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

Supporting information
1. Materials and methods

1.1 Materials

Ce6 and ICG were from Sigma-Aldrich Co., LLC (USA). CCK-8 was obtained from Dojindo Molecular Technologies (Japan). Singlet oxygen sensor green (SOSG) was purchased from Life Technologies (China). Lecithin, cholesterol and Propidium Iodide (PI) were obtained from Aladdin Reagents Co., LTD (China).

1.2 Preparation and characterization of lip(Ce6), lip(ICG), and lip(Ce6$_1$+ICG$_2$) (m$_{Ce6}$/m$_{ICG}$=1:2) and lip(Ce6$_1$+ICG$_4$) (m$_{Ce6}$/m$_{ICG}$=1:4)

Lip(Ce6) was prepared by homogeneity. Briefly, liposome colloidal suspensions were prepared by dissolving the lipid mixture (soya lecithin: 70mg, and cholesterol: 30mg) in chloroform. The organic solvent was removed via rotavapor to form a thin lipid film on the glass vial. The lipid film was then hydrated with an aqueous solution containing 0.4mg Ce6 by sonication for 30min in ice bath. Then the liposomes were homogenized by a Nano homogenize machine (15cycles, 800bar, ATS Engineering Inc.). After that, the excess Ce6 was removed by ultrafiltration. The concentrated liposomes were filtrated by 220nm filter (Millipore). The concentration of Ce6 in liposomes was detected by UV-Vis spectrum.

Similarly, liposome (ICG), lip(Ce6$_1$+ICG$_2$) and lip(Ce6$_1$+ICG$_4$) were prepared via the same method. 0.675mg Ce6 and 2mg ICG were added for hydration to obtained lip(Ce6$_1$+ICG$_2$) with m$_{Ce6}$/m$_{ICG}$ equaling to 1:2.01. And 0.35mg Ce6 and 2mg ICG were added for hydration to obtained lip(Ce6$_1$+ICG$_4$) with m$_{Ce6}$/m$_{ICG}$ equaling to 1:4.03.

TEM Size, Zeta Potential, and UV-Vis spectrum investigation:

The morphology of lip(Ce6$_1$+ICG$_4$) was characterized by transmission electron microscopy (TEM, H-7650, Hitachi, Japan). Particle size distribution and zeta potential of liposomes were detected by Nanoparticle Size Analyzer (ZEN3600, Malvern). UV-Vis spectrum of liposomes was detected by a UV-vis spectrometer (UV 2450, SHIMADZU).

1.3 Fluorescence and Singlet oxygen generation

Fluorescence spectrum:
Fluorescent spectrums of lip(Ce6) (C_{Ce6}=1\mu g/ml), lip(ICG) (C_{ICG}=4\mu g/ml), lip(Ce6$_1$+ICG$_2$) (C_{Ce6} = 1\mu g/ml) and lip(Ce6$_1$+ICG$_4$) (C_{Ce6} = 1\mu g/ml) were measured by RF-5301 Spectrofluorophotometer (Shimadzu Scientific Instruments) with the excitation wavelength at 400 nm. After that, lip(Ce6$_1$+ICG$_4$) solutions were irradiated by 808nm laser (1W/cm$^2$) for 1, 2, 3, 4 and 5 minutes, respectively. Fluorescent spectrums were also recorded after each irradiation.

_Singlet oxygen quenching by ICG:_

Singlet oxygen sensor green (SOSG) was employed to evaluate the singlet oxygen generation (SOG) of lip(Ce6), lip(Ce6$_1$+ICG$_2$) and lip(Ce6$_1$+ICG$_4$) upon laser irradiation. In these liposomes, ICG served as singlet oxygen quenching agent. Singlet oxygen sensor green was dissolved in methanol (5mM) and diluted in water before use. Different liposomes containing the same concentration of Ce6 (1.25\mu g/ml, 200\mu l) were mixed with SOSG (1\mu M, 20\mu l) solutions, and irradiated by a 660nm laser (0.1W/cm$^2$) for 0min, 1min, 2min, 4min and 8min, respectively. Then SOSG fluorescence (EX 504nm, EM 525nm) in different liposomes was detected to evaluate singlet oxygen generation. Fluorescence of free SOSG was set as control and subtracted from Fluorescence of each group. Each point was set as $\Delta$ Fluorescence.

In addition, singlet oxygen generation of different liposomes, in which singlet oxygen quenching agent (ICG) was removed by NIR laser irradition (808nm, 1W/cm$^2$), was also detected by SOSG. Three liposomes containing the same Ce6 (1.25\mu g/ml) and different ICG (0, 2.5 and 5\mu g/ml) were firstly irradiated by NIR laser (808nm, 1W/cm$^2$) for 5min to remove ICG molecules. After that, SOSG (1\mu M) was added into the above liposomes, irradiated by 660nm laser (0.1W/cm$^2$) for 8min. Then SOSG fluorescent detection was conducted.

_Quantification of the generation of $^1$O$_2$ from each sample:_

The singlet oxygen quantum yield of each sample was also evaluated according to the described protocol $^1$. Briefly, the $^1$O$_2$ quantum yield of different samples was estimated by comparing the reaction rate of a known photosensitizer using SOSG after photosensitization in PBS. In the present study, the $^1$O$_2$ quantum yield of different samples was determined, with respect to Ce6 as a standard photosensitizer as following:

$$\Phi_{\Delta sample} = \frac{\gamma_{sample}/A_{sample}}{\gamma_{Ce6}/A_{Ce6}} \cdot \Phi_{Ce6}$$
γsample and γCe6 are the reaction rates of the SOSG with \(^1\)O\(_2\) generated from photosensitization of the samples and Ce6, respectively. A\(_{\text{sample}}\) and A\(_{\text{Ce6}}\) are the absorbance of the samples and Ce6, respectively. \(\Phi \Delta \) Ce6 is the \(^1\)O\(_2\) quantum yield of Ce6, which is defined as 0.65\(^2\).

1.4 Temperature increase

Liposome (ICG) (\(C_{\text{ICG}}=5\mu\text{g/ml}\)), lip(Ce6\(_1\)+ICG\(_4\)) (\(C_{\text{ICG}}=5\mu\text{g/ml}\)) and water in 24-well plates were irradiated upon an 808 nm laser at power density of 1W/cm\(^2\). The temperature of solutions (1ml) was measured with temperature probe at 30s intervals for a total of 5 min. Each solution was measured for three times.

1.5 Cell viability

MCF-7 cells were cultured in Roswell Park Memorial Institute 1604 medium (RPMI 1640, Life Science, Shanghai, China) supplemented with 10% fetal bovine serum (FBS, Life Science) at 37°C in a 5% CO\(_2\) humidified incubator. Cell-viability of lip(ICG) (\(C_{\text{ICG}}=5\mu\text{g/ml}\)), lip(Ce6) (\(C_{\text{Ce6}}=1.25\mu\text{g/ml}\)) and lip(Ce6\(_1\)+ICG\(_4\)) (\(C_{\text{Ce6}}=1.25\mu\text{g/ml}\)) in dark was determined by CCK-8 assay. Briefly, MCF-7 cells were plated in 96-well plates with a concentration of 3,000 cells per well and allowed to grow overnight prior to incubation with liposomes. PBS was set as control. After 4 hours, culture medium containing liposomes was changed with fresh Medium. 48 hours later, CCK-8 was added to each well for an additional 2 hours. The cell viability was determined by measuring the absorption at 450nm using a microplate reader (Safire, TECAN, USA).

In addition, cell viability of lip(Ce6), lip(Ce6\(_1\)+ICG\(_2\)) and lip(Ce6\(_1\)+ICG\(_4\)) (all \(C_{\text{Ce6}}=1.25\mu\text{g/ml}\)) irradiated by 660nm laser was detected. MCF-7 cells were plated in 96-well flat-bottomed plates with a concentration of 3,000 cells per well and allowed to grow overnight prior to incubation with different liposomes for 4hours. Then these cells were irradiated by 660nm laser (0.1W/cm\(^2\)) for 8min. After that, culture medium containing liposomes was changed with fresh DMEM Medium, and the cells were incubated for another 48hours. The cell viability was determined by incubation with CCK-8 for 2hours and measuring the absorption at 450nm. Cells incubated with PBS without any laser irradiation were set as control.
To assess the cell viability after removing $^1$O$_2$ quenching agent, lip(Ce6$_1$+ICG$_4$) irradiated by 808nm laser, lip(Ce6$_1$+ICG$_4$) irradiated by 660nm laser and lip(Ce6$_1$+ICG$_4$) irradiated by 808nm and 660nm lasers successively were conducted. MCF-7 cells were plated in 96-well flat-bottomed plates with a concentration of 3,000 cells per well and allowed to grow overnight prior to incubation with different liposomes for 4 hours. Then cells incubated with lip(Ce6$_1$+ICG$_4$) were irradiated by 808nm laser (1W/cm$^2$) for 5 min. Cells incubated with lip(Ce6$_1$+ICG$_4$) was irradiated by 660nm laser (0.1W/cm$^2$) for 8 min. Cells in another group incubated with lip(Ce6$_1$+ICG$_4$) were successively irradiated by 808nm laser (1W/cm$^2$) for 5 min and 660nm laser (0.1W/cm$^2$) for 8 min. Cells incubated with PBS without any laser irradiation were set as control. After that, culture medium containing liposomes was changed with fresh DMEM Medium, and the cells were incubated for another 48 hours. The cell viability was determined by incubation with CCK-8 for 2 hours and measuring the absorption at 450 nm.

The cell viability of four groups (lip(Ce6) plus 660nm laser irradiation, lip(Ce6$_1$+ICG$_4$) plus 660nm laser irradiation, lip(ICG) plus 808nm laser irradiation and lip(Ce6$_1$+ICG$_4$) plus 808nm and 660nm laser irradiation) was also evaluated by 2.5μg/ml PI staining. After incubation with fresh medium for 4 hours, dead cells were stained with PI, respectively.

1.6 Localization of the liposome in the cells

$5 \times 10^4$ MCF-7 cells were seeded in glass-bottom dishes and incubated at 37°C for overnight. After attachment, lip(Ce6$_1$+ICG$_4$) (10μM) was added into medium and the cell was treated for 4 hours. Thereafter, the cells were washed with PBS and treated with lysotracker Green DND-26 (75nM) which was used to track lysosomes for 1 hour. After washing with PBS, images were captured with confocal microscope (Olympus FV1000) and merged to determine the intracellular localization of the liposome.
2. Results

Tab.S.1: Size and zeta potential of lip(Ce6$_1$+ICG$_2$), lip(Ce6) and lip(ICG).

<table>
<thead>
<tr>
<th></th>
<th>Size(nm)</th>
<th>Zeta Potential(mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lip(Ce6$_1$+ICG$_2$)</td>
<td>101.6±21.4</td>
<td>-11.5±5.1</td>
</tr>
<tr>
<td>Lip(Ce6)</td>
<td>73.8±18.4</td>
<td>-13.6±5.8</td>
</tr>
<tr>
<td>Lip(ICG)</td>
<td>92.6±14.6</td>
<td>-2.1±3.2</td>
</tr>
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</table>

Tab.S.2: $^1$O$_2$ quantum yield of different liposomes upon laser irradiation

<table>
<thead>
<tr>
<th></th>
<th>660nm</th>
<th>808nm+660nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lip(Ce6)</td>
<td>0.72</td>
<td>0.63</td>
</tr>
<tr>
<td>Lip(Ce6$_1$;ICG$_2$)</td>
<td>0.26</td>
<td>0.67</td>
</tr>
<tr>
<td>Lip(Ce6$_1$;ICG$_4$)</td>
<td>0.03</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Fig.S.1: UV-vis absorption of ICG exposed to 808nm laser irradiation for 0, 1, 2, 3, 4 and 5 min, respectively.
Fig.S.2: UV-vis absorption of Ce6 in lip(Ce6₁+ICG₄) and lip(Ce6) exposed to sunlight for 11 days.
Fig.S.3: Fluorescent images of MCF-7 cells stained by propidium iodide (PI). From top to bottom: lip(Ce6) plus 660nm laser irradiation, lip(Ce6_{1}+ICG_{4}) plus 660nm laser irradiation, lip(ICG) plus 808nm laser irradiation and lip(Ce6_{1}+ICG_{4}) plus 808nm and 660nm laser irradiation. PI is a popular red-fluorescent nuclear and chromosome counterstain. Since PI is not permeant to live cells, it is commonly used to detect dead cell.
Fig.S.4: Cell viability of MCF-7 cells treated with or without laser irradiation. There is no significant difference between the viability of cells treated with 660nm laser irradiation (8min), 808nm laser irradiation (5min), or 808nm laser irradiation (5min) plus 660nm laser irradiation (8min). Therefore, we selected the cells treated without any laser irradiation as the control group in the present study. NS: no significance.

Fig.S.5: Localization of lip(Ce6+ICG) in MCF-7 cells. After incubating with the liposome, the cells were treated with Lysotracker which was used to track lysosomes. The obtained results indicated that the liposomes were localized in the lysosome (with arrow).
Fig.S.6: Measurement of the size of lip(Ce6₁+ICG₄) in distilled water for 12 days. Please note that the size of the liposomes did not change significantly.
Reference:
