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

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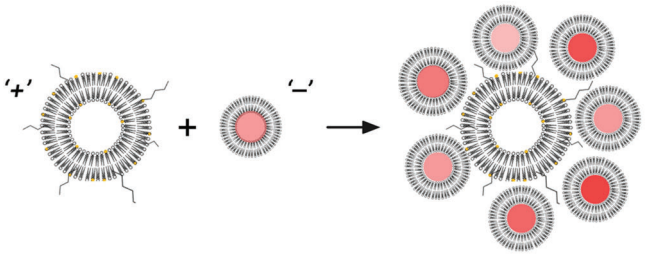
**Small anionic liposomes were electrostatically adsorbed on the surface of larger cationic liposomes thus forming multi-compartment complexes composed exclusively of natural and synthetic lipids. The complexes contained two dozen anionic liposomes per a single cationic liposome and showed low cytotoxicity and ability to enzyme-induced biodegradation. The liposomal multi-compartment complexes demonstrate great application potential as containers for drug encapsulation and delivery.**




*Keywords*: liposome, multi-liposomal complex, multi-compartment container, biodegradation, cytotoxicity.

Increasing the efficiency of drug delivery to target cells and organs is a key task of pharmacology.[1–5] This can be done *via* incorporating drugs into a polymer matrix[6] or binding them on the surface of colloidal particles.[7] These techniques allow concentrating drugs in a small volume, reducing their toxic effects and increasing their bioavailability.[8] Bilayer lipid vesicles (liposomes) are among the drug carriers that have been intensively used for drug immobilization.[9–11] The hydrophilic drugs are dissolved in the inner water pool of liposomes, the hydrophobic drugs are embedded in the liposomal membrane.[12–14] Anionic liposomes can be electrostatically adsorbed on the surface of cationic colloidal particles[15] that results in multi-liposomal and multi-functional core–shell carriers capable of carrying a mixture of various drugs in the desirable ratio;[6] this is actually a way to design therapeutic agents for treatment of a particular patient.

Anionic liposomes are known to be non-toxic and biodegradable objects.[16] Key points concern the possible toxicity of a cationic core taken for liposome binding and an ability of the core to decompose down to small fragments after its transport function is completed. It was suggested to form the cationic core from synthetic polymeric microspheres,[7] amphiphilic copolymers[17] or cross-linked polysaccharides.[18–20] However, most of them did not meet the above requirements being either toxic or non-degradable, or the resulting core–shell complex showed a size exceeding recommended value of 200–400 nm.[19,21]

In the current paper, the multi-liposomal construct is prepared *via* adsorption of small anionic liposomes onto the surface of larger cationic liposomes. Both small and large liposomes were made from native or synthetic lipids recommended as carriers for biological material like nucleic acids.[22] This allowed us to minimize the problems associated with toxicity and controlled destruction of multi-liposomal carriers.

We have shown previously[18] that anionic liposomes retain their integrity when they are adsorbed onto the soft hydrogel surface. The latter can be either the outer border of the micro-sized hydrogel particles or the hydrogel layer over the solid polymeric microsphere. In other words, the stability of adsorbed liposomes is due to the hydrophilic interlayer between liposomes and the sorbent. Taking this into account, the larger core liposomes were synthesized from a mixture of cationic 1,2-dioleoyl-3-trimethylammonium-propane ($DOTAP^{1+}$) and electroneutral dipalmitoylphosphatidylcholine (DPPC), which additionally contained 1,2-dipalmitoyl-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DPPE-PEG). The PEGylated lipid formed a soft hydrophilic layer on the cationic liposome surface. The smaller shell liposomes were synthesized from a mixture of anionic diphosphatidylglycerol (cardiolipin, $CL^{2-}$) and electroneutral dioleoylphosphatidylcholine (DOPC). Chemical formulas of lipids and compositions of cationic and anionic liposomes are shown in Figure 1. The cationic and anionic liposomes were prepared conventionally by sonication (see Online Supplementary Materials). Size of liposomes fluctuated from sample to sample but always remained within a $40 \pm 10$ nm range for anionic liposomes and $160 \pm 20$ nm for cationic liposomes.

Electrostatic complexation between anionic $CL^{2-}$/DOPC liposomes and cationic $DOTAP^{1+}$/DPPC/DPPE-PEG liposomes was performed in an aqueous phosphate buffer solution with pH 7. The complexation was monitored using laser microelectrophoresis and dynamic light scattering. The former allowed measuring electrophoretic mobility (EPM) of the resulting complexes composed of cationic liposomes covered by anionic liposomes [Figure 2(*a*)]. Electroneutral complexes were obtained at the total lipid concentration used for ionic liposome preparation $C(-)_{EPM=0} = 0.5$ g dm$^{-3}$. At higher $C(-)$ concentrations, negatively charged complexes are formed, which carried an excess of anionic liposomes.

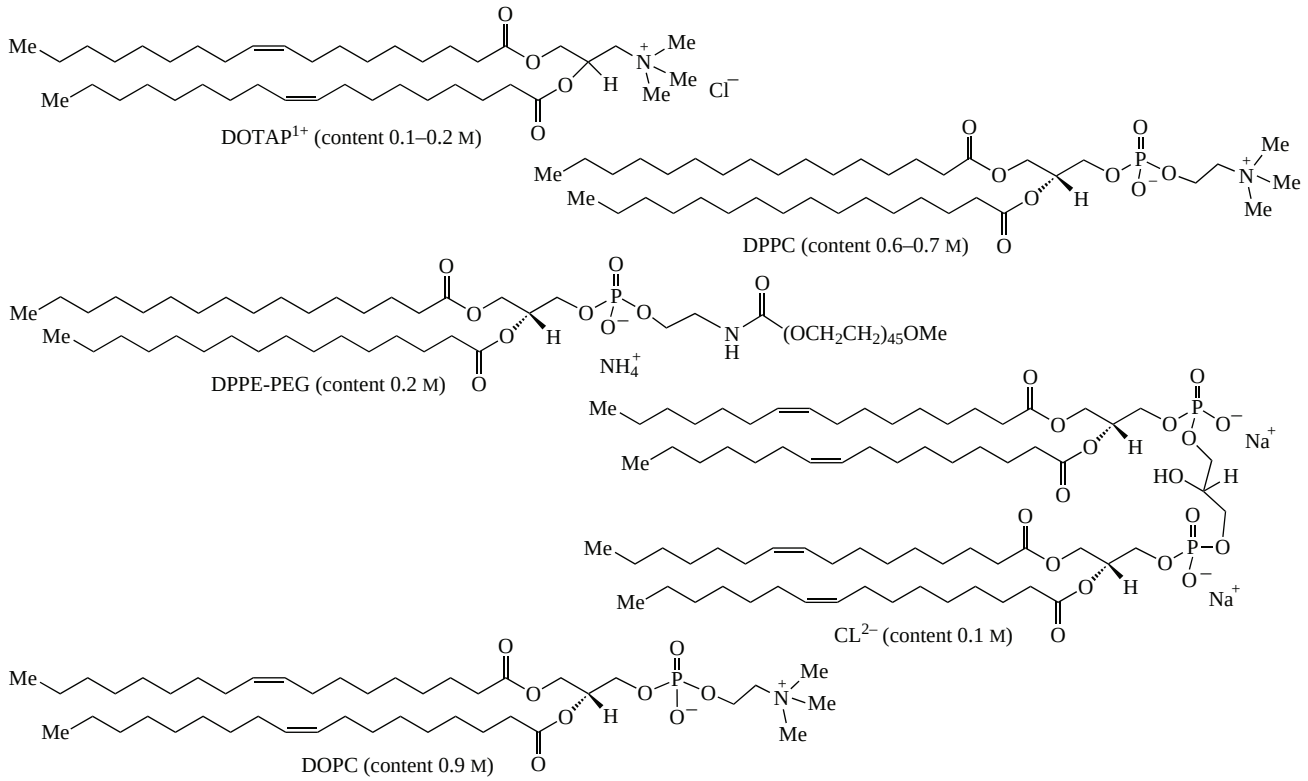



**Figure 1** Lipids for liposome preparation. Their molar contents in liposomes are given in parentheses; for $CL^{2-}$, the value relates to both anionic headgroups $\nu_{CL} = 2[CL]/(2[CL] + [DOPC])$.

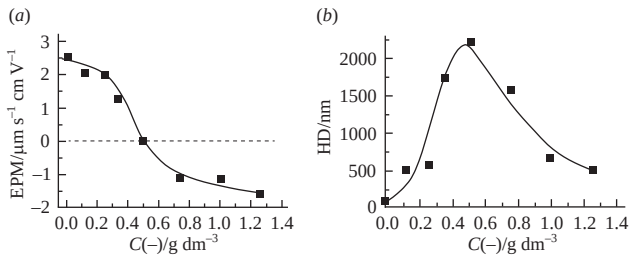



**Figure 2** (*a*) Electrophoretic mobility (EPM) and (*b*) hydrodynamic diameter (HD) of the complexes *vs*. the total lipid concentration used for anionic liposome preparation *C*(–). Cationic DOTAP$^{1+}$/DPPC/DPPE-PEG (2 : 7 : 1) liposomes and anionic $CL^{2-}$/DOPC (1 : 9) liposomes. Total lipid concentration used for cationic liposome preparation *C*(+) = 1 g dm$^{-3}$ in $10^{-2}$ M phosphate buffer with pH 7.2.

In parallel, size of complexes as a function of *C*(–) was detected [see Figure 2(*b*)]. Neutralization of the cationic liposome charge by the charge of anionic liposomes resulted in destabilization of the resulting complexes and their aggregation. The maximum size was observed at a mutual neutralization of cationic and anionic lipids, *i.e*. at EPM = 0. Further increase in *C*(–) was accompanied by a decrease in size due to a negative charge brought in by abundant anionic liposomes. It was also found that no significant decrease in the particle size was observed when a low molecular weight electrolyte of concentration 0.15–0.18 M was added to the suspension of complex particles. This indicated that the complexes were stable in water–salt media at physiological salt concentrations.

It was shown in a separate experiment (see details in Online Supplementary Materials) 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 ones, and no free anionic liposomes were found in the suspension. This allowed the ultimate amount of anionic liposomes to be capable to binding to a single cationic liposome $N_{max}$, *i.e*. the capacity of cationic liposomes to the anionic, based on the experimentally found anionic-to-cationic liposome binding plot. An average lipid molecular mass in the cationic liposomes is a sum of molecular masses of individual lipids multiplied by their contents in the cationic liposomes:

$$M(+)_{lip} = 0.1\,M(\text{DOTAP}^{1+}) + 0.7\,M(\text{DPPC}) + 0.2\,M(\text{DPPE-PEG}) = 1140. \quad (1)$$

Taking an average surface occupied by a single lipid molecule in liposomes[23] $S$ = 0.60 nm$^2$ and a diameter of cationic liposomes $D$ = 160 nm, an amount of cationic liposomes per liter of solution was calculated as

$$N(+)_L = [C(+)SN_A]/[2\pi D^2 M(+)_{lip}], \quad (2)$$

where *C*(+) is the total lipid concentration used for cationic liposome preparation (1 g dm$^{-3}$), and $N_A$ is the Avogadro's number.

Using similar considerations, an amount of anionic liposomes per liter of solution was calculated as

$$N(-)_L = [C(-)SN_A]/[2\pi d^2 M(-)_{lip}], \quad (3)$$

where *C*(–) is the total lipid concentration used for anionic lipid preparation at the saturation of 0.9 g dm$^{-3}$, *d* is a diameter of anionic liposomes 40 nm, and $M(-)_{lip}$ is an average lipid molecular mass in the anionic liposomes, which is a sum of molecular masses of individual lipids multiplied by their contents in the anionic liposomes:

$$M(-)_{lip} = 0.1\,M(\text{CL}^{2-}) + 0.9\,M(\text{DOPC}) = 770. \quad (4)$$

Since all added anionic liposomes were quantitatively bound to the cationic liposomes up to the saturation at *C*(–) of 0.9 g dm$^{-3}$ (for details, see Online Supplementary Materials), the $N_{max(exp)}$ is equal to

$$N_{max(exp)} = N(-)_L/N(+)_L = [C(-)D^2 M(+)_{lip}]/[C(+)d^2 M(-)_{lip}] = 22. \quad (5)$$

On the other hand, the capacity of cationic liposomes to anionic liposomes can be assessed from the geometrical

considerations. The surface area of a single cationic liposome is $S(+)_L = \pi D^2$; the surface area occupied by a single adsorbed liposome was taken to be equal to $S(-)_L = d^2$. Then the calculated $N_{max}$ is determined as

$$N_{max(cal)} = S(+)_L/S(-)_L = 50. \quad (6)$$

The experimentally found $N_{max(exp)}$ value is lower than calculated $N_{max(cal)}$ value. This is apparently due to random (disordered) adsorption of anionic liposomes on the surface of cationic liposomes and strong repulsion between adsorbed liposomes, each of which carries several thousand negative charges.

The next step was to confirm the integrity of anionic liposomes after their conjugation with the cationic ones. Recall that the preservation of the integrity was expected to be due to a PEG coating on the cationic liposome surface, which would work like a soft hydrophilic interlayer between oppositely charged liposomes. Small anionic liposomes with 1 M NaCl solution in the inner pool were complexed with the cationic PEG-modified liposomes. The appearance of defects in the membrane of anionic liposomes should be accompanied by a leakage of salt from liposomes into the surrounding solution and an increase in the electrical conductivity of the suspension. In the experiment, no change in the conductivity was observed within 24 h after the complexation that definitely indicated the preservation of the anionic liposome integrity in the resulting complex. This allows the conclusion about formation of multi-liposomal complex *via* simple electrostatic adsorption of two dozen native (unbroken) small anionic liposomes on the surface of each bigger cationic liposome. We also prepared anionic liposomes filled with antitumour antibiotic doxorubicin (Dox, for details see Online Supplementary Materials). Then Dox loaded small anionic liposomes were complexed with the cationic ones. The release of Dox from liposomes was detected by fluorescent spectroscopy. The experiment revealed no changes in the fluorescence within 24 h after the complex formation. This result also confirmed the integrity of anionic liposomes under the complexation with cationic ones.

Since all components – native and synthetic lipids – are subject to biodegradation, it was reasonable to expect the biodegradation of the entire complex. The lipase enzyme (from porcine pancreas) was added to an aqueous suspension of the saturated complex, and the size of particles in the system was monitored. The lipase would hydrolyze ester bonds in lipids being active within a pH range of 4.5–8, with pH optimum being between 6.5–7.5. So, it could attack lipids in both anionic and cationic liposomes thus reducing the size of complex particles. The kinetics of the size alteration (Figure 3) shows no change in the size of complex in the absence of enzyme (curve *1*) and a progressive decrease in the particle size after injection of the lipase down to 10–20 nm particles (curve *2*), which can be easily removed from the body.[24]

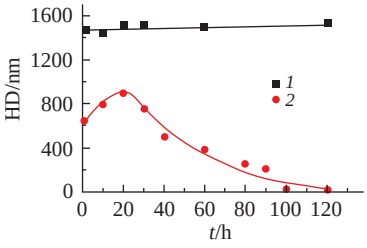



**Figure 3** Time-dependent change in size (hydrodynamic diameter, HD) of complex particles (*1*) in the absence and (*2*) in the presence of lipase. Cationic DOTAP$^{1+}$/DPPC/DPPE-PEG (1:7:2) liposomes and anionic CL$^{2-}$/DOPC (1:9) liposomes. Total lipid concentration used for cationic liposome preparation $C(+) = 1$ g dm$^{-3}$, total lipid concentration used for anionic liposome preparation $C(-) = 1$ g dm$^{-3}$. Lipase concentration is $5 \times 10^{-2}$ g dm$^{-3}$ in $10^{-2}$ M phosphate buffer with pH 7.2.

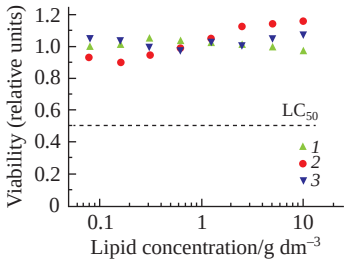



**Figure 4** Viability of MCF-7 cells *vs.* lipid concentration. (*1*) Anionic CL$^{2-}$/DOPC (1:9) liposomes, (*2*) cationic DOTAP$^{1+}$/DPPC/DPPE-PEG liposomes (1:7:2), and (*3*) saturated complex.

Finally, the cytotoxicity of the individual anionic and cationic liposomes and the entire complex was examined using MCF-7 breast carcinoma cells. To detect viable cells, the conventional MTT test was used. A concentration of added liposomes, which ensured 50% cell death ($LC_{50}$), was taken as a measure of cytotoxicity.[17] Anionic and cationic liposomes did not show the cytotoxicity up to lipid concentration of 10 g dm$^{-3}$ (Figure 4, curves *1* and *2*, respectively), the maximum lipid concentration achieved in the experiments. The same was true for the saturated complex (curve *3*).

Additionally, we estimeted cytotoxicity of liposomes and the complex hydrolyzed (destroyed) by lipase. For this, a suspension of complex was mixed with enzyme, then samples were taken after 30 min, one day and three days, which were tested for the cytotoxicity with the MTT assay. Figure 5 shows the viability of MCF-7 cells in the presence of hydrolyzed complex. For all samples, no cytotoxicity or negligible cytotoxicity was observed. Thus, the initial saturated complex and products of its destruction did not exhibit cytotoxicity at all stages of the biodegradation process, which indicates great potential of the multi-liposomal complex for biomedical applications.

Summarizing, the multi-liposomal complexes have been obtained *via* electrostatic adsorption of small anionic liposomes on the surface of larger cationic liposomes. The complexes are composed exclusively of natural and synthetic lipids, that provide biocompatibility, biodegradability and low toxicity of the resulting multi-liposomal constructs. As a result of the study, the range of colloidal biodegradable objects that can be used for delicate immobilization of liposomes is expanded. The complexes are capable of enzyme-induced degradation down to nanometer particles. The complex and products of its biodegradation showed no or negligible cytotoxicity up to 10 g dm$^{-3}$ lipid concentration. The multi-liposomal complex with two dozen anionic liposomes per a single cationic liposome has great application potential as a multi-compartment container for immobilization and delivery of bioactive compounds.

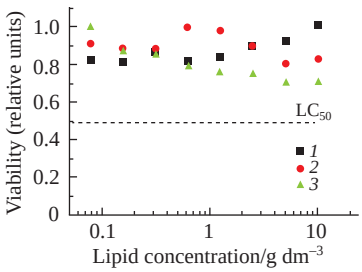



**Figure 5** Viability of MCF-7 cells *vs.* lipid concentration after addition of lipase. Time after lipase addition: (*1*) 0.5, (*2*) 36 and (*3*) 72 h. Saturated complex from anionic CL$^{2-}$/DOPC (1:9) liposomes and cationic DOTAP$^{1+}$/DPPC/DPPE-PEG liposomes (1:7:2). Lipase concentration is $5 \times 10^{-2}$ g dm$^{-3}$.

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*Online Supplementary Materials*

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2023.02.023.

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