ACS Medicinal Chemistry Letters

Letter

Therapeutic nanosystem consisting of singlet-oxygen-responsive pro-drug and photosensitizer excited by two-photon light

Yi Lin, Xiao-Fang Jiang, Xiangyan Duan, Fang Zeng, Bo Wu, and Shuizhu Wu

ACS Med. Chem. Lett., **Just Accepted Manuscript** • DOI: 10.1021/acsmedchemlett.7b00394 • Publication Date (Web): 22 Dec 2017

Just Accepted

ACS Publications

ACS Medicinal Chemistry Letters is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Therapeutic nanosystem consisting of singlet-oxygen-responsive prodrug and photosensitizer excited by two-photon light

Yi Lin, Xiao-fang Jiang, Xiangyan Duan, Fang Zeng*, Bo Wu and Shuizhu Wu*

State Key Laboratory of Luminescent Materials and Devices, College of Materials Science and Engineering, South China University of Technology (SCUT), Guangzhou, 510640, China

KEYWORDS: prodrug; liposomal nanosystem; drug delivery; photodynamic therapy; chemotherapy

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 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.

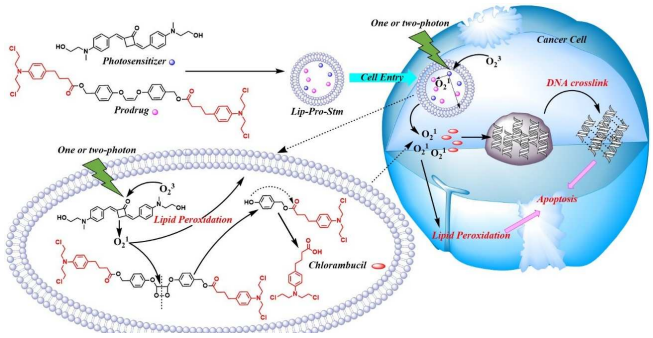
As an important first-line treatment option for cancers, chemotherapy does show its life-saving potential; however, in many cases, it may as well be notorious for its serious side-effects[1-3] (e.g. high toxic effects and non-specificity) resulted from its damage to normal cells and tissues/organs. Therefore, 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 anti-cancer 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]. On the other hand, light has long been used to induce the generation of singlet oxygen (O_2^1)[10], heat[11], nitric oxide[12] and etc., which served as the active agents 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]. While 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). And 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-phonton 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 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

Scheme 1. Schematic overview of the prodrug stimulated by light to generate singlet oxygen which triggers the drug release and the related anti-cancer action.



chlorambucil (CHB)[27] 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**. And 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 (^{1}H NMR) and mass spectrometry (MS) (Figures S1 - S9). And the liposomal prodrug system (hereinafter referred to as LIP-Pro-Stm) was obtained by loading the pro-

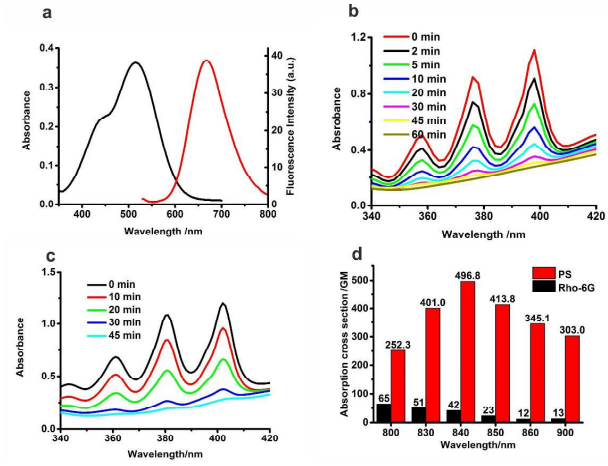


Figure 1. (a) Absorption and Fluorescence spectra of the PS (5 mM). (b) Photobleaching of ABDA (10 mM) by singlet oxygen generated by PS (5 mM) solution over different periods of time under light irradiation (500 nm, 30mW·cm^{-2}). (c) Photobleaching of ABDA (10 mM) by singlet oxygen generated by PS (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.

drug 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), the absorption spectra, fluorescence spectra, two-photon excited fluorescence and the generation of O_2[1] 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 band at about 630 - 670

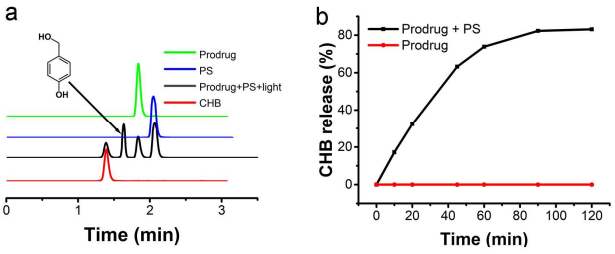


Figure 2. (a) Typical HPLC chromatogram of the prodrug, the PS, and the mixture of the prodrug (6 μM) and the PS (4 μM) with one-photon light irradiation (500 nm and 30mW·cm^{-2}) for 60 minutes, and CHB. The mobile phase was 80/20 methanol/water and the flow rate was 1.0 mL / min. (b) Percentage of 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, 30mW·cm^{-2}).

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 O_2[1](Figure 1b, Figure 1c, Figures S13 and S14).

As the generation of O_2[1] 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 minutes. 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 that the active

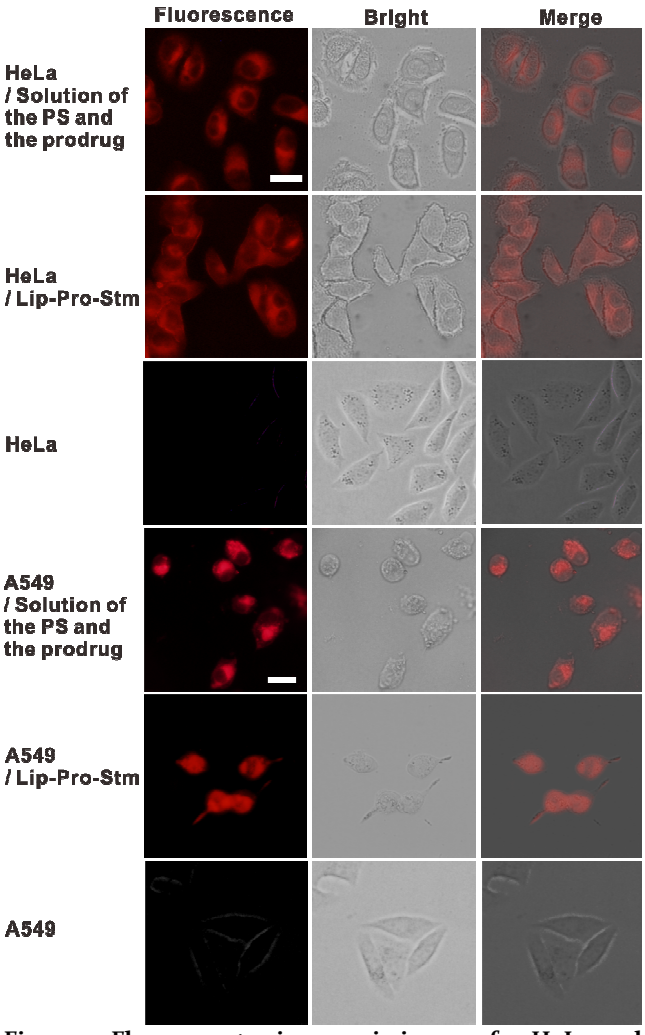


Figure 3. Fluorescent microscopic images for HeLa and A549 cells incubated in culture media with solution (4 μM PS and 6 μM prodrug) or Lip-Pro-Stm (50 μg/mL) with the incubation time of 2 hours, respectively. The control groups are placed in the 3rd and 6th row. Scale bar: 25 μm.

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 min, 1.63 min, 1.83 min and

2.06 min in HPLC chromatogram. For the prodrug (6 μM) and the PS (4 μM) with one-photon light irradiation (500 nm and 30mW·cm^{-2}) for 60 minutes, the peak intensities at 1.83 min 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 by-product) can be observed. And 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). And the results are shown in Figure 3. For each cell line, we treated the cells with both the solution (containing the prodrug 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, 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 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 as 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 experiments[31] 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

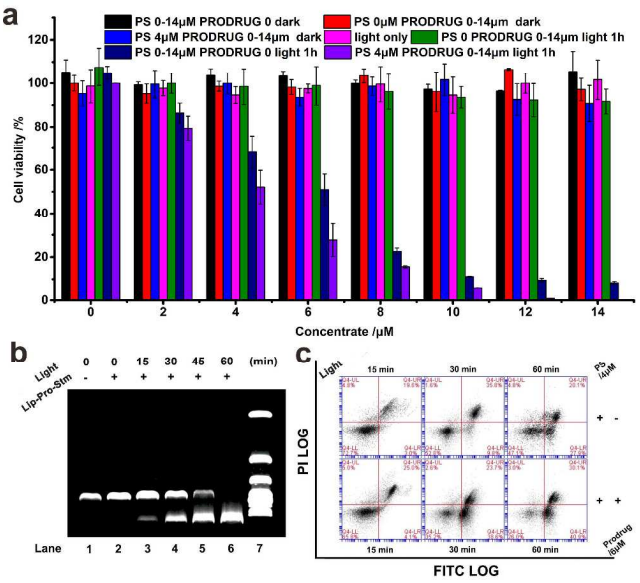


Figure 4. (a) Viabilities of HeLa cells upon treatment with different formulations of varied components and concentrations, as evaluated with MTT assay; the cells were incubated in culture media with --- black bar: PS 0-14 μM, dark; red bar: prodrug 0-14 μM, dark; blue bar: PS 4 μM, prodrug 0-14 μM, dark; pink bar: 500 nm light irradiation for 60 minutes; green bar: prodrug 0-14 μM 500 nm light irradiation for 60 minutes; indigo bar: PS 0-14 μM, 500 nm light irradiation for 60 minutes; purple bar: PS 4 μM, prodrug 0-14 μM, 500 nm light irradiation for 60 minutes. (b) Agarose gel electrophoresis assay for linear DNA (0.5 μg pBR322) crosslinking formation upon treatment with different formulations; the DNA were incubated in culture media with --- lane 1: buffer, lane 2-6: 50 μg/mL LIP-Pro-Stm (equivalent to 4.07 μM of the PS and 12.7 μM of the CHB) for 2 h after various time (15 min, 20 min, 30 min, 45 min 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 μM PS in the first row, with 4 μM PS and 6 μM prodrug in the second row after of 500 nm light irradiation and then further incubated in dark for 2h.

crosslinking caused by the drug CHB which was released from the prodrug. As a result, the linear DNA which was crosslinked 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) anti-cancer effect of the solution containing both the prodrug and the PS as well as the PS solution only. And 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. And the IC_{50} of the PS (Figure 4a) and the Lip-Pro-Stm (Figure S21) were calculated to be 6.03μM and 35.8 μg/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 which 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 anti-cancer 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

The Supporting Information is available free of charge on the ACS Publications website.
Materials and methods, and supplemental Tables and Figures (PDF)

AUTHOR INFORMATION

Corresponding Author

* Tel: 86-20-22236262; E-mail: mcfzeng@scut.edu.cn; shzhwu@scut.edu.cn

Author Contributions

Y. Lin, F. Zeng and S. Wu conceived the project and designed the research strategy and experimental approach; Y. Lin synthesized the compounds and performed the experiments, with assistance from X. Jiang, X. Duan and B. Wu. The data was analyzed by Y. Lin. The manuscript was written by Y. Lin, F. Zeng and S. Wu. All authors have given approval to the final version of the manuscript. F. Zeng and S. Wu supervised the research.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

We acknowledge the financial support by NSFC (21574044, 21474031, and 51673066), the Science and Technology Planning Project of Guangzhou (Project No. 201607020015), the Science and Technology Planning Project of Guangdong (Project No. 2014A010105009), the Natural Science Foundation of Guangdong Province (2016A030312002) and the Fundamental Research Funds for the Central Universities (2015ZY013).

ABBREVIATIONS

O_2^1, singlet oxygen; CHB, chlorambucil; PS, photosensitizer; LIP-Pro-Stm, the liposomal prodrug system; ^{1}H 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.

REFERENCES

[1] C. Shi, D. Guo, K. Xiao, X. Wang, L. Wang, J. Luo, A drug-specific nanocarrier design for efficient anticancer therapy, Nat. Commun. **2015**, 6, 7449.

[2] C. Li, A targeted approach to cancer imaging and therapy, Nat. Mater. **2014**, 13(2), 110-115.

[3] J. Savard, H. Ivers, M.H. Savard, C.M. Morin, Cancer treatments and their side effects are associated with aggravation of insomnia: results of a longitudinal study, Cancer **2015**, 121(10), 1703-1711.

[4] B. Li, P. Liu, H. Wu, X. Xie, Z. Chen, F. Zeng, S. Wu, A bioorthogonal nanosystem for imaging and in vivo tumor inhibition, Biomaterials **2017**, 138, 57-68.

[5] F. Huang, R. Cheng, F. Meng, C. Deng, Z. Zhong, Micelles based on acid degradable poly (acetal urethane): preparation, pH-sensitivity, and triggered intracellular drug release, Biomacromolecules **2015**, 16(7), 2228-2236.

[6] Z. Wang, H. Wu, P. Liu, F. Zeng, S. Wu, A self-immolative prodrug nanosystem capable of releasing a drug and a NIR reporter for in vivo imaging and therapy, Biomaterials **2017**, 139, 139-150.

[7] W.J. Fairbrother, A. Ashkenazi, Designer proteins to trigger cell death, Cell **2014**, 157(7), 1506-1508.

[8] C. Bao, L. Zhu, Q. Lin, H. Tian, Building biomedical materials using photochemical bond cleavage, Adv. Mater. **2015**, 27(10), 1647-1662.

[9] H. Xiao, G.T. Noble, J.F. Stefanick, R. Qi, T. Kiziltepe, X. Jing, B. Bilgicer, Photosensitive Pt (IV)–azide prodrug-loaded nanoparticles exhibit controlled drug release and enhanced efficacy in vivo, J. Control. Release **2014**, 173, 11-17.

[10] J. A. González-Delgado, P. J. Kennedy, M. Ferreira, et al. Use of Photosensitizers in Semisolid Formulations for Microbial Photodynamic Inactivation, J. Med. Chem. **2015**, 59(10), 4428-4442.

[11] E. Carrasco, B. del Rosal, F. Sanz□Rodríguez, Á.J. de la Fuente, P.H. Gonzalez, U. Rocha, K.U. Kumar, C. Jacinto, J.G. Solé, D. Jaque, Intratumoral Thermal Reading During Photo□Thermal Therapy by Multifunctional Fluorescent Nanoparticles, Adv. Funct. Mater. **2015**, 25(4), 615-626.

[12] J. Xu, F. Zeng, H. Wu, S. Wu, A mitochondrial-targeting and NO-based anticancer nanosystem with enhanced photo-controllability and low dark-toxicity, J. Mat. Chem. B **2015**, 3(24), 4904-4912.

[13] D. Luo, K.A. Carter, D. Miranda, J.F. Lovell, Chemophototherapy: an emerging treatment option for solid tumors, Adv. Sci. **2017**, 4(1), 1600106

[14] X. Yao, X. Chen, C. He, L. Chen, X. Chen, Dual pH-responsive mesoporous silica nanoparticles for efficient combination of chemotherapy and photodynamic therapy, J. Mater. Chem. B **2015**, 3(23), 4707-4714.

[15] W. Fan, B. Shen, W. Bu, F. Chen, Q. He, K. Zhao, S. Zhang, L. Zhou, W. Peng, Q. Xiao, A smart upconversion-based mesoporous silica nanotheranostic system for synergetic chemo-/radio-/photodynamic therapy and simultaneous MR/UCL imaging, Biomaterials **2014**, 35(32), 8992-9002.

[16] J. Fan, G. Fang, F. Zeng, X. Wang, S. Wu, Water□Dispersible Fullerene Aggregates as a Targeted Anticancer Prodrug with both Chemo□and Photodynamic Therapeutic Actions, Small **2013**, 9(4), 613-621.

[17] R. Lv, P. Yang, F. He, S. Gai, G. Yang, Y. Dai, Z. Hou, J. Lin, An imaging-guided platform for synergistic photodynamic/photothermal/chemo-therapy with pH/temperature-responsive drug release, Biomaterials **2015**, 63, 115-127.

[18] W.-H. Chen, G.-F. Luo, W.-X. Qiu, Q. Lei, L.-H. Liu, S.-B. Wang, X.-Z. Zhang, Mesoporous silica-based versatile theranostic nanoplatform constructed by layer-by-layer assembly for excellent photodynamic/chemo therapy, Biomaterials **2017**, 117, 54-65.

[19] G. Saravanakumar, J. Lee, J. Kim, W.J. Kim, Visible light-induced singlet oxygen-mediated intracellular disassembly of polymeric micelles co-loaded with a photosensitizer and an anticancer drug for enhanced photodynamic therapy, Chem. Commun. **2015**, 51(49), 9995-9998.

[20] G. Yang, X. Sun, J. Liu, L. Feng, Z. Liu, Light□Responsive, Singlet□Oxygen□Triggered On□Demand Drug Release from Photosensitizer□Doped Mesoporous Silica Nanorods for Cancer Combination Therapy, Adv. Funct. Mater. **2016**, 26(26), 4722-4732.

[21] P. Thapa, M. Li, M. Bio, P. Rajaputra, G. Nkepang, Y. Sun, S. Woo, Y. You, Far-red light-activatable prodrug of paclitaxel for the combined effects of photodynamic therapy and site-specific paclitaxel chemotherapy, J. Med. Chem. **2016**, 59(7), 3204-3214.

[22] M. Bio, P. Rajaputra, G. Nkepang, S.G. Awuah, A.M. Hossion, Y. You, Site-specific and far-red-light-activatable prodrug of combretastatin A-4 using photo-unclick chemistry, J. Med. Chem. **2013**, 56(10), 3936-3942.

[23] M.Y. Jiang, D. Dolphin, Site-specific prodrug release using visible light, J. Am. Chem. Soc. **2008**, 130(13), 4236-4237.

[24] D. Luo, N. Li, K.A. Carter, C. Lin, J. Geng, S. Shao, W.C. Huang, Y. Qin, G.E. Atilla□Gokcumen, J.F. Lovell, Rapid Light□Triggered Drug Release in Liposomes Containing Small Amounts of Unsaturated and Porphyrin–Phospholipids, Small **2016**, 12(22), 3039-3047.

[25] Qian, C., Yu, J., Chen, Y., Hu, Q., Xiao, X., Sun, W., Wang, C., Feng, P., Shen, Q., Gu, Z. Light□Activated Hypoxia□Responsive Nanocarriers for Enhanced Anticancer Therapy. Adv. Mater. **2016** 28(17), 3313-3320.

[26] Qian, C., Feng, P., Yu, J., Chen, Y., Hu, Q., Sun, W., Xiao, X., Hu, X., Bellotti, A., Shen, Q., Gu, Z. Anaerobe□Inspired Anticancer Nanovesicles. Angew. Chem. Int. Ed. **2017,** 56(10), 2588-2593.

[27] V. Goede, K. Fischer, R. Busch, A. Engelke, B. Eichhorst, C.M. Wendtner, T. Chagorova, J. de la Serna, M.-S. Dilhuydy, T. Illmer, Obinutuzumab plus chlorambucil in patients with CLL and coexisting conditions, N. Engl. J. Med. **2014**, 370(12), 1101-1110.

[28] A. Ayala, M.F. Muñoz, S. Argüelles, Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal, Oxid. Med. Cell. Longevity **2014**, 360438, 31.

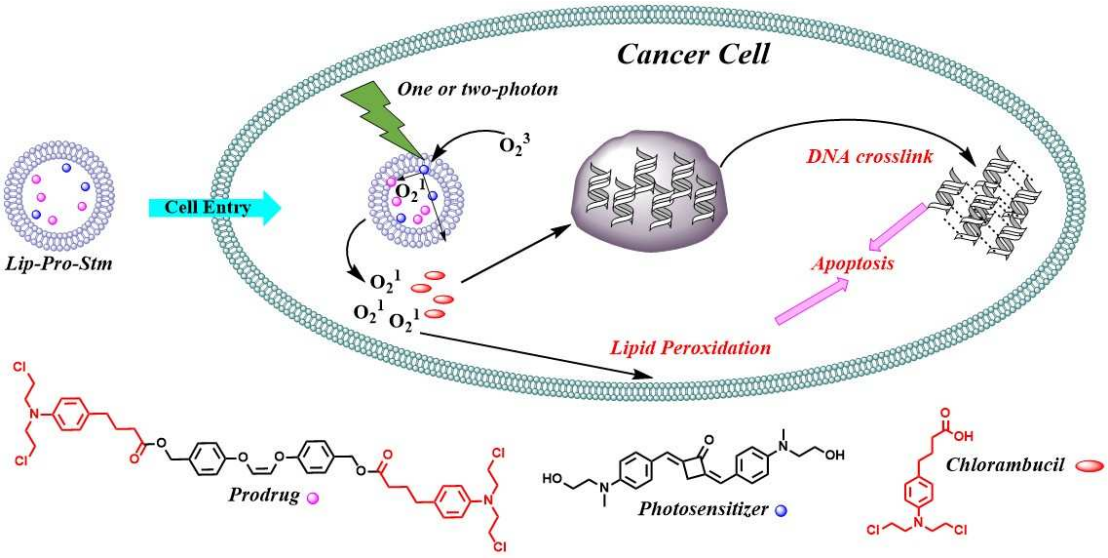
[29] M. Zamadar, G. Ghosh, A. Mahendran, M. Minnis, B.I. Kruft, A. Ghogare, D. Aebisher, A. Greer, Photosensitizer drug delivery via an optical fiber, J. Am. Chem. Soc. **2011**, 133(20), 7882-7891.

[30] J. Xu, F. Zeng, H. Wu, C. Yu, S. Wu, Dual-targeting nanosystem for enhancing photodynamic therapy efficiency, ACS Appl. Mater. Interfaces **2015**, 7(17), 9287-9296.

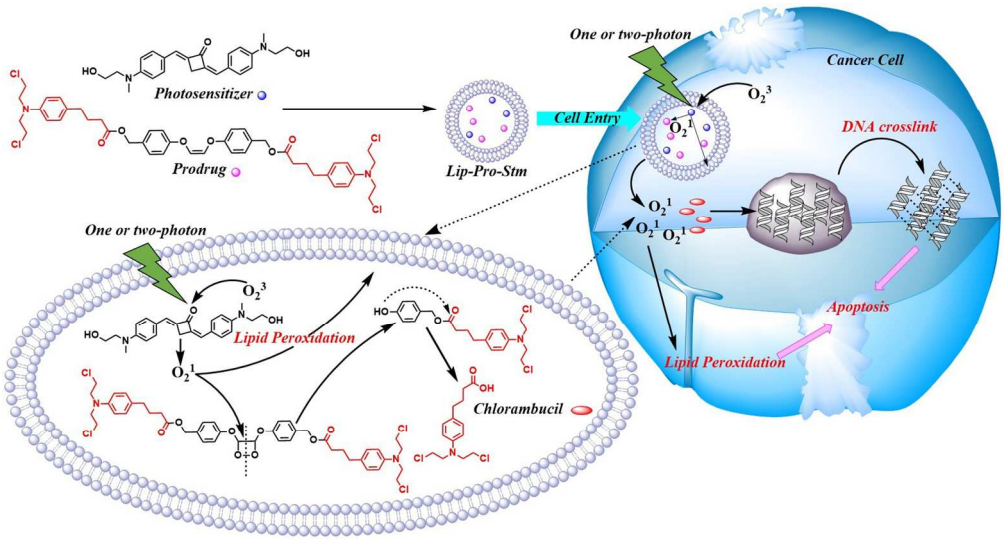
[31] C. Barckhausen, W. Roos, S. Naumann, B. Kaina, Malignant melanoma cells acquire resistance to DNA interstrand cross-linking chemotherapeutics by p53-triggered upregulation of DDB2/XPC-mediated DNA repair, Oncogene **2014**, 33(15), 1964.

[32] M. Fan, X. Liang, Z. Li, H. Wang, D. Yang, B. Shi, Chlorambucil gemcitabine conjugate nanomedicine for cancer therapy, Eur. J. Pharm. Sci. **2015**, 79, 20-26.

TOC



A liposomal therapeutic nanosystem features one- or two-photon light-responsive drug release to activate both chemotherapy and photodynamic therapy as well as self-imaging.



Scheme 1. Schematic overview of the prodrug stimulated by light to generate singlet oxygen which triggers the drug release and the related anti-cancer action.

168x90mm (300 x 300 DPI)

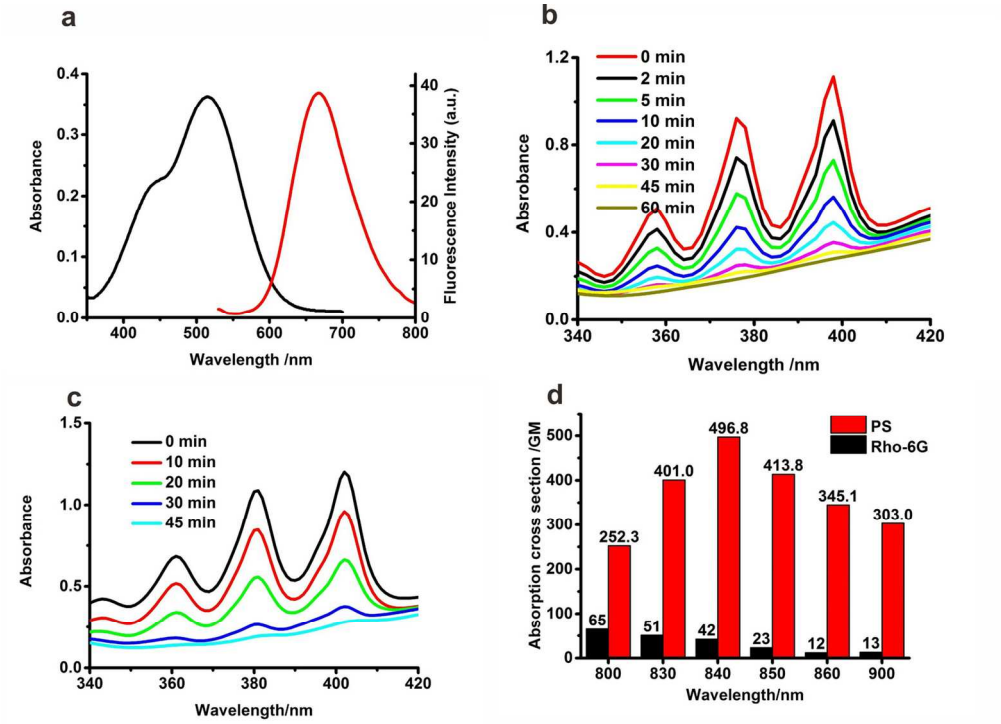


Figure 1. (a) Absorption and Fluorescence spectra of the PS (5 mM). (b) Photobleaching of ABDA (10 mM) by singlet oxygen generated by PS (5 mM) solution over different peri-ods of time under light irradiation (500 nm, 30mW·cm^{-2}). (c) Photobleaching of ABDA (10 mM) by singlet oxygen generated by PS (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.

146x105mm (300 x 300 DPI)

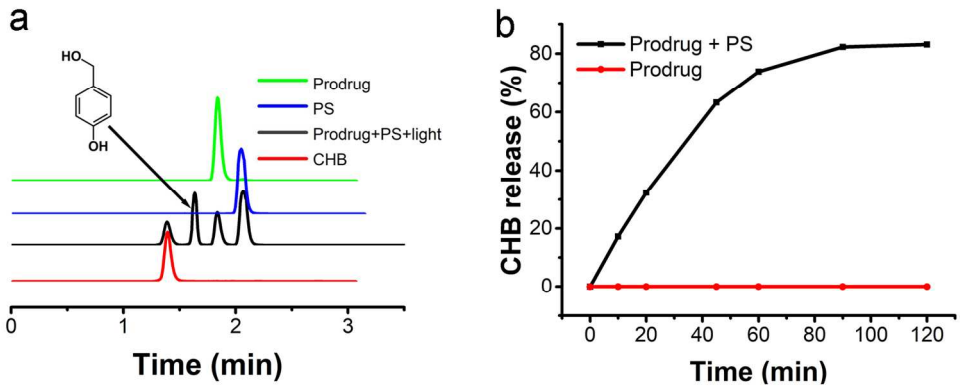


Figure 2. (a) Typical HPLC chromatogram of the prodrug, the PS, and the mixture of the prodrug (6 μM) and the PS (4 μM) with one-photon light irradiation (500 nm and 30mW·cm^{-2}) for 60 minutes, and CHB. (b) Percentage of CHB (as determined by HPLC) released from the prodrug (6 μM) as a function of time in the presence or absence of the PS (4 μM) upon light irradiation (500 nm, 30mW·cm^{-2}).

146x59mm (300 x 300 DPI)

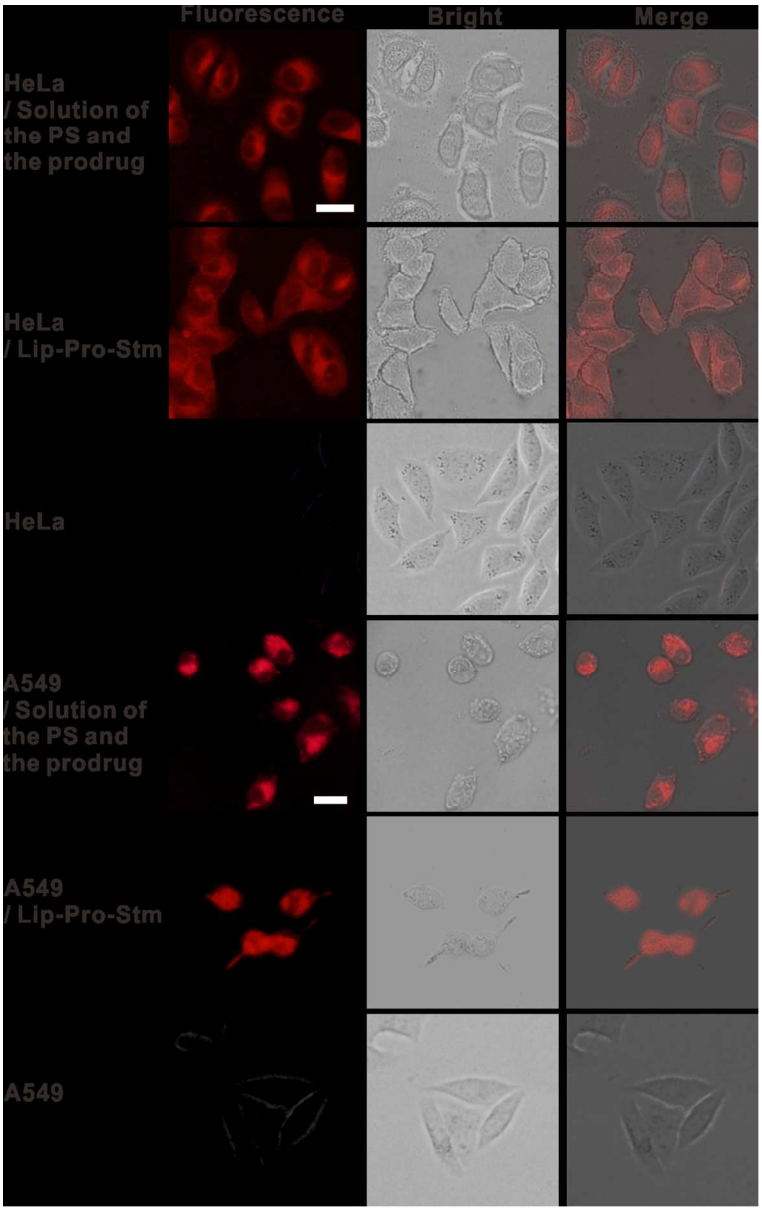


Figure 3. Fluorescent microscopic images for HeLa and A549 cells incubated in culture media with solution (4 μM PS and 6 μM prodrug) or Lip-Pro-Stm (50 μg/mL) with the incubation time of 2 hours, respectively. The control groups are placed in the 3rd and 6th row. Scale bar: 25 μm.

84x134mm (299 x 299 DPI)

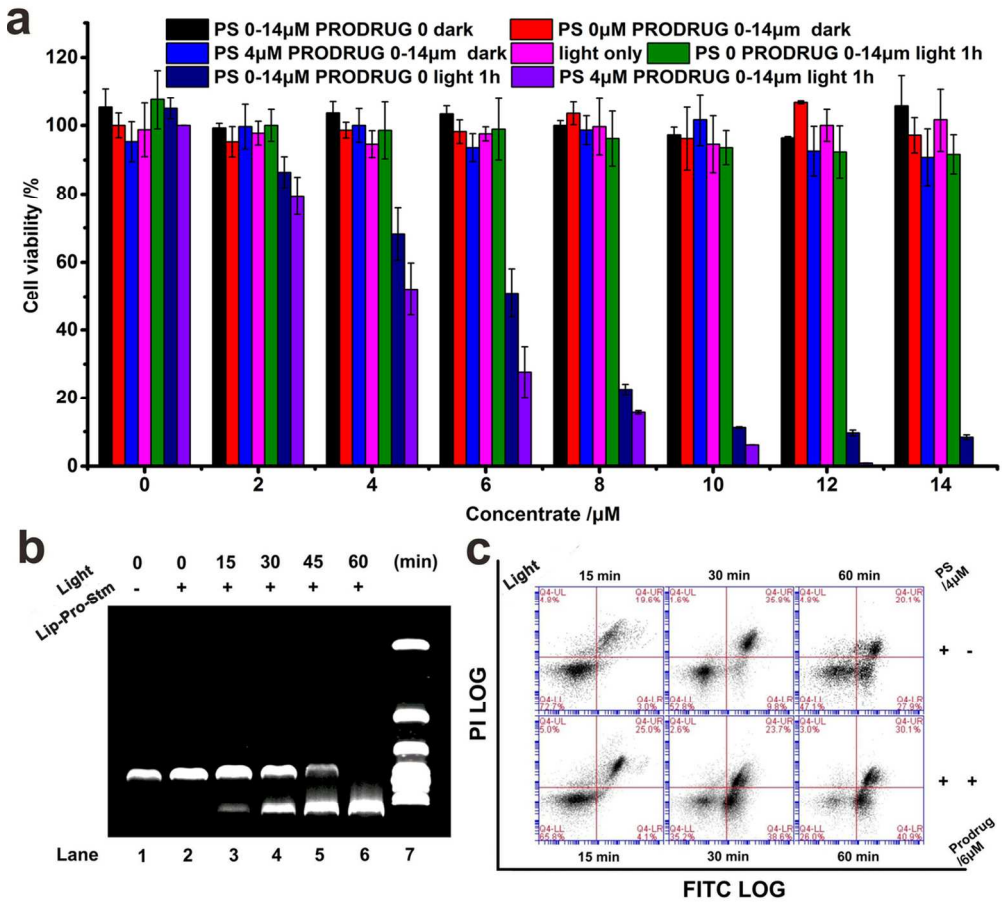
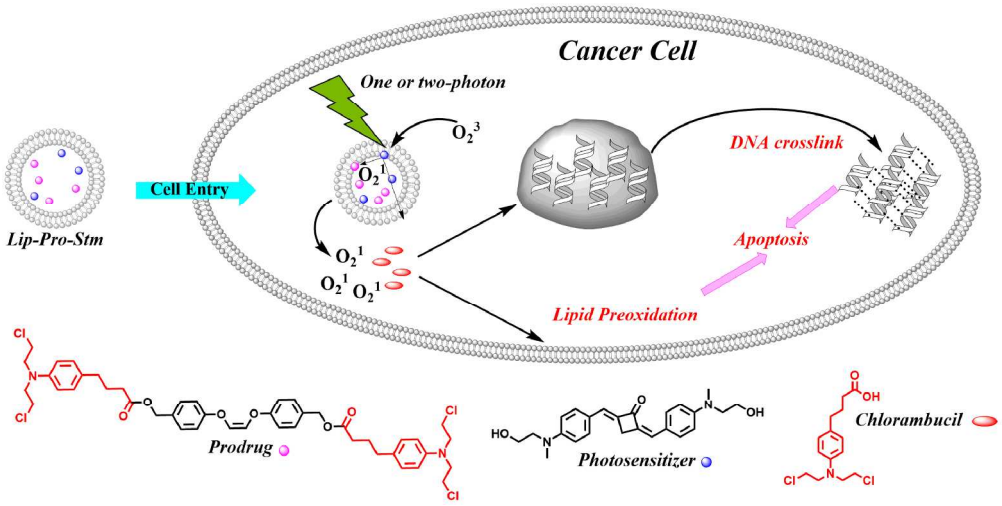


Figure 4. (a) Viabilities of HeLa cells upon treatment with different formulations of varied components and concentrations, as evaluated with MTT assay; the cells were incubated in culture media with --- black bar: PS 0-14 μM, dark; red bar: prodrug 0-14 μM, dark; blue bar: PS 4 μM, prodrug 0-14 μM, dark; pink bar: 500 nm light irradiation for 60 minutes; green bar: prodrug 0-14 μM 500 nm light irradiation for 60 minutes; indigo bar: PS 0-14 μM, 500 nm light irradiation for 60 minutes; purple bar: PS 4 μM, prodrug 0-14 μM, 500 nm light irradiation for 60 minutes. (b) Agarose gel electrophoresis assay for linear DNA (0.5 μg pBR322) crosslinking formation upon treatment with different formu-lations; the DNA were incubated in culture media with --- lane 1: buffer, lane 2-6: 50 μg/mL LIP-Pro-Stm (equivalent to 4.07 μM of the PS and 12.7 μM of the CHB) for 2 h after various time (15 min, 20 min, 30 min, 45 min 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 μM PS in the first row, with 4 μM PS and 6 μM pro-drug in the second row after of 500 nm light irradiation and then further incubated in dark for 2h.

115x104mm (300 x 300 DPI)



209x105mm (300 x 300 DPI)