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Protease-Mediated Phototoxicity of a Polylysine–Chlorin$_{e6}$ Conjugate

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Type II photosensitizers generate cytotoxic singlet oxygen ($^1O_2$) by energy transfer from the triplet excited state to neighboring oxygen molecules.[1] Owing to this action, target tissues such as tumors can be selectively destroyed by local illumination following intravenous administration of a photosensitizer. Although many photosensitizers accumulate to some degree in tumors, they also distribute to normal tissues and show undesired phototoxicity such as skin photosensitivity brought on by bright indoor light or sunlight.[2] Thus, it is recommended that patients avoid exposure to sunlight for several weeks to months following photodynamic therapy (PDT). Herein, we report a novel design of protease-mediated photosensitization by which phototoxicity can be selectively turned on through tumor-associated proteases (Figure 1a). As normal tissues exhibit highly regulated protease expression, phototoxicity in normal tissues can be minimized.

To obtain convertible phototoxicity, multiple copies of the photosensitizer chlorin$_{e6}$ (Ce6), were conjugated onto a poly-L-lysine backbone, similar to previously developed protease-activated near-infrared (NIR) fluorescent probes for cancer imaging.[3] Ce6, a commercially available second-generation photosensitizer, was chosen for conjugation because of its reactivity and the significant overlap between its emission and absorption spectra (Figure 1b). The overlapping spectra and close geometry between the conjugated photosensitizers make self-quenching efficient,[4] thereby prohibiting the process of energy transfer between the photosensitizer and neighboring oxygen, and thus inhibiting the generation of cytotoxic singlet oxygen. When the peptide linkages of the polylysine backbone are cleaved by tumor-associated enzymes (such as cathepsins), the degraded probes become highly phototoxic and fluorescent because no more resonance energy transfer occurs between the photosensitizers. A second possibility for the induction of the self-quenching is the formation of intramolecular aggregates of the conjugated photosensitizers. Porphyrin-based photosensitizers have been shown to have decreased fluorescence intensity upon aggregation at increased concentrations. The decrease in fluorescence intensity is directly proportional to the decrease in the production of triplet state intermediates and singlet oxygen.[5–7] Based on these observations, we hypothesized that conjugation of multiple Ce6 molecules onto a polymer backbone would induce aggregation of the conjugated Ce6 depending on the conjugation ratio within the polymer backbone, resulting in diminished fluorescence and singlet oxygen generation.

Ce6 was conjugated to poly-L-lysine grafted with monomethoxy-poly(ethylene glycol) (L-PGC) at various ratios (Supporting Information). To optimize the quenching-to-activation ratio, four substitution ratios (SR) of the L-PGC conjugate were prepared: 0.9 ± 0.7, 5.9 ± 0.1, 15.0 ± 1.2, and 36.4 ± 0.8 Ce6 molecules per L-PGC chain, referred to as L-SR1, L-SR6, L-SR15, and L-SR36, respectively. The same polymeric template, but with non-natural D-lysine residues (D-PGC), was used as a control for Ce6 conjugation because the D-form polypeptide is not readily degraded by natural proteases. Three similar substitution ratios of D-PGC conjugate were prepared with 4.3 ± 0.3, 16.2 ± 0.6, and 39.8 ± 0.5 Ce6 molecules per D-PGC chain (D-SR4, D-SR16, and D-SR40, respectively).

The fluorescence properties of the conjugates were compared at an equimolar concentration of Ce6 in phosphate buffer solution (10 mM, pH 7.0). It was found that the fluorescence intensity of the conjugates decreased with increasing substitution ratios of Ce6 (Figure 2a). When the L-PGC conjugates were digested by the lysine-recognizing protease trypsin, fluorescence intensities were increased twofold for L-SR6 and L-SR36, and 4.2-fold for L-SR15. No change in the fluorescence intensity was observed for L-SR1, indicating that there was no quenching in the native state. In contrast, D-PGC conjugates also showed SR-dependant fluorescence quenching, but there was no significant fluorescence change following protease treatment (Figure 2b).

Singlet oxygen generation (SOG) of the conjugates showed trends similar to the fluorescence properties discussed above (Figure 2c and d). SOG of both L- and D-PGC conjugates decreased with increasing SR of Ce6. The SOG of L-SR6 was 32% of that of free Ce6 at equimolar concentrations, and that of L-SR15 was further decreased to 12%. No SOG was observed with L-SR36. Similar trends were observed for D-PGC conjugates. Importantly, SOG was recovered by proteolysis. Treatment of the conjugates L-SR6 and L-SR15 with trypsin resulted in 2.7 and 5.4-fold increases in SOG, which represent 86 and 65% recovery of total phototoxicity, respectively (Figure 2c). As observed in fluorescence activation experiments, no improvement in SOG was observed with L-SR36. None of the D-PGC conjugates showed changes in SOG with enzyme treatment, as their peptide backbones are nondegradable (Figure 2d).

Comparison of the UV/Vis absorption spectra of L-SR15, L-SR36, and free Ce6 in phosphate buffer solution indicates the presence of aggregation after conjugation, as shown in Figure 3a and b.[8,9] Both conjugates showed significant broadening of the Soret band region of the spectrum, whereas L-SR36 showed a broader spectrum than that of L-SR15. Following trypsin treatment for 4 h, the absorption spectrum of L-SR15 narrowed and approximated the spectrum of free Ce6 (Figure 3a). In contrast, L-SR36 still showed minor changes in the UV/Vis spectrum following trypsin treatment (Figure 3b). By in-

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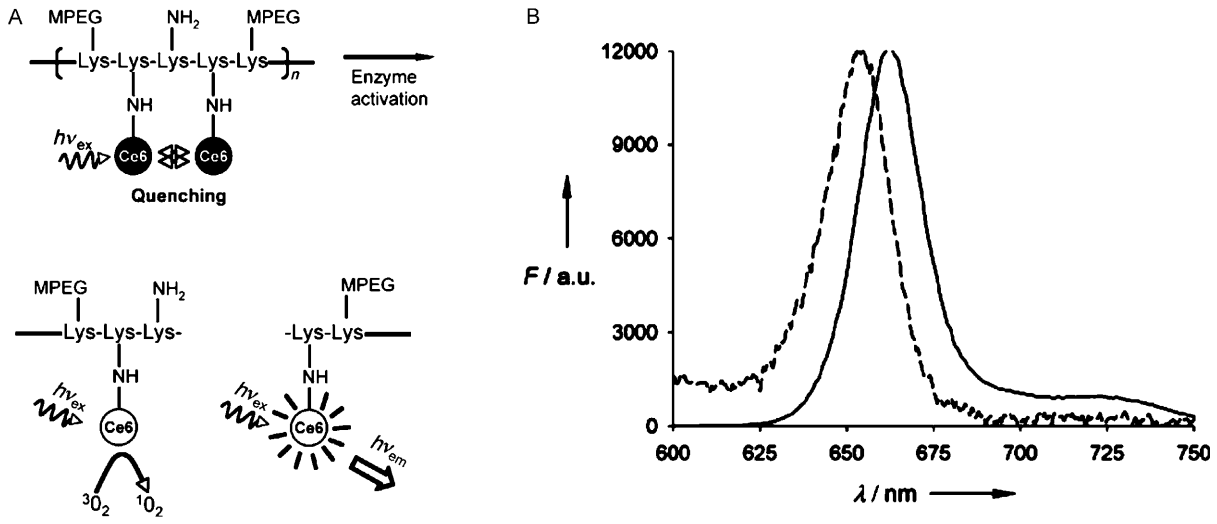

Figure 1. A) Activation of fluorescence signal and generation of singlet oxygen by protease activity; B) absorption (-----) and emission (——) ($\lambda_{ex}=400$ nm) profiles of Ce6 in phosphate buffer (10 mM, pH 7.4).

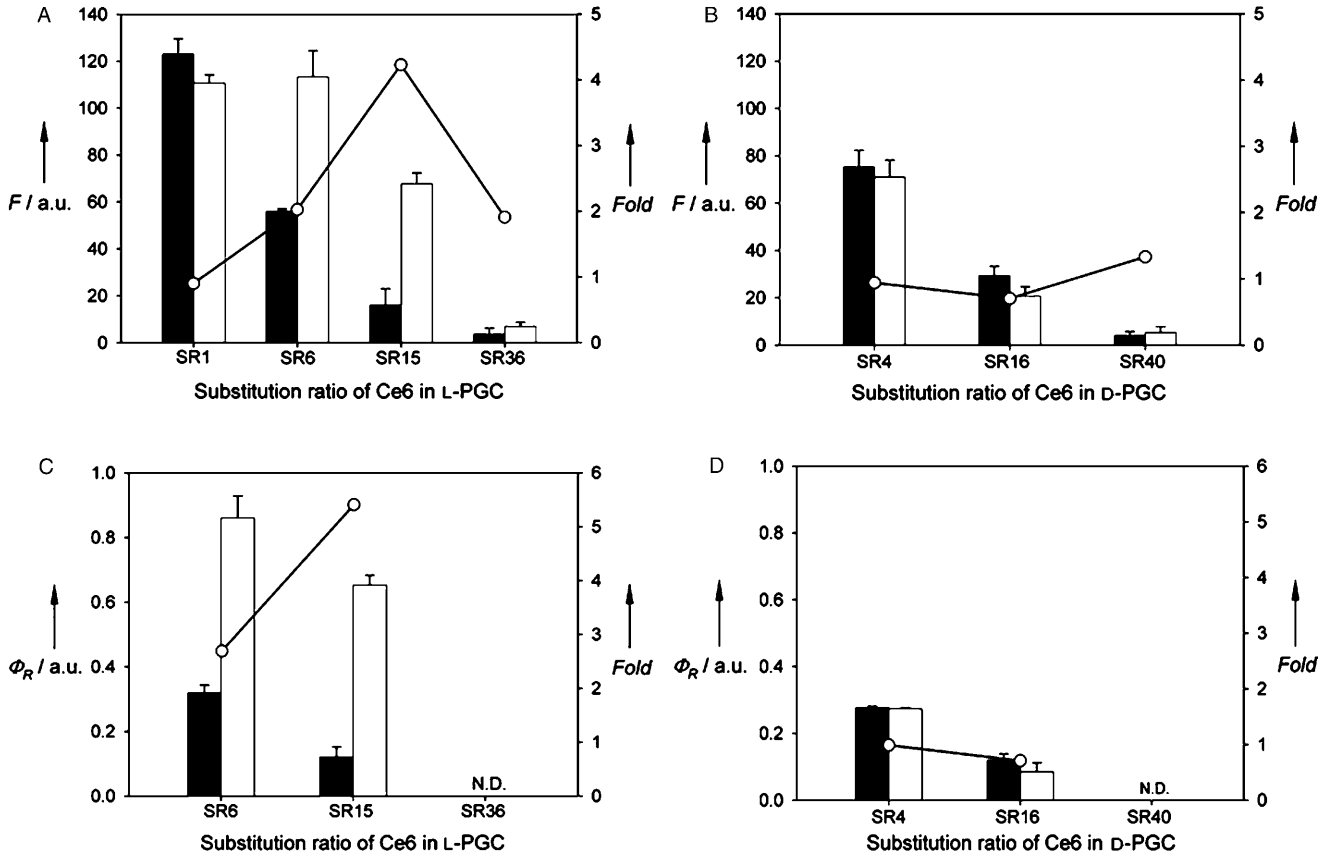

Figure 2. Trypsin-induced changes of fluorescence (A and B) and singlet oxygen generation (SOG; C and D). L-PGC conjugates (A and C) and D-PGC conjugates (B and D) treated with trypsin (open bars) or with phosphate buffer only (filled bars). Fluorescence was measured at $\lambda_{ex}=650$ nm and $\lambda_{em}=670$ nm, and the SOG was determined by irradiation at $\lambda=650$ nm. The *y*-axes show fold increase (○) in the fluorescence signal and SOG. Experiments were performed in triplicate, mean ± SD. N.D. = not determined.

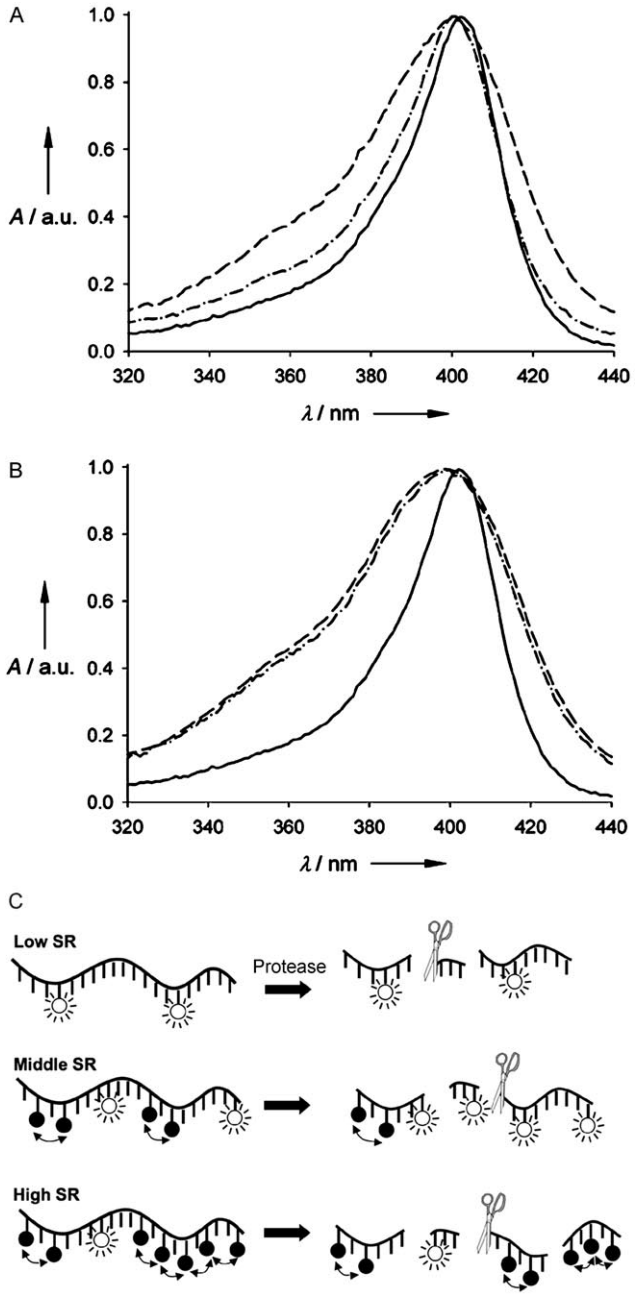

Figure 3. Normalized UV/Vis absorption spectra of A) L-SR15 and B) L-SR36. Spectra show conjugates (-----), trypsin-treated conjugates (–•–•), and free Ce6 (——) in phosphate buffer solution. C) SR-dependant fluorescence activation after enzyme treatment (arrow indicates energy transfer between photosensitizers).

creasing the SR of Ce6 in L-PGC, the number of free lysine residues in the poly-L-lysine backbone is decreased, resulting in fewer sites for enzymatic cleavage. This results in larger degradation products, which are still partially quenched (Figure 3c). This would explain why the fluorescence intensities and SOG were not fully recovered at higher SRs.

The activation of fluorescence signal was subsequently studied in cell culture. Previously, we demonstrated that the PGC backbone can be degraded by trypsin-like cysteine proteases, including cathepsins B, L, and S.[3] Incubation of HT1080 fibrosarcoma cells with the L-PGC conjugates at concentrations corresponding to 1 μM Ce6 for 4 h gave SR-dependant decreases in fluorescence intensity with increasing SR of L-PGC conjugates, as observed by confocal microscopy of the cells (Figure 4). No fluorescence signal was observed in the cells incubated with free Ce6. Previously, it was reported that the cellular uptake of porphyrin derivatives, including Ce6, is significantly lower in the presence of serum than it is in the absence of serum, because nonspecific binding to serum prevents intracellular uptake of the photosensitizers.[10,11] Therefore, the results reported herein indicate that conjugation of Ce6 with L-PGC is helpful to overcome this shortcoming. Prior studies indicate that up to 5% of the injected dose of PGC accumulates in tumors[12] as a result of the enhanced permeability and retention (EPR) effect.[13]

The correlation between fluorescence intensity and phototoxicity was further investigated in cell studies. From the in vitro phototoxicity study, L-PGC conjugates with lower SR showed better phototoxicity than those with higher SR. Cell viability (at a light dose of 10 J cm^{-2}) was 53.9 ± 3.1% for SR6, 70.5 ± 2.9% for SR15, 80.2 ± 1.1% for SR36, and 95.1 ± 6.4% for free Ce6 (Figure 5). Significant differences ($p < 0.01$) in cell viability were observed between all groups. The cell viability data correlated well with confocal microscopy data.

The above data indicate that chemical optimization is an essential step in preparing protease-mediated photosensitizers. Although the preparation with a high SR ratio, such as L-SR36, showed near complete quenching of fluorescence and SOG, it could also not be activated. L-SR15 showed the highest activation ratio of the conjugates tested in this study. This lead conjugate had only 12% phototoxicity in its initial state, but proteolysis increased its phototoxicity by greater than fivefold.

The results of this study show that the inhibition of SOG can be achieved by conjugating multiple Ce6 photosensitizers onto a polypeptide backbone, and that the photosensitivity of Ce6 can be recovered by proteolytic activity. We expect that higher increases in SOG after enzyme treatment and better enzyme selectivity can be obtained by inserting other protease-selective peptide substrates between the photosensitizers and the polymeric backbone, such as previously reported with protease-sensitive probes.[14] As porphyrin-based photosensitizers show fluorescence quenching and decreased SOG at increased concentration, this protease-mediated approach to PDT may be applied not only to Ce6 but also to other porphyrin derivatives. In addition, this protease-activated design may be useful to treat specific types of diseases in which a targeted protease is overexpressed, while prohibiting photosensitivity to normal tissues.

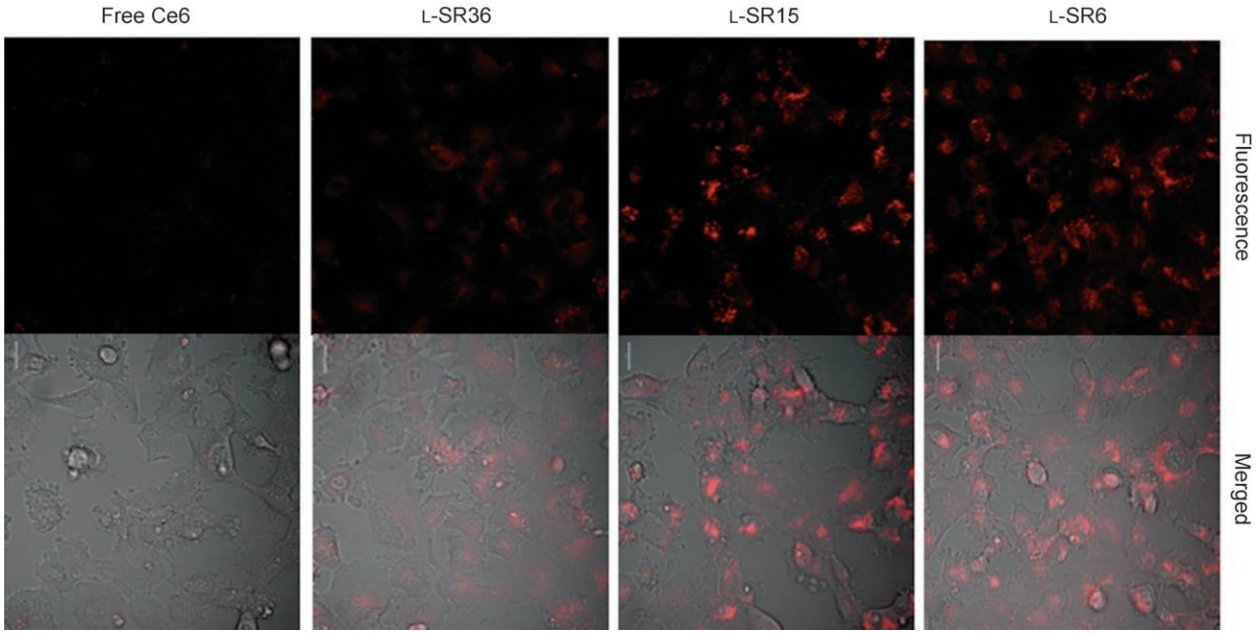

Figure 4. Confocal microscopy images of unfixed HT1080 cells incubated with free Ce6 or L-PGC conjugates (1 μM) with different SRs. Top row: fluorescence images of the cells. The fluorescence signals are from Ce6. Bottom row: transmitted light images merged with the fluorescence image above. Magnification: 40×.

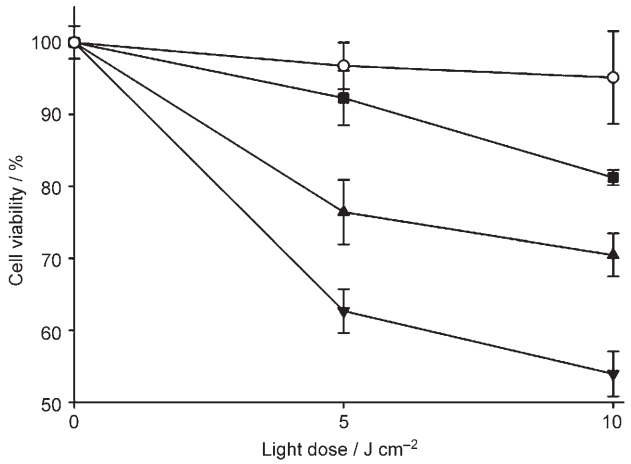

Figure 5. In vitro phototoxicity of free Ce6 and L-PGC conjugates. Photosensitizers (equivalent to 1 μM Ce6) were incubated for 4 h, then treated with laser light ($\lambda = 650$ nm, $n = 5$–6) at varying doses. Symbols represent cells incubated with free Ce6 (○), L-SR36 (■), L-SR15 (▲), and L-SR6 (▼). Significant differences ($p < 0.01$) in the cell viability were observed between all groups at both light doses (5 and 10 J cm^{-2}). Experiments were performed in triplicate, mean ± SD.

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