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Conjugation of a Photosensitizer to an Oligoarginine-Based Cell-Penetrating Peptide Increases the Efficacy of Photodynamic Therapy

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To improve the efficiency of intracellular delivery of photosensitizers and the efficacy of photodynamic therapy, a membrane-penetrating arginine oligopeptide (R_7) was conjugated to 5-[4-carboxyphenyl]-10,15,20-triphenyl-2,3-dihydroxychlorin (TPC). The resulting conjugate (R_7–TPC) enhanced intracellular TPC uptake, which increased proportionally with the incubation time of the conjugate. The water solubility of the highly hydrophobic TPC photosensitizer was also improved after conjugation. Increased phototoxicity of R_7–TPC was observed after an incubation time of only 30 min. Tumor cells mainly underwent apoptosis at lower concentrations of the photosensitizer–polyarginine conjugate, whereas necrotic cell damage became prevalent at higher concentrations.

Introduction

Photodynamic therapy (PDT) is a relatively new modality for the treatment of cancers and other nonmalignant conditions.[1] It involves the administration of a photosensitizing agent, usually a porphyrin-based compound, and subsequent illumination of the tissue by a visible, nonthermal light source of the appropriate wavelength. This light exposure excites the photosensitizer, which is then able to interact with its surroundings. In oxygenated environments, the energy of the excited state is often dissipated by transfer to molecular oxygen, which leads to the formation of the highly reactive and cytotoxic singlet oxygen species. When this process occurs within tissues, it results in cellular damage.[2] As this effect is observed only in the presence of light, PDT is locally selective, thereby minimizing the damage to surrounding healthy tissue. When injected, porphyrin-based photosensitizers are found to be taken up by malignant or dysplastic tissues with some selectivity; however, the hydrophobic nature of photosensitizers often causes them to accumulate in healthy tissues, resulting in prolonged photosensitivity.[3] Cases of skin and eye photosensitivity have been observed in clinical trials, requiring patients to avoid sunlight exposure for several weeks or months.[1,3]

Cellular localization is important to the efficacy of PDT agents, as singlet oxygen has a short lifetime (<0.04 μs) and a radius of action (<0.02 μm) that is small in comparison with the diameter of tumor cells (≥ 10 μm).[4] Although many photosensitizers in current use tend to accumulate within the plasma membrane of cancer cells as a result of their lipophilicity,[5,6] some subcellular sites have been shown to be more sensitive to photodynamic damage than the plasma membrane.[7] Nevertheless, various delivery systems, such as nuclear localization signals and receptor targeting, have been suggested to enhance subcellular accumulation.[7,8]

Recently, arginine-rich peptides, originating from the HIV-1 Tat protein and other proteins, have been reported as cell-penetrating signals.[9] These oligoarginine peptides have been applied to the delivery of various chemical agents and drugs into cells.[9–11] Previous studies have demonstrated that the conjugation of *meso*-tetraphenylporphyrin to positively charged peptides containing up to three arginine residues showed increased cellular uptake, yet the photodynamic efficacy of this delivery system has not been demonstrated.[10] As it has been shown that longer polyarginine chains (heptamers and nonamers) undergo more efficient cellular uptake than do monomers, dimers, or trimers,[11,12] we hypothesized that the conjugation of an arginine heptamer oligopeptide (R_7) to a potent chlorin-based photosensitizer, 2,3-*vic*-dihydroxy-*meso*-tetraphenylchlorin,[13] would drastically increase the effectiveness of tumor cell killing by improving the aqueous solubility and cellular uptake of the conjugate. Thus, this novel class of cell-penetrating peptide-based photodynamic therapy agents may permit a decrease in the dose of photosensitizer required for the treatment of cancers.

Results

Synthesis and characterization of the photosensitizer–peptide conjugate

The photosensitizer used in this study was chosen for its optical properties and its reactivity. 5-[4-Carboxyphenyl]-10,15,20-

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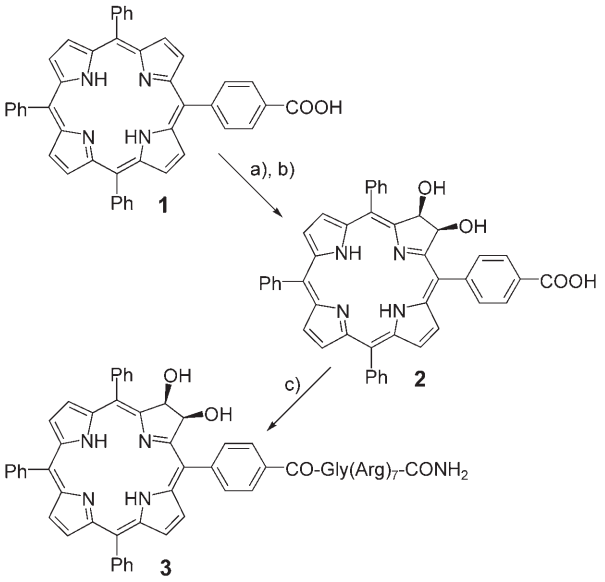
triphenyl-2,3-dihydroxychlorin (TPC) was synthesized by the osmium tetroxide oxidation of the corresponding porphyrin (Scheme 1).[14] This reaction is one of the few known pathways by which a porphyrin can be directly converted into a chlorin,



Scheme 1. Preparation of the R_7–TPC conjugate: a) OsO_4, pyridine; b) H_2S; c) solid-phase peptide synthesis.

and as such, is more efficient than other multi-step, total syntheses of chlorins or extraction from natural sources.[15] TPC was purified by HPLC, as column chromatography with silica gel was rendered unsuccessful from impurities of similar polarity. The identity of the product was confirmed by mass spectrometry ($m/z = 693.2492$ $[M+H]^+$) and ^1H and ^{13}C NMR spectroscopy. The ^1H NMR spectra showed peaks characteristic of diol chlorins, namely a singlet at 3.3 ppm, which is exchangeable with D_2O and attributable to the protons of the hydroxy groups, and a singlet at 6.5 ppm that corresponds to the methynic protons of the partially saturated pyrrolic ring. In the ^{13}C NMR spectra, the carbon atoms of the partially saturated pyrrolic ring are observed at 74 ppm, while the resonance for the carbon atom of the acid moiety is at 168 ppm. Purified TPC was coupled to the N terminus of the arginine oligopeptide on solid support. The final R_7–TPC product was purified by HPLC and confirmed by MALDI-TOF mass spectrometry ($m/z =$ 1842 $[M+H]^+$).

The UV/Vis spectrum of TPC (Figure 1 A) is similar to that observed for analogous chlorins, having a broadened Soret band with a slightly lower extinction coefficient (1.0×10^5 L mol^{-1} cm^{-1}) than that of the starting porphyrin (1.6×10^5 L mol^{-1} cm^{-1}). Moreover, the longest-wavelength side band for TPC is sevenfold greater than that of porphyrin (1.5×10^4 L mol^{-1} cm^{-1} for TPC versus 2.0×10^3 L mol^{-1} cm^{-1} for the starting porphyrin). Surprisingly, upon TPC conjugation to the oligopeptide, the extinction coefficient of the Soret band increases (1.6×10^5 L mol^{-1} cm^{-1}, Figure 1 B). This contrasts with what is usually observed for the dissolution of relatively hydrophobic porphyrins in aqueous solution, as aggregation often occurs and leads to decreased and broadened absorption.[16] This may indicate that the conjugation of TPC to the peptide results in a product that is favorably solvated, although there is a slight decrease ($\approx$33 %) in the extinction coefficient of the longest-wavelength side band (1.0×10^4 L mol^{-1} cm^{-1}).

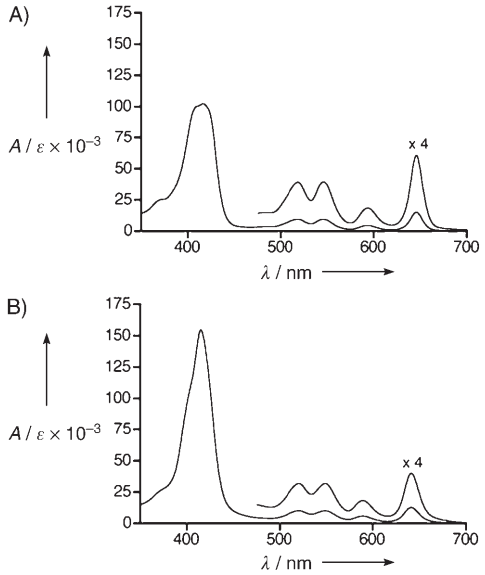


Figure 1. UV/Vis spectra of A) TPC in CH_2Cl_2/MeOH (99:1 *v/v*), and B) R_7–TPC in water.

Singlet oxygen quantum yields (ϕ_Δ) were calculated for both TPC and R_7–TPC with 1,3-diphenylisobenzofuran (DPBF) as the probe molecule in *N*,*N*-dimethylformamide (DMF) (Table 1).[17,18] DPBF, a fluorophore, has been shown to chemically quench singlet oxygen to yield a nonfluorescent species. Therefore, the singlet oxygen quantum yield for each compound was obtained by comparing the initial slope of the decrease in fluorescence intensity versus time for the molecule of interest against that of a standard. The singlet oxygen quantum yield was not altered after conjugation, which indicates that phototoxicity was retained.

Table 1. Physical properties of chlorins used in this study and the corresponding singlet oxygen quantum yields.

Compd	λ_{max} [nm]	log ε[a]	ϕ_Δ
TPC	646[b]	4.18	0.65[e]
R_7–TPC	642[c]	4.00	0.69[e]
chlorin e6	664[d]	4.60	0.75[f]

[a] Extinction coefficients are for the longest-wavelength absorptions. [b] In CH_2Cl_2/MeOH (99:1 *v/v*). [c] In H_2O. [d] In acetone. [e] In DMF. [f] In phosphate-buffered saline.[27,28]

Cellular uptake of photosensitizers

Cellular uptake of the photosensitizers chlorin e6 (C_{e6}) and R_7–TPC was quantified by fluorescence measurement at different

time points (Figure 2). It was found that only a trace amount of C_{e6} was taken up by MDA-MB-468 cells, and that longer incubation times did not lead to improved internalization. However, R_7–TPC showed an almost linear relationship between

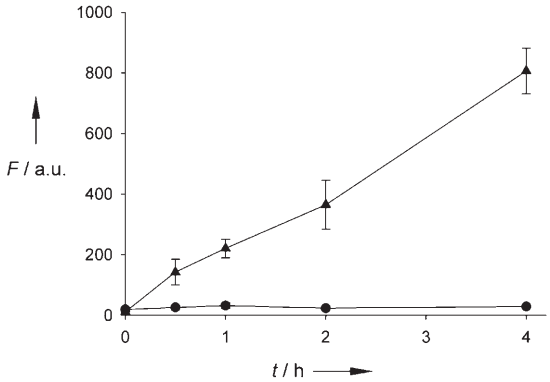


Figure 2. Intracellular uptake of C_{e6} (●) and R_7–TPC (▲) at different incubation times. Photosensitizers were dissolved in serum containing growth medium at a final concentration of 1 μM and distributed to a 24-well plate for 30 min, 1 h, 2 h, and 4 h ($n=4$, a.u.: arbitrary units).

uptake and incubation time. About 5.78 ± 0.55 % of the added conjugate was internalized within 4 hours as determined by calculation from a calibrated standard solution. In contrast, the cells took up only 0.06 ± 0.03 % of C_{e6} within 4 hours. Internal distribution of R_7–TPC was further observed by confocal microscopy (Figure 3). In less than 30 min, a characteristic endosomal distribution of the conjugate was observed. Longer incubation led to greater accumulation in the cell. In particular, a significant amount of R_7–TPC was found along the nuclear membrane, but not within the nucleus. As expected, minimal fluorescence was observed for cells incubated with C_{e6}. However, during image acquisition, changes in cell morphology were observed (Figure 3). Repetitive laser scanning of cells may excite the photosensitizers and thus cause cell damage.

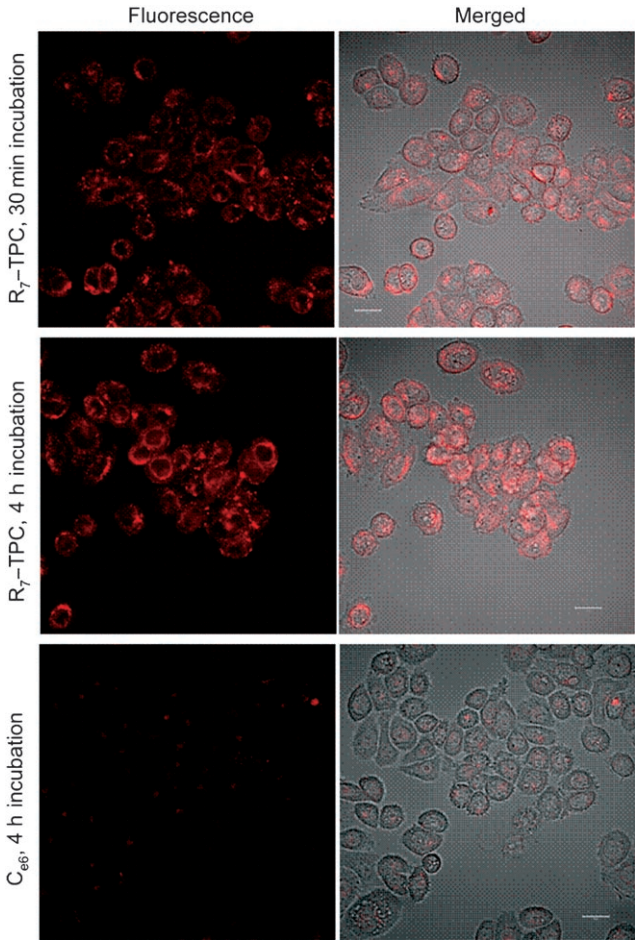


Figure 3. Confocal microscopy images of unfixed MDA-MB-468 cells incubated with C_{e6} or R_7–TPC (1 μM) at various incubation times. Left column: fluorescence images; fluorescence signals were from chlorin. Right column: Transmitted light images merged with the respective fluorescence image. Magnification: 40×; scale bars: 20 μm.

Light-induced phototoxicity

To demonstrate the effectiveness of PDT with the R_7–TPC conjugate, cells were incubated with C_{e6} or R_7–TPC (1 μM) for various times. After incubation, the cells were washed, illuminated with light, and then incubated for an additional 24 hours. Cell survival was then determined with an MTT assay. For the R_7–TPC treated groups, cell survival rates were 20.5 ± 3.7, 13.7 ± 2.7, 10.1 ± 1.7, and 11.5 ± 3.4 % for 0.5, 1, 2, and 4 hours incubation time, respectively (Figure 4). As expected, cells incubated with C_{e6} showed no significant cell death. At the tested concentrations of both photosensitizers, dark toxicity was not observed in the absence of light exposure (data not shown). Cells incubated with the R_7 peptide for 4 hours and treated with light also showed no cell death, which indicates that R_7 alone does not affect cell viability.

The possible pathways of cell damage were studied by staining the cells with Hoechst 33342 and PI fluorescent dyes. The Hoechst dye is known to stain all nuclei, whereas PI only stains

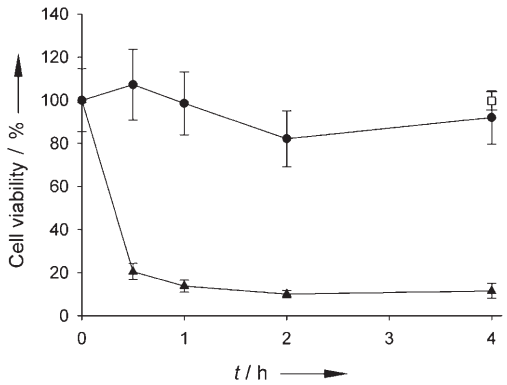


Figure 4. Phototoxicity at varying incubation times for R_7 (□), R_7–TPC (▲), and C_{e6} (●). Cells were incubated with photosensitizers (1 μM) for 0.5, 1, 2, and 4 h, then treated with laser light ($\lambda=650$ nm, $n=4$). Cells were treated with R_7 (1 μM) for 4 h, then exposed to light ($n=4$). Cell survival rate was measured by the MTT assay. Significant difference ($p<0.01$) in cell viability between groups treated with C_{e6} and R_7–TPC was observed at all incubation time points.

necrotic cells. As shown in Figure 5, cells treated with R_7–TPC plus PDT show intense nuclear PI signal, which indicates necrosis. As expected, longer incubation with R_7–TPC induced

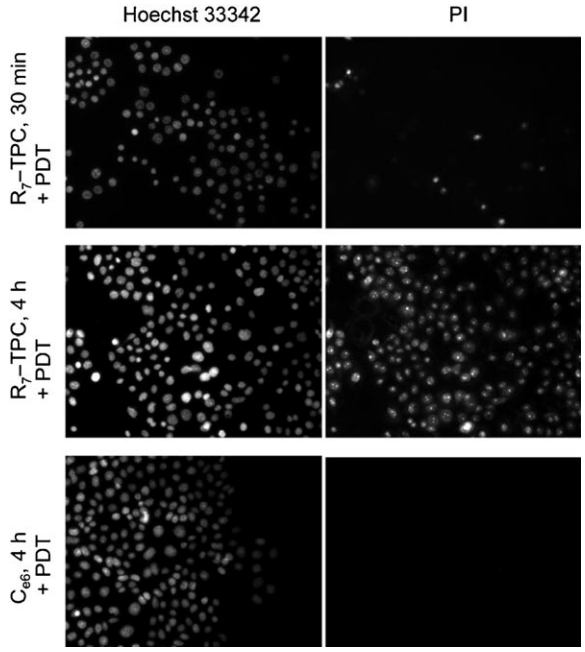


Figure 5. Representative fluorescence microscopy images illustrating necrotic cell damage, which occurred during PDT. Cells were stained with both Hoechst 33342 (left column) and PI (right column) immediately after PDT.

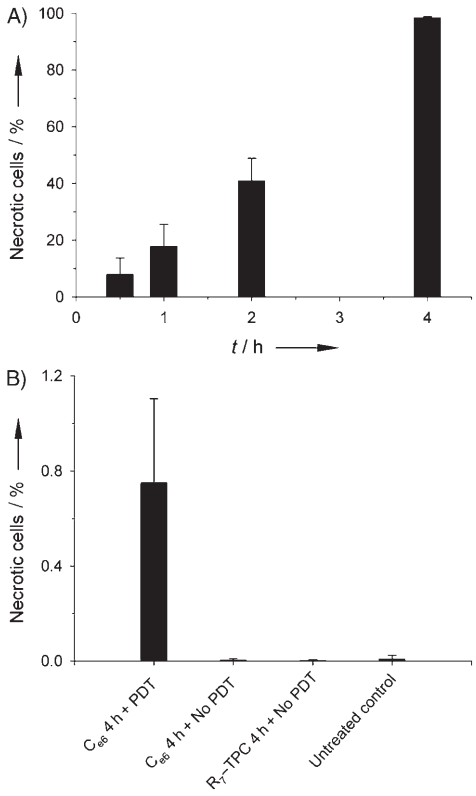


Figure 6. Quantitation of necrotic cells ($n = 3$). A) Necrotic damage to the cells treated with R_7–TPC (1 μM) for varying incubation times; cells were stained with Hoechst 33342 and PI immediately after PDT to quantify necrotic damage of the cells. B) Necrotic damage for C_{e6}-treated and control groups. Dark toxicity of cells incubated with photosensitizers or cell growth media for 4 h without light treatment showed no sign of necrosis.

more cell necrosis (Figure 6). Incubation times of 0.5, 1, 2, and 4 hours gave 7.9, 17.8, 40.9, and 98.4% necrotic cells, respectively. In contrast, PI signal was not observed in the cells incubated with C_{e6} (Figure 5 and 6B). Only 1.2% of the cells were damaged under treatment with C_{e6} for 4 hours and exposure to light. Less than 0.1% of the necrotic damage was observed for cells treated with either light or photosensitizers alone.

Discussion

The hydrophobic nature of most photosensitizers limits their application in vivo. Special formulations, such as mixtures of ethanol/polyethylene glycol 400/water, have been proposed for the administration of hydrophobic photosensitizers.[19] TPC, for example, is completely water-insoluble and as such, has little clinical relevance. Our approach of appending an R_7 oligopeptide to TPC improves not only the delivery, but also the aqueous solubility of the sensitizer. The solubility of the R_7–TPC conjugate in phosphate buffered saline (pH 7.4) is greater than 10 mM.

The uptake of R_7–TPC was observed to have a near-linear relationship with concentration in the presence of 10% FBS, whereas the uptake of C_{e6} was negligible even after 4 hours of incubation (Figure 2). It has been reported previously that the cellular uptake of photosensitizers, including C_{e6}, is significantly lower in the presence of serum than it is in the absence of serum as a result of nonspecific binding to serum components.[20–22] Our results indicate that the R_7 oligopeptide conjugated TPC overcomes this limitation and allows effective cellular uptake.

Confocal microscopy shows that the R_7–TPC conjugate enters cells efficiently, as the fluorescence of R_7–TPC was observed in almost all cells after an incubation time of only 30 min (Figure 3). Photodynamic treatment of these cells with light ($\lambda = 650$ nm) caused necrotic membrane damage of 8% of the cells, as observed by staining with Hoechst 33342 and PI immediately after light exposure (Figures 5 and 6). However, MTT assay results showed that about 80% of the cells were nonviable 24 hours later (Figure 4). We therefore conclude that approximately 70% of cell death occurred through an apoptotic mechanism, as PDT is known to elicit both necrosis and apoptosis, depending on the sensitizer used and its subcellular localization. By increasing incubation time to 4 hours, the cellular concentration of R_7–TPC was increased by about sixfold; most cells then turned necrotic upon treatment with light. As photodamage is limited to within 0.02 μm of the site of photoactivation owing to the short half-life of reactive singlet oxygen (< 0.04 μsec),[4] our results indicate that low concentrations of R_7–TPC induce apoptosis, yet higher concentrations of R_7–TPC are needed to cause necrosis.

In summary, the therapeutic efficiency of a photosensitizer can be significantly improved by conjugation to a cell-pene-

trating peptide, as has been demonstrated. The highly charged R_7 oligopeptide not only imparts solubility to the hydrophobic TPC in aqueous solution, but also transports TPC into cells. Following illumination with light of the appropriate wavelength, the internalized conjugate is able to kill cells through both necrotic and apoptotic pathways, depending on the concentration of the sensitizer.

Experimental Section

General. All solvents and reagents were reagent grade and used as received. HPLC was performed with a Vydac 218TP Series C-18 reversed-phase column (particle size = 10 μm, i.d. = 22 mm, *l* = 250 mm). Buffer A consisted of 0.1% trifluoroacetic acid (TFA) in deionized water; buffer B was acetonitrile/buffer A (9:1 *v/v*). UV/Vis spectra were recorded on a Cary 50 spectrophotometer (Varian, Palo Alto, CA) and fluorescence spectra, on a Hitachi F-4500 fluorescence spectrophotometer (Danbury, CT, USA).

5-[4-Carboxyphenyl]-10,15,20-triphenyl-2,3-dihydroxychlorin (compound 2). The synthesis of TPC **2** was performed as described by Brückner et al.[14] (Scheme 1). OsO_4 (250 mg, 0.984 mmol) was added to a solution of 5-(4-carboxyphenyl)-10,15,20-triphenylporphyrin **1**[23] (500 mg, 0.760 mmol) in CH_2Cl_2/pyridine (3:1 *v/v*, 100 mL). The flask was sealed, and the reaction proceeded for 48 h. H_2S gas was then bubbled through the solution for 5 min. The system was closed again for 45 min, after which time N_2 was bubbled through the system to purge off extraneous H_2S. The solution was evaporated to dryness in vacuo, dissolved in buffer B, and purified by preparative HPLC by using a linear gradient from 65% buffer B to 80% buffer B over 45 min at a flow rate of 6 mL min^{-1}. The product eluted at $t_R = 18$ min. The fractions containing the product were combined and evaporated to dryness to yield TPC as a green-purple film. Purity was assessed by analytical HPLC, and a Beer's law plot was used to determine the extinction coefficients of the product. R_f (silica, CH_2Cl_2/MeOH (95:5 *v/v*)): 0.20; UV/Vis (CH_2Cl_2/MeOH (99:1 *v/v*)) λ_{max} (log ε): 416 (5.01), 517 (3.98), 546 (3.98), 594 (3.67), 646 nm (4.18) (Figure 1); ^{1}H NMR (400 MHz, [D_7]DMF): $\delta = -1.74$ (s, 2H), 6.36 (s, 2H), 7.73–7.77 (m, 6H), 7.83–7.86 (m, 4H), 8.21–8.24 (m, 4H), 8.33–8.40 (m, 2H), 8.43–8.48 (m, 4H), 8.51 (s, 2H), 8.75 (d, $J = 4.9$ Hz, 1H), 8.79 ppm (d, $J = 5.0$ Hz, 1H); ^{13}C NMR (100 MHz, [D_7]DMF): $\delta = 74.0$, 74.1, 114.5, 114.6, 121.1, 122.5, 124.7, 124.9, 127.1, 127.4, 127.9, 128.1, 130.8, 132.1, 132.3, 132.6, 134.0, 134.3, 134.9, 135.4, 140.6, 141.7, 146.2, 152.1, 152.7, 164.5, 164.6, 167.6 ppm; ESI MS (70 V, CH_3CN) *m/z* $[M+H]^+$: 693; HRMS (ES+ of $[M+H]^+$) calcd for $C_{45}H_{32}N_4O_4$: 693.2496, found: 693.2492.

Arginine oligopeptide synthesis and R_7–TPC conjugation (compound 3). Synthesis of peptide GR_7 was performed on an automated solid-phase peptide synthesizer (433A, Applied Biosystems, Foster City, CA, USA) by using the traditional Fmoc (9-fluorenylmethyloxycarbonyl) methodology on Rink amide resin (405 mg, 0.25 mmol). All amino acids, Fmoc–Gly and Fmoc–Arg(Pbf) (4 equiv; Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl) were attached to the Rink amide (0.1 mmol) resin by stepwise elongation using 2(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 4 equiv)/*N*-hydroxybenzotriazole (HOBt, 4 equiv)/*N*-methyl morpholine (8 equiv) as the coupling reagents in DMF (10 mL). Upon completion of synthesis, TPC (30 mg, 0.043 mmol, 0.87 equiv) was added to the resin-bound peptide (0.05 mmol) in DMF (4 mL). The resin was allowed to swell for 15 min, at which time HOBt (6.8 mg, 0.05 mmol, 1 equiv), HBTU (18 mg, 0.05 mmol, 1 equiv), and diisopropylethylamine (DIPEA, 1 mL) were added. The reaction proceeded for 16 h, at which point it was filtered to collect the resin-peptide conjugate. This was then washed twice with CH_2Cl_2 and twice with methanol to remove excess reagents. The conjugate was cleaved from the resin with TFA/triisopropylsilane (TIS)/H_2O (95:2.5:2.5 *v/v/v*), filtered to remove the resin, and precipitated in methyl *tert*-butyl ether. The precipitate was dissolved in buffer A and purified by HPLC using a linear gradient of buffer B (20→80%, 45 min, flow rate = 6 mL min^{-1}). The product eluted at 22 min, and fractions containing the product were combined and lyophilized to yield R_7–TPC as a green powder (23 mg, 0.012 mmol, 29%): UV/Vis (H_2O) λ_{max} (log ε): 416 (5.19), 520 (3.90), 549 (3.90), 589 (3.65), 642 nm (4.00) (Figure 1); MALDI-TOF MS *m/z* $[M+H]^+$: 1842.

Singlet oxygen quantum yields. Quantum yields were calculated by using a modification of the technique described by Kochevar and Redmond.[17] In brief, stock solutions of the photosensitizers with optical densities of 0.03, as well as a solution of 1,3-diphenylisobenzofuran (DPBF, 0.25 M), all in DMF, were mixed and kept in the dark. Stock solution of the photosensitizer (2.0 mL) containing the DPBF solution (8 μL, final concentration, 1 mM) was added into a fluorescence cuvette before irradiation at $\lambda = 650$ nm (60 mW) in a fluorescence spectrophotometer under constant stirring. Simultaneously, the fluorescence emission intensity of DPBF was monitored (excitation $\lambda = 471$ nm, emission $\lambda = 495$ nm). Singlet oxygen quantum yields were then calculated from the initial slope of the decrease in fluorescence intensity with the following equation:

$$\Phi_\Delta(U) = \Phi_\Delta(St) \times {}^{S(U)}/_{S(St)}$$

in which *U* and *St* denote unknown and standard, and *S* represents the slope. *meso*-Tetraphenylporphyrin was used as the standard.

Cellular uptake. MDA-MB-468 cells (human breast carcinoma, American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's medium (DMEM, Cellgro, Mediatech, Washington DC, USA) supplemented with 10% fetal bovine serum (FBS, Cellgro) and 1% penicillin/streptomycin at 37 °C under a humidified atmosphere with 5% CO_2. Cellular uptake of photosensitizers was measured as previously published, with slight modification.[24] MDA-MB-468 cells (10^5 cells) in DMEM (1 mL) with FBS (10%) were seeded into each well of 24-well plates and incubated at 37 °C in a humidified CO_2 atmosphere (5%) for 24 h. Fresh medium with FBS (10%) containing either C_{e6} or R_7–TPC (1 μM, 1 mL) was added, and cells were incubated for 30 min, 1 h, 2 h, or 4 h. The cells were then washed three times with Hank's balanced salt solution (HBSS, Mediatech, Herndon, VA, USA) and dissociated from the plates by incubating the cells with trypsin–EDTA (1 mL) for 15 min at 37 °C. The resulting cell suspension was centrifuged, and the cell pellets were then dissolved in a solution of sodium hydroxide (0.1 M, 1.5 mL)/sodium dodecyl sulfate (SDS, 1%) for at least 24 h at room temperature to give a homogeneous solution. The fluorescence was measured and compared with a standard curve. Standard solutions of C_{e6} and R_7–TPC at known concentrations were prepared in 0.1 M NaOH/1% SDS, and the fluorescence of the solutions was measured after 24 h incubation at room temperature.

Cellular distribution by confocal microscopy. MDA-MB-468 cells (10^5 cells) in DMEM (0.5 mL) with FBS (10%) were seeded into each well of a Lab-Tek II chambered cover glass (Nalge Nunc, Naperville, IL, USA) and incubated at 37 °C in a humidified atmosphere (5% CO_2) for 24 h. C_{e6} or R_7–TPC were dissolved in fresh DMEM medium with 10% FBS (1 μM, 0.5 mL), added to the cells, and incubated for 30 min or 4 h. The cells were washed three times with HBSS before

imaging, and intracellular drug uptake was observed with a confocal microscope (Zeiss Axiovert 200, Thornwood, NY, USA) fitted with a Zeiss LSM Pascal Vario Laser Module (argon, 458/488/514 nm; HeNe, 543/633 nm). The HeNe laser ($\lambda = 543$ nm) paired with a long-pass emission filter for $\lambda = 650$ nm was used to visualize photosensitizers inside cells. It has been reported previously that fixation could affect cellular distribution.[25] Therefore, all experiments were performed with live cells without fixation.

Cell damage during PDT. MDA-MB-468 cells (10^5 cells) in DMEM (1 mL) with FBS (10%) were seeded into each well of 24-well plates and incubated at 37 °C in a humidified atmosphere (5% CO_2) for 24 h. Fresh medium with 10% FBS, containing C_{e6} or R_7–TPC (1 μM, 1 mL) was added, and the cells were incubated for 30 min, 1 h, 2 h, or 4 h. Thereafter, cells were washed three times with HBSS, fresh medium was added, and the cells were exposed to light ($\lambda = 650$ nm) delivered from a diode laser (B&W TEK, Newark, DE, USA) to give a total fluence of 10 J cm^{-2} at 42.1 mW cm^{-2}. To investigate the necrotic damage of cell membranes during PDT, nuclei of the cells were stained with Hoechst 33342 and propidium iodide (PI). The stained cells were then viewed in phase-contrast or fluorescence mode with an inverted epifluorescence microscope (Zeiss Axiovert, Thornwood, NY, USA). A cooled CCD camera (Sensys Photometrics, Tucson, AZ, USA) adapted with a narrow-bandpass filter was used for image capture. Three wells were used for each experimental group, with at least 350 cells in four random fields counted on each well. Cells without photosensitizer or cells with photosensitizer but without light illumination were also stained for comparison.

Cell survival assay at 24 h post-PDT. In total, 5000 cells in 0.2 mL DMEM with 10% FBS were seeded in each well of 96-well plates and cultured for 24 h until 70% confluent. The cells were incubated with fresh complete medium containing R_7, R_7–TPC or C_{e6} (1 μM, 0.2 mL) for different time periods. Thereafter, the cells were washed three times with HBSS, fresh medium was added, and the cells were exposed to light ($\lambda = 650$ nm) delivered from a diode laser to give a total fluence of 10 J cm^{-2} at 42.1 mW cm^{-2}. Cells were then incubated for a further 24 h, and the MTT microculture assay was used to measure cell viability (MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide).[26] Untreated cells served as the gauge for 100% viability, whereas media served as background. Cells incubated with photosensitizers for 4 h but without light illumination were also evaluated.

Statistical analysis. The mean ± SD values were used for the expression of data. Statistical analyses of data were performed by using the Student *t* test. Differences were considered statistically significant with $p < 0.05$.

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Keywords: arginine · chlorin e6 · membranes · peptides · photodynamic therapy

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