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# An electrostatic conjugate composed of liposomes, polylysine and a polylactide micelle: a biodegradability–cytotoxicity relationship

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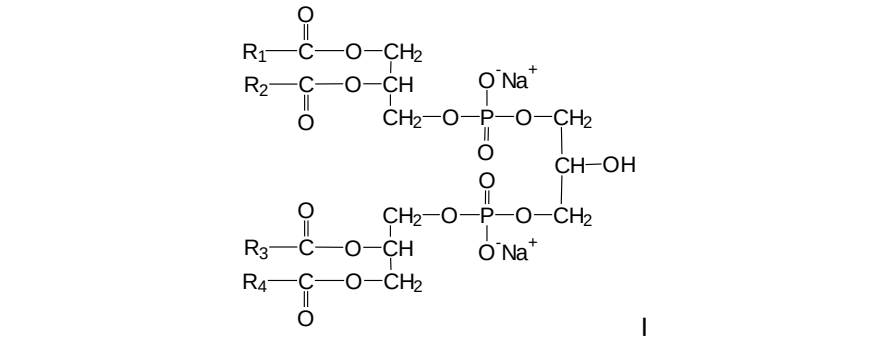
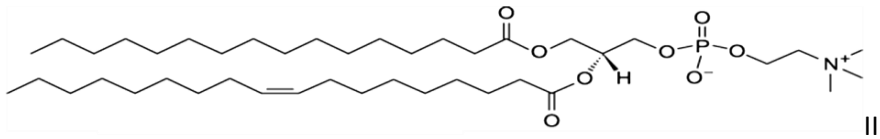


I



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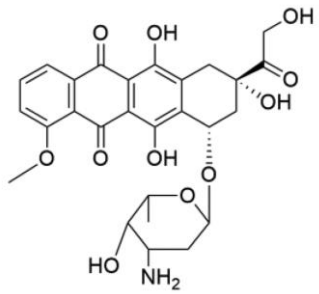

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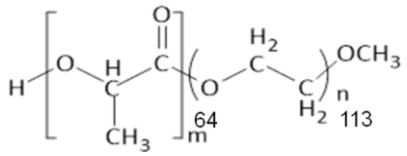

IV

**Figure S1** The structure of the compounds: diphosphatidyl glycerol (cardiolipin, $CL^{2-}$) (I), phosphatidylcholine (PC) (II), doxorubicin (Dox) (III), PLA−PEG diblock copolymer ($PLA_{64}$-$PEG_{113}$) (IV).

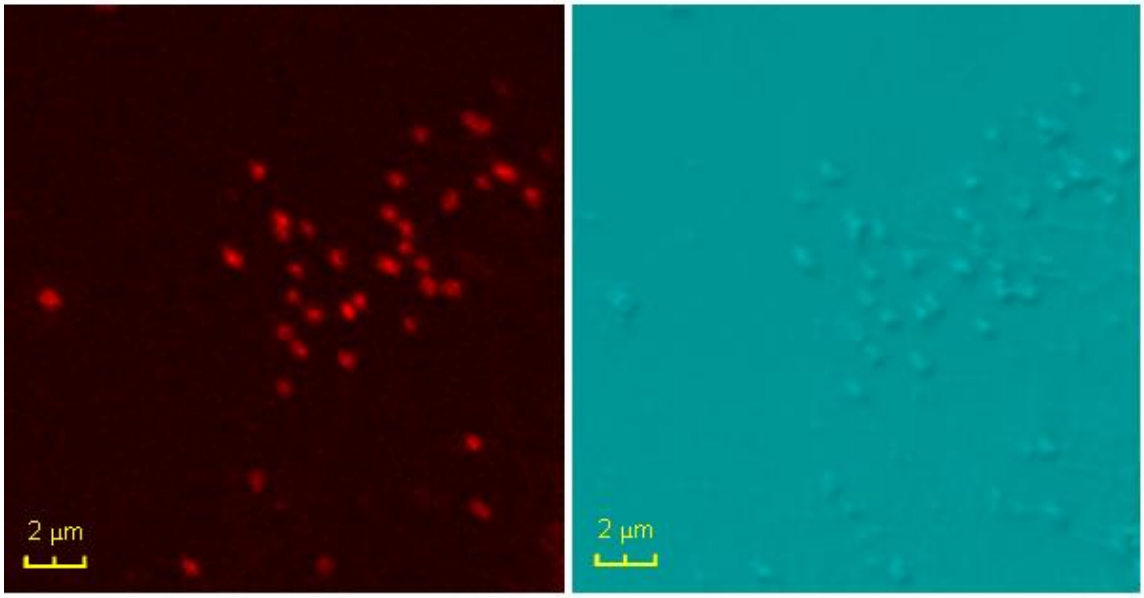


**Figure S2** Laser Confocal Microscopy image of Dox-loaded liposome-colloidal complex particles. (a) Fluorescence mode, (b) differential interference contrast (DIC) mode.

**Procedure S1.** The small unilamellar mixed PC/$CL^{2-}$ liposomes were performed by standard sonication technique from mixture of anionic $CL^{2-}$ and electroneutral PC. The required amounts of lipid solutions in a methanol-chloroform mixture (1:1 wt/wt) were mixed, and the organic solvent was removed on a vacuum rotary evaporator at 30°C. The formed thin film was dispersed in a $10^{-2}$ M Tris buffer with pH 7, and then sonicated with a Cole-Parmer 4710 ultrasonic homogenizer for 400 s (2 × 200 s) at 55 °C. The resulting liposomes were separated from titanium dust via centrifugation in a J-11 centrifuge (Beckman) for 5 min at 12000 rpm.
Size of liposomes, measured by quasi-elastic light scattering was within a 40-50 nm interval.

**Procedure S2.** The aqueous dispersion of copolymer micelles was prepared by replacing the solvent from organic to water as follows [1]. Block copolymer was dissolved in THF. After one day, distilled water was added dropwise to the solution under intense stirring to make the water content as high as 10 vol %, and after another day, the water content were brought to 20 vol %. Aqueous−organic mixture was dialyzed for a week against water to remove the organic solvent. As a result, aqueous dispersion of diblock copolymer micelles was obtained. For our experiments we chose diblock copolymer that demonstrated the highest aggregative stability: $PLA_{64}$-$PEG_{113}$.

**Procedure S3.** The biodegradation of the liposome/PL/polylactide micelle ternary complex was initiated via addition of a Morikrase proteolytic complex [2], prepared from the hepatopancreas of the Kamchatka crab Paralithodes camchatica (OAO Trinita, Russia). 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.

**Procedure S4.** Cell survival (cytotoxicity) of the samples towards human breast adenocarcinoma MCF-7/R cells was evaluated with a methyl-tetrazolium blue assay [3]. Briefly, the day before the experiment, MCF-7/Adr cells were seeded on a 96-well plate (Costar, USA) at a density 3000–4000 cells per well. The next day, the culturing medium was removed and 0.2 mL of the solutions of liposomes or Liposome-colloid complexes at varying concentrations in the serum-free medium were placed in the wells for 1 h. In control wells (100% of surviving cells), the assayed compounds were replaced with equal volume of the appropriate medium. Then, the polymer solutions were removed and the cells were cultured in 0.2 mL of fresh complete medium containing 10% of serum for 3 days. The amount of remaining living cells was assayed by addition of 0.05 mL of MTT solution (1 mg/mL) in the MEM culturing medium for 3–4 h. Then, the medium was removed, violet crystals of formazan were dissolved in 0.1 mL of DMSO, and the optical density at 550 nm was measured on a Multiscan photometer (Titertek, USA). The portion of survived cells was calculated as a ratio of optical density in well with certain polymer concentration to that in control well. All runs were carried out in quadruplicates.

**Procedure S5.** Loading of liposomes with Dox was performed according to the modified procedure described previously [4]. 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 smaller (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 a 0.15 M citrate buffer (pH=3). The resulting suspension was dialyzed extensively for 4.5 h against a 20 mM Hepes buffer (pH=7) supplemented with 0.15M NaCl for compensation of osmotic gradient. Hepes buffer was renewed every 45 minutes. Addition of 50 μM solution of Dox in the external buffer to these vesicles resulted in a considerable quenching of Dox fluorescence ($\lambda$em = 557 nm, $\lambda$ex = 490 nm) indicating its accumulation in the internal cavity.

**Procedure S6.** The cells were seeded on coverslips at a density about 70000 of cells per sample the day prior to the experiment. The cells were treated with DOX-loaded liposomes or liposome-colloid complexes for 1 h in the serum-free medium at 37°C under atmosphere containing 5% $CO_2$, washed thoroughly with cold PBS and fixed in 4% solution of formaldehyde in PBS (+4°C, 15 min). Then the samples were analyzed by Confocal Scanning Laser Microscopy using

FluoView FV1000microscope (Olympus Corp., Japan) equipped with spectral versionscan unit and transmitted light detector. The wavelength of excitation was 488 nm (multiline Argon laser) and the fluorescence was collected using an emission window set at 500–600 nm. Transmitted light Nomarski differential interference contrast (DIC) signal was detected simultaneously. All signals collected were adjusted to remain within the linear range of the detectors. Images were collected and treated for publication using the FV10 ASW 1.7 software (Olympus Corp., Japan).

**References**

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