Photodynamic therapy via FRET following bioorthogonal click reaction in cancer cells

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ABSTRACT

Longer wavelength light (650–800 nm) is desired to treat large tumors in photodynamic therapy (PDT). However, shorter wavelength light is needed in PDT for thin tumors, not to cause undesirable local side effects. We proposed a strategy for stepwise optical imaging and PDT using a bioorthogonal click chemistry and fluorescence resonance energy transfer (FRET). We prepared azidyl rhodamine (Rh-N_3, clickable FD) and cyclooctynyl phthalocyanine [Pc-(DIBAC), clickable PS], with which, here, we demonstrate that the non-catalytic click chemistry is rapid and efficient in cancer cells and FRET from a fluorescence dye (FD) to a photosensitizer (PS) is sufficient to generate enough singlet oxygen killing cancer cells by using shorter wavelength light.

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Photodynamic therapy (PDT) has much less side effects than more established treatments like chemotherapy and radiation therapy in treating local tumors. PDT is a unique treatment modality involving a photosensitizer (PS) that absorbs photons upon illumination, converting the photonic energy to chemical energy.[1–4] The photodynamic process generates cytotoxic singlet oxygen that damages tumors. PDT was approved for the clinical treatment of diseases including the wet form of age-related macular degeneration, esophageal and lung cancers, and actinic keratosis.

Bladder cancer, in particular non-muscle invasive bladder cancer (NMIBC), was a prime target for PDT in the early PDT era due to easy accessibility of light to bladder lumen through the urethral track.[5,6] However, PDT for NMIBC was not successful due to bladder dysfunction after PDT.[7] PDT with red light (630 nm) for exciting Photofrin, although NMIBC resides in thin layers of urothelium and lamina propria,[8,9] damaged bladder muscle.[10] For such thin tumors, shorter wavelength light can be more beneficial in minimizing damage of normal tissues under the tumors, which is counterintuitive to the conventional PDT approach where longer wavelength light is preferred for deeper tissue penetration. In the bladder PDT, high specificity of PSs to tumors is also required to minimize collateral damage to normal epithelium because a whole bladder is illuminated to treat multifocal cancers.[11]

Recently, optical imaging has emerged as a very useful tool for detecting small invisible cancers to improve surgical outcome.[12–14] FDA approved HAL (hexyl-5-aminolevulinic acid) was approved for the fluorescent diagnosis of NMIBC in 2010.[15,16] It is intravesically administered for cancer cells to accumulate protoporphyrin IX. Although it was demonstrated to improve detection sensitivity of small tumors, further improvement is necessary. PpIX is not an ideal fluorescence probe because it has low fluorescence quantum yield as well as poor solubility in water causing aggregation and fluorescence self-quenching.

To overcome such problems in detection and treatment of NMIBC, we proposed a new strategy where the fluorescence imaging and PDT are performed stepwise. First, tumors can be detected by fluorescence imaging using fluorescence dye (FD). After a bioorthogonal click reaction of FD and PS to form FD–PS conjugate in cancers, singlet oxygen can be generated from FD–PS excited by either shorter wavelength light *hv1* or direct activation using longer wavelength light *hv3* of PS (Fig. 1). Use of shorter wavelength light *hv1* will minimize muscle damage in treating NMIBC. There are three key questions to be addressed for this strategy to be successful. (1) Can the FD be specifically delivered to cancer cells using bladder cancer targeting vectors? (2) *Can the click reaction be accomplished in a short period time (∼1 h) in cancer cells?* (3) *Can singlet oxygen be generated from FD–PS enough via the FRET to kill cancer cells after exciting FD with shorter wavelength light hv1?* In this Letter, we provide clear answers for keys questions # (2) and # (3).

We used azide-functionalized rhodamine (Rh-N_3, clickable FD) and cyclooctyne-functionalized phthalocyanine [Pc-(DIBAC), clickable PS] for a bio-orthogonal copper-free click reaction.[17–26] We chose the combination of Rh and Pc because effective energy
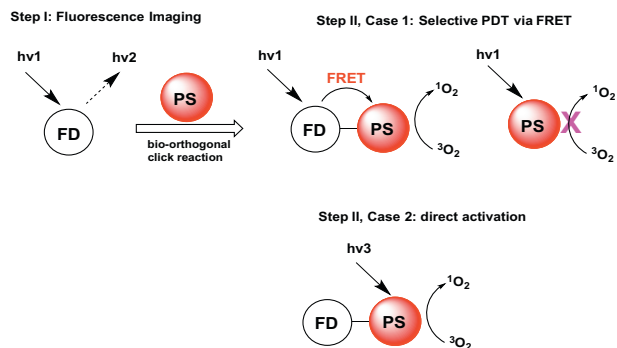
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Figure 1. Selective activation of FD–PS via FRET using *hv1*, avoiding activation of non-conjugated PS or direct activation (using *hv3*) of PS. If FD is a FRET donor and PS is an acceptor, wavelength of *hv1* is shorter than wavelength of *hv3*.

transfer from Rh (a FRET donor) to Pc (a FRET acceptor) has been demonstrated.[27]

The synthesis of Rh-N$_3$ and Pc-(DIBAC) involved two facile and high-yielding reactions for each compound (Scheme 1A). For Rh-N$_3$, the first step was azide formation using sodium azide, followed by esterification of rhodamine B and 3-azidopropan-1-ol using DCC and DMAP. Pc-(DIBAC) was synthesized by amide formation between DBCO acid and compound **2**. The purity of Rh-N$_3$ and Pc-(DIBAC) was confirmed by HPLC, >95% (Figs. S8 and S9).

We first confirmed the FRET from Rh to Pc in Rh–(DIBAC)-Pc by observing a dramatic decrease of Rh fluorescence. The absorption spectra of Rh–(DIBAC)-Pc showed the characteristic absorption peaks of its individual components Pc-(DIBAC) and Rh-N$_3$ (Fig. 2A). There was no significant change in electronic absorption of Rh and Pc chromophores in Rh–(DIBAC)-Pc at the ground state. However, we observed a dramatic decrease of Rh fluorescence emission in Rh–(DIBAC)-Pc compared with Rh-N$_3$ at the equimolar concentration, most likely due to FRET from Rh to Pc (Figs. 1B and 2B).
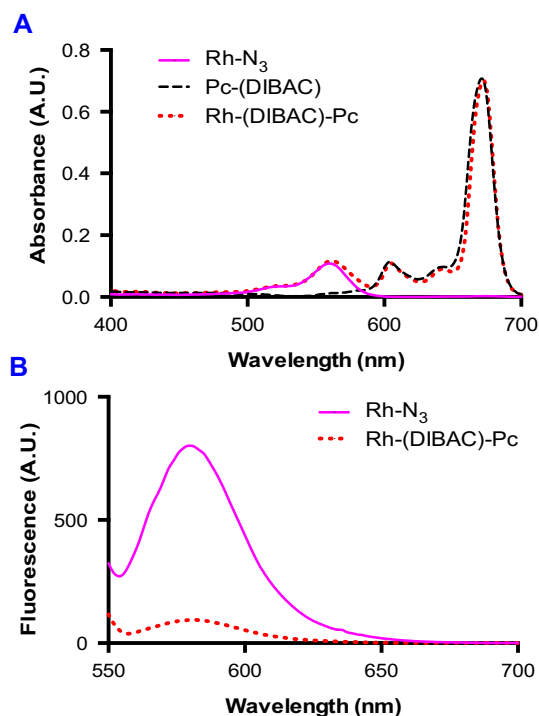

Figure 2. (A) Absorption spectra of Rh-N$_3$, Pc-(DIBAC) and Rh–(DIBAC)-Pc in acetonitrile. (B) Fluorescence emission spectra of Rh-N$_3$ and Rh–(DIBAC)-Pc in acetonitrile at 525 nm excitation (both at 0.05 µM). Inset—UV–vis spectra of Rh-N$_3$ and Rh–(DIBAC)-Pc at the same absorbance at the Rh peak.

We then monitored the progress of the click reaction of Rh-N$_3$ and Pc-(DIBAC) in acetonitrile using fluorescence emission changes; that is, examining the decrease in Rh emission due to FRET. The fluorescence emission from the Rh group was highest at the beginning of the reaction before the click reaction. However, as the reaction between Rh-N$_3$ and Pc-(DIBAC) proceeded, the fluorescence of Rh gradually decreased due to the FRET from Rh to the Pc (Fig. 3A). [*There was no photobleaching of Rh-N$_3$ by excitation at 525 nm for fluorescence determination (Fig. S10). The Rh fluorescence decrease was not due to photobleaching of Rh moiety.*] After the bioorthogonal click reaction, Rh–(DIBAC)-Pc was formed (yield, 90%, ^{1}H NMR and high mass in Figs. S11 and S13). In contrast, the fluorescence of Pc (Fig. 3B) remained unchanged, meaning there was no FRET from Pc to Rh in the Rh–(DIBAC)-Pc, consistent with a previous report.[22]

We were able to monitor the click reaction in the cultured cells (T-24 cells, human bladder carcinoma cells). We observed rapidly diminished Rh fluorescence in the cells after the addition of Pc-(DIBAC), due to the click reaction. The emission of Rh of the cells decreased in a time-dependent manner after the addition of Pc-(DIBAC) into the cells having Rh-N$_3$ (Fig. 4A). The reduction of Rh fluorescence reached maximum in 60 min. Confocal imaging of the cells provided further evidence of the click reaction in the cells (Fig. 4B). Most of the Rh fluorescence was quenched after 50 min without observable morphological change of the cells (Fig. 4Bb').

Singlet oxygen generation of Rh–(DIBAC)-Pc via FRET was evaluated using DPBF (1,3-diphenylisobenzofurance).[28] Light, Rh-N$_3$, or Pc-(DIBAC) alone did not produce any significant DPBF oxidation by 531 nm laser illumination, exciting Rh moiety. Only Rh–(DIBAC)-Pc produced rapid DPBF oxidation due to FRET from Rh to Pc (Scheme 1B). After 14 min of illumination, the illuminated Rh–(DIBAC)-Pc with DPBF showed significantly lower DPBF absorption than the control (DPBF only; *p* <0.001, Fig. 5). The generation of singlet oxygen via FRET [Rh–(DIBAC)-Pc + *hv* (531 nm at
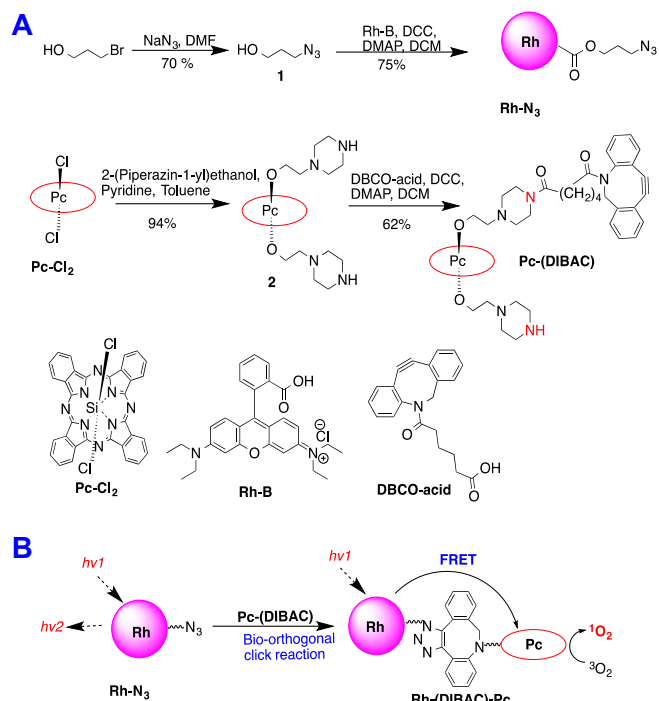
Scheme 1. (A) Reaction sequence and prepared donor fluorophore (Rh-N$_3$) and acceptor PS (Pc-(DIBAC)); (B) bioorthogonal click reaction process.