Multiliposomal nanocontainers based on anionic solid liposomes and spherical polycationic brushes

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Abstract. The incorporation of cholesterol in membrane of liposomes in gel state opens the gates of their usage as cargo-carrying component in complex multiliposomal nanocontainers.

1. Introduction
For last decades bilayer lipid vesicles (liposomes) have been used as nanocontainers for encapsulation and delivery of biologically active substances [1-4]. Recently in order to increase the efficacy of liposome uptake by cells and therapeutic effect of a liposomal drug it was proposed to adsorb electrostatically anionic liquid liposomes on the surface of polystyrene microspheres with grafted polycationic chains (spherical polycationic brushes) [5,6]. It is well known that the presence of unsaturated bonds in the hydrocarbon chains leads to the decrease in the thermodynamic stability of liposomes and acceleration of the processes of lipid oxidation [7-10]. In order to increase stability of liposomes in complex with polycation the fraction of unsaturated bonds in fatty tails of lipids should be drastically decreased. On the other hand increase of saturated tails in liposomes may shift phase state of lipid bilayer from liquid crystalline (“liquid”) to gel (“solid”). Phase state of liposomal membrane plays significant role in formation of complexes of lipid vesicles with polymers and physico-chemical properties of the resulting compounds. In the present study we describe complexation and properties of multiliposomal nanocontainers based on solid liposomes composed of zwitter-ionic dipalmitoylphosphatidylcholine (DPPC), anionic phosphatidylserine (PS) and spherical polycationic brushes (SPB).

2. Experimental Section
DPPC, PS, N-fluoresceinisothiocyanldipalmitoylphosphatidylethanolamine (FITC-DPPE) and cholesterol from Avanti were used as received. Small unilamellar liposomes 40-60 nm in diameter, including those loaded by NaCl solution, were prepared by the standard sonication procedure [11]. The molar ratio of PS was equal to 0.1. Liposomes with a fluorescent dye incorporated into the bilayer, were prepared by the same procedure, except 0.1 wt.% of FITC-DPPE was added to the lipid mixture before organic solution evaporation. Liposomes with sodium chloride solution in the inner water cavity were prepared via dispersion of the lipid film in a $10^{-3}$ M Tris buffer additionally containing 1 M NaCl. The resulting suspension was dialyzed for 1.5 h against a $10^{-3}$ M Tris buffer that was renewed every 30 minutes.

SPBs samples were provided by M. Ballauff, the details of synthesis and characterization of SPBs were described elsewhere [12].
Mean hydrodynamic diameters of SPBs, liposomes, and SPB/liposome complexes and their electrophoretic mobility (EPM) were determined at Brookhaven Zeta Plus. The fluorescence intensity was measured using a F-4000 Hitachi fluorimeter. Permeability of the liposomal membranes towards inorganic ions was investigated by measuring the conductivity of NaCl-loaded vesicle suspensions with a CDM83 conductometer (Radiometer) as described in [13].

For the lipids and SPB structures see Supplement (S1).

3. Results and Discussion

Formation of complexes was studied as follows. A suspension of SPBs was mixed with a suspension of fluorescent-labeled FITC-DPPE liposomes, and 5 min after a SPB/liposome complex was separated by centrifugation. The fluorescence intensity of the supernatant upon concentration of liposomes added to SPBs is presented in Figure 1. It follows from the figure that all added liposomes were bounded to SPBs up to a concentration 1.6 mg/ml; at higher concentrations free (unbound) liposomes could be detected in the supernatant.

![Figure 1. Fluorescence intensity of DPPC/PS liposomes in supernatant after separation of SPB/liposome complex vs total liposome concentration.](image)

It was reported earlier that complexation of anionic “solid” liposomes with linear polycations is accompanied by formation of defects in the liposomal membrane which initiate a leakage of the content from liposomes to surrounding solution [14] and thus restricts the use of these constructions as containers. So we controlled the integrity of liposomes complexed with SPBs by conductometry. The relative conductivity of the NaCl-loaded liposome suspension after SPB addition is shown in Figure 2. Complexation is followed by increase of conductivity of the system indicating that electrostatic sorption of DPPC/PS liposomes on SPBs results in formation of defects in membrane. At the same time no change in conductivity of initial DPPC/PS liposomes suspension unbound to SPBs was detected. So the modification of membrane is needed to prevent leakage of internal cargo.
Figure 2. Time-dependent change in the conductivity of DPPC/PS liposomes, loaded with 1 M NaCl solution. \( \nu_{PS} = 0.1 \); total liposome concentration 1 mg/ml; \([SPB+] = 10^{-4} \text{ M} \); 10\(^{-3} \) M Tris buffer, pH 7. The maximum possible increase in conductivity due to the irreversible destruction of liposomes was achieved by addition of 10-fold excess of Triton X-100 surfactant.

Incorporation of cholesterol (Chol) into liposomal membrane allows one to decrease defectiveness of vesicles and prevent an early release of encapsulated drugs [15,16]. So the membrane of DPPC/PS liposomes was modified with Chol with molar fraction equal 0.2 to prevent formation of defects after complexation with SPBs.

An effect of Chol on the permeability of the liposomal membrane in complex with SPB was examined by using conductometry as described above. Time-dependent relative conductivity of the DPPC/PS/Chol liposome suspension after SPB addition was measured. No salt leakage was detected after 200 minutes of investigation. Chol incorporation into membrane led to a gradual healing of defects in the membrane. Thus, by incorporation of Chol into membrane the leakage of salt from DPPC/PS solid liposomes complexed with SPB can be completely suppressed.

The key question is whether addition of Chol to DPPC/PS liposomal membrane changes character of interaction of vesicles with SPBs or not. As electrostatic binding determines properties of the complexes between anionic liposomes and polycationic brushes the complexation of DPPC/PS and DPPC/PS/Chol liposomes with SPB was studied by measuring of electrophoretical mobility (EPM). For the both types of liposomes the addition of liposomes to SPBs resulted in decrease of surface charge. In Figure 3 the dependence of EPM of the complexes upon concentration of vesicles is presented. The point of electroneutrality (EPM = 0) for the DPPC/PS and DPPC/PS/Chol was found to be 1.5 mg/ml. This important result indicates that incorporation of less than 0.2 molar fraction of Chol in membrane of DPPC/PS does not affect on complexation with SPBs.

We see therefore that, by Chol incorporation into the solid liposomal membrane we can control the integrity of liposomes complexed with SPBs. However the presence of Chol did not affect the possibility of the liposomal membrane to interact with oppositely charged SPBs. This result is of interest for preparing liposomal containers for drug encapsulation.
Figure 3. EPM of SPB particles vs liposome concentration for DPPC/PS (1) and DPPC/PS/Chol (2) liposomes. \( v_{PS} = 0.1; \ v_{Chol} = 0.2; \ [SPB+] = 10^{-4} \text{ M}; \ 10^{-2} \text{ M Tris buffer, pH 7.} \)

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