SUPPORTING INFORMATION

Premature Drug Release from Polyethylene Glycol (PEG)-Coated Liposomal Doxorubicin via Formation of the Membrane Attack Complex

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Supplemental Figure 1. Drug release in mouse serum

6.3 (mouse anti-PEG IgG1) and AGP4 (mouse anti-PEG IgM) were mixed with serum from BALB/c (A) or C57bl/6 (B) mice containing Doxisome to give a final concentration of 27.5 µg/mL of 6.3, 93 µg/mL of AGP4 and 10 µg/mL of Doxisome, respectively. The same quantity of non-binding IgG1 and IgM were used as negative controls. 100% lysis was determined by adding Triton X-100 to mouse serum and Doxisome instead of antibodies. Drug release is plotted as fluorescence.
Supplemental Figure 2. Correlation between anti-PEG IgG antibody binding avidity and Doxisome lytic activity

The concentration of anti-PEG IgG antibody that lysed 10% of Doxisome was plotted against the binding EC\textsubscript{50} concentrations of the anti-PEG antibodies. Antibodies with greater binding avidity (lower EC\textsubscript{50}) could lyse Doxisome at lower concentrations.
Supplemental Figure 3. Anti-PEG antibody-induced drug release of doxorubicin from Doxisome is complement-dependent

50 µg/mL of mouse anti-PEG (A), rat anti-PEG (B) or humanized anti-PEG (C) antibodies were incubated with 10 µg/mL Doxisome in the presence untreated sera (yellow bars), CVF-treated sera (orange bars) or sera heated to 56 °C (red bars). Error bars show standard deviation, n = 3. Significant differences between untreated serum and CVF or heat treated serum are indicated: *, p<0.05; **, p<0.005; ***, p<0.0005.
Supplemental Figure 4. Release of doxorubicin from Doxisome and Doxil

Comparison of drug release induced by 50 µg/mL of humanized anti-PEG IgG and IgM incubated in human serum and Doxisome (red) or Doxil (pink). Percentage drug release was calculated in comparison to 100% drug lysis with Triton X-100, and 0% drug lysis with PBS. Results show mean values ± SD, n = 3. Significance is reported as p > 0.01 (ns).
Supplemental Figure 5. Comparison of drug release from Doxisome and Lipo-Dox

Humanized anti-PEG antibodies were serially diluted in PBS and human serum containing 20 µg/mL of (A) Doxisome or (B) Lipo-Dox were added to the dilutions. Mixtures were incubated for 30 min before fluorescence of released doxorubicin was measured. Bars show standard deviation, n = 3.
Supplemental Figure 6. Drug release by pre-existing human anti-PEG IgG

50 µl of human serum containing 15 µg/mL of anti-PEG IgG (Donor 1) was serially diluted two-fold, then added to an equal volume of human serum without anti-PEG antibody containing 20 µg/mL Doxisome. Hu6.3 and control IgG₁ antibody were used as positive and negative controls, respectively. Drug release is plotted as fluorescence. Error bars show standard deviation, n = 8.
Supplemental Figure 7. Control cryo-EM images of Doxisome.

Supplemental Figure 8. Wide-view cryo-EM images of Doxisome incubated under various conditions.

A) Doxisome in PBS. B) Doxisome in PBS and hu6.3. C) Doxisome in human serum and hu6.3. Reaction mixtures of Doxisome/human serum/hu6.3 were passed through a Sephadex CL-4B column to remove free antibody and observe C5b-9 complexes binding to Doxisome. D) Doxisome incubated with hu6.3 and heat-inactivated serum. E) Doxisome in human serum and control IgG1. Scale bars represent 100 nm.
Supplemental Figure 9. Proportion of empty liposomes

Wide-view cryo-EM pictures were analysed by counting the number of liposomes with and without visible Doxorubicin nanorods present. Empty liposomes are expressed as a percentage of total liposomes.
Supplemental Figure 10. Doxorubicin release from liposomes by different subclasses of humanized anti-PEG IgG.

The ability of a series humanized anti-PEG IgG subclasses to cause drug release from Doxisome was measured as described in methods and materials. Results show mean values ± SD, n = 8.
Supplemental Figure 11. Rate of doxorubicin release from liposomes

Doxisome was mixed to 20 µg/mL in Wistar rat serum and added to a black 96-well plate. The plate’s fluorescence was detected at 490/590nm with a microplate reader (Tecan Infinite® M1000 Pro) every 15 seconds for five minutes at 37 °C. At time zero, an equal volume of 100 µg/mL r33G, 6.3 or E11, or 20 µg/mL 6.3 was added to the wells in triplicate. As controls, anti-PEG antibodies were replaced with either 100 µg/mL control IgG1, PBS or 1% Triton X-100 in PBS and the fluorescence was recorded for another 15 minutes.
Supplemental Figure 12. Influence of Triton X-100 on doxorubicin fluorescence

Rat serum containing 20 µg/mL of doxorubicin was serially diluted two-fold in rat serum in a 96-well black plate. An equal volume of PBS or 1% Triton X-100 was added, and the fluorescence of the wells was measured. Error bars show standard deviation, n = 3.
Supplemental Figure 13. Drug release in PBS

For cryo-EM images, antibodies were diluted in PBS rather than GHBS++. We show here that drug release can still be detected under this condition. Bars show standard deviation, n = 3.
Supplemental Figure 14. Release of doxorubicin from PLD in rats

A-D) Male Wistar rats were i.v. injected with a control rat IgG or r33G rat anti-PEG IgG to achieve the indicated serum concentrations of antibodies. After 1 h, the rats were i.v. injected with the indicated doses of LC-101 PLD. Plasma samples obtained from the rats within 5 min of LC-101 injection were serially diluted in PBS and the fluorescence of either untreated plasma (solid columns) or Dowex-treated plasma (dotted columns) was measured. E) The fluorescence of free doxorubicin versus LC-101 fluorescence. The concentrations of doxorubicin in PLD and free doxorubicin were identical at each point. The slope of the line (12.0) corresponds to the ratio of free doxorubicin fluorescence as compared to LC-101 fluorescence. F) Comparison of the amount of free doxorubicin in plasma of rats treated as in panel D by two methods. The first method uses plasma treated with or without Dowex as
described in materials and methods (no lysis). The second method uses plasma treated with or without Dowex but then lyses LC-101 in all samples with 1% Triton X-100 to measure total doxorubicin in the samples (Lysis). Comparison of the calculated doxorubicin release by the two methods was not significantly different, indicating good estimation of doxorubicin release.
Supplemental Figure 15. Validation of the Dowex assay

A) A constant amount of LC-101 PLD (50 µg) in 800 µL of 25% rat serum/PBS was spiked with the indicated concentrations of free doxorubicin. The samples were untreated or treated to remove free doxorubicin by addition of 100 mg Dowex resin for 20 min at 4°C. The fluorescence of the samples in 96-well black fluorescence plates was measured on a fluorescence reader. Dowex treatment removed free doxorubicin so only the fluorescence of the remaining LC-101 PLD is observed. B) The indicated concentrations of LC-101 was added to 600 µL of 25% rat serum/PBS containing a constant amount of free doxorubicin (1 µg/mL). The samples were untreated or treated to remove free doxorubicin by addition of 100 mg Dowex resin for 20 min at 4°C. The fluorescence of the samples in 96-well black fluorescence plates was measured on a fluorescence reader. The continually increasing fluorescence is due to the presence of LC-101, which is not adsorbed by Dowex resin.
Supplemental Table 1. List of anti-PEG antibodies

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<tr>
<th>Antibody name</th>
<th>Fc region</th>
<th>Subclass</th>
<th>Epitope</th>
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<td>Mouse</td>
<td>IgG₁</td>
<td>PEG backbone</td>
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<td>Mouse</td>
<td>IgG₂b</td>
<td>Methoxy group</td>
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<td>r33G</td>
<td>Rat</td>
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<td>rAGP6</td>
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Supplemental Table 2. Relative binding of anti-PEG antibodies to Doxisome. Results show the concentrations of antibody producing 50% maximal signal when binding to Doxisome in ELISA.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Class</th>
<th>Species</th>
<th>Binding EC$_{50}$ (nM)</th>
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Supplemental Table 3. Comparison of PLD used in this study

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Doxil</th>
<th>Doxisome</th>
<th>Lipo-Dox</th>
<th>LC-101</th>
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<tr>
<td>Diameter</td>
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<td>Doxorubicin concentration</td>
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<td></td>
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<td>Lipid</td>
<td>HSPC</td>
<td>HSPC</td>
<td>DSPC</td>
<td>HSPC</td>
</tr>
<tr>
<td>Lipid (molar %)</td>
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<td>56%</td>
<td>56%</td>
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<tr>
<td>Cholesterol (molar %)</td>
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<td>39%</td>
<td>39%</td>
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<td>Manufacturer (country)</td>
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<td>Taiwan Liposome Company (Taiwan)</td>
<td>TTY Biopharm (Taiwan)</td>
<td>Lipocue (Israel)</td>
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Supplemental Table 4. List of commercial antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalogue no.</th>
<th>Supplier</th>
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<td>Jackson Immunoresearch</td>
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<tr>
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<td>HRP-donkey anti-mouse IgG</td>
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