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COMMUNICATION

A simple FRET-based modular design for diagnostic probes†

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In recent years, there has been a massive effort to develop molecular probes with optical modes of action. Probes generally produce detectable signals based on changes in fluorescence properties. Here, we demonstrate the potential of selfimmolative molecular adaptors as a platform for Turn-On probes based on the FRET technique. The probe is equipped with identical fluorophore pairs or a fluorophore/quencher FRET pair and a triggering substrate. Upon reaction of the analyte of interest with the triggering substrate, the self-immolative adaptor spontaneously releases the two dye molecules to break off the FRET effect. As a result, a new measurable fluorescent signal is generated. The fluorescence obtained can be used to quantify the analyte. The modular structure of the probe design will allow the preparation of various chemical probes based on the FRET activation technique.

Molecular probes based on fluorescence signal generation are widely used for various diagnostic applications. Several different classes of Turn-On optical probes have been described in the literature for detection/imaging of chemical and biological factors.¹⁻⁴ Among the methods used to obtain an emitted fluorescence signal upon detection or imaging of a specific reactivity, Förster resonance energy transfer (FRET), which is based on a fluorophorequencher interaction or fluorophores' self-quenching, is one of the most common and efficient options.^{5,6} Herein, we demonstrate a simple modular approach for design of activatable fluorescent probes using the FRET technique. One method for turning off the fluorescence of a dye molecule is to force close proximity of a quencher and a fluorophore through a covalent linkage. Under such circumstance, the excited fluorophore can transfer its excitation energy to the nearby quencher-chromophore in a nonradiative fashion through long rang dipole-dipole interactions. Disconnection of the linkage results in diffusion of the fluorophore away from the quencher and generation of a measurable fluorescent signal. Similar effects can be obtained by self-quenching of two identical fluorophores (Homo-FRET). Based on this known

concept, we sought to develop a modular design for preparation of Turn-On optical probes. A schematic representation of our probe design is illustrated in Fig. 1. The head of an adaptor molecule is attached to a protecting group that can be cleaved by a specific analyte. The tails of the adaptor are attached to a fluorophore and to a quencher unit (Fig. 1A). Removal of the protecting group by the analyte of interest results in spontaneous release of the fluorophore and the quencher from the molecular adaptor. Consequently, the FRET process is no longer active and the fluorescent signal is turned on. The same idea is illustrated with two identical fluorophores, which quench each other through a Homo-FRET interaction (Fig. 1B).

There are quite a few examples of small organic molecules that can act as molecular adaptors with chemical reactivity as illustrated in Fig. 1.7,8 Our group has reported the use of several such adaptors for construction of self-immolative dendritic molecules.9 These molecules can translate a single cleavage event of a protective group at the head of the dendritic molecule into the release of multiple tail-units from the periphery. The disassembly mechanism of such an adaptor (molecule 1a) is depicted in Fig. 2. In general any phenol or aniline derivative with substituents at the ortho or para benzylic positions can be used to release tail-units. Upon removal of a protective group from the head of the molecular adaptor, a phenolate is obtained (molecule 1a). The latter can undergo two consecutive quinone-methide-type eliminations (molecules 1b and 1d) to release the two tail-units from the main adaptor moiety. Thus, a single cleavage event at the head of the adaptor is amplified with the release of two units from the tails. Similar molecular reactivity can be achieved by using aniline or aromatic thiol derivatives instead of the phenol. We have used the structural properties of self-immolative dendritic adaptors to demonstrate molecular amplification in the fields of drug delivery and diagnostics.9-13

Initially, we sought to explore the capability of an AB₂ selfimmolative dendritic adaptor to serve as a platform for a Homo-FRET with the design presented in Fig. 1B. Probe **2** is based on an aniline AB₂ self-immolative adaptor with substituents at the *ortho* or *para* benzylic positions (Fig. 3). The probe is equipped with a protecting group (phenylacetamide) that is a substrate for cleavage by the protease penicillin-G-amidase (PGA). We have used two molecules of a Cy5 dye as the fluorophore.¹⁴ This dye molecule has a small Stokes shift and thus can significantly quench the fluorescence of a neighboring molecule.¹⁵ Removal of the phenylacetamide group by PGA followed by 1,6 azaquinonemethide elimination results in formation of aniline **2a**. The latter can

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Fig. 1 Modular design of a FRET-based optical probe. (A) Modular probe with a fluorophore–quencher pair. (B) Modular probe with fluorophore pair.



Fig. 2 Disassembly pathway of an AB₂ self-immolative dendritic adaptor upon removal of a protective group.

undergo consecutive 1,6 and 1,4 eliminations to release the two Cy5 molecules.

The synthesis of probe **2** was performed as shown in Fig. 4. Compound **3** was prepared as previously described.¹⁶ Activation of the hydroxybenzyl groups of diol **3** with 4-nitrophenyl-chloroformate afforded dicarbonate **3a**. The latter was reacted with 2 eq. of Cy5amine derivative **3b** to afford probe **2**.

The fluorescence spectrum of probe **2** was measured and compared to that of free Cy5 (see ESI†). Expectedly, the Homo-FRET quenching effect was observed. To evaluate the capability of probe **2** for detection of the protease PGA, the probe was incubated in PBS 7.4 with and without the enzyme, and the fluorescence emission was monitored with a spectrofluorometer (Fig. 5). In the presence of PGA, a significant fluorescence emission was observed,

whereas emission remained at background levels in absence of PGA.

Next, we prepared two additional probes based on the FRETpair design presented in Fig. 1A. These probes were equipped with a triggering group, phenyl-boronic ester, which is known to undergo cleavage by hydrogen peroxide.¹⁷⁻²⁰ The protecting group was linked to a phenol derivative of an AB₂ self-immolative adaptor with two substituents at the *ortho* benzylic positions. Probe 4 contained fluorescein dye (4a) as a fluorophore and disperse red 1 (DR1) dye as a quencher (Fig. 6).²¹ Probe 5 contained Cy5 dye as a fluorophore and compound 5a as a quencher (Fig. 7).²² Both probes are designed to release both fluorophore and quencher upon removal of the protecting group, similarly to the reaction shown in Fig. 2. The phenol obtained





Fig. 3 Activation of a Homo-FRET probe by PGA to release Cy5 fluorophores.



Fig. 4 Chemical synthesis of probe 2.



Fig. 5 NIR fluorescence ($\lambda_{Ex} = 630$ nm, $\lambda_{Em} = 670$ nm) emitted upon incubation of probe **2** [10 μ M] in the presence (red) or absence (blue) of PGA [0.1 mg ml⁻¹] in PBS, pH 7.4.

after cleavage of the phenyl-boronic ester is known to release its end-units through double *ortho*-quinone-methide elimination.⁹

The synthesis of probe 4 was achieved as depicted in Fig. 8. 4-Hydroxybenzoic acid 6a was coupled with allyl bromide to protect the carboxylic acid in the form of allyl ester 6b. The latter was reacted with formaldehyde to generate dibenzylalcohol 6c, which was then protected with two equivalents of tbutyldimethylsilylchloride to give phenol derivative 6d. The phenol was alkylated with iodide 6e to give ether 6f. Deprotection of 6f with p-toluene sulfonic acid afforded diol 6g. Activation of diol 6g with two equivalents of 4-nitrophenyl-chloroformate gave dicarbonate 6h, which was selectively reacted with one equivalent of disperse red 1 to yield compound 6i. Reaction of **6i** with fluorescein derivative **6** (see ESI[†]) generated compound 6j, which was deprotected using tetrakis(triphenylphosphine)palladium afford and tributyltin hydride to probe 4.

The synthesis of probe **5** was performed according to the scheme shown in Fig. 9. Compound **7a** was synthesized as previously described.⁹ Alkylation of phenol **7a** with iodide **6e** afforded ether **7b**, which was then deprotected with *p*-toluene sulfonic acid to give



Fig. 6 Activation of a FRET-pair probe by hydrogen peroxide.



Fig. 7 Activation of a FRET-pair probe by hydrogen peroxide.

diol **7c**. Activation of diol **7c** with two equivalents of 4-nitrophenylchloroformate gave compound **7d**. Reaction of one equivalent of Cy5 amine derivative **7f** with dicarbonate **7d** gave compound **7e**, which was further reacted with quencher **5a** (see ESI†) to afford probe **5**.

The fluorescence spectra of probes 4 and 5 compared to those of the free fluorophores confirmed the FRET quenching effect (see ESI[†]). Both probes were incubated with and without hydrogen peroxide in PBS pH 7.2 for probe 4, and pH 8.3 for probe 5, and the emission was monitored using a spectrofluorometer. Fig. 10 shows emission from probe 4 as a function of time after addition of hydrogen peroxide. A significant increase of the emitted fluorescence was observed within minutes after addition of hydrogen peroxide, whereas no change in fluorescence was observed in the absence of hydrogen peroxide. Similar results were obtained when probe 5 was activated with hydrogen peroxide (Fig. 11).

In this study, we evaluated two designs of Turn-On probes activated by the FRET technique. One was based on separation of two identical fluorophores that quench each other. This option is most suitable for fluorophores with relatively small Stokes shifts. The second design was based on a fluorescent molecule and a quenching dye. Activation of the probe dissociated the two moieties and resulted in measurable fluorescent signal. These two options were tested in a probe system constructed with an AB_2 self-immolative dendritic adaptor.

Usually FRET-based probes are composed of FRET-pair dyes tethered through a peptide linker. Cleavage of the linker between the dye molecules, by a protease for example, results in generation of fluorescence.²³ In this design, the dyes must be linked through the terminal functional groups of the peptide substrate. To achieve a quenching effect in the probe, the absorption spectrum of the quencher must partially overlap with the fluorescent spectrum of the fluorophore. Therefore, **DR1** (absorption band between 400–600 nm) was selected for probe **4** and quencher **5a** (absorption band between 600–900 nm) for probe **5**.

The self-immolative dendritic adaptor is linked to the substrate through one side only, while the other remains open. This feature increases the flexibility of probe design and allows use of various molecules as substrates for detection of either enzyme or chemical



Fig. 9 Chemical synthesis of probe 5.



Fig. 10 Fluorescence ($\lambda_{Ex} = 490 \text{ nm}$, $\lambda_{Em} = 530 \text{ nm}$) emitted upon incubation of probe **4** [40 µM] in the presence (red) or absence (blue) of hydrogen peroxide [40 µM] in PBS, pH 7.2 (12% DMSO as a co-solvent).



Fig. 11 NIR fluorescence ($\lambda_{Ex} = 630 \text{ nm}$, $\lambda_{Em} = 670 \text{ nm}$) emitted upon incubation of probe 5 [30 µM] in the presence (red) or absence (blue) of hydrogen peroxide [30 µM] in PBS, pH 8.3 (30% DMSO as a co-solvent).

analytes. We have demonstrated that our probes can be used to detect the enzymatic activity of PGA and the chemical reactivity of the analyte hydrogen peroxide.²⁴

In summary, we have shown a simple and general modular approach for design of FRET-based probes with a Turn-On mechanism. The probe platform is based on an AB_2 self-immolative dendritic adaptor that releases two end-groups upon a single activation event at the adaptor's head by the analyte of interest. In a self-quenching approach, the probes were equipped with two identical fluorophores. In second design, a fluorophore and a quencher were attached to the probe. The modular structure may be adapted to incorporate a variety of fluorophores and various protecting groups as triggering substrates. This design allows the facile construction of probes for detection of various analytes simply by installing a specific protecting group as an activator. The modular concept presented in this report should be useful for preparation of many other chemical probes based on the FRET activation technique.

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