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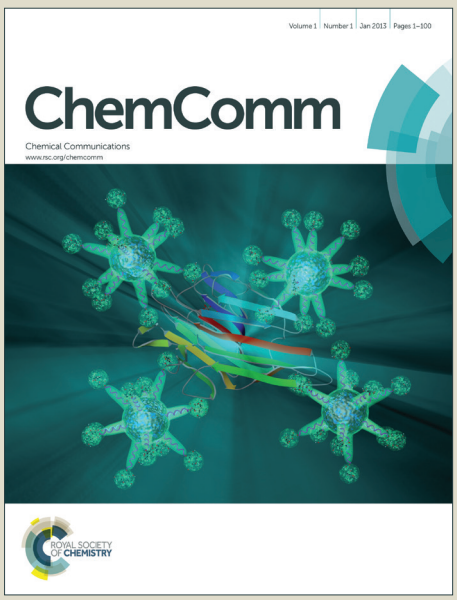

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COMMUNICATION

Activatable photodynamic destruction of cancer cells by NIR dye/photosensitizer loaded liposomes

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The phototoxicity of Chlorin e6 (Ce6) for photodynamic therapy (PDT) was found to be effectively suppressed by indocyanine green (ICG), a near infrared 10 (NIR) dye. Upon NIR laser irradiation at 808 nm, ICG in the liposomes containing ICG and Ce6 could be degraded while the phototoxicity of Ce6 could be recovered. In addition, we demonstrate that this newly developed liposomal component can be successfully used for activatable PDT to destruct cancer cells in vitro.

Photodynamic therapy (PDT) is a promising strategy for treatment of cancers[1]. Upon appropriate wavelength, photosensitizer molecules can transfer energy of photon to surrounding molecular oxygen to generate singlet oxygen ($^1O_2$) which could effectively damage the structure and function of cancer cells[2]. Compared with conventional chemotherapy, PDT shows remarkable improved selectivity and reduced side effects[3]. Chlorin e6 (Ce6), a widely studied photosensitizer[4-6], is promising for PDT because it could be activated by 660nm laser irradiation which has the capacity to penetrate tissue deeper than the 630nm laser irradiation used for conventional photosensitizers, such as Photofrin and Verteporfin[7, 8]. When irradiated, Ce6 has high $^1O_2$ quantum yield to generate sufficient $^1O_2$. In addition, low dark toxicity and ease of synthesis and production make Ce6 an ideal photosensitizer for PDT[9].

However, inevitable distribution of Ce6 in normal tissues, especially skin, was observed in clinical practices. Under sunlight, Ce6 could generate $^1O_2$ and subsequently induce skin photosensitivity, which might result in serious sunburn or pain[10, 11]. One of the potential reasons for the undesired phototoxicity is that Ce6 is always "ON" (the designation ''ON'' indicates that Ce6 is always active, even in normal tissues)[12] .

To overcome this flaw, some strategies have been developed. For instance, photosensitizers are designed to be quenched (turned "OFF") before administration. After accumulating in target tissues, photosensitizers could be turned "ON" (de-quenched) by physical stimuli (e.g. near infrared (NIR) light[12, 13]), chemical stimuli (e.g. pH[14] and redox[15]) or biological stimuli (e.g. enzymes[16]) to prevent the potential side effects of active photosensitizers. Among them, NIR laser has attracted increasing interests in recent years due to its non-invasive deeper tissue penetration.

NIR sensitive nanocarriers such as graphene oxide and gold nanoparticles have been used to turn "OFF" photosensitizers[12, 13]. They exhibit unique strong surface plasmon resonance absorption in the NIR region, and could simultaneously serve as effective quencher of photosensitizers. Taking gold nanorod as an example, the generation of $^1O_2$ by the photosensitizer could be effectively suppressed when the photosensitizer is located near gold nanorod. After irradiating by NIR laser, the photosensitizer would be released from the gold nanorod surface and become highly phototoxic[12]. Although the above methods have already shown high efficacy in quenching photosensitizers in preclinical studies, all these materials are non-biodegradable and might retain in the body for long periods of time, which limits their further clinical application[17]. Therefore, to discover alternative materials with great biodegradability and biocompatibility for NIR irradiation based activatable PDT is urgently necessary.

Indocyanine green (ICG), which was approved by Food and Drug Administration (FDA) in 1959, is a cyanine dye used for determining cardiac output, hepatic function, and ophthalmic angiography in clinical practices. The safety of ICG has been well described. In the present study, we firstly found that the phototoxicity of Ce6 could be effectively suppressed when Ce6 is located near ICG. Interestingly, the phototoxicity Ce6 would be recovered when ICG was degraded upon NIR irradiation. The quenching and de-quenching of Ce6 regulated by ICG and NIR irradiation make it possible to develop a NIR irradiation based activatable PDT. When loaded with ICG, Ce6 is turned "OFF" to prevent the potential side effects of Ce6 to normal tissues. After accumulating in target tissues, the phototoxicity of Ce6 could be turned "ON" for the treatment of cancers by degrading ICG using NIR irradiation.

To develop the NIR irradiation based activatable PDT, Ce6 was encapsulated into liposomes with ICG to turn "OFF" Ce6 (Fig. 1). Upon irradiation at 660nm, $^1O_2$ generation by Ce6 was effectively suppressed by ICG molecules. Upon irradiation at 808nm, ICG was degraded and the phototoxic of Ce6 was turned "ON". In addition, ICG could produce heat upon NIR irradiation to kill cancer cells, which could be used as photothermal therapy (PTT). Therefore, our designed liposomes containing ICG and Ce6 could be used as an activatable PDT combined with PTT for the treatment of cancers.

In this study, liposomes were synthesized via homogeneity and ultrafiltration. Transmission electron microscope (TEM) images showed that lip($Ce6_1$+ICG_4) (Ce6 and ICG were

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encapsulated into liposome in the weight ratio of 1:4) were spherical vesicles (Fig. 2A). The average hydrodynamic diameter is 140.9 nm (Fig. 2C) according to the results obtained from dynamic light scattering (DLS). The zeta potential of lip($Ce6_1$+ICG_4) was -12.1mV (Fig. 2D). The UV-vis spectrum showed that lip(Ce6) exhibited two absorption bands of 405nm and 670nm while lip(ICG) exhibited one absorption band of about 798nm (Fig. 2B). Lip($Ce6_1$+ICG_4) exhibited three absorption peaks (405nm, 670nm and 798nm), suggesting Ce6 was successfully encapsulated with ICG in the liposomes.

To determine the effect of ICG in turning "OFF" Ce6, fluorescent spectra and $^1O_2$ generation of the liposomes were measured. Firstly, fluorescence spectra of liposomes were investigated (Fig. 3A). lip(Ce6), lip(ICG), lip($Ce6_1$+ICG_2) and lip($Ce6_1$+ICG_4), in which the ratio of Ce6 to ICG was 1:0, 0:1, 1:2, and 1:4 (w/w) respectively, were prepared for investigation. Lip(Ce6) showed strong fluorescence at 400nm excitation wavelength while lip(ICG) did not. The maximal fluorescence intensity of Ce6 was quenched by 58.1% and 83.5% in lip($Ce6_1$+ICG_2) and lip($Ce6_1$+ICG_4) respectively, indicating that ICG could quench the fluorescence of Ce6 effectively. After exposing to laser irradiation at 808nm, the fluorescence of Ce6 was recovered (Fig. 3B) while the fluoresence of ICG decreased (Fig.S.1). With the irradiation time prolonged more than 5min, the recovery of the Ce6 fluorescence was very limited. Therefore, 5min was selected as the irradation time of 808nm laser for the further study.

Next, we evaluated the $^1O_2$ generation in different samples upon laser irradiation at 660nm (Fig.3C). $^1O_2$ was evaluated by the fluorescence of singlet oxygen sensor green (SOSG, λ_{Ex}=504nm, λ_{Em}=525nm). Upon laser irradiation at 660nm, the generation of $^1O_2$ in lip(Ce6) group increased rapidly. With the time of irradiation prolonged, the amount of generated $^1O_2$ by Ce6 also increased. In lip($Ce6_1$+ICG_2), the generation of $^1O_2$ was lower than those in lip(Ce6). In lip($Ce6_1$+ICG_4), the content of ICG increased and almost no $^1O_2$ could be observed (Fig. 3C). The $^1O_2$ generation from each sample was also quantified based on the singlet oxygen quantum yield of Ce6 (Tab.S.2). These data indicated that ICG could be an effective quencher to turn "OFF" the phototoxicity of Ce6. As well demonstrated, Ce6 could transfer energy of photon to surrounding molecular oxygen to generate ^{1}O2 upon 660nm laser irradiation. In the present study, the capacity of Ce6 to generate ^{1}O2 was effectively suppressed when encapsulated into liposome with ICG. It might be because the photon energy of Ce6 was absorbed by ICG instead of the surrounding oxygen.

To prevent the potential phototoxicity of Ce6 to normal tissues, ICG should be stable in the liposome until the degradation of Ce6. As shown in Fig.S.2, the Ce6 in lip(Ce6) degraded when exposed to sunlight. However, when ICG was encapsulated with Ce6, the degradation of Ce6 was significantly suppressed, suggesting the effects of ICG in preventing the degradation of Ce6 under sunlight. Combined with the stability of the liposome (Fig.S.6), it's reasonable to speculate that ICG could be stable for a long time in the liposome to turn "OFF" Ce6.

To determine the effect of NIR irradiation in turning "ON" Ce6, lip(Ce6), lip($Ce6_1$+ICG_2) and lip($Ce6_1$+ICG_4) were exposed to NIR irradiation (808nm, 1W/cm^2) to degrade ICG before exposing to laser irradiation at 660nm (0.1W/cm^2). After ICG was degraded, the phototoxicity of Ce6 was recovered as expected (Fig. 3D). In lip($Ce6_1$+ICG_2), the generation of $^1O_2$ recovered from about 70% to almost 100% after degradation of ICG. In lip($Ce6_1$+ICG_4), the generation of $^1O_2$ increased from 10% to 68%. These results indicate that the suppressed $^1O_2$ by ICG can be recovered by degrading ICG using NIR irradiation.

The photothermal effects of liposomes were also evaluated (Fig. 4A). Upon 808nm laser irradiation (1W/cm^2), the temperature of liposomes was recorded at intervals of 30 seconds for 5min. Similar temperature increase (about 9℃) were observed in lip(ICG) and lip($Ce6_1$+ICG_4), while slight temperature increase (2.5 °C) was observed in water.

In vitro cell viability was used to determine the effect of our designed liposomes in destructing cancer cells. Cells without any treatment were set as the control group according to our preliminary results (Fig.S.4). As shown in Fig.S.5, the liposomes were localized in lysosomes. As shown in Fig. 4B, without irradiation, lip($Ce6_1$+ICG_4) exhibited almost no toxicity to cells, indicating the safety of this formulation. We further evaluated the cytotoxicity of liposomal photosensitizers under laser irradiation at 660nm. Lip($Ce6_1$+ICG_4) showed much less toxic than both lip (Ce6) and lip($Ce6_1$+ICG_2), indicating that ICG can quench the generation of $^1O_2$ from Ce6 and decrease its phototoxicity (Fig. 4C). Next, lip($Ce6_1$+ICG_4) was exposed to 808nm laser to degrade ICG before exposing to 660nm laser. The phototoxicity of lip($Ce6_1$+ICG_4) was significantly increased since almost all cells were destructed (Fig. 4D, Fig.S.3). These indicate that degradation of ICG by 808nm laser irradiation could turn "ON" the phototoxicity of Ce6. In addition, the laser irradiation at 808nm also caused about 40% cell death, which might be due to the photothermal property of ICG. Taken together, lip($Ce6_1$+ICG_4) provides a PTT combined activatable PDT. However, for further in vivo or clinical studies, the biological stability of the delivery system and the optimal ratio between Ce6 and ICG need to be investigated, which is a limitation of the present study.

Conclusions

We developed a NIR irradiation based activatable PDT to prevent the side effects of active Ce6. In the ICG/Ce6 loaded liposomes, ICG were located around Ce6 to turn "OFF" the phototoxicity of Ce6. After degrading ICG by NIR irradiation, the phototoxicity of Ce6 was turned "ON". In addition, the temperature increase due to the exposure of ICG to NIR irradiation could serve as PTT. Therefore, the designed ICG/Ce6 loaded liposome could be used as a PTT combined activatable PDT.

Acknowledgement

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Inserting Graphics

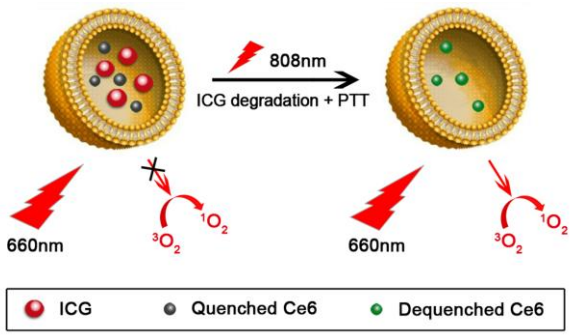

Fig 1: Schematic illustration of ICG/Ce6 loaded liposomes as PTT combined activatable PDT. Upon irradiation at 660nm, the phototoxicity of Ce6 was effectively supressed by ICG. Upon irradiation at 808nm, ICG was degraded and the $^1O_2$ generation capacity of Ce6 was recovered.

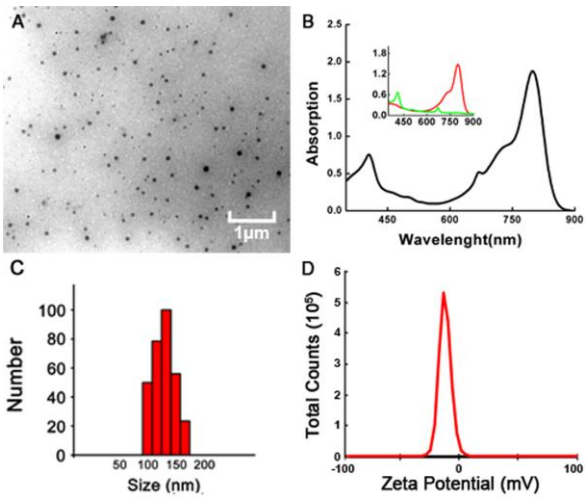

Fig 2: (A) TEM images of lip(Ce6$_1$+ICG$_4$), (B) UV-vis-NIR absorbance spectrum of lip(Ce6$_1$+ICG$_4$). Insets are the absorbance spectrum of lip(Ce6) (green) and lip(ICG) (red). (C) Size distribution and (D) Zeta Potential of lip(Ce6$_1$+ICG$_4$).

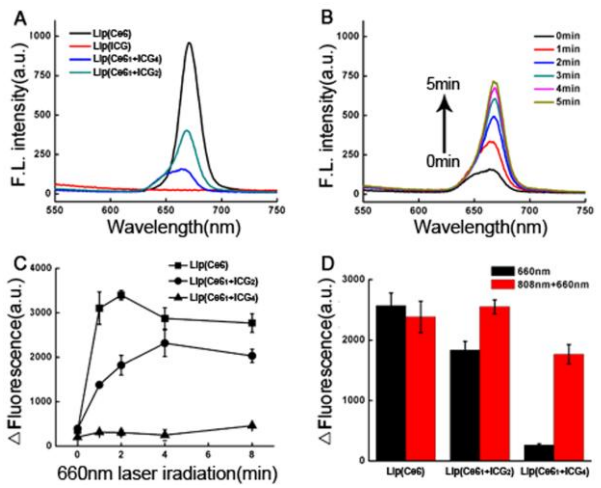

Fig 3: (A) Fluorescence spectra (λ_{Ex}=400nm) of lip(Ce6), lip(ICG), lip(Ce6$_1$+ICG$_2$) and lip(Ce6$_1$+ICG$_4$). (B) Fluorescence spectra (λ_{Ex}=400nm) of lip(Ce6$_1$+ICG$_4$) after laser irradiation at 808nm for 0, 1, 2, 3, 4 and 5min respectively. (C) Fluorescence of sensor green ($^1O_2$ probe, λ_{Ex}=504nm, λ_{Em}=525nm) in lip(Ce6), lip(Ce6$_1$+ICG$_2$) and lip(Ce6$_1$+ICG$_4$) after laser irradiation at 660nm for 0, 1, 2, 4, 8min respectively. (D) Fluorescence of sensor green in lip(Ce6), lip(Ce6$_1$+ICG$_2$) and lip(Ce6$_1$+ICG$_4$) after laser irradiation at 660nm or 808nm+660nm.

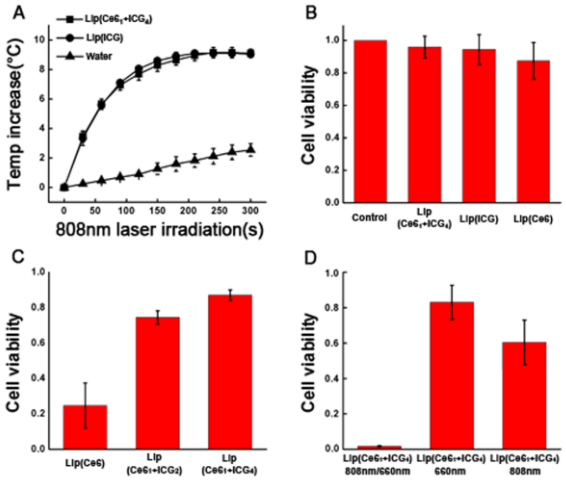

Fig 4: (A) Photothermal effect of lip(ICG), lip(Ce6$_1$+ICG$_4$) and water by 808nm laser irradiation for 5min. (B) Cell viability of lip(Ce6), lip(ICG) and lip(Ce6$_1$+ICG$_4$) in dark. (C) Cell viability of lip(Ce6), lip(Ce6$_1$+ICG$_2$) and lip(Ce6$_1$+ICG$_4$) exposed to 660nm laser irradiation. (D) Cell viability of lip(Ce6$_1$+ICG$_4$) exposed to 808nm/660nm, 660nm and 808nm laser irradiation.

Notes and references

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1. D. E. Dolmans, D. Fukumura and R. K. Jain, *Nat Rev Cancer*, 2003, **3**, 380-387.
2. T. Maisch, J. Baier, B. Franz, M. Maier, M. Landthaler, R. M. Szeimies and W. Baumler, *Proc Natl Acad Sci U S A*, 2007, **104**, 7223-7228.
3. M. B. Ericson, A. M. Wennberg and O. Larko, *Ther Clin Risk Manag*, 2008, **4**, 1-9.
4. H. Park and K. Na, *Biomaterials*, 2013, **34**, 6992-7000.
5. Z. Li, C. Wang, L. Cheng, H. Gong, S. Yin, Q. Gong, Y. Li and Z. Liu, *Biomaterials*, 2013, **34**, 9160-9170.
6. C. Tu, L. Zhu, P. Li, Y. Chen, Y. Su, D. Yan, X. Zhu and G. Zhou, *Chem Commun (Camb)*, 2011, **47**, 6063-6065.
7. M. Panjehpour, R. C. DeNovo, M. G. Petersen, B. F. Overholt, R. Bower, V. Rubinchik and B. Kelly, *Lasers Surg Med*, 2002, **30**, 26-30.
8. Y. Miyamoto, D. Nishikiori, F. Hagino, M. Wakita, I. Tanabe and M. Toida, *Laser therapy*, 2011, **20**, 135-138.
9. L. Huang, T. Zhiyentayev, Y. Xuan, D. Azhibek, G. B. Kharkwal and M. R. Hamblin, *Lasers Surg Med*, 2011, **43**, 313-323.
10. J. Wang, M. You, G. Zhu, M. I. Shukoor, Z. Chen, Z. Zhao, M. B. Altman, Q. Yuan, Z. Zhu, Y. Chen, C. Z. Huang and W. Tan, *Small*, 2013.
11. J. Wang, G. Zhu, M. You, E. Song, M. I. Shukoor, K. Zhang, M. B. Altman, Y. Chen, Z. Zhu, C. Z. Huang and W. Tan, *ACS Nano*, 2012, **6**, 5070-5077.
12. B. Jang, J. Y. Park, C. H. Tung, I. H. Kim and Y. Choi, *ACS Nano*, 2011, **5**, 1086-1094.
13. B. Tian, C. Wang, S. Zhang, L. Feng and Z. Liu, *ACS Nano*, 2011, **5**, 7000-7009.
14. J. Shi, Y. Liu, L. Wang, J. Gao, J. Zhang, X. Yu, R. Ma, R. Liu and Z. Zhang, *Acta Biomater*, 2013.
15. Y. Cho and Y. Choi, *Chem Commun (Camb)*, 2012, **48**, 9912-9914.
16. Y. Cho, H. Kim and Y. Choi, *Chem Commun (Camb)*, 2013, **49**, 1202-1204.
17. L. Guo, I. Panderi, D. D. Yan, K. Szulak, Y. Li, Y. T. Chen, H. Ma, D. B. Niesen, N. Seeram, A. Ahmed, B. Yan, D. Pantazatos and W. Lu, *ACS Nano*, 2013, **7**, 8780-8793.

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