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A nonfluorescent, broad-range quencher dye for Förster resonance energy transfer assays

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ABSTRACT

We report here a novel, water-soluble, nonfluorescent dye that efficiently quenches fluorescence from a broad range of visible and near-infrared (NIR) fluorophores in Förster resonance energy transfer (FRET) systems. A model FRET-based caspase-3 assay system was used to test the performance of the quencher dye. Fluorogenic caspase-3 substrates were prepared by conjugating the quencher, IRDye® QC-1, to a GDEVDGAK peptide in combination with fluorescein (emission maximum ~540 nm), Cy3 (~570 nm), Cy5 (~670 nm), IRDye 680 (~700 nm), IRDye 700DX (~690 nm), or IRDye 800CW (~790 nm). The Förster distance R_0 values are calculated as 41 to 65 Å for these dye/quencher pairs. The fluorescence quenching efficiencies of these peptides were determined by measuring the fluorescence change on complete cleavage by recombinant caspase-3 and ranged from 97.5% to 98.8%. The fold increase in fluorescence on caspase cleavage of the fluorogenic substrates ranged from 40 to 83 depending on the dye/quencher pair. Because IRDye QC-1 effectively quenches both the NIR fluorophores (e.g., IRDye 700DX, IRDye 680, IRDye 800CW) and the visible fluorophores (e.g., fluorescein, Cy3, Cy5), it should find broad applicability in FRET assays using a wide variety of fluorescent dyes.

Nonfluorescent quenching dyes, also known as dark quenchers, have been commonly used in Förster resonance energy transfer (FRET)[1] -based fluorogenic probes for protease activity detection [1–13], nucleic acid hybridization [14,15], and real-time polymerase chain reaction (PCR) [16–18]. In FRET-based systems, a specific quencher is normally able to quench the fluorescence only from those fluorophore donors that have significant overlap of their emission spectra with the absorption spectrum of the quencher when the donor and quencher are brought into proximity. To design a donor/quencher FRET system, the quenching range information and/or careful comparison of the donor's fluorescence spectrum with the quencher's absorption spectrum are required [1].

Although nonfluorescent dyes that efficiently quench visible fluorescent donors have been described, there is an unmet need for an efficient nonfluorescent quencher for near-infrared (NIR) dyes. In addition, it would be advantageous to have a nonfluorescent dye with broad capability to quench both visible and NIR donors. Broad quenching ability would simplify assay development, and applicability to NIR dyes would take advantage of the low assay background characteristics of this spectral region.

There are several advantages to working in the NIR region. Although FRET-based assays using red-shifted fluorophores such as rhodamine, Cy3, and Cy5 can reduce background compared with traditional assays using shorter wavelength donor/quencher pairs, longer wavelength NIR fluorescence assays can virtually eliminate background fluorescence due to the extremely low autofluorescence in the NIR [19,20]. For in vivo imaging applications, NIR assays also benefit from the enhanced tissue penetration of light near 650 to 900 nm [21].

To develop NIR FRET assays, it is essential to have a well-matched NIR fluorophore and quencher. Considerable efforts in developing NIR FRET assays have been reported. Pham and coworkers reported an NIR fluorescence probe for sensing matrix metalloproteinase 7 (MMP-7) protease activity using a Cy 5.5 donor paired with a fluorescent NIRQ820 dye as the acceptor [21]. The probe showed a limited working range due to a maximum sevenfold fluorescence increase after complete proteolytic cleavage. Furthermore, an NIR caspase-3 assay using a nonfluorescent azulene dye and Alexa Fluor 680 was also reported but showed only a fourfold fluorescence increase [22].

We report here a novel, water-soluble, monoreactive nonfluorescent dye, IRDye QC-1, that efficiently quenches fluorescence

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[1] *Abbreviations used:* FRET, Förster resonance energy transfer; PCR, polymerase chain reaction; NIR, near-infrared; MMP-7, matrix metalloproteinase 7; NHS, *N*-hydroxysuccinimide; TCEP·HCl, tris(2-carboxyethyl)phosphine hydrochloride; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; LC/MSD, liquid chromatography/mass selective detection; UV, ultraviolet; ICG, indocyanine green; DOTCI, 3,3′-diethyloxatricarbocyanine iodide; DMF, dimethylformamide; DIPEA, diisopropylethylamine; TFA, trifluoroacetic acid; TEAA, triethylammonium acetate; EDTA, ethylenediaminetetraacetic acid; *S/N*, signal-to-noise; FBS, fetal bovine serum; NaOAc, sodium acetate; BSA, bovine serum albumin; LOD, limit of detection.

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from a wide range of fluorophores spanning the visible to NIR spectrum (~500–800 nm). We synthesized a series of fluorogenic caspase-3 peptide substrates using IRDye QC-1 paired with various fluorophore donors and measured the fluorescence quenching efficiencies by cleaving these substrates with human recombinant caspase-3. IRDye QC-1 showed efficient quenching with all dyes tested in the model system and showed fluorescent signal increases ranging between 40-fold and 83-fold on complete cleavage of the fluorogenic substrates. The broad applicability of IRDye QC-1 should simplify FRET assay development and enable development of sensitive NIR assays.

Materials and methods

General aspects

Unless otherwise noted, all general chemical reagents were purchased from commercial suppliers and used without further purification. Resin-bound peptide H_2 N-Gly-Asp(OtBu)-Glu(OtBu)-Val-Asp(OtBu)-Gly-Ala-Lys(Boc)-NovaSyn TGA resin was custom synthesized by Pi Proteomics (Huntsville, AL, USA). IRDye QC-1, IRDye 800CW, IRDye 680, and IRDye 700DX *N*-hydroxysuccinimide (NHS) ester dyes were obtained from LI-COR Biosciences (Lincoln, NE, USA). Cy3 and Cy5 NHS ester dyes were purchased from Amersham Biosciences (Buckinghamshire, UK). 5-Carboxyfluorescein succinimidyl ester dye was obtained from Invitrogen/Molecular Probes (Eugene, OR, USA). Human recombinant caspase-3 was purchased from Upstate Biotechnology (Rochester, NY, USA) and contained 300 to 400 U/μg enzyme, where 1 U = 1 nmol DEVD-pNA substrate cleavage per hour at 37 °C at saturated substrate concentrations. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Phosphate-buffered saline (PBS) was purchased as the 10× concentrate from Sigma (St. Louis, MO, USA) and diluted 10-fold prior to use.

Reverse-phase high-performance liquid chromatography (HPLC) was conducted with an Agilent 1100 series HPLC system using a ZORBAX 300SB-C18 column (Agilent Technologies, Santa Clara, CA, USA). Mass spectra were obtained on an Agilent 1100 series liquid chromatography/mass selective detection (LC/MSD) ion trap mass spectrometer. Ultraviolet (UV)-visible spectra were measured using an Agilent 8453 spectrophotometer. Fluorescence spectra were measured using a PTI QuantaMaster luminescence spectrometer from Photon Technology International (Birmingham, NJ, USA). Microtiter plate images were obtained with Falcon 96-well plates from BD Biosciences (San Jose, CA, USA) using an Aerius® Infrared Imager (LI-COR Biosciences). The fluorescence quantum yield φ_{sample} was measured using indocyanine green (ICG, Aldrich, Milwaukee, WI, USA) as the fluorescence standard in methanol solution ($\varphi_{ST} = 0.043$) [23] and calculated according to Eq. (1)

$$\phi_{sample} = \frac{A_{ST}}{A_{sample}} \times \frac{F_{sample}}{F_{ST}} \times \frac{\eta^2_{sample}}{\eta^2_{ST}} \times \phi_{ST}, \quad (1)$$

where F_{sample} and F_{ST} are the integrated fluorescence intensity of the full corrected emission spectra of the sample and standard (solvent blank corrected), respectively; A_{sample} and A_{ST} are the absorbance of the sample and standard, respectively; and η_{sample} and η_{ST} are the refractive indexes of the solvents for the sample and standard, respectively. We used 3,3′-diethyloxatricarbocyanine iodide (DOTCI, Eastman Kodak, Rochester, NY, USA) as a secondary standard to check the validity of the quantum yield measurements.

Fluorogenic caspase-3 substrate synthesis

The structure of IRDye QC-1 is shown schematically in Fig. 1. The structure of the dye was verified by mass spectrometry and nuclear magnetic resonance. The preparation of the dual-labeled peptides is shown in Fig. 2. Resin-bound peptide was mixed with either IRDye 800CW NHS ester or IRDye QC-1 NHS ester in dimethylformamide (DMF) and diisopropylethylamine (DIPEA) at room temperature overnight. The resulting IRDye 800CW or IRDye QC-1-conjugated peptide-tethered resin was washed with DMF and methanol and then dried. The resin was treated with a solution containing trifluoroacetic acid (TFA, 95%), water (2.5%), and triisopropylsilane (2.5%) to remove all of the protecting groups on the side chains of the peptide and to cleave the peptide from the resin at the same time. The product was isolated by filtration and ethyl ether precipitation. Further purification by reverse-phase C18 preparative HPLC using acetonitrile and 50 mM triethylammonium acetate (TEAA) buffer (pH ~5.5) gradient provided the intermediate product, IRDye 800CW–GDEVDGAK or IRDye QC-1–GDEVDGAK.

IRDye 800CW–GDEVDGAK peptide was reacted with IRDye QC-1 NHS ester in 0.4 M phosphate buffer (pH 8.0) at room temperature for 3 h. Reporter–quencher-labeled peptide product was purified by reverse-phase C18 preparative HPLC using acetonitrile and 50 mM TEAA buffer (pH 5.5) gradient. The product was subjected

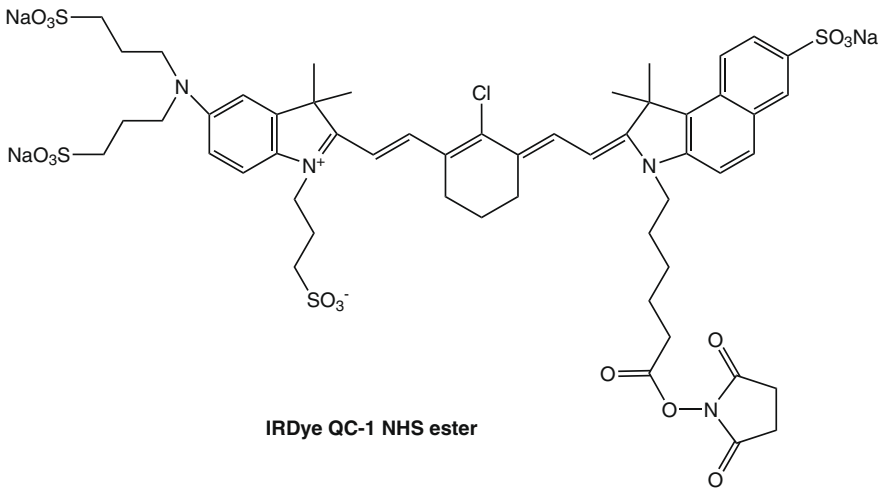

Fig. 1. Chemical structure of IRDye QC-1 NHS ester.