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Multi-compartment containers from a mixture of natural and synthetic lipids

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Methods

Mean hydrodynamic diameter of liposomes was measured by dynamic light scattering (DLS) at a fixed scattering angle (90°) in a thermostatic cell with a Brookhaven Zeta Plus instrument (USA). Software provided by the manufacturer was employed to calculate diameter values (error ±7%). Electrophoretic mobility (EPM) of liposomes was determined in a thermostatic cell by laser microelectrophoresis by using Brookhaven Zeta Plus instrument with the corresponding software (error ±3-5%).

The pH values of solutions were measured with a Radiometer pHM 83 pH meter (Denmark) equipped with a measuring P1041 glass electrode and a K4041 calomel reference electrode (error ±0.02 units).

The conductivities of solutions were determined with a Radiometer CDM 83 conductometer (Denmark) equipped with a PP1042 platinum electrode (error ±0.01 units).

Fluorescence intensities of solutions were determined using a F-4000 spectrofluorometer (Hitachi). The measurements were carried out in 1 cm quartz cuvettes (error ±5%).

All experiments were carried out in quadruplicates. Double-distilled water was used for making solutions after additionally treating it with a Milli-Q Millipore system.

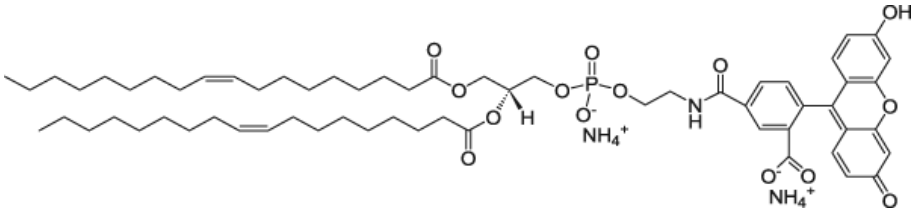
Experimental procedures

Synthesis of cationic and anionic liposomes

Liposomes were prepared *via* mixing of methanol-chloroform (1:1 w/w) solutions of lipids, evaporation of the organic solvent under vacuum at 55°C, dispersion of the lipid film a 10^{-2} M buffer solution with appropriate pH followed by final sonication of the resulting lipid/water mixture with a Cole-Parmer 4710 ultrasonic homogenizer for 400 s (2 × 200 s) at 20 °C. The resulting liposomes were separated from titanium dust via centrifugation in a J-11 centrifuge (Beckman) for 5 min at 10000 rpm.

Liposomes loaded with 1 M NaCl were prepared *via* dispersion of the lipid film in a 10^{-3} M buffer solution containing 1 M NaCl and 2 h dialysis of the suspension against the 10^{-3} M buffer that was renewed every 30 minutes.

Liposomes with a fluorescent label embedded in the bilayer were obtained by adding of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(carboxyfluorescein) ammonium salt (from Avanti polar lipids) (0.1 wt % of the total amount of lipids) to the mixture of lipid solutions:



Further procedure is similar to that described above.

Anionic liposomes with antitumor antibiotic doxorubicin (Dox) were synthesized according the following procedure. First, the pH-gradient anionic liposomes were prepared with pH 3 inside and pH 7 in surrounding solution. To this end, lipid film was dispersed in a 0.15M citrate buffer (pH=3). The resulting suspension was dialyzed extensively for 4.5 h against a $2x10^{-2}$ M Hepes buffer (pH=7) supplemented with 0.15M NaCl for compensation of osmotic gradient. Hepes buffer was renewed every 45 min. Addition of Dox solution to the pH-gradient liposomes resulted in penetration of Dox through the liposomal membrane, accumulation of the drug in the internal water pool of liposomes and self-quenching of the Dox fluorescence (λ_{em}=557 nm, λ_{ex}=490 nm) indicating its accumulation in the internal cavity. The Dox-captured liposomes were separated from uncaptured Dox via dialysis against a 10^{-2} M buffer solution. The release of Dox from liposomes was detected with fluorescence. Inside liposomes the fluorescence of Dox decreased because of selfquenching; a release of Dox from liposomes was accompanied by an increase in the Dox fluorescence at 490 nm. According to fluorescence spectroscopy data , the degree of doxorubicin loading was 95%.

Degree of binding of anionic liposomes with the cationic ones

To determine degree of binding of anionic liposomes with the cationic ones, the obtained complexes are separated from the solution by centrifugation, and then the supernatant is analyzed for the dendrimer content by fluorescent spectroscopy.

To determine whether all anionic liposomes bind to cationic ones, the following experiment was carried out. Anionic liposomes with a fluorescent label embedded in the bilayer were obtained and complexed with cationic in different ratio. The obtained complexes were separated from the solution by centrifugation. The experiment was carried out as follows. The suspension of the complex of anionic liposomes with cationic ones was separated by centrifugation in Eppendorf tubes containing a filter with a pore size of 0.2 μm in a J-11

centrifuge (Beckman) for 5 min at 12000 rpm. Then the fluorescence intensity of the label in the suspension that passed through the pores was detected by fluorescence spectroscopy. It was shown in a separate control experiment that free liposomes did not precipitate on the Eppendorf filter under these conditions of centrifugation. Analyzing the obtained results we found out that the quantitative binding of anionic liposomes was observed up to $C(-)_{max}$ = 0.9 g dm^{-3}. Until this concentration all added anionic liposomes were complexed with the cationic and no free anionic liposomes were found in the suspension.

Evaluation of cytotoxicity

Cytotoxicity of the liposomes towards human breast adenocarcinoma MCF-7 cells was evaluated with a methyl-tetrazolium blue assay. The dye, penetrated into the living cells and attacked by redox enzymes, is oxidized to formazan and precipitates as dark-blue crystals whereas in dead cells such transformation does not occur. The standard procedure involves incubation of the test suspension (solution) with the cells, addition of the dye solution, dissolution of formazan crystals in DMSO, measurement of the optical density of the resulting solution and comparison of the result with the calibration curve in the absence of the tested objects. Briefly, the day before the experiment, MCF-7 cells were seeded on a 96-well plate (Biofil, China) at a density 3800 cells per well (in 0.1 mL DMEM/F12, PanEco, Russia), 10% (v/v) fetal bovine serum (Hiclone, USA), 1% (v/v) L-glutamax (Sigma) and 1% (v/v) antibiotic solution (penicillin, streptomycin)(PanEco, Russia)). The next day, the culturing medium was removed, and the solutions of liposomes (0.1 mL) 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 solutions were removed and the cells were cultured in 0.1 mL of complete medium for 3 days. The amount of living cells was assayed by addition of 0.1 mL of MTT solution (0.375 mg mL^{-1}) in the culturing medium for 4 h. Then, the medium was removed, violet crystals of formazan were dissolved in DMSO (0.1 mL), and the optical density at 570 nm was measured on a VersaMax microplate reader (USA). The reference wavelength at 630 nm was used. The portion of survived cells was calculated as a ratio of optical density in well with certain concentration to that in control well. All runs were carried out in quadruplicates.