Imaging Agents

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# A Smart Polysaccharide/Drug Conjugate for Photodynamic Therapy**

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Recent improvements in drug-carrier design for photodynamic therapy (PDT) have brought about significant advances for treating skin, breast, and lung tumors.[1–3] The local high-dose strategy of PDT suggests beneficial therapeutic efficacy with high selectivity when using photosensitizing drugs for the target site, as well as reduced side effects for normal tissues. A variety of drug-carrying vehicles, such as nanoparticles, drug conjugates, and polymeric micelles have frequently exhibited characteristics that may make possible the successful delivery of photosensitizing drugs, thus improving cell entry and residence in tumor sites.[1–3] However, these approaches have, thus far, achieved rather limited success, owing primarily to the practical obstacles inherent to natural in vivo conditions.[1–4] In this study, we describe a novel molecular "Trojan horse" system that quickly switches into an aggressive molecule for tumor destruction within the environment of the tumor. Advances in functionality have enabled our system to exhibit an intelligent switch from a three-dimensional supramolecular assembly (i.e., self-quenched state of photosensitizing drugs) into extended random molecules (i.e., dequenched state for singlet-oxygen production), which corresponds to a change in surface charge (Figure 1). This system may be more significant than any known photosensitizing drug conjugate thus far developed.

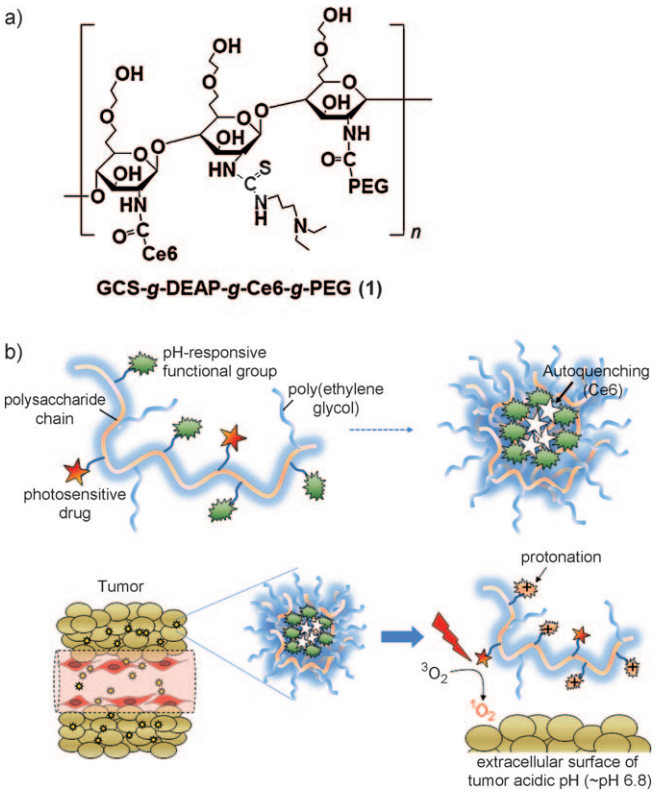


***Figure 1.*** Schematic concept for a proposed polysaccharide/drug conjugate. a ) GCS-*g*-DEAP-*g*-Ce6*g*-PEG (**1**). b) At high pH values **1** undergoes autoquenching, and upon reaching the more acidic surface of the tumor cell protonation occurs and singlet oxygen is generated, thereby destroying the cell.

Figure 1 shows that our polysaccharide/drug conjugate consists of a glycol chitosan backbone (GCS, $M$w = 86 kDa), a functional 3-diethylaminopropyl isothiocyanate (DEAP) block,[5] a chlorine e6 block (Ce6, used as a photosensitizing model drug),[6] and a poly(ethylene glycol) block (PEG, $M$w = 2 kDa). GCS was grafted with DEAP, Ce6, and PEG (hereafter termed GCS-*g*-DEAP-*g*-Ce6-*g*-PEG (**1**); Figure 1). We have previously shown that the p$K_b$ value of a glycol chitosan conjugate with DEAP is near 6.8.[5] This characteristic should be able to provide a pH-induced "intelligence" to the polysaccharide/drug conjugate. The synthesized polysaccharide/drug conjugate, GCS-*g*-DEAP-*g*-Ce6-*g*-PEG (**1**), was characterized using $^1$H NMR spectroscopy. The degree of substitution (defined as the number of DEAP or Ce6 or PEG blocks per primary amine of GCS) was estimated as 0.40 for the DEAP block, 0.20 for the Ce6 block, and 0.38 for the PEG block (see the Supporting Information). Compound **1** is

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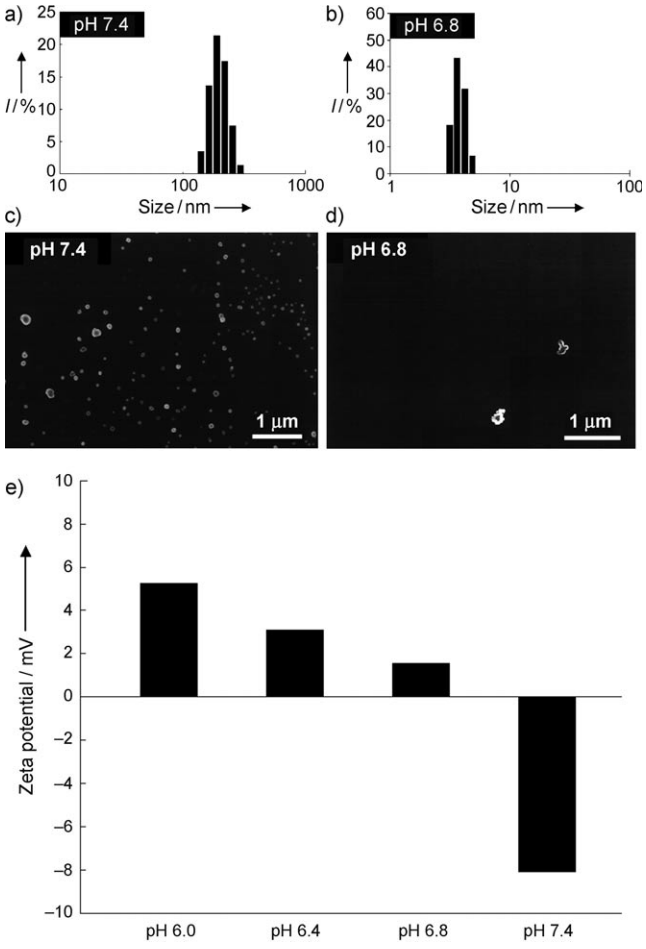


***Figure 2.*** Characteristics of the polysaccharide/drug conjugate **1**. Particle-size distribution of **1** (0.1 mg mL$^{-1}$) at a) pH 7.4 (PBS 150 mM) and b) pH 6.8 (PBS 150 mM) by Zetasizer. *I* = intensity. Field-emission scanning electron microscope (FE/SEM) images of **1** at pH 7.4 (c) and pH 6.8 (d). e) Zeta-potential change for **1** (0.1 mg mL$^{-1}$, pH 7.4–6.0, PBS 150 mM) at different pH values.

self-organized in aqueous solution: the GCS and PEG blocks are on the hydrophilic outer shell, and the DEAP and Ce6 blocks are within the hydrophobic inner core. In particular, the incorporation of a PEG block may improve the stability of the drug conjugate in serum and the penetration into an in vivo tumor vasculature.[6] Upon encountering the tumor environment, the polysaccharide/drug conjugate will undergo conformational changes into the uncoiled structure. This unique trait of **1** was confirmed experimentally (Figure 2a–d). The magnitude of the particle-size changes for **1** was large between pH 7.4 and pH 6.8; that is, the particle size was 150 nm in diameter at pH 7.4 and 3.4 nm at pH 6.8. Figure 2c shows that **1** was almost spherical in shape at pH 7.4. However, it becomes disentangled at pH 6.8, although very few aggregates were observed in this case (Figure 2d). Moreover, the zeta potential of **1** was changed from −8.0 mV to +1.3 mV as the pH of the solution decreased from pH 7.4 to 6.8; the negative value originated from the PEG block at pH 7.4, and was offset by the protonation of the DEAP block at pH 6.8 (Figure 2e). It is known that the extracellular pH value ($pH_e$) in most clinical tumors is more acidic (pH, 6.5–7.0) than in normal tissues (ca. pH 7.4).[7,8] The different response of **1** at pH 7.4 (normal tissue pH) and at pH 6.8 ($pH_e$ in tumors) presents a new route for the functionalization of photosensitizing drug conjugates (Figure 1).

Figure 3 shows the changes in photoactivity of **1** as the pH value is changed. In this study, we employed 9,10-dimethylanthracene (DMA) as an extremely rapid chemical trap for singlet oxygen,[9] to confirm the generation of singlet

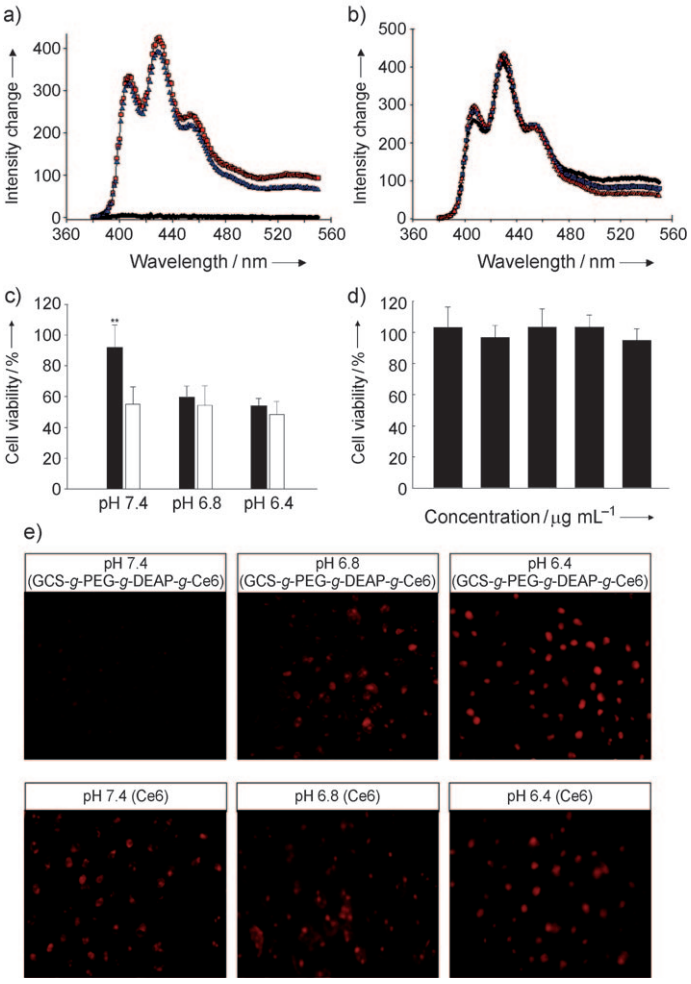


***Figure 3.*** pH sensitivity for **1** targeting an acidic, solid tumor. The 9,10-dimethylanthracene (DMA) fluorescence change of a) GCS-*g*-DEAP-*g*-Ce6-*g*-PEG (**1**;0.1 mg mL$^{-1}$, PBS 150 mM) and b) GCS-*g*-Ce6-*g*-PEG (0.1 mg mL$^{-1}$, PBS 150 mM) at pH 7.4 (●, black), 6.8 (■, red), 6.4 (▲, blue). The change in DMA fluorescence intensity (at $\lambda_{ex}$ 360 nm and $\lambda_{em}$ 380–550 nm) was plotted after subtracting each of the sample fluorescence spectra from the full DMA fluorescence spectra (for DMA in PBS, indicating no singlet oxygen). c) Phototoxicities determined by a Cell Counting Kit-8 (CCK-8) of HeLa cells treated with **1** (equivalent Ce6 1 μg mL$^{-1}$; filled bars) and free Ce6 (1 μg mL$^{-1}$; unfilled bars). All cells were irradiated for 10 min at a light intensity of 5.2 mW cm$^{-2}$ using a 670 nm laser source and were then incubated for an additional 12 h ($n = 7$) (** $p < 0.01$ compared to free Ce6). d) Cell viabilities of HeLa cells treated with **1** (1-400 μg mL$^{-1}$) without irradiation for 24 h ($n = 7$). e) Fluorescence images of HeLa cells treated with **1** (equivalent Ce6 1 μg mL$^{-1}$) and free Ce6 (1 μg mL$^{-1}$). Annexin V/propidium iodide stain (red) depicts apoptotic cells.

oxygen from **1** with changes in the pH value. Solutions of **1** having different pH values (pH 7.4–6.4) were each mixed with DMA, and then irradiated for 10 minutes at a light intensity of 5.2 mW cm$^{-2}$ using a 670 nm laser source. Figure 3a reveals that the reduced generation of singlet oxygen at pH 7.4, owing to the self-quenching event of **1**, was recovered at pH 6.8 or 6.4, which is a comparable result to the pH-independent singlet-oxygen generation for the drug conjugate without DEAP (GCS-*g*-Ce6-*g*-PEG; Figure 3b) or free Ce6 (data not shown). In particular, the different amounts of singlet oxygen that were generated by **1** at different pH values allowed higher phototoxicity for human epithelial carcinoma HeLa cells at pH 6.8 and 6.4 than at pH 7.4 (Figure 3c), especially when considering that before irradiation **1** has no cytotoxic effects when present in up to 200 μg mL$^{-1}$ over 24 hours (Figure 3d). Similarly, **1** led to higher levels of apoptosis for HeLa cells at pH 6.8 and 6.4 than at pH 7.4 (Figure 3e). However, no noticeable differences in cell apoptosis with changes in pH values and in the presence of free Ce6 (Figure 3e) or the drug conjugate without DEAP (GCS-*g*-Ce6-*g*-PEG; data not shown) were observed.

Figure 4 presents an impressive contrast for in vivo fluorescent intensity for the various cases.[10] We determined the tumor specificity of the polysaccharide/drug conjugate **1**, the drug conjugate without DEAP (GCS-*g*-Ce6-*g*-PEG), and free Ce6 in BALB/c nu/nu female mice harboring HeLa tumor cells. Compound **1** (equivalent Ce6 0.1 mg kg$^{-1}$ body) was administered intravenously to nude mice and resulted in a strong fluorescent signal for Ce6 in the tumor site, thus providing a clearer image of the tumor (Figure 4a). For comparison images taken for mice treated with the drug conjugate without DEAP (GCS-*g*-Ce6-*g*-PEG, equivalent Ce6 0.1 mg kg$^{-1}$ body) and the free Ce6 (2.5 mg kg$^{-1}$ body) are shown. In particular, despite high-dose administration of free Ce6 (up to 2.5 mg kg$^{-1}$ body), a very weak fluorescent Ce6 signal was observed in the tumor site, thereby reflecting inefficiency of targeting the tumor. Interestingly, **1** exhibited a fluctuating fluorescent Ce6 signal for 24 hours (Figure 4b); for example, the intensity observed after 30 minutes was higher than that observed after 1 hour, and the intensity increased again after 6 hours. This behavior is comparable to that of most GCS nanoparticles having persistent fluorescent signals in the tumor site for 2–3 days.[11] One possible hypothesis for this behavior is that the dequenching of **1** within acidic tumor sites resulted in a strong fluorescent signal, and then once **1** was cleared or diffused freely the fluorescence intensity decreased. The fluorescence intensity of free Ce6 in the tumor site decreased gradually over time (Figure 4c), and this may possibly have been the result of enhanced renal clearance.[11]

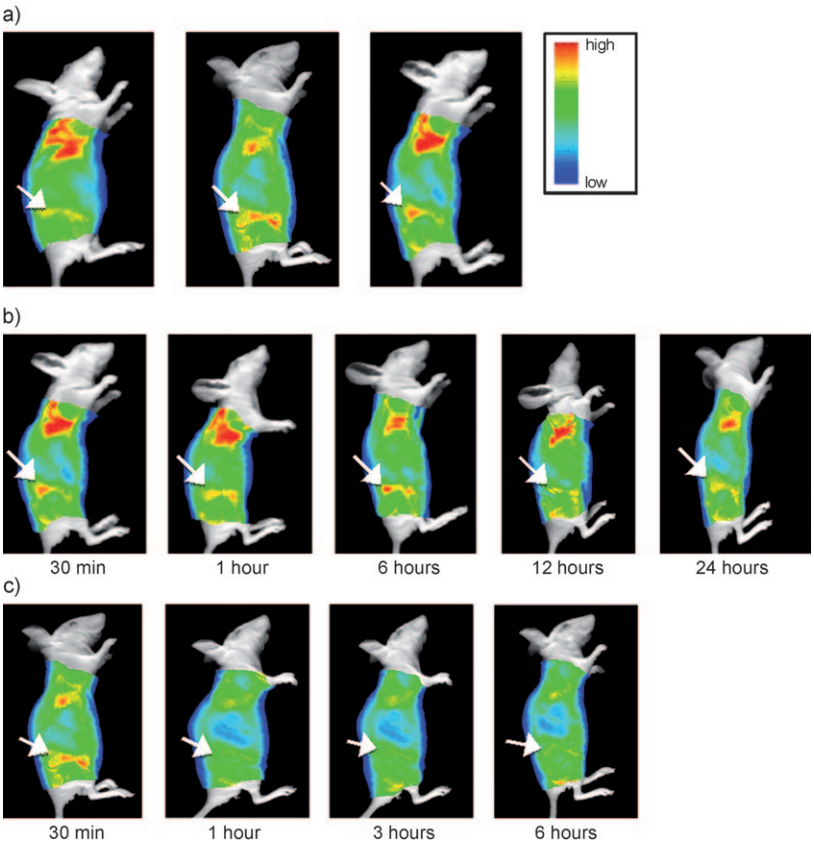


***Figure 4.*** In vivo non-invasive fluorescent imaging of nude mice having HeLa tumors. a) Fluorescent images obtained 30 min after mice having a HeLa tumor were intravenously injected through a tail vein with GCS-*g*-DEAP-*g*-Ce6-*g*-PEG (**1**; equivalent Ce6 0.1 mg kg$^{-1}$ body; right), GCS-*g*-Ce6-*g*-PEG (equivalent Ce6 0.1 mg kg$^{-1}$ body; middle), and free Ce6 (2.5 mg kg$^{-1}$ body; left). Whole body images of HeLa-tumor-bearing mice taken over time. The mice were treated with **1** (b) and GCS-*g*-Ce6-*g*-PEG (c).

In this study, we demonstrated the potential of a polysaccharide/drug conjugate (GCS-*g*-DEAP-*g*-Ce6-*g*-PEG; **1**) for PDT. We anticipate the following potential advantages for future in vivo and clinical applications: 1) targeting specific tumors using a polysaccharide/drug conjugate that responds to different pH values might prove highly valuable in the treatment of various solid tumors with acidosis,[7,8] and 2) a tailormade polysaccharide/drug conjugate could be potentially beneficial as an advanced therapeutic platform. Overall, a polysaccharide/drug conjugate is expected to represent a more effective approach to tumor therapy, providing targeted high-dose cancer therapy while ensuring the safety of normal tissues.

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