Therapeutic Nanosystem Consisting of Singlet-Oxygen-Responsive Prodrug and Photosensitizer Excited by Two-Photon Light

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S Supporting Information

ABSTRACT: Using light as the sole stimulus and employing the generated singlet oxygen as a therapeutic agent and the trigger to activate chemo-drug release could serve as an elegant way to bring into full play the advantageous features of light and enhance therapeutic efficacy through a combination of chemotherapy and photodynamic therapy. Herein a liposomal drug system has been developed by embedding a fluorescent photosensitizer and a prodrug into phospholipid vesicles. Upon one- or two-photon light irradiation, the photosensitizer generates singlet oxygen, which removes the protecting group of the prodrug and subsequently causes the release of the active drug chlorambucil. With the combined action of O_2^1 and chlorambucil, highly controllable cytotoxicity toward cancer cells was achieved. In addition, the fluorescent photosensitizer gives out fluorescent signal acting as the drug monitoring agent. This strategy may provide an efficient approach for cancer treatment and some useful insights for designing light-stimulated on-demand therapeutic systems.

KEYWORDS: Prodrug, liposomal nanosystem, drug delivery, photodynamic therapy, chemotherapy

As an important first-line treatment option for cancers, chemotherapy does show its life-saving potential; however, in many cases, it may as well be notorious for serious side-effects^{1–3} (e.g., high toxic effects and non-specificity) resulting from its damage to normal cells and tissues/organs. Therefore, effective chemotherapy should be able to distinguish between cancer cells and normal cells, specifically killing cancer cells while maintaining normal cells intact. In order to achieve highly selective killing of cancer cells and avoid side-effects, an ideal drug delivery system is needed to control the release of anticancer drugs only at the tumor site. To date, several approaches, including both the internal and external stimuli,^{4–7} have been used to trigger the drug release at the target locations. As an external stimulus, light is particularly attractive due to the fact that it can trigger various biological events in a space- and time-controlled manner. In some prodrugs, the active drug molecules were coupled with a light-labile protecting group, which temporarily deactivated the active drug; upon light stimulation, the active drug was released and subsequently unleashed its therapeutic action.^{8,9} However, light has long been used to induce the generation of singlet oxygen (O_2^1),¹⁰ heat,¹¹ nitric oxide,¹² etc., which served as the active agent for disease treatment.

To further enhance therapeutic efficacy, the combinatorial approaches comprising chemotherapy and photodynamic therapy were also employed.¹³ In these combination therapy systems, usually a photosensitizer and a chemotherapy drug were incorporated into the same drug delivery system; and

singlet oxygen was generated by the photosensitizer via light stimulation, while the drug was usually released by the action of another stimulant such as pH, enzymes, or degradation/disintegration of drug carrier.^{14–18} Using light as the sole stimulus and employing the generated singlet oxygen as both therapeutic agent and the trigger to activate drug release could serve as an elegant way to bring into full play the advantageous features of light and enhance therapeutic efficacy through combination of chemotherapy (the released drug) and photodynamic therapy (singlet oxygen). Several drug delivery systems based on this approach have been developed and proved to be feasible and effective;^{19–24} these systems employed bis(alkylthio)alkene,^{19,20} aminoacrylates,^{21–23} unsaturated phospholipid,²⁴ and 2-nitroimidazole (bioreducing agent activated via hypoxia or the singlet-oxygen generation process)^{25,26} as the singlet-oxygen-sensitive moiety; despite of the progress in this regard, new singlet-oxygen responsive moieties are still highly desired in terms of high drug loading and overall performance as well as the remarkable two-photon properties. Toward this aim, in this study, we employed diphenoxyethene as the singlet-oxygen-responsive moiety, which has symmetrical structure thus ensuring two drug molecules can be incorporated simultaneously, and developed a liposomal therapeutic system, which consists of

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a singlet-oxygen-responsive prodrug and a fluorescent photosensitizer excitable by one- or two-photon light. In this system, two sequential activations were involved: singlet oxygen was generated by the photosensitizer as a result of light irradiation, followed by the removal of the protecting group and the subsequent release of active drug. In addition, the fluorescent photosensitizer also gives out fluorescent signal acting as the drug monitoring agent and can be employed for imaging.

As shown in Scheme 1, the prodrug molecule designed herein contains two anticancer drug chlorambucil (CHB)²⁷

Scheme 1. Schematic Overview of the Prodrug Stimulated by Light To Generate Singlet Oxygen, Which Triggers the Drug Release and the Related Anticancer Action

moieties, which can be activated and released by the reaction between the prodrug with singlet oxygen. While for the photosensitizer (PS), a bis(arylidene)-cyclobutanone derivative was designed to afford effective two-photon excited photodynamic therapy and highly fluorescent property. Since liposomes can be degraded via the lipid peroxidation process,²⁸ in this study, the molecular prodrug (diphenoxyethene derivative) and the photosensitizer were encapsulated into liposome, the resultant liposomal prodrug system can be stimulated by visible light or infrared two-photon laser to generate singlet oxygen, and then the prodrug can be triggered rapidly by singlet oxygen to release active drug molecules from liposomes (Scheme 1).

The synthesis route for the prodrug (compound 6) and the photosensitizer (compound 7) are shown in Schemes S1 and S2. Compounds 1–5 were synthesized according to previously reported methods.²⁹ Compound 6 was obtained through an esterification reaction between CHB and compound 5. Compound 7 was synthesized from cyclobutanone and *N*-methyl-*N*-(2-hydroxyethyl)-4-aminobenzaldehyde. The structures of the intermediates and the final product were confirmed by proton nuclear magnetic resonance spectroscopy (¹H NMR) and mass spectrometry (MS) (Figures S1–S9). The liposomal prodrug system (hereinafter referred to as LIP-Pro-Stm) was obtained by loading the prodrug and the PS into the liposomes with the particle size of around 90 nm (Figure S10).

To demonstrate the photophysical property and the singlet-oxygen generating capability of the fluorescent photosensitizer (cyclobutanone derivant), absorption spectra, fluorescence spectra, two-photon excited fluorescence, and generation of O₂¹ by the photosensitizer solutions were determined under physiological conditions (Figure 1; Figures S11–S14). The absorption and fluorescence spectra are shown in Figure 1a (and Figures S11 and S12). It is clear that the photosensitizer has a strong absorption band at around 510 nm and emission

Figure 1. (a) Absorption and fluorescence spectra of the PS (5 mM). (b) Photobleaching of ABDA (10 mM) by singlet oxygen generated by PS (5 mM) solution over different periods of time under light irradiation (500 nm, 30 mW·cm^{−2}). (c) Photobleaching of ABDA (10 mM) by singlet oxygen generated by PS (5 mM) solution over different periods of time under light irradiation (840 nm 55 mW·cm^{−2} femtosecond laser). (d) Two-photon absorption cross-section of the PS using Rho-6G as a reference.

band at about 630–670 nm. We then determined the fluorescence quantum yield of the fluorescent photosensitizer using rhodamine 6G (Rho-6G) as a reference as well as its two-photon absorption cross-section (Figure 1; Figures S15 and S16). As shown in Figure 1d, the PS has the strongest absorption cross-section of about 500 GM at 840 nm. In vitro reactive oxygen species (ROS) generation by the PS was demonstrated using 9,10-anthracenediyl-bis(methylene)-dimalonic acid (ABDA)³⁰ as the fluorescence probe for O₂¹ (Figure 1b,c; Figures S13 and S14).

As the generation of O₂¹ was confirmed via both one-photon and two-photon excitation, we then used it as a trigger to test whether the active drug CHB could be released from the prodrug. First, we performed high-resolution mass spectrometry (HR-MS) measurement for the mixture solution containing the PS and the prodrug upon 500 nm light irradiation for 60 min. As shown in Figure S17, the molecular weights of CHB, the PS, and the prodrug can be clearly found. Furthermore, we carried out high-performance liquid chromatography (HPLC) tests (Figure 2a) so as to further confirm

Figure 2. (a) Typical HPLC chromatogram of the prodrug, the PS, the mixture of the prodrug (6 μM) and the PS (4 μM) with one-photon light irradiation (500 nm and 30 mW·cm^{−2}) for 60 min, and CHB. The mobile phase was 80/20 methanol/water, and the flow rate was 1.0 mL/min. (b) Percentage of CHB (as determined by HPLC) released from the prodrug (6 μM) as a function of time in the presence or absence of the PS (4 μM) upon light irradiation (500 nm, 30 mW·cm^{−2}).

that the active drug CHB can be released from the prodrug molecule in the presence of PS upon light irradiation. The retention times of CHB, 4-(hydroxymethyl)phenol, the prodrug, and the PS are identified at 1.38, 1.63, 1.83, and 2.06 min in the HPLC chromatogram. For the prodrug ($6\ \mu\text{M}$) and the PS ($4\ \mu\text{M}$) with one-photon light irradiation ($500\ \text{nm}$ and $30\ \text{mW}\cdot\text{cm}^{-2}$) for 60 min, the peak intensities at 1.83 and 2.06 min (corresponding to the prodrug and the PS, respectively) decrease and a new strong peak emerges at 1.38 min, which corresponds to that for the active drug CHB; also another peak corresponding to 4-(hydroxymethyl) phenol (the byproduct) can be observed. The percentage of CHB (as determined by HPLC) released from the prodrug as a function of time in the presence or absence of the PS was shown in Figure 2b. All these results indicate that the active drug CHB can be released upon light irradiation and the fluorescence of the photosensitizer can be utilized as the reporting signal.

Subsequently, we investigated the cell uptake of the prodrug and PS in cancer cells (in both HeLa cell line and A549 cell line). The results are shown in Figure 3. For each cell line, we treated the cells with both the solution (containing the prodrug

Figure 3. Fluorescent microscopic images for HeLa and A549 cells incubated in culture media with solution ($4\ \mu\text{M}$ PS and $6\ \mu\text{M}$ prodrug) or Lip-Pro-Stm ($50\ \mu\text{g}/\text{mL}$) with the incubation time of 2 h, respectively. The control groups are placed in the 3rd and 6th row. Scale bar: $25\ \mu\text{m}$.

and photosensitizer) and the Lip-Pro-Stm (encapsulated with the prodrug and photosensitizer). In Figure 3, compared to the control (without being treated with the solution of prodrug or Lip-Pro-Stm), all the treated groups show strong red fluorescence of the PS in the cells, and the results indicate that both the solution containing the prodrug and the PS as well as the Lip-Pro-Stm can be transported into the cancer cells efficiently and can be applied for imaging the cancer cell clearly.

The viabilities of HeLa and A549 cells upon treatment with the prodrug system were assessed using MTT assays (Figure 4a); the results show that there is little cytotoxicity when

Figure 4. (a) Viabilities of HeLa cells upon treatment with different formulations of varied components and concentrations, as evaluated with MTT assay; the cells were incubated in culture media: black bar, PS 0–14 μM , dark; red bar, prodrug 0–14 μM , dark; blue bar, PS 4 μM , prodrug 0–14 μM , dark; pink bar, 500 nm light irradiation for 60 min; green bar, prodrug 0–14 μM 500 nm light irradiation for 60 min; indigo bar, PS 0–14 μM , 500 nm light irradiation for 60 min; purple bar, PS 4 μM , prodrug 0–14 μM , 500 nm light irradiation for 60 min. (b) Agarose gel electrophoresis assay for linear DNA ($0.5\ \mu\text{g}$ pBR322) cross-linking formation upon treatment with different formulations; the DNA were incubated in culture media: lane 1, buffer; lane 2, $6:50\ \mu\text{g}/\text{mL}$ LIP-Pro-Stm (equivalent to $4.07\ \mu\text{M}$ of the PS and $12.7\ \mu\text{M}$ of the CHB) for 2 h after various time (15, 20, 30, 45, and 60 min) of 500 nm light irradiation; lane 7, DNA ladder. (c) Annexin V-FITC/propidium iodide (PI) dual staining of HeLa cells for detecting the time related apoptosis by flow cytometry. The HeLa cells were incubated in culture media for 15–60 min with $4\ \mu\text{M}$ PS in the first row, with $4\ \mu\text{M}$ PS and $6\ \mu\text{M}$ prodrug in the second row after of 500 nm light irradiation, and then further incubated in dark for 2 h.

treated with either the prodrug or the PS alone (without light irradiation), or even with both as long as it is under the dark environment (namely, without light irradiation). With light irradiation, there is still little cytotoxicity when the prodrug alone was added. These results indicate that the prodrug alone is stable in both dark environment and under light irradiation. However, under light irradiation, when only the photosensitizer was incubated with cells, obvious cytotoxicity can be observed, indicating that the PS can generate singlet oxygen upon light stimulation, which is the key factor leading to cell

apoptosis. While the prodrug was added during cell incubation together with the PS and under proper light irradiation, the viability of the cancer cells dropped remarkably; this result indicates that the generated O_2^1 together with the released active drug triggered by singlet oxygen can greatly enhance cell apoptosis. In Figure 4b, the interstrand DNA cross-linking experiments³¹ were assessed by alkaline agarose gel electrophoresis. As for the prodrug in the presence of the PS (LIP-Pro-Stm) with the proper light irradiation, we can see the linear DNA cross-linking caused by the drug CHB, which was released from the prodrug. As a result, the linear DNA, which was cross-linked, had a lower migration rate as shown in lanes 3–6 (the quantification of Figure 4b and the control group are shown in Figures S18–19). Thereafter, we used the flow cytometry (Figure 4c) to further demonstrate the time-related (15 to 60 min) anticancer effect of the solution containing both the prodrug and the PS as well as the PS solution only. The flow cytometry of the control, CHB, liposome with equivalent amount of CHB were also tested as shown in Figure S20. Upon light irradiation, as for the prodrug in the presence of the photosensitizer, the apoptotic percentage increases as the irradiation time is lengthened. However, for the control (with light irradiation only), the percentage for apoptotic cells is only 3.1%. These results clearly display that the prodrug and the photosensitizer together with light irradiation can induce significant cell apoptosis and could realize highly controllable toxicity toward cancer cells. The IC_{50} of the PS (Figure 4a) and the Lip-Pro-Stm (Figure S21) were calculated to be 6.03 μM and 35.8 $\mu g/mL$ (equivalent to 2.91 μM of the PS and 4.55 μM of the CHB), which is lower than that of CHB, as reported.³² In addition, the cytotoxicity of the Lip-Pro-Stm with two-photon light was tested in Figure S21, showing that the two-photon light can trigger the prodrug system as well.

In summary, we have successfully developed a prodrug system that can be sequentially triggered by light (to generate singlet oxygen) and O_2^1 (to activate the release of active drug molecules). The system can be activated by one-photon or two-photon light irradiation to release CHB active drug molecules; and the fluorescent photosensitizer also serves as the tracking and imaging agent. With the combined action of O_2^1 and CHB, the cancer cells undergo apoptosis rapidly. This new strategy is the first to employ light as the stimulant and simultaneously employ the fluorescent photosensitizer (singlet oxygen) as the trigger for drug release (covalent bond cleavage) in cancer cells, therapeutic agent, and imaging agent in combination with anticancer drug; and it may provide an efficient approach for cancer treatment and some useful insights for designing other light-stimulated on-demand drug release systems.

■ ASSOCIATED CONTENT

Supporting Information

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Materials and methods, and supplemental Tables and Figures (PDF)

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Author Contributions

Y.L., F.Z., and S.W. conceived the project and designed the research strategy and experimental approach. Y.L. synthesized the compounds and performed the experiments, with assistance from X.J., X.D., and B.W. The data was analyzed by Y.L. The manuscript was written by Y.L., F.Z., and S.W. All authors have given approval to the final version of the manuscript. F.Z. and S.W. supervised the research.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

O_2^1 , singlet oxygen; CHB, chlorambucil; PS, photosensitizer; LIP-Pro-Stm, the liposomal prodrug system; 1H NMR, proton nuclear magnetic resonance spectroscopy; MS, mass spectrometry; Rho-6G, rhodamine 6G; ROS, reactive oxygen species; ABDA, 9,10-anthracenediyl-bis(methylene)dimalonic acid; HR-MS, high-resolution mass spectrometry; HPLC, high-performance liquid chromatography

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