was first examined by incubation of mouse liver lysates with various concentrations of the construct, followed by resolution of the proteins on SDS-PAGE. We managed to visualize the bands by overnight incubation of the gel in acidic fixing solution (MeOH/H_2O/acetic acid), thereby lowering the pH to allow in-gel fluorescence scanning. Labeling is concentration-dependent and the profile corresponds well with that seen for our previously synthesized probe, with a MW-shift of 3–4 kDa compared to BODIPY-DCG-04[3] (β-DCG-04; Fig. S2, ESI‡), consistent with the molecular weight of the

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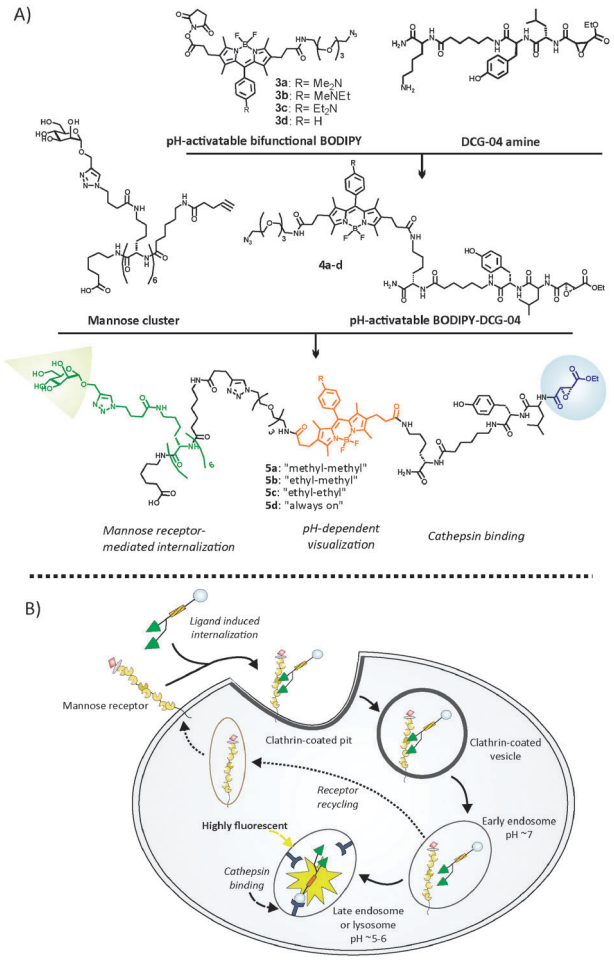

Fig. 1 Synthesized pH-activatable mannose cluster-BODIPY-DCG-04 constructs (A) and a schematic overview of their expected internalization and fluorescence properties in mannose receptor expressing cells (B).

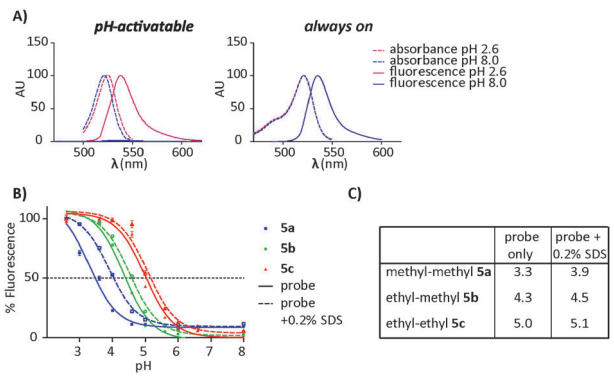

Fig. 2 pH-dependency of compounds **5a–c**. (A) Normalized absorption and emission spectra of pH-activatable compounds **5a–c** and the "always on" control **5d**. (B) Fluorescence measurements as a function of pH in citrate/phosphate buffer with or without 0.2% SDS. (C) Apparent pK_a values (50% fluorescence) as determined by the curves shown in (B).

probes (Fig. 3A, Fig. S2, ESI‡).[3] In a competition experiment with β-DCG-04 it was shown that the constructs competed for the same set of cysteine proteases.

Next, the behaviour of probes **5a–d** in living cells was investigated. Immature mouse dendritic cells (DC's) were incubated with 1 μM of probe and studied with live-cell fluorescence microscopy. Brightly fluorescent vesicles appeared inside the cells after 30 min of incubation, with increasing fluorescence upon prolonged exposure to the probe (Fig. S1A, ESI‡). In contrast to the "always on" construct **5d**, no background signal was detected for the pH-activatable probes **5a–c** and therefore cells could be imaged without any additional washing steps (Fig. S1C, ESI‡). In accordance with the observed pK_a values, the signal for the methyl–methyl (**5a**) and methyl–ethyl (**5b**) probes was less strong and took longer to appear. This clearly indicates that the probes were indeed trafficked to increasingly acidic cellular compartments over time. Subsequent cell lysis and SDS-PAGE analysis showed a labeling profile of cysteine proteases identical to that found in lysates (Fig. 3C and Fig. S2B, ESI‡). Dendritic cells, being professional antigen-presenting cells, can take up macromolecules from the environment by macropinocytosis.[17] Therefore, incubation times for follow-up experiments were set to two hours, to diminish the amount of probe internalized by means other than mannose receptor-mediated internalization. Indeed, under these conditions, pre-incubation of the cells with mannan, a naturally occurring polymannoside that binds the mannose receptor, abolished the uptake of the probes and subsequent labeling of cathepsins (Fig. 3B/C, cond. *ii*). Pre-incubation of the cells with non-fluorescent azido-DCG-04 did not prevent the probes from entering the cell, as seen by live-cell imaging. However, no labeling of cathepsins was seen on SDS-PAGE, indicating that the probes were taken up by the mannose receptor, trafficked to the lysosomes, but unable to bind to, the already blocked, cathepsins (Fig. 3B/C, cond. *iii*). Interestingly, in-cell fluorescence remained even after prolonged washing of the cells (Fig. 3B, cond. *vi*). To further investigate this, cells were lysed after washing and analyzed by SDS-PAGE. As shown in Fig. 3C, cond. *vi* (lane 8), labeling of cathepsins was largely restored. Also, more cathepsins were labeled after overnight washing compared to immediate lysis in the control experiment (Fig. 3C, cond. *v*, lane 7 and Fig. S2B, ESI‡). Because of their size, the probes were probably retained in the cells, where they could bind any newly formed cathepsins.

In a next experiment, the intracellular pH was increased by addition of ammonium chloride to the medium.[18] Ammonium chloride passively diffuses into the cell and acts as a weak base, thereby increasing the pH up to 6 in lysosomes in a reversible manner.[16,19] Almost no in-cell fluorescence was detectable for both the ethyl-ethyl (**5c**) and control probe (**5d**) after pre-incubation with 10 mM NH_4Cl, even after extensive washing at normal pH, indicating that the levels of internalized probe were low (Fig. 3B, cond. *iv*, Fig. S3, ESI‡). This is in accordance with the postulation of Tietze *et al.*, that an increased cellular pH decreases receptor recycling, which leads to lower levels of cell surface receptors.[20] SDS-PAGE analysis on the other hand, revealed that not all cathepsin activity was gone, as seen by, albeit weak, labeling on gel (Fig. 3C, cond. *iv*, lane 6 and Fig. S2, ESI‡). This finding illustrates the efficiency with which the probes were internalized and processed in the endocytic pathway.

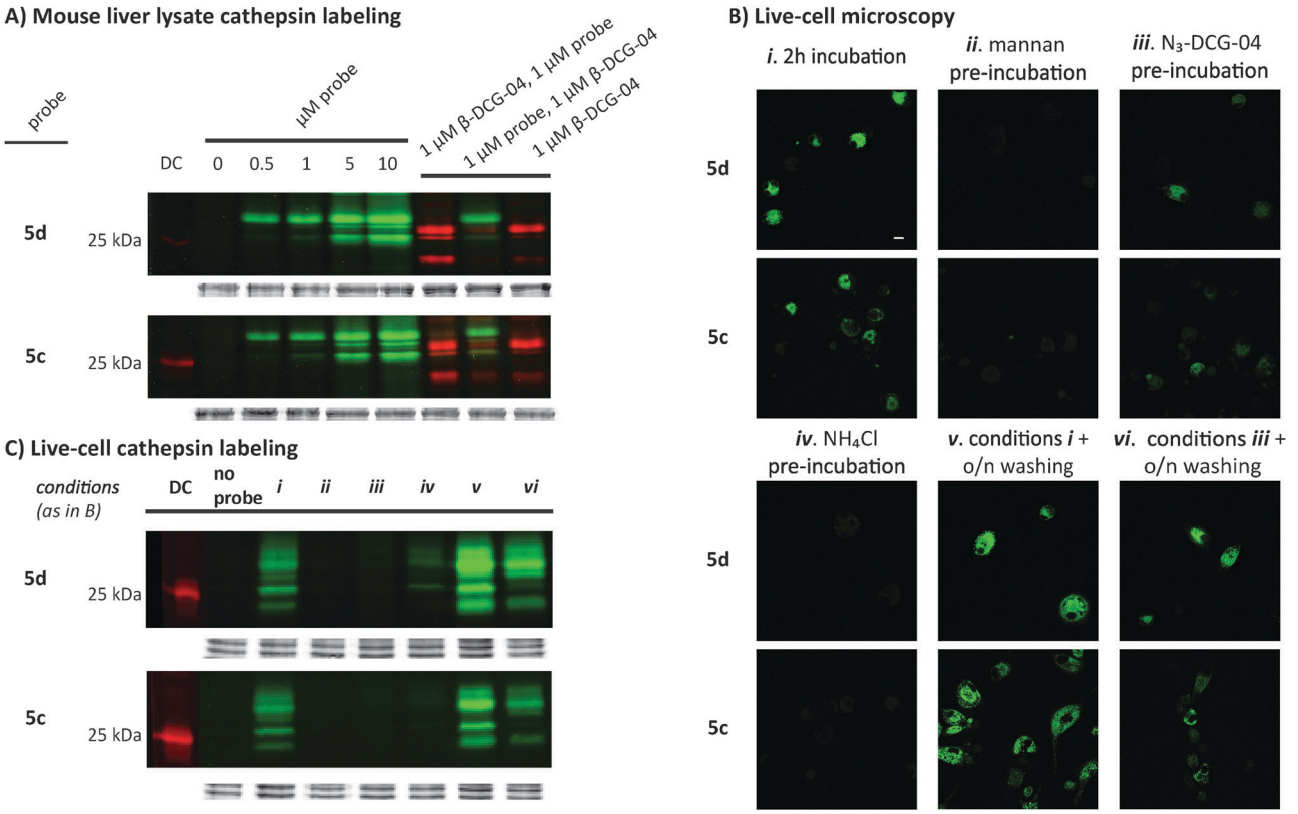

Fig. 3 Biological evaluation of the constructs **5c–d**. (A, C) 12.5% SDS-PAGE analysis; fluorescence scanning images (upper) and coomassie staining for total proteins (lower) are shown. DC: dual color prestained protein marker (see also Fig. S4, ESI‡). (A) Incubation of mouse liver lysate shows concentration dependent labeling of cathepsins (lanes 2–6). The probes compete for the same set of cathepsins as the known cathepsin inhibitor β-DCG-04 (red bands, lanes 7–9) and the bands are shifted consistent with the molecular weight of the probes. (B) Confocal microscopy images of DC's incubated for 2 h with probes **5c** or **5d**, under different conditions (*i–vi*) show mannose receptor dependent uptake of the constructs. Scale bar (white) corresponds to 10 μm. (C) Cell lysis and analysis of cells treated as in (B) shows inhibition of cathepsin labeling by azido-DCG-04.

In summary, in the present study we showed that pH-activatable fluorophores can be incorporated in large mannose cluster containing ABPs, thereby facilitating their imaging without influencing their uptake or distribution in dendritic cells. We believe that these kind of molecules might find use in studies concerning antigen cross-presentation and the role of cysteine proteases herein. Moreover, the ease of access to our panel of bifunctional, tunable, pH-activatable dyes should allow their incorporation in bioconjugates quite different from those presented here. In this fashion, we foresee that alternative receptors can be targeted to specifically deliver cargo to endocytic compartments with the aim to interfere with a variety of subcellular targets.

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