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Porphyrin FRET Acceptors for Apoptosis Induction and Monitoring

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Supporting Information

ABSTRACT: Photodynamic therapy (PDT) stands to benefit from improved approaches to real-time treatment monitoring. One method is to use activatable photosensitizers that can both induce cell death (via singlet oxygen) and monitor it (via caspase detection). Here, we report porphyrins as caspase-responsive Forster Resonance Energy Transfer (FRET) *acceptors* to organic fluorophore donors. Compared to porphyrin FRET *donor* constructs, singlet oxygen generation was unquenched prior to caspase activation, resulting in more efficient photosensitization in HT-29 cancer cells. The donor 5-Carboxy-X-Rhodamine (Rox) formed a robust FRET pair with the pyropheophorbide (Pyro) acceptor. The large dynamic range of the construct enabled ratiometric imaging (with Rox excitation) of caspase activation in live, single cells following induction of cell death (with Pyro excitation) using a single agent. Quantitative, unquenched activatable photosensitizers (QUaPS) hold potential for new feedback-oriented PDT approaches.

Photodynamic therapy (PDT) is a clinical procedure that combines photosensitizers and light to destroy target tissues through singlet oxygen generation.[1] Since it is difficult to assess tissue damage during the course of treatment, PDT dosimetry has been the subject of significant research efforts.[2] Several approaches have been explored, including some that provide immediate feedback, such as directly measuring singlet oxygen luminescence or measuring photosensitizer photobleaching. Unfortunately, such feedback methods can only provide an empirical and indirect proxy for presumed cellular damage. Other cell properties can be used to gauge apoptosis, such as the intracellular viscosity of photosensitizers.[3] Another approach is to monitor feedback with molecular markers of programmed cell death, such as caspases; proteases that are activated during apoptosis.[4] The onset of caspase activation leads to a proteolytic cascade culminating in cell death. Activatable photosensitizers (aPS), which are quenched prior to activation, have been developed which are cleaved and activated by caspases.[5] Fluorescence approaches have been used to detect caspase activity in cells, making use of self-quenched polymers and nanoparticles, quantum dots, dark and FRET quenchers, and optical nanoprobes.[6−11] aPS face a paradoxical limitation with respect to caspases because the aPS requires caspase activation to become unquenched, but, in cells, caspase activation requires an unquenched aPS. Thus, for the purpose of inducing apoptosis in cancer applications, unquenched singlet oxygen-generating constructs are desirable. Furthermore, attributing an observed increase in aPS fluorescence intensity to caspase activation is difficult, since the signal is also affected by cellular beacon uptake and release, which changes over time and with treatment. To address these two shortcomings, we have developed a new class of quantitative, unquenched activatable photosensitizer (QUaPS) that use a porphyrin as the FRET acceptor.

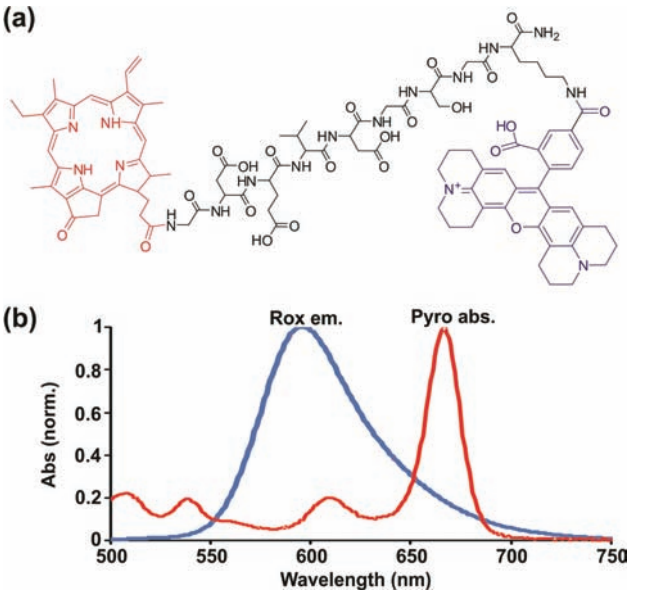

Figure 1. (a) Structure of a QUaPS with Pyro shown in red and Rox shown in blue. (b) Normalized spectra of Rox donor emission and Pyro acceptor Q-band absorption.

To synthesize the QUaPS, the fluorescent photosensitizing porphyrin pyropheophorbide (Pyro) was conjugated to the N-terminus of a caspase-3 specific peptide sequence (GDEVDGSGK) on solid phase support.[12] The peptide was deprotected and cleaved, then the fluorophore 5-Carboxy-X-Rhodamine (Rox) was conjugated to the epsilon amine on the C terminus lysine residue. The QUaPS was then purified by reverse phase HPLC, and purity and identity were confirmed with analytical HPLC-MS (Figure S1 in the Supporting Information). The spectral overlap between ROX emission and Pyro absorption suggested that Pyro could be an effective acceptor for ROX emission (Figure 1b). Analysis of the

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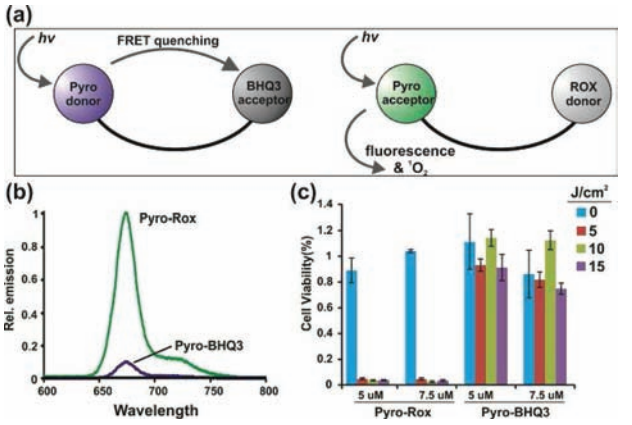

Figure 2. Increased brightness and photosensitization of QUaPS compared to a Pyro donor aPS. (a) Differences between FRET donor or FRET acceptor aPS. (b) Fluorescence of 250 nM peptide linked Pyro-ROX (Pyro-acceptor) and Pyro-BHQ3 (Pyro-donor). (c) PDT efficiency in HT-29 cancer cells of peptide linked Pyro-ROX and Pyro-BHQ3.

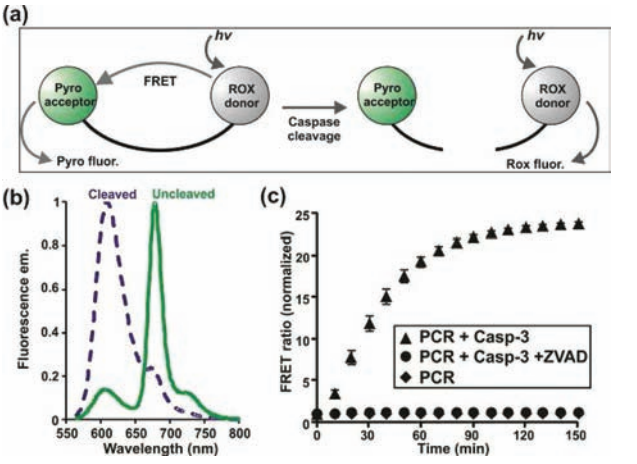

Figure 3. Ratiometric sensing using QUaPS. (a) Schematic representation of FRET response to caspase cleavage. (b) Fluorescence emission spectra of QUaPS before and after proteolytic cleavage. (c) Normalized FRET ratio change in response to recombinant Caspase-3 with or without ZVAD-fmk, a caspase inhibitor.

spectral overlap integral showed that Rox and Pyro have a theoretical Forster radius of ~50 Å, a distance much greater than the expected separation distance for a 9 amino acid linker (for context, a 9 residue alpha helical peptide would span 13.5 Å).

Since Rox donor absorption had no significant overlap with Pyro emission, there was minimal energy transfer from the Pyro to the Rox and Pyro remained unquenched (Figure 2a). Following Pyro excitation, the fluorescence emission of QUaPS was 10 times greater than the previously reported and corresponding Black Hole Quencher 3 (BHQ3) FRET acceptor linked to a Pyro donor (Figure 2B).[4] It has been shown that fluorescence intensity measurements can be used as a proxy for singlet oxygen detection for photodynamic molecular beacon unquenching.[13] To validate that QUaPS was a more potent photosensitizer, Rox-Pyro (QUaPS) or the corresponding Pyro-BHQ3 construct were incubated with HT-29 colorectal cancer cells and then subjected to 660 nm light for Pyro to generate singlet oxygen (Figure 2c). At all the concentrations and nonzero light doses examined, the QUaPS probe generated sufficient singlet oxygen to destroy all the cancer cells, whereas Pyro-BHQ3 was ineffective, due to the quenching of singlet oxygen generation by the dark quencher. This demonstrated that QUaPS generated singlet oxygen more effectively compared to Pyro donor caspase aPS, which were not activated in healthy cells.

We hypothesized that because Rox was an effective FRET donor for Pyro, the integrity of the peptide linkage between them could be queried by directly exciting Rox and examining the ratio of Rox and Pyro emission (Figure 3A). In solution, when QUaPS was intact, Rox excitation led to FRET and emission of Pyro at 680 nm (Figure 3B). As expected, following proteolytic digestion, FRET efficiency diminished and Rox excitation led primarily to direct Rox emission at 610 nm with minimal Pyro emission. In solution, the FRET ratios of the intact and cleaved QUaPS were consistent over the range of concentrations examined from 0.1 to 5 uM (Figure S2). When recombinant Caspase-3 was added to QUaPS, the emission ratio of Rox to Pyro increased over 150 min (Figure 3c). When Caspase-3 was omitted, or when ZVAD-fmk, a pan-caspase inhibitor was included, no change in the emission ratio was observed. Thus, by comparing the fluorescence of Rox to Pyro, a ratiometric readout of caspase activity based on QUaPS cleavage was obtained. The

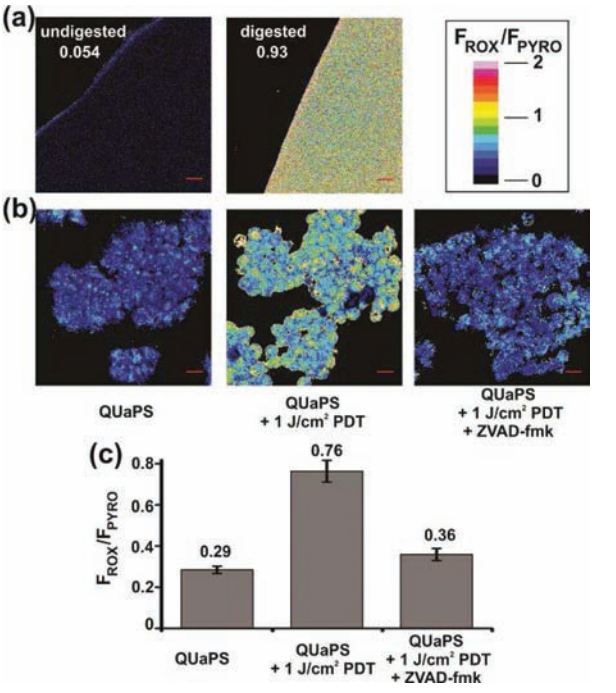

Figure 4. QUaPS as an inducer and ratiometric sensor of capase activation. (a) Fluorometric confocal microscope image of a 2 μM QUaPS solution, with or without proteolytic digestion. Numbers represent the Rox:Pyro average intensity ratio for the solution. FRET ratio legend is indicated. Scale bars represent 20 μm. (b) Induction of cell death and ratiometric imaging of caspase activation. Cells were incubated with 5 μM QUaPS and treated as indicated. Confocal microscopy was used to image the cells 2 h later. (c) Single cell analysis of caspase activation. Error bars show mean ± std. dev. for 30 cells for each group from 3 separate experiments.

normalized FRET ratio increase following caspase-3 cleavage was over 20-fold, high enough to easily distinguish the cleaved and uncleaved QUaPS.

Rox and Pyro emission maxima were spectrally well-separated by over 50 nm, and multichannel confocal microscopy could readily

be used to examine both fluorophores following Rox excitation. With the microscope settings used, the ratio of Rox to Pyro fluorescence displayed a 17-fold dynamic range when 2 μM QUaPS was incubated in solution following proteolytic digestion (Figure 4a). Next, 5 uM QUaPS was incubated with HT-29 cells for 3 h. The cells were then subjected QUaPS-mediated PDT using 660 nm light. Two hours later, caspase activation was also probed with QUaPS by exciting Rox and examining the Rox:Pyro FRET ratios. Without light treatment, the average Rox:Pyro ratio based on single cell analysis was just below 0.3 (Figure 3b, 3c). This value was significantly higher than the ratio for QUaPS in solution (0.054), suggesting that a moderate background level of peptide degradation occurred during the 3 h incubation. However, when the QUaPS was subjected to 1 J/cm^2 of 660 nm light, the ratiometric images demonstrated that QUaPS had been cleaved by activated caspases and the ratio increased to 0.76, well above the light-free treatment levels. Spatially, the greatest caspase activation was seen in the center of the cells. In some locations, the ratio increased to greater than unity, the highest value expected based on solution cleavage of QUaPS. An explanation for this phenomenon could be that following cleavage the peptide linker, differential subcellular partitioning in the cell occurred, leading to spatial separation of Pyro and ROX in some pixels. By including the caspase inhibitor ZVAD-fmk, caspase activity was reduced following PDT and the average ratio based on single cell analysis decreased to just above light-free treatment levels to 0.36. One potential limitation of QUaPS is that any singlet oxygen mediated Pyro self-bleaching would result in a decrease in Pyro emission and possibly an increase in Rox emission, giving the false appearance of proteolytic cleavage. In solution, a light dose of 2.5 J/cm^2 (two and a half times greater than that used with the cells) only minimally increased the QUaPS ratio by 4% (Figure S3). Fifteen J/cm^2 light treatment caused the ratio to increase by 60%. However these were relatively insignificant compared to the greater than 4000% change induced upon proteolytic cleavage of QUaPS. Therefore, at the low light doses used for these in vitro studies, bleaching effects were minimal. However, special care must be taken to account for any changes in the QUaPS ratio induced directly by singlet oxygen for potential *in vivo* situations requiring higher light doses.

In summary, QUaPS represents a simple approach that uses porphyrin FRET acceptors to produce greater singlet oxygen generation and provide practical ratiometric apoptosis sensing. By performing PDT until QUaPS is activated, the minimal amount of light could be used that places target cells into an apoptotic (as opposed to nonapoptotic or necrotic) state. By designing new QUaPS with red-shifted fluorescence donors and porphyrin acceptors, light of longer wavelength could be used to allow for deeper light penetration into the tissues of interest. QUaPS holds potential for new clinical scenarios in which direct molecular feedback is used to ensure the targeted treatment areas are treated with an appropriate light and photosensitizer dose.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures, HPLC-MS analysis of QUaPS, and FRET ratio stability with respect to concentration and light exposure. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Dougherty, H.; Dougherty, H.; Gomer, C. J.; Jori, G.; Kessel, D.; Korbelik, M.; Moan, J.; Peng, Q. *J. Natl. Cancer Inst.* **1998**, *90*, 889–905.
(2) Jarvi, M. T.; Niedre, M. J.; Patterson, M. S.; Wilson, B. C. *Photochem. Photobiol.* **2006**, *82*, 1198–1210.
(3) Kuimova, M. K.; Botchway, S. W.; Parker, A. W.; Balaz, M.; Collins, H. A.; Anderson, H. L.; Suhling, K.; Ogilby, P. R. *Nat. Chem.* **2009**, *1*, 69–73.
(4) Stefflova, K.; Chen, J.; Marotta, D.; Li, H.; Zheng, G. *J. Med. Chem.* **2006**, *49*, 3850–3856.
(5) Lovell, J. F.; Liu, T. W. B.; Chen, J.; Zheng, G. *Chem. Rev.* **2010**, *110*, 2839–2857.
(6) Mizukami, S.; Kikuchi, K.; Higuchi, T.; Urano, Y.; Mashima, T.; Tsuruo, T.; Nagano, T. *FEBS Lett.* **1999**, *453*, 356–360.
(7) Messerli, S. M.; Prabhakar, S.; Tang, Y.; Shah, K.; Cortes, M. L.; Murthy, V.; Weissleder, R.; Breakefield, X. O.; Tung, C.-H. *Neoplasia* **2004**, *6*, 95–105.
(8) Kim, K.; Lee, M.; Park, H.; Kim, J.-H.; Kim, S.; Chung, H.; Choi, K.; Kim, I.-S.; Seong, B. L.; Kwon, I. C. *J. Am. Chem. Soc.* **2006**, *128*, 3490–3491.
(9) Medintz, I. L.; Clapp, A. R.; Brunel, F. M.; Tiefenbrunn, T.; Tetsuo Uyeda, H.; Chang, E. L.; Deschamps, J. R.; Dawson, P. E.; Mattoussi, H. *Nat. Mater.* **2006**, *5*, 581–589.
(10) Bullok, K.; Piwnica-Worms, D. *J. Med. Chem.* **2005**, *48*, 5404–5407.
(11) Kasili, P. M.; Song, J. M.; Vo-Dinh, T. *J. Am. Chem. Soc.* **2004**, *126*, 2799–2806.
(12) Chen, J.; Stefflova, K.; Niedre, M. J.; Wilson, B. C.; Chance, B.; Glickson, J. D.; Zheng, G. *J. Am. Chem. Soc.* **2004**, *126*, 11450–11451.
(13) Lovell, J. F.; Chen, J.; Jarvi, M. T.; Cao, W.-G.; Allen, A. D.; Liu, Y.; Tidwell, T. T.; Wilson, B. C.; Zheng, G. *J. Phys. Chem. B* **2009**, *113*, 3203–3211.