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Synthesis and evaluation of new NIR-fluorescent probes for cathepsin B: ICT versus FRET as a turn-ON mode-of-action

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ABSTRACT

Recent years have seen tremendous progress in the design and study of molecular imaging geared towards biological and biomedical applications. The expression or activity of specific enzymes including proteases can be monitored by cutting edge molecular imaging techniques. Cathepsin B plays key roles in tumor progression via controlled degradation of extracellular matrix. Consequently, this protease has been attracting significant attention in cancer research, and many imaging probes targeting its activity have been developed. Here, we describe the design, synthesis and evaluation of two novel near infrared (NIR) fluorescent probes for detection of cathepsin B activity with different turn-ON mechanisms. One probe is based on an ICT activation mechanism of a donor-two-acceptor π-electron dye system, while the other is based on the FRET mechanism obtained by a fluorescent dye and a quencher. The two probes exhibit significant fluorescent turn-ON response upon cleavage by cathepsin B. The NIR fluorescence of the ICT probe in its OFF state was significantly lower than that of the FRET-based probe. This effect results in a higher signal-to-noise ratio and consequently increased sensitivity and better image contrast.

Protease activity is an important necessary factor for cell proliferation. Numerous tumor types have been shown to contain elevated levels of proteolytic enzymes, presumably in adaptation to rapid cell cycling and for secretion in order to sustain invasion, establish metastasis and promote angiogenesis.[1,2] Cathepsin B is a lysosomal cysteine protease involved in cellular protein turnover and degradation, which is overexpressed in many tumors, as well as by host cells at the tumor microenvironment. Its high expression at tumor sites compared with that of its basal expression in healthy tissues makes it an attractive target for tumor-selective treatment and diagnosis.[3] Recent years have seen tremendous progress in the design and study of molecular imaging geared towards biological and biomedical applications Expression and activity of specific enzymes including proteases can now be monitored by various molecular imaging techniques,[4] including optical imaging in the near-infrared (NIR) region.[2] This technique enables detection of molecular activity intravitally thanks to high penetration of NIR photons through organic tissues and low autofluorescence background. Various NIR imaging probes were designed and applied for diagnostics of a variety of diseases. Among them are enzyme-sensitive probes, which can change their signal intensity in response to specific enzymes at targeted sites. Cyanine dyes are widely employed as fluorescence markers for NIR-imaging probes, since they are compounds with large extinction coefficient, relatively high quantum yield and photostability.[5]

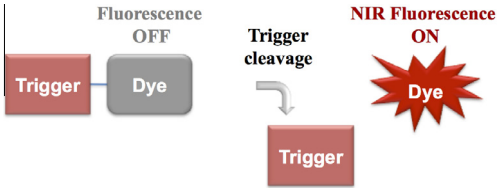

Figure 1. Illustration of the general design and mode of action of an ICT-based fluorescent probe.

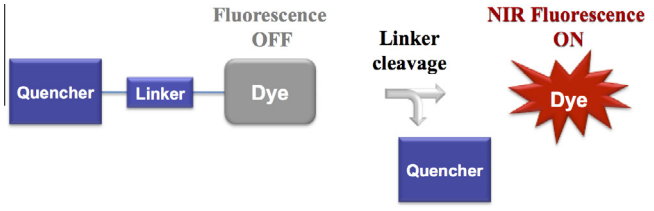

Figure 2. Illustration of the general design and mode of action of a FRET-based turn-ON probe.

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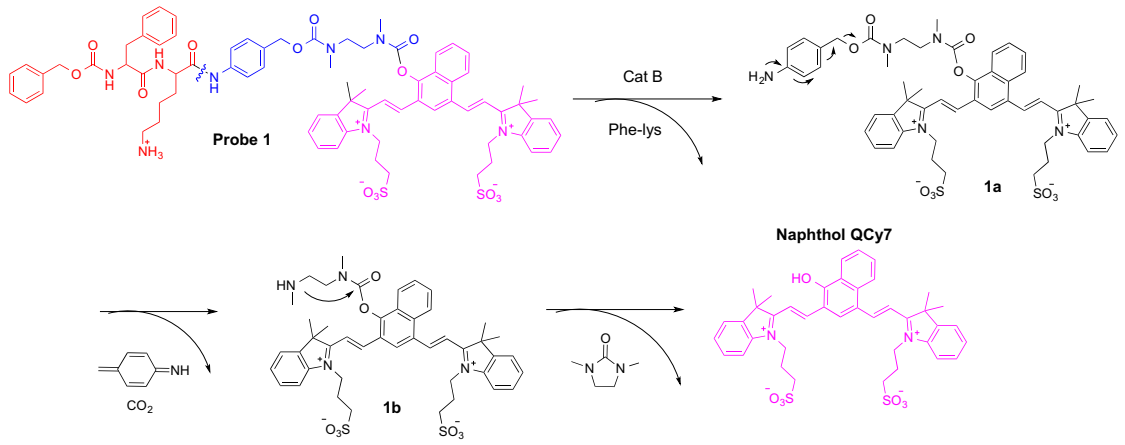

Figure 3. Chemical structure and activation mechanism of the ICT-based fluorescent probe for cathepsin B with the cyanine dye naphthol-QCy7.

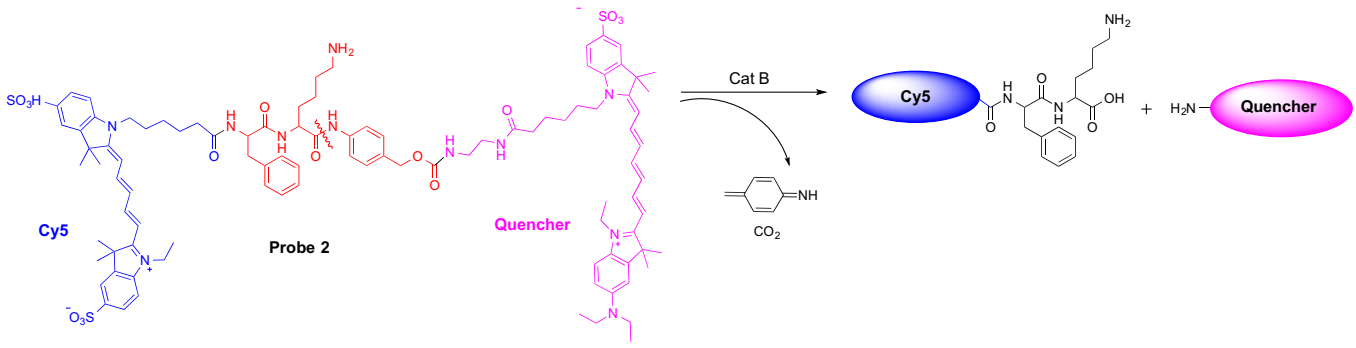

Figure 4. Chemical structure and activation mechanism of a FRET-based cathepsin B fluorescent probe with the cyanine dye Cy5.

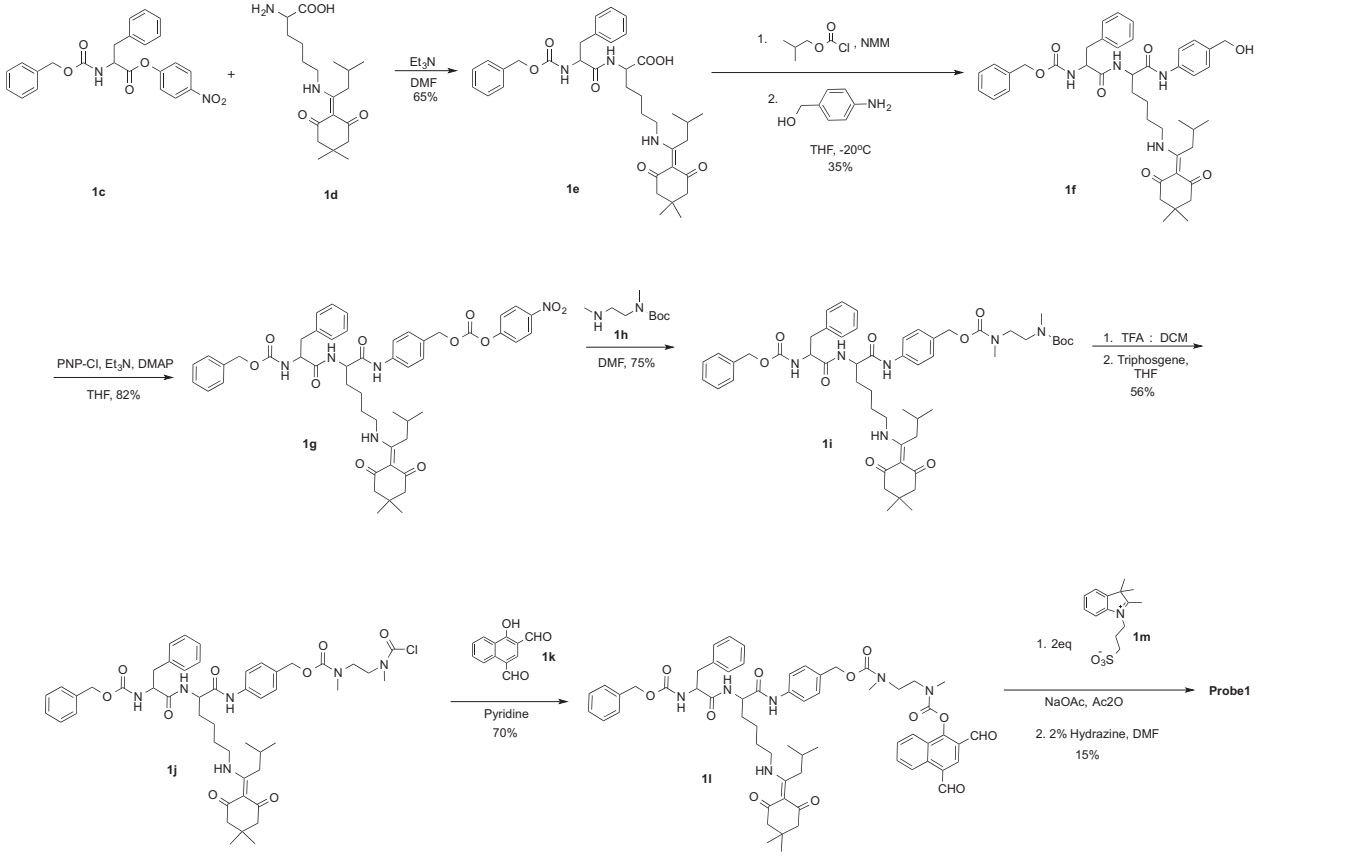

Figure 5. Chemical synthesis of probe **1**.

There are several known approaches to turn-ON a fluorescent probe such as Förster Resonance Energy transfer (FRET), Photo–induced Energy Transfer (PET), and Internal Charge Transfer (ICT).[6–8] Here, we report the synthesis and activity evaluation of two new NIR-fluorescent cyanine-probes for detection of cathepsin B; one is based on an ICT turn-ON mode and the other on a FRET turn-ON mechanism.

A fluorescent ICT-based probe is usually composed of a dye, masked by a substrate that acts as a trigger (Fig. 1). The substrate is attached to a functional group of the dye and thus, masks the optical signal by decrease of π-electrons conjugation. Removal of the triggering substrate by an analyte or an enzyme of interest, results in release of the free dye and activation of an optical signal, such as NIR fluorescence.

FRET-based probes can be composed of a fluorescent dye attached through a specific linker to a quencher (Fig. 2).[9,10] Under such circumstances, the excited fluorophore transfers its excitation energy to the nearby quencher-chromophore in a non-radiative manner through long rang dipole–dipole interactions. Cleavage of the linker moiety by the analyte or enzyme of interest, results in diffusion of the fluorescent dye away from the quencher and thereby, generation of a measurable fluorescent signal.

We have recently reported a novel strategy for the design of long-wavelength fluorogenic probes with a turn-ON option.[11,12] Their design is based on a donor-two-acceptor π-electron cyanine dye that can undergo an internal charge transfer to form a new fluorochrome with an extended π-conjugated system. This strategy was translated into synthesis of a library of dyes with fluorescence emission in the NIR region.[13] One dye, naphthol-QCy7, composed of naphthol donor and two indolium acceptors, was found to have superb optical characteristics in terms of NIR fluorescence efficiency.[14] Thus, we have selected naphthol-QCy7 as a fluorescent dye for preparation of a turn-ON probe for cathepsin B. The chemical structure and activation mechanism of the probe are presented in Figure 3. Probe **1** is composed of the dipeptide Z-Phe-Lys (red) as a substrate for cathepsin B, attached to naphthol-QCy7 (pink) through a self-immolative linker[15] (blue). Cleavage of the amide linkage between the dipeptide and the linker by cathepsin B, results in the formation of aniline **1a**. The latter undergoes 1,6-elimination followed by decarboxylation to release amine **1b**. This amine spontaneously undergoes cyclization reaction to form a cyclic urea and to release the free naphthol-QCy7.

Next, we sought to prepare a FRET-activated probe for cathepsin B with a cyanine dye. The chemical structure and the activation mechanism of such a probe based on a FRET technique are presented in Figure 4. The probe is composed of Cy5 fluorescent dye attached through a cathepsin B substrate to a quencher dye. Similarly, as was described for the ICT-probe, the selected cathepsin B substrate was the dipeptide Phe-Lys. The NH_2-terminus of the dipeptide was linked to Cy5 and the COOH-terminus was linked through a short self-immolative spacer[16,17] to the quencher (Fig. 4). Cleavage of the amide linkage after the lysine, followed by 1,6-elimination of the self-immolative spacer, results in separation between the Cy5 and the quencher. Consequently, a turn-ON NIR fluorescent signal should be obtained from the emission of the Cy5 dye.

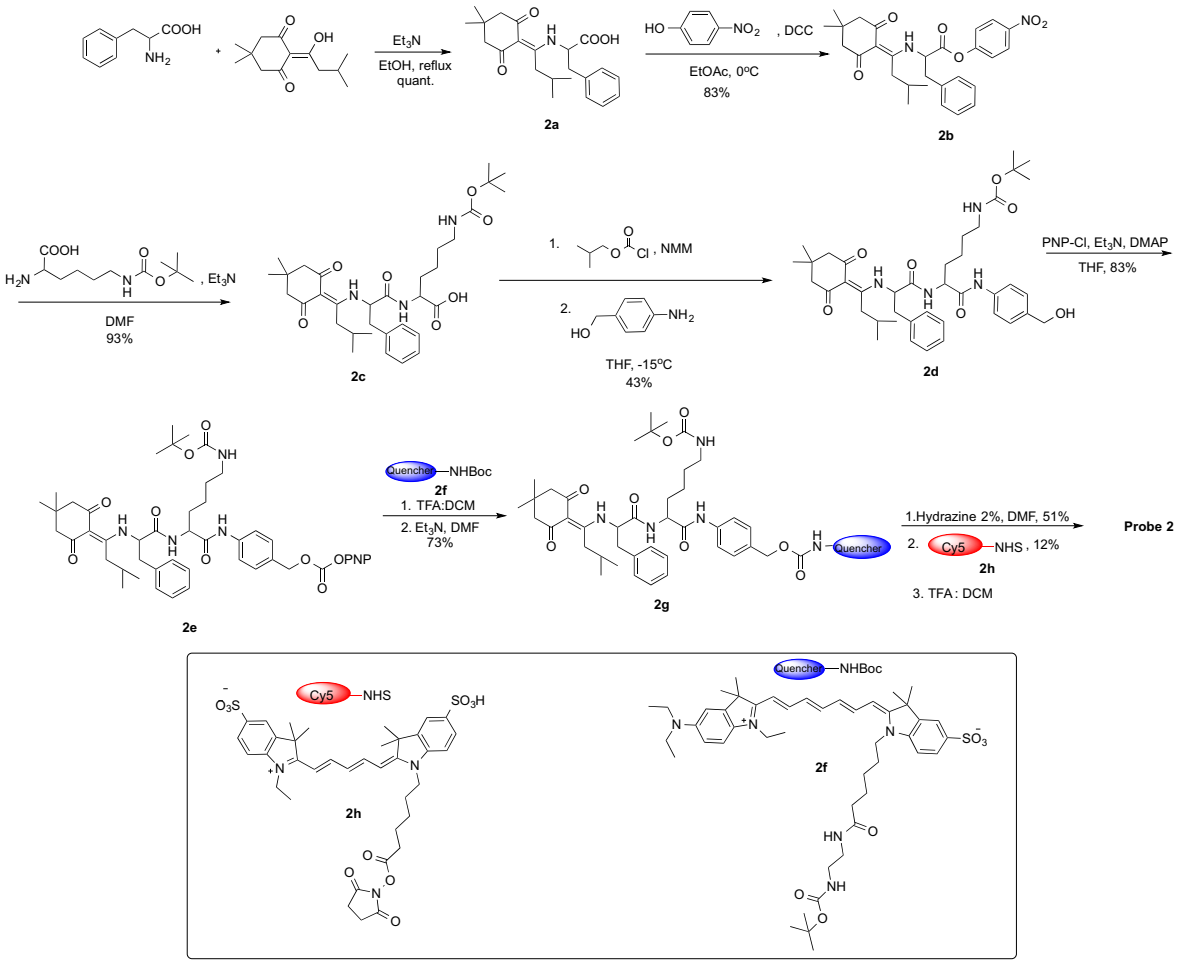

Figure 6. Chemical synthesis of probe **2**.

The synthesis of probe **1** was achieved as presented in Figure 5. 4-Nitrophenyl-ester **1c** was reacted with lysine derivative **1d** to afford dipeptide **1e**. The latter was treated with 4-amino-benzylalcohol in the presence of *N*-methyl-morpholine and isobutyl-chloroformate to generate benzylalcohol **1f**. The obtained benzylalcohol was reacted with 4-nitrophenyl chloroformate to give carbonate **1g**, which was then treated with amine derivative **1h** to afford carbamate **1i**. Compound **1i** was first treated with trifluoreoacetic acid to remove the Boc-protecting group, and then with triphosgene to produce carbamoyl chloride **1j**. The latter was reacted with naphthol dialdehyde **1k** to afford compound **1l**, which was then condensed with two equivalents of indolium-propane-sulfonate **1m**. The obtained crude product was subjected to 2% of hydrazine solution in DMF for removal of amino-lysine protecting group to afford probe **1**.

The chemical synthesis of probe **2** is presented in Figure 6. The phenylalanine amino acid was initially reacted with 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde) protecting group to afford compound **2a**. The latter was coupled with 4-nitrophenol using *N*,*N*′-dicyclohexylcarbodiimide coupling reagent to obtain ester **2b**. Compound **2b** was reacted with H-lys (Boc)-OH to give dipeptide **2c**. The latter was treated with 4-amino-benzylalcohol in the presence of *N*-methyl morpholine and isobutyl-chloroformate to give benzylalcohol **2d**. The alcohol was activated with 4-nitrophenyl chloroformate to afford carbonate **2e**. The Quencher moiety **2f** was deprotected with trifluoroacetic acid and then reacted with carbonate **2e** to afford carbamate **2g**. Compound **2g** was first deprotected using 2% of hydrazine in solution of DMF and then reacted with Cy5-NHS derivative **2h**. The obtained crude product was treated with trifluoroacetic acid to remove the Boc-protecting group and to afford probe **2**.

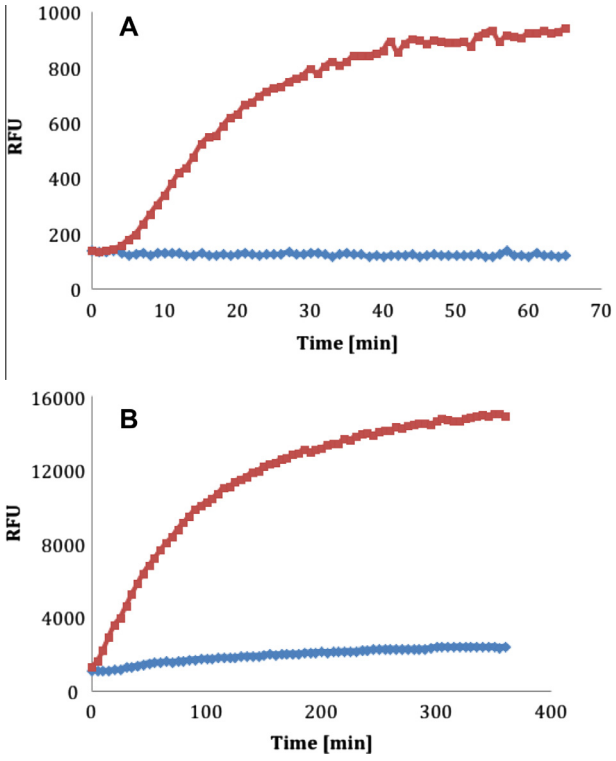

Figure 7. (A) NIR fluorescence (λex = 680 nm, λem = 740 nm) emitted upon incubation of probe **1** [50 μM] in the presence (blue) or absence (red) of cathepsin B [1.4 U/ml] in activity buffer (pH = 6.0) solution. (B) NIR fluorescence (λex = 620 nm, λem = 670 nm) emitted upon incubation of probe **2** [25 μM, 10% DMSO] in the presence (red) or absence (blue) of cathepsin B [1.4 U/ml] in activity buffer (pH = 6.0) solution.

With probes **1** and **2** in hand, we initially sought to evaluate their turn-ON response upon reaction with cathepsin B. Probes **1** and **2** were incubated with cathepsin B in activity buffer solution (pH = 6.0) and the NIR fluorescence emission was monitored using a spectrofluorometer. Figure 7A shows the increase of the emitted fluorescence at wavelength of 740 nm indicating the release of free naphthol-QCy7 fluorophore as a function of time upon addition of cathepsin B. No change in the NIR fluorescence emission was observed in the background reaction over more than 1 h where no enzyme was added. Figure 7B shows the increase of the emitted fluorescence at wavelength of 670 nm upon incubation of probe **2** with cathepsin B, whereas hardly, no change in fluorescence was observed in the absence of the enzyme.

To evaluate the capability of the probes to serve as imaging agents, we assessed their turn-ON response upon reaction with cathepsin B by the CRI Maestro™ imaging system (Fig. 8). Figure 8A and C show images of probes **1** and **2** in the presence and the absence of cathepsin B versus the free naphthol-QCy7 and Cy5 fluorophores, respectively. The images were acquired 1 min following addition of cathepsin B. Figure 8B and D show the increase of the emitted fluorescence of probe **1** and **2**, 4 h following the addition of cathepsin B.

Under these conditions both probes exhibit no emission of NIR fluorescence in their OFF state. Probe **1** shows strong emission of NIR fluorescence, 4 h after exposure to cathepsin B, while no fluorescence is observed in the absence of the enzyme at this time point. Probe **2** also exhibits strong emission of NIR fluorescence, 4 h. after exposure to cathepsin B, however, in the absence of the enzyme, the background signal of the probe clearly reveals certain fluorescence emission.

Ultimately, we have performed intravital imaging evaluation of probes **1** and **2** turn-ON response. We have studied the probes' fluorescence dependency on endogenous cathepsin B activity upon intra-tumoral injection to cathepsin B-overexpressing 4T1 mammary adenocarcinoma cells. Probes **1** and **2** were injected intratumorally and their NIR fluorescence emission was monitored over 4 h. Intravital non-invasive imaging and quantification of time-dependent fluorescence signal within tumors are presented in Figure 9.

Probes **1** and **2** present a distinguishable increase of their NIR fluorescence over 4 h. after the injection into the tumors. The time-dependent fluorescence signal and the images of the tumors following injection of the probes clearly show considerable higher initial background signal emitted from FRET-based probe **2** in comparison with ICT-based probe **1**. This observation is in agreement with the in vitro images of the probes shown in Figure 8.

Both probes described in this study are composed of cyanine molecule as the fluorogenic dye. Cyanine dyes are widely employed as fluorescence labels for NIR imaging, because they are compounds with large extinction coefficient and relatively high quantum yield. These qualities allow their fluorescence to penetrate deep tissues and thus, cyanines serve as attractive probes for intravital non-invasive imaging.[18,19] In order to generate a turn-ON system for a cyanine molecule, a FRET approach is usually applied. To incorporate a cyanine molecule in a FRET-based probe, an additional dye must be included in the molecular system, similarly as was applied in probe **2**. An alternative approach, to turn ON a fluorophore, could be achieved by applying changes in the pull–push conjugated π-electron system of the dye. This approach is demonstrated in the chemical design of probe **1**. The naphthol-QCy7 fluorophore belongs to the recently developed donor-two-acceptor dye family that is activated through an ICT. When the phenol donor is masked by the cathepsin B-cleavable substrate, the NIR fluorescence emission of the dye is almost completely turned OFF. This outcome is responsible for a low background signal of the probe and therefore, high signal-to-noise ratio is

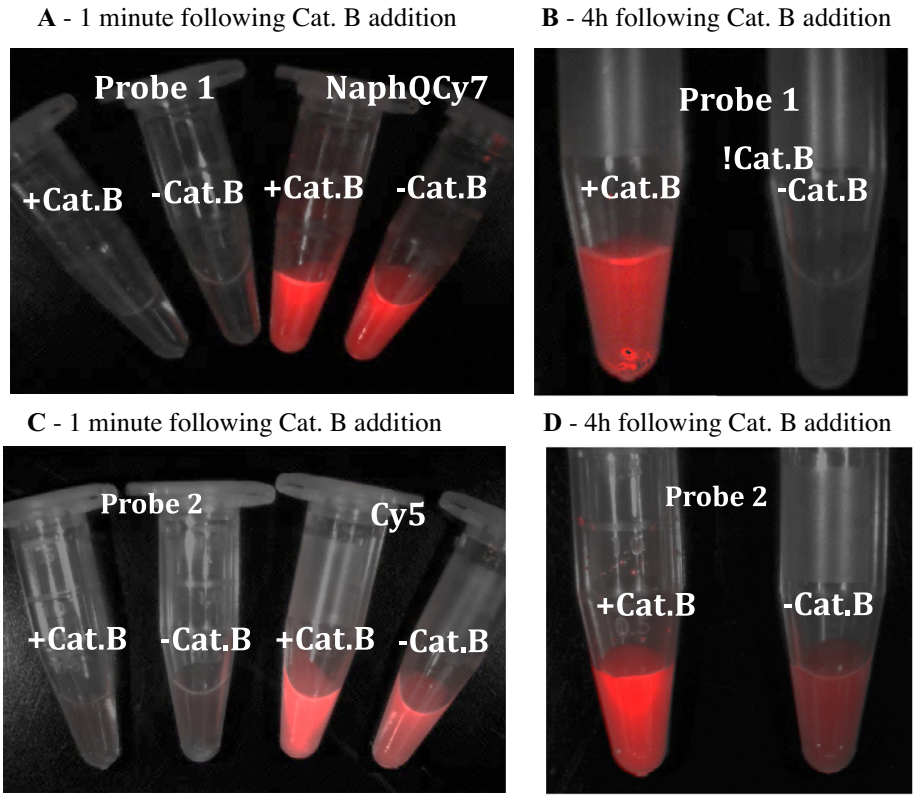

Figure 8. NIR fluorescence turn-ON response of probes **1** and **2** upon reaction with cathepsin B (solutions in activity buffer pH 6.0). (A) Images of probe **1** [0.01 mM] and naphthol-QCy7 [0.01 mM] in the presence (1 min after addition) and in the absence of cathepsin B [10 U/ml]. (B) Images of probe **1** in the presence (4 h after addition) and in the absence of cathepsin B [10 U/ml]. (C) Images of probe **2** [0.01 mM] and Cy5 [0.01 mM] in the presence (1 min after addition) and in the absence of cathepsin B [10 U/ml]. (D) Images of probe **2** [0.01 mM] in the presence (4 h after addition) and in the absence of cathepsin B [10 U/ml]. Images were taken by CRI Maestro™ Imaging system. Filter set: naphthol-QCy7 and Probe **1**-excitation at 661 nm, emission cut-off filter of 750 nm, Cy5 and Probe **2**- excitation at 635 nm, emission cut-off filter of 675 nm.

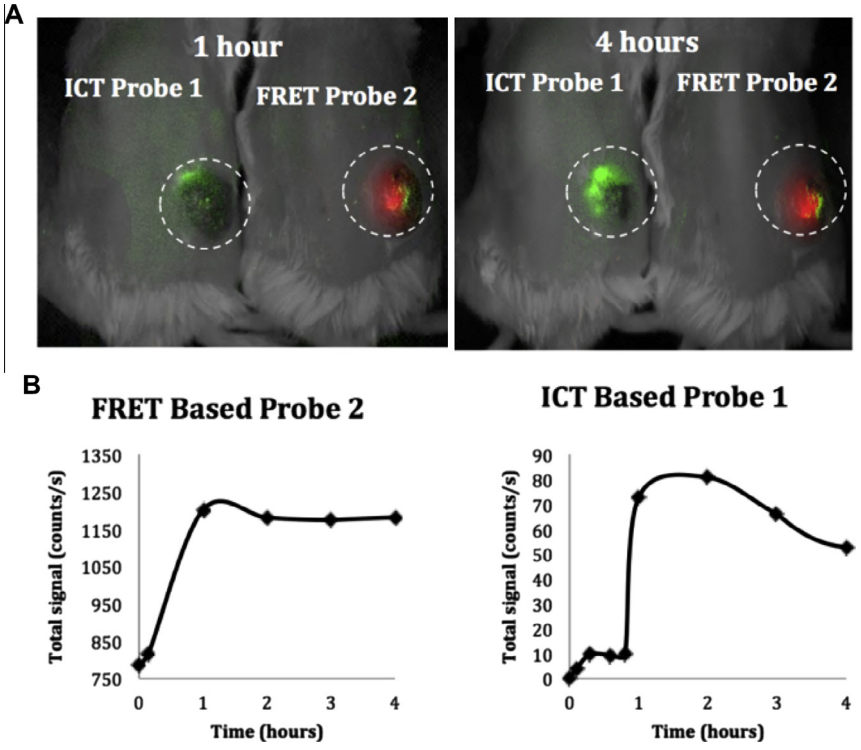

Figure 9. Intravital non-invasive imaging of probes **1** and **2** upon activation with cathepsin B. (A) Representative images of Probes **1** and **2** fluorescence dependency on endogenous cathepsin B activity upon intratumoral injection into cathepsin B-overexpressing 4T1 mammary adenocarcinoma [50 μl; 0.01 mM]. (B) Quantification of time-dependent fluorescence signal within tumors. Images were acquired and quantified using non-invasive intravital CRI Maestro™ imaging system. Filter set: ICT Based probe **1**-excitation at 661 nm, emission cut-off filter of 750 nm; FRET Based probe **2**-excitation at 635 nm, emission cut-off filter of 675 nm.

obtained from probe **1** after activation by the enzyme. On the other hand in probe **2**, the NIR fluorescence emission in the OFF state is noticeably higher than that of probe **1**. The limited ability of the quencher dye in probe **2** to absorb the emission of Cy5, generates certain background fluorescence, which results in lower signal-to-noise ratio. This outcome is also reflected in the in vitro and in vivo images of probes **1** and **2** activated by cathepsin B (see Figs. 8 and 9).

In summary, we have described the design, synthesis and evaluation of two novel NIR fluorescent probes for detection of cathepsin B with different turn-ON mechanisms. One probe is based on an ICT activation mechanism of a donor-two-acceptor π-electron dye system, while the other is based on FRET mechanism obtained by a fluorescent dye and a quencher. The two probes exhibit significant fluorescence turn-ON response upon reaction with cathepsin B, whereas almost no fluorescence change is observed in the absence of the target enzyme. The probes demonstrated potential to image cathepsin B activity in vivo, when evaluated with cathepsin B overexpressing 4T1 mammary adenocarcinoma subcutaneously-inoculated tumors. The NIR fluorescence of the ICT probe in its OFF state was significantly lower than that of the FRET-based probe. This effect leads to a higher signal-to-noise ratio and consequently increased sensitivity and better contrast of images. The obtained in vitro and in vivo results indicate that both probes could be suitable for detection of cathepsin B activity and potential imaging, detection and progression of tumors.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.04.022.

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