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A FRET-based probe with a chemically deactivatable quencher†

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A new concept of a chemically deactivatable quencher is proposed for a FRET-based probe that turns-on its fluorescence by either an enzymatic cleavage or a chemical reagent (sodium dithionite). This concept allowed us to quantify the caspase-3 cleavage activity in solution and to reveal unreacted probes in cell experiments.

Fluorescence is widely used to study biological processes in living cells. Recently, turn-on probes have been developed to measure cell enzymatic activity, pH alterations and to detect singlet oxygen, nitric oxide and highly reactive oxygen species (hROS).[1]

Common turn-on probes are designed by linking a fluorescent dye and a quencher together *via* a biologically labile covalent bond. The most frequently used quencher moieties are non-emitting dyes, which act as FRET acceptors for the covalently bound fluorescent dye. The fluorescence is often turned-on by the enzymatic cleavage of the linker, resulting in separation of the fluorescent dye from the quencher (Scheme 1A). This strategy was used to image different enzymes activities such as cathepsins, caspases and MMPs.[2] Recently, this approach was also used to image free thiols in cells by the cleavage of either a disulfide bridge[3] or a vinyl sulfide group.[4]

Currently used turn-on fluorescent probes are not detectable until activation leading to a high signal-to-noise ratio, which is advantageous for live cell imaging. However, in the case where there is no or weak signal, it is impossible to assess whether this is due to weak biological activity, insufficient probe concentration or inappropriate cellular localization of the probe. Having the ability to detect the substrate entering within the cells would have far reaching applications in biology.

The strategy developed here relies on the design of a probe whose fluorescence can be turned-on either by a biological stimulus or by a chemical reagent. The probe contains a central biosensitive bond that links a fluorescent dye and a chemically deactivatable quencher. When the labile bond is cleaved, the fluorescence is turned-on enabling the biological stimulus to be detected (Scheme 1A). Then, a chemical reagent capable of deactivating the quencher can be used to turn-on fluorescence of the unreacted probe (Scheme 1B), allowing estimation of biological cleavage. This is particularly important for intracellular studies where the probe localisation and concentration are difficult to predict.

We selected an azo-based molecule as a suitable quencher[5] and dithionite as a chemical reagent of relatively low toxicity.[6] The azo-groups are known to react readily and under mild reduction conditions with dithionite[7] leading to N=N bond cleavage. Consequently, when the azo-quencher is linked to a fluorescent dye, dithionite can deactivate the quencher and turn-on the fluorescence.

Dabcyl (4-(4′-dimethylamino-phenylazo)benzoic acid) is a common azo-based quencher widely used in a variety of biomolecular applications and nucleic acid probes.[8] To the best of our knowledge no information on Dabcyl reduction with dithionite has been published. The **Dabcyl** dye was then incubated with a 1 mM sodium dithionite solution in phosphate buffer (pH 7.4, 100 mM) and the azo bond cleavage was monitored using UV spectrophotometry at 460 nm. The half-life of **Dabcyl** was longer than 13 minutes (Fig. S1, ESI†) and could not be transposed to live cells. To overcome this low reactivity, we used a highly reactive (4-hydroxy-2-methoxy-phenylazo) benzoic acid **CDQ** (Fig. 1) as a suitable chemically deactivatable quencher candidate.[9] This molecule has a UV profile similar to **Dabcyl**, but undergoes a faster cleavage. With a 1 mM sodium dithionite solution, this compound exhibited a half-life of less than 1 second and total cleavage was achieved in less than 15 seconds (Fig. S1 and S2, ESI†).

Considering these results, we linked the chemically deactivatable quencher (CDQ) to a fluorescent dye. Its absorption spectrum overlaps with the emission of the 7-diethylaminocoumarin-3-carboxylic acid (DEAC), which has a 470-nm emission maximum (Fig. S3, ESI†). The corresponding chemically deactivatable probe **2** (Fig. 1) was synthesized in five steps with an overall yield of 19% (see ESI†).

Quencher deactivation kinetics was investigated by adding dithionite (1 mM) to the FRET-based probe **2**. A rapid 17-fold increase of fluorescence intensity was observed following cleavage of **2**, with a short half life of ~10 seconds (Fig. 2A and B). We verified the photostability of the fluorescent dye **1** and its stability towards dithionite (Fig. 2A). The deactivation of **2** in the presence of dithionite was complete, as it can be seen from the disappearance of its absorption peak at 490 nm (Fig. S4, ESI†). Since **2** showed very good quencher deactivation properties, we used this couple of CDQ/DEAC groups in the design of a fluorometric dual turn-on caspase-3 sensitive probe.[10]

We synthesized a dual probe: CDQ-DEVD-G-DEAC **3**. This probe consists of three parts: an L-amino acid effector caspase-3 recognition sequence (DEVD)[11] that is flanked by

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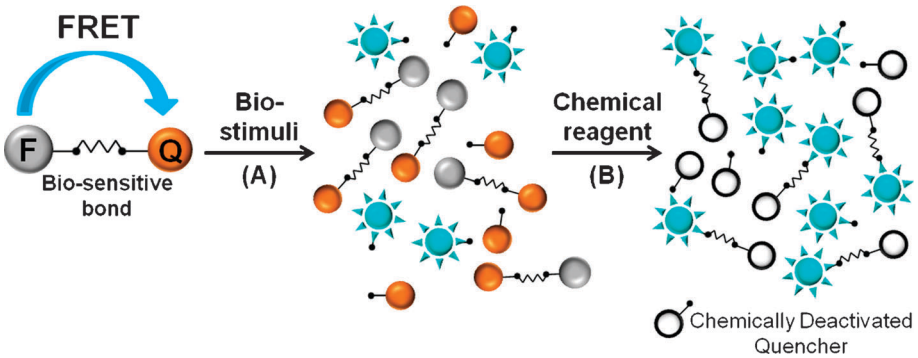

Scheme 1 Principle of FRET-based probe with a chemically deactivatable quencher. (A) In the presence of biological stimuli, the labile bond is cleaved and the fluorescence is turned-on enabling the biological stimuli to be detected. (B) The introduction of a chemically deactivatable quencher allows us to reveal the presence of an inactivated probe by treatment with a chemical reagent.

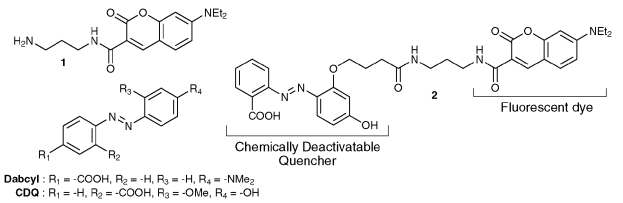

Fig. 1 Structures of cleavable quenchers (**Dabcyl** and **CDQ**), modified DEAC **1**, and probe **2**.

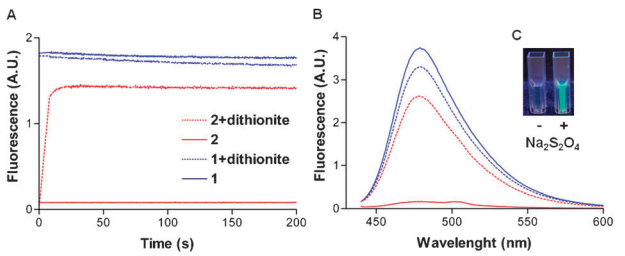

Fig. 2 Quencher deactivation analysis in FRET-based probe. (A) Deactivation kinetics of **1** (dashed blue line) and **2** (dashed red line) with 1 mM of sodium dithionite. Photo-stability of **1** (solid blue line) and **2** (solid red line). Excitation and emission wavelengths were 430 and 476 nm, respectively. (B) Emission fluorescence spectra of compounds **1** (blue lines) and **2** (red lines) before (solid lines) and after deactivation with 1 mM of dithionite solution (dashed lines). All data were recorded with a probe concentration of 2 μM in phosphate buffer (pH 7.4, 100 mM). (C) Cuvettes under a UV-lamp containing **2** (20 μM) without and with dithionite (1 mM).

the chemically deactivatable quencher (CDQ) and the fluorescent dye (DEAC). Probe **3** was synthesized in 12 steps using Fmoc chemistry in solution (see ESI†). After the quencher moiety of **3** was deactivated by dithionite, the fluorescence signal was increased by 22-fold (Fig. S5, ESI†). Thus, the quenching efficacy and therefore the turn-on property of probe **3** was as good as those of probe **2**, despite the longer peptide linker length in the former.

In vitro enzyme kinetic studies were performed using human recombinant caspase-3 enzyme, probe **3**, and the commercially available turn-on fluorescent substrate Ac-DEVD-AMC **4**, used as control (AMC = 7-amino-4-methylcoumarin). Both probes were incubated with caspase-3 at 25 °C in buffer and showed comparable fluorescence enhancement kinetics (Fig. 3A and Fig. S6, ESI†). This result indicates that the CDQ/DEAC pair does not perturb the enzymatic activity of caspase-3. After 300 minutes, when saturation curves were

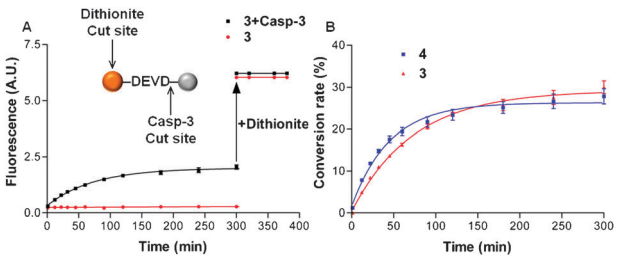

Fig. 3 Response to caspase-3 cleavage and dithionite quencher deactivation. (A) Cleavage kinetics of **3** at 2 μM by human recombinant caspase-3 at 25 or 0 ng mL^{-1} (black and red lines, respectively). The arrow indicates the addition of dithionite (1 mM for 1 min) to cleave probe excess. (B) Conversion rates for **3** and **4** calculated from dithionite cleavage or AMC standard, respectively.

observed, dithionite (1 mM, 1 min) was added to the reaction mixture with **3**. A 3.1-fold fluorescence increase was observed, indicating that some unreacted probe was still present. This incomplete conversion might be imputed to enzyme inhibition by the reaction product. The chemical deactivation of the quencher enables us to calculate the total concentration and the conversion rate of the fluorescent substrate (Fig. 3B). After 300 minutes, 30% of **3** had been enzymatically cleaved by the recombinant caspase-3. To estimate the amount of uncleaved probe **4**, the fluorescence intensity was compared to the free fluorescent dye, AMC, at the same concentration (2 μM). A 3.5-fold difference was recorded, which is similar to the signal observed for probe **3** (Fig. S6, ESI†). Thus, the chemically deactivatable quencher allows direct estimation of the probe conversion rate.

In the control experiment, a competitive inhibition study of caspase-3 was performed using a commercially available inhibitor, Ac-DEVD-CHO **5** (Fig. S7, ESI†). As expected, an increase in the inhibitor concentration slowed down the enzymatic cleavage.

The chemical activation of **3** was then studied in tissue culture. HeLa cells were incubated for 1 hour with **3**. After washing out probe excess, cells were incubated with dithionite. In the control experiment, cells were treated with HEPES buffer, instead of dithionite (Fig. 4A). The quencher deactivation was monitored using live-cell imaging (Fig. 4). Fluorescence could be clearly seen in dithionite treated cells after 15 minutes and the intensity continued to increase for 45 minutes. After 45 minutes, a maximum intensity was reached indicating that all the probes were chemically turned-on. It confirms that dithionite can abolish the

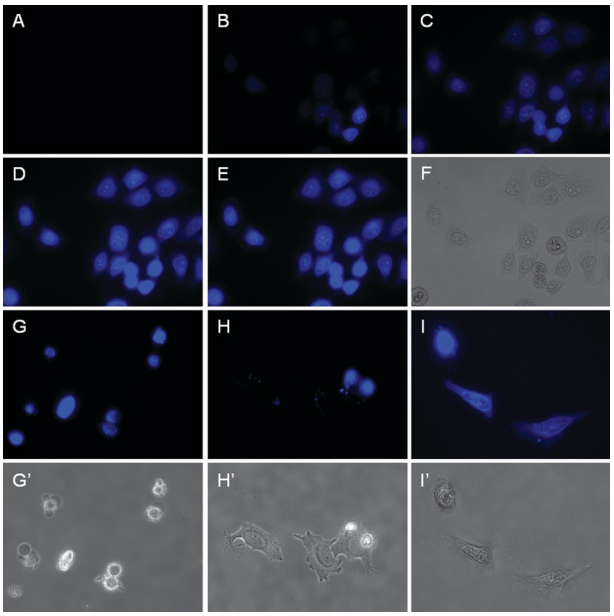

Fig. 4 Fluorescence and brightfield microscopy images of HeLa cells treated with **3** (40 μM, 1 h). (A–F) Quencher deactivation kinetics with dithionite (10 mM) at different time points: 15 (B), 30 (C), 45 (D), 60 min (E). In the control experiment (A), the cells were incubated with HEPES buffer. (F) Brightfield image of cells after 60 min of dithionite treatment. (G–H′) Response of **3** to apoptotic cells (pre-treated with Actinomycin D (0.5 μg mL^{-1}, 18 h)). (I and I′) Quencher deactivation with dithionite (10 mM, 45 min) in apoptotic cells. The images were acquired with the same camera settings, but with a different fluorescence scale ranging from 2700 to 7360 (G–H) and from 7880 to 36 010 (all others). The image size was 219 × 163 μm (A–H′) and 146 × 109 μm (I and I′).

FRET-quenching in **3** in healthy cells. Compared to an *in vitro* experiment, *in vivo* dithionite-mediated quencher deactivation is much slower due to the low dithionite membrane permeability. It should be noted that the cells treated with dithionite showed slightly different morphology, which could be connected with cell shrinkage (Fig. S8, ESI†). However, after dithionite treatment the morphology remained unchanged for the experiment timescale. According to luciferase ATP assay, the dithionite cytotoxicity was low, even at a 10 mM concentration (Fig. S9, ESI†). Numerous reports showed that dithionite can be used as a reductive agent in cell culture, without strong cytotoxicity effects at the millimolar range.[12]

Then we assessed cell internalization of probe **3** as a function of incubation time. Cells were treated with 40 μM of **3** for different times (15, 30, 60 and 120 min), washed and incubated with 10 mM of dithionite for 45 min (Fig. S8, ESI†). Results showed an increase in the probe internalization during the first hour of incubation, and then reached a plateau. This allowed us to determine the optimal incubation time of the cells with the probe, which would not be possible for classical turn-on probes.

The biological activation of **3** in live HeLa cells was then tested. Apoptosis was induced to activate caspase-3, which can cleave the DEVD peptide linker in **3**. Cells were incubated with Actinomycin D for 18 hours and then with **3** for 1 hour. Cells undergoing apoptosis were recognized by their rounded morphology and bright blue fluorescence, indicating that **3** had been cleaved in these cells. In addition, we observed different levels of fluorescence intensity for the Actinomycin-D-treated cells, which is most likely due to the cells being in different stages of apoptosis (Fig. 4G and G′). In contrast, cells with a healthy morphology had only very low levels of fluorescence (Fig. 4H and H′), in line with data on non-treated HeLa cells (Fig. 4A). The quencher was further deactivated in the cells by 10 mM dithionite treatment for 45 minutes. A strong increase in fluorescence was observed in both apoptotic and healthy cells (Fig. 4I and I′), indicating the presence of uncleaved probe **3** in the both cell populations and confirms the presence of the probe in non-fluorescent healthy cells. Thus, we have shown that the fluorescence of probe **3** is only turned-on in apoptotic cells, and the "chemically deactivatable quencher" confirms the presence of the probe in all cells.

In conclusion, we have developed a caspase-3 activity-sensitive probe containing a DEVD peptide which was used to link a fluorescent dye and a "chemically deactivatable quencher". The synthesized probe was shown to turn-on its fluorescence in response to both recombinant and endogenous caspase-3 stimuli, whereas it can be turned-on independently by dithionite deactivation of the quencher. The advantages of this novel strategy over traditional "turn-on" probes include control for internalization and localization of the probe in living cells, irrespective of whether they contain the biological stimulus or not. The "chemically deactivatable quencher" concept can be applied to all "turn-on probes" with a fluorescent dye-linker-quencher structure for assaying different biological stimuli. These results pave the way for the development of a new generation of enzymatic probes containing an internal control.

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