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Biodegradable liposome–chitosan complexes: enzyme-mediated release of encapsulated substances

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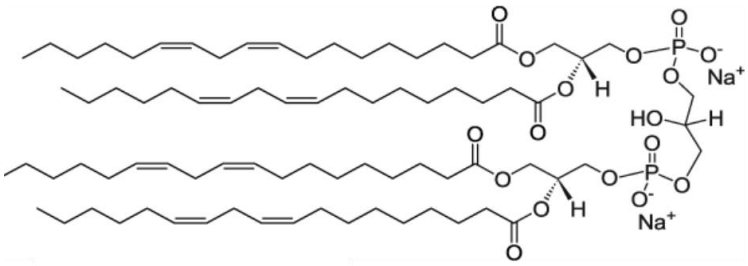

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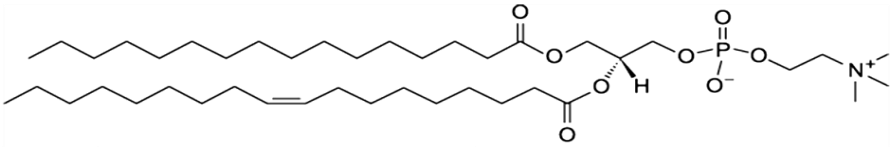

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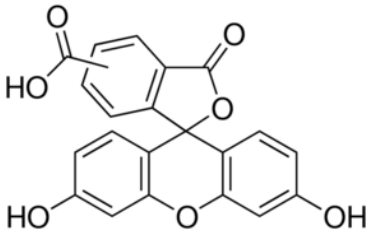

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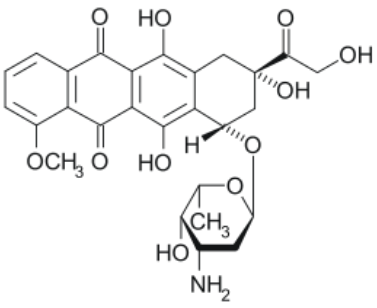

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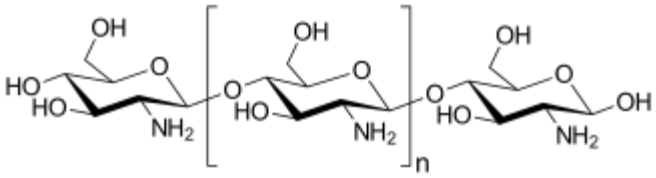

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Figure S1 The structure of diphosphatidyl glycerol (cardiolipin, CL^{2-}) **1**, phosphatidylcholine (PC) **2**, 5(6)-carboxyfluorescein (CF) **3**, doxorubicin (Dox) **4** and chitosan **5**.

Procedure S1. Small unilamellar liposomes were prepared by the standard sonication technique from mixture of anionic CL^{2-} and electroneutral PC (Procedure S1). The required amounts of lipid solutions in a methanol-chloroform (1:1 wt/wt) were mixed, and the organic solvent was removed by vacuum rotary evaporator at 30°C. The formed thin film was dispersed in TRIS buffer (10^{-2} M, pH 7), and then sonicated with a Cole-Parmer 4710 ultrasonic homogenizer for 400 s (2 × 200 s). The resulting liposomes were separated from titanium dust *via* centrifugation in a J-11 centrifuge (Beckman) for 5 min at 10000 rpm.

To prepare liposomes loaded by NaCl the lipid film was suspended in NaCl solution (1 M) in TRIS buffer (10^{-3} M). The resulting suspension was dialyzed for 1.5 h in TRIS buffer (10^{-3} M), the outer TRIS buffer (10^{-3} M) solution was changed every 30 min. To prepare liposomes loaded by CF, the lipid film was suspended in CF solution (1mg ml^{-1}) in TRIS buffer (10^{-3} M). Then the suspension was dialyzed for 12 h in TRIS buffer (10^{-3} M), the outer buffer was changed every 2 h.

Loading of liposomes with antitumor Dox was performed according to the modified procedure originally described previously. It is based on the weakly basic properties of Dox which contains an amino group with pKa 8.6, so at pH 7 about 2.5% of Dox molecules are uncharged and can incorporate into the lipid membrane. If the internal cavity of the liposome is loaded with an acidic buffer, Dox desorbs from the membrane and accumulates inside the liposomes. This obviously shifts the equilibrium: $NH_2(Dox) + H^+ \rightleftharpoons NH_3^+(Dox)$ to the uncharged form of Dox in the surrounding solution, thus ensuring the transmembrane migration of a major part of the initial Dox. The internal volume of the liposomes is much fewer (approximately 1000-fold) than the total solution volume. So, a substantial concentrating of Dox inside the vesicles occurs. This results in the self-quenching of Dox fluorescence. Briefly, a suspension of pH-gradient PC/CL^{2-} liposomes with pH 7 outside and pH 3 inside was prepared. To this end, lipid film was dispersed in citrate buffer (0.15 M, pH=3). The resulting suspension was dialyzed extensively for 4.5 h in Hepes buffer (20 mM, pH 7) supplemented with NaCl (0.15 M) for compensation of osmotic gradient. Hepes buffer was renewed every 45 minutes. Addition of Dox solution (50 μM) in the

external buffer to these vesicles resulted in considerable quench of Dox fluorescence (λem = 557 nm, λex = 490 nm) indicating its accumulation in the internal cavity.
Size of liposomes, measured by quasi-elastic light scattering was within 40-50 nm interval. Double-distilled water was used for making solutions after additionally treating it with a Milli-Q Millipore system.

Procedure S2. The biodegradation of the liposome/chitosan complex was initiated *via* addition of Morikrase proteolytic complex, prepared from the hepatopancreas of the Kamchatka crab Paralithodes camchatica (OAO Trinita, Russia) or the enzyme lipase from *Rhizopus sp.* (Serva, Germany) or lysozyme from Chicken Egg (Sigma-Aldrich, USA). Morikraza is a mixture of enzymes (serine proteinase, collagenase, metalloproteinase, etc.) capable of ester, peptide, and amide bond splitting that demonstrates enzyme activity in the pH range 6.0−9.0 with the maximum at pH 7.5. Lipase, which catalyzes the hydrolysis of ester bonds in lipid molecules, is active in the pH range from 4.5 to 8.0, with the optimum at pH 6.5−7.5. Lysozyme is one of enzymes present in the human body that can hydrolyze the β-(1-4)-bonds between N-acetylglucosamine and glucosamine in chitosan with the optimum at pH 5−7.

Procedure S3. Mean hydrodynamic diameters of particles were determined by dynamic light scattering at the fixed scattering angle (90°) in a thermostatic cell with a Brookhaven Zeta Plus instrument. Software provided by the manufacturer was employed to calculate diameter values.