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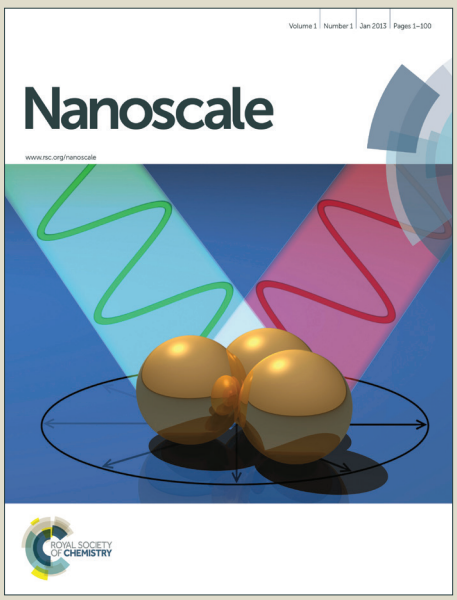

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ARTICLE TYPE

Capacious and Programmable Multi-Liposomal Carriers

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Spherical polycationic brushes (SPBs) were synthesized by grafting polycationic chains onto 100 nm polystyrene particles. These particles were exposed to unilamellar egg-lecithin (EL) liposomes with mean diameter 40 nm, that had been rendered anionic via the presence of 10 molar % of phosphatidlyserine (PS^{1-}). The liposomes also contained 30 mole-% of a morpholinocyclohexanol-based lipid (MOCH) that undergoes a conformational flip when the pH is decreased from 7.0 to 5.0. Mixtures of SPBs and liposomes at pH 7 gave an electrostatically-driven complex possessing, on average, about 40 liposomes for each SPB particle. It was found that the bound liposomes rapidly release much of their their contents when the pH is reduced from 7.0 to 5.0 owing mostly to a MOCH conformational change that creates defects in the bilayer membrane. The drop in pH does not, however, induce a separation of the liposomes from the SPBs. Around 50 – 60% of the liposomes contents escapes before, it is reasoned, lateral and trans-membrane motion of the membrane components heals the defects and prevents further release. Remarkably, the liposomes complexed with SPB release their cargo much faster than the identical but non-complexed liposomes.

Introduction

Liposomes represent an important category of nano-scale systems for encapsulation, delivery, and release of biologically active agents.[1] Hydrophobic guests can be incorporated within the liposomal membrane, whereas hydrophilic guests reside in the aqueous liposomal cavity.[2] The scientific and patent literature describes antiviral, antifungal, and antitumor liposomal drugs [1,3,4] among the most cited of which are liposomal forms of the antitumor antibiotic doxorubicin, Doxil and Caelyx.[5]

Various techniques are used for improving the therapeutic effect of drugs incorporated into liposomes: (a) increasing the circulation time of liposomes in the body by coating them with a hydrophilic polymer, e.g. polyethylene glycol or poly(N-vinylpyrrolidone);[1,6] (b) modifying the liposome surface by "vector" molecules that interact with complimentary receptors on the surface of target cells;[7] (c) enlarging the liposome size up to 200-400 nm, thus ensuring their selective penetration in the capillaries of tumors and other inflammation areas ("passive targeting");[8] (d) tacking on a specific peptide or phosphatidylethanolamine (PE) to the membrane, both capable of facilitating liposome fusion with cells;[9] (e) targeted releasing the liposome content in response to certain characteristics of a disease;[10] and (f) applying an external stimulus such as heat or light.[11,12] A decrease in pH value (increase of acidity) is typical for pathological physiological pathways, e.g. inflammation, solid tumor progression, ischemic injuries of the heart and brain tissues, etc.[13-17] Thus, the drop in pH can serve as an attractive stimulus to trigger release in a drug delivery system. In contrast with liposomal containers that "open" in response to radiation or a temperature increase,[11,18,19] the pH-sensitive liposomes need no additional medical device for their activation. The release from the container provides by changing of lipids phase induced by pH-sensitive agent incorporated in membrane. Different agents have been proposed, including combination of PE with negatively charged lipids,[20,21] pH-cleavable lipid derivatives,[22] pH-dependent fusogenic peptides,[23,24] and specific pH-sensitive polymers.[25-27] These agents destabilize the lipid bilayer thus inducing leakage of liposome-entrapped compounds when exposed to lower pH. Liposomes with entrapped pH-sensitive agents have been evaluated as delivery vehicles in several contexts, ranging from therapy to diagnostic applications. Encouraging results were obtained in gene delivery with use of the lipoplex technology[28-30] and cancer therapy.[31-33]

Recently we have shown that anionic liposomes effectively complex with "spherical polycationic brushes" (SPB) composed of 100 nm polystyrene particles with grafted cationic macromolecules on their surface.[34] Importantly, the liposomes retain their integrity after the complexation because the grafted polymer layer prevents direct contact of liposomes with the colloidal core of the brush and subsequent liposome disruption.[35] By varying the size of the cationic colloidal particles and the anionic lipid content in the liposomal membrane, we can manipulate the amount of bound liposomes and size of the resulting complex[36,37] the key parameters to determine the therapeutic effect of the multi-liposomal constructs.

In this paper we describe multi-liposomal containers created by coating of SPB with a special type of pH-sensitive liposomes. Such liposomes consist of anionic/zwitterionic lipid mixture and an additional synthetic lipid-like compound, a "flipid" capable of flipping its conformation when pH of a medium is lowered from 7 down to 5 (details given later) as developed by one of the authors (VVS with colleagues).[38-40] The protonation-triggered conformational flip loosens the lipid packing in the liposomal membrane and induces a release of entrapped content from the liposomes to the surrounding solution.[38-40] We show that SPB-

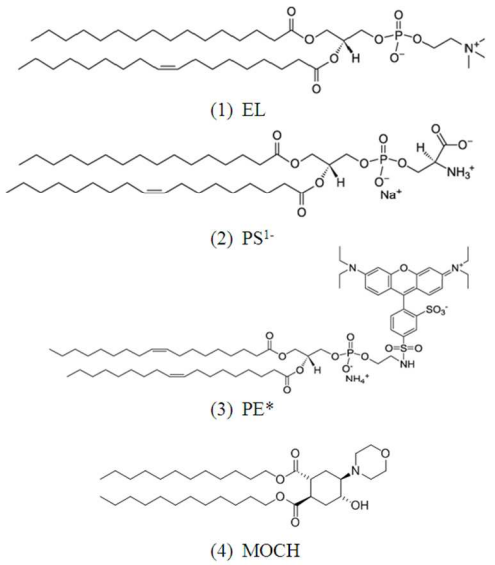

Fig.1. Lipids (1-3) and the lipid-like conformational switch (4).

bound, pH-sensitive liposomes retain their original size, shape and encapsulating power. The capacity of SPBs toward anionic liposomes, the stability of resulting complexes in aqueous salt solutions, and the nanostructure of the complexes were also examined.

Results and discussion

SPBs were synthesized by graft polymerization of a cationic monomer, (2-methyl-propenoyloxyethyl)trimethylammonium chloride, on the surface of monodispersed polystyrene (PS) latex particles ca. 100 nm in diameter (see ESI S1).[34] With dynamic light scattering a mean hydrodynamic diameter of the brush was found to be 230 nm, which gave a thickness of a cationic corona equal to (230-100)/2 = 65 nm. Zwitter-ionic phosphatidylcholine (egg lecithin, EL), anionic phosphatidylserine (PS^{1-}), and fluorescent labeled 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) ammonium salt (PE*) from Avanti were used as received (see lipid structures in Figure 1). The MOCH-flipid, trans-4,5-di(dodecyloxycarbonyl)-trans-2-morpholynocyclohexanol, was synthesized as described in Refs. 24,41. More details may be found in Experimental section.

The key concept in the recently suggested novel design of pH-sensitive liposomes is the construction of a liposome membrane using lipid-like molecules with an incorporated pH-responsive conformational switch.[38-40] Thus, the predominant conformation of the trans-2-morpholinocyclohexanol-based pH-sensitive MOCH-flipid (Figure 2, left) has a free amino group and a neighboring hydroxy group in axial positions. Protonation with acid generates a strong intramolecular hydrogen-bond between these groups, forcing them to adopt an equatorial conformation (Figure 2, right). This change is mechanically transmitted via the conformational flip of the ring, thus forcing the remote COOR groups into axial positions and increasing their separation. This protonation-induced spreading of lipophilic tails ("peacock effect" [40]) was shown to be functional after incorporation of

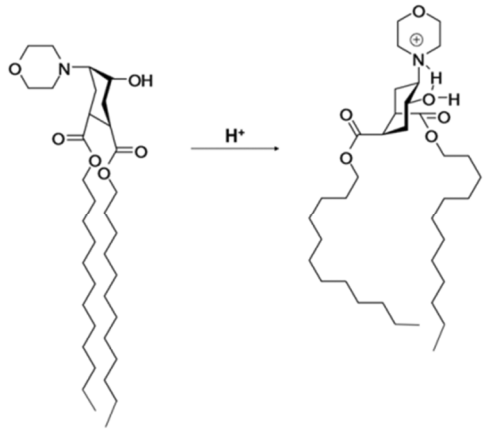

Fig.2. The protonation-triggered conformation switch in MOCH.

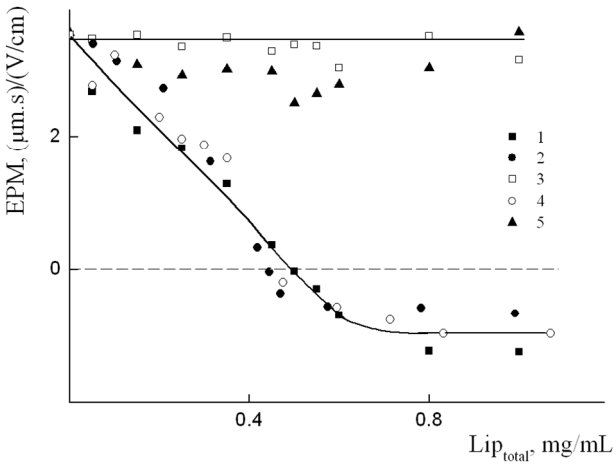

Fig.3. EPM of SPB particles vs. liposome concentration. EL/PS^{1-}/MOCH (6/1/3) liposomes, pH 7 (1) and 5 (3); EL/PS^{1-} (9/1) liposomes, pH 7 (2) and 5 (4); EL/PS^{1-}/MOCH (6/1/3) liposomes were complexed with SPBs at pH 7 and the resulting complexes were transferred to a pH 5 solution (5). [SPB+] = 10^{-4} M.

MOCH into the liposomal membrane, thus making MOCH-containing liposomes (fliposomes [39,40]) pH-sensitive. The simultaneous conformational change of many molecules disturbs the lipid packing and triggers a quick release of the fliposome contents.[38-40]

In this paper we describe the complexation between cationic SPBs and liposomes comprising zwitter-ionic EL, anionic PS1- and MOCH, taken at a EL/PS^{1-}/MOCH = 6/1/3 molar ratio. The expectation was that anionic PS1- would ensure electrostatic binding of liposomes to cationic SPBs at neutral pH (where MOCH has no impact on the liposome surface charge), and that the complexation would be maintained when adjusting the surrounding solution down to pH 5.

As detected by electrophoretic mobility (EPM), addition of anionic EL/PS^{1-}/MOCH liposomes to a suspension of cationic SPBs in a pH 7 buffer was accompanied first by neutralization of the SPB surface charge and then by an overall change from a positive to negative charge at high liposome concentrations (Figure 3, curve 1). Figure 3, curve 2 characterizes the change in the SPB surface charge upon addition of EL/PS^{1-} (9/1) binary

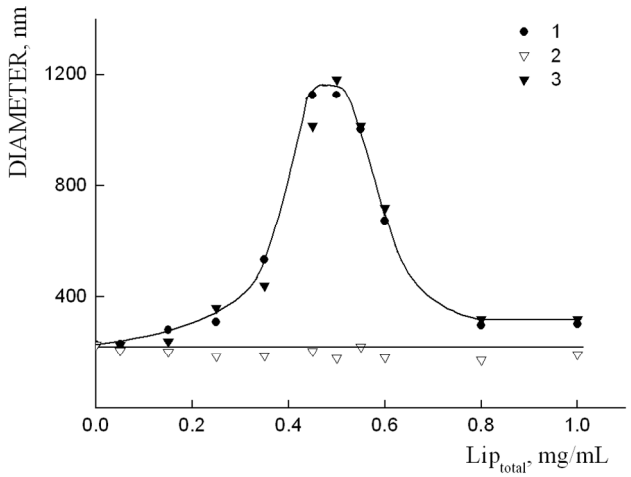

Fig.4. Hydrodynamic diameter of SPB particles vs. anionic liposome concentration. EL/PS^{1-}/MOCH (6/1/3) liposomes, pH 7 (1) and 5 (2); EL/PS^{1-}/MOCH (6/1/3) liposomes were complexed with SPBs at pH 7 and the resulting complexes were transferred to a pH 5 solution (3). [SPB+] = 10^{-4} M.

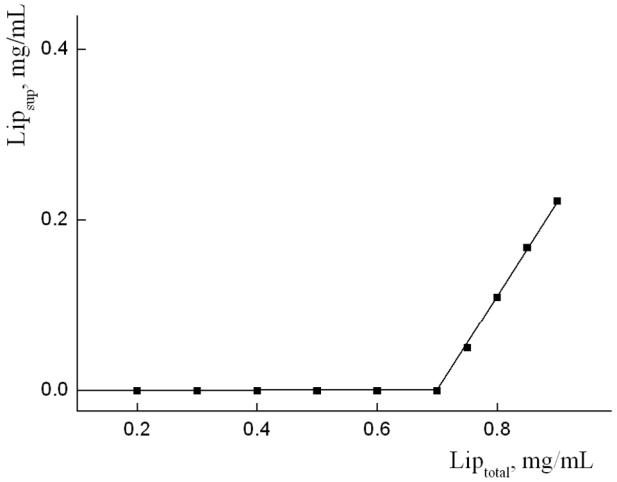

Fig.5. Concentration of fluorescent-labeled EL/PS^{1-}/MOCH (6/1/3) liposomes in supernatant after separation of SPB/liposome complex vs. total liposome concentration. [SPB+] = 10^{-4} M.

liposomes lacking any MOCH. It is seen that curves 1 and 2 coincide, proving that MOCH plays no role in complexation at pH 7. Note that curves 1 and 2 level off when, at high liposome concentration, the SPB particles become saturated with liposomes.

Complexation was next studied in a buffer solution with pH 5. As expected, the anionic EL/PS^{1-} binary liposomes, whose charge remained constant with a decrease in pH, adsorbed on the surface of cationic brushes in acidic solution. The EPM vs. liposome concentration plot at pH5 (Figure 3, curve 4) overlaps with the plot for the same system obtained at pH 7 (curve 2). In contrast to this, the EL/PS^{1-}/MOCH ternary liposomes did not complex with the brushes in a pH 5 solution (curve 3). The complexation was obviously suppressed by the overall positive charge of the ternary liposomes owing to protonation of MOCH amino groups (see ESI S2).

Finally, a series of complexes with different ternary liposome/SPB ratios were prepared in a pH 7 solution then the pH of the samples was decreased down to pH 5. Only positive particles were detected in the pH-adjusted system (curve 5). This result might reflect either a positive charge of bare SPBs following desorption of the now positive ternary liposomes or a positive charge of the entire SPBs/protonated MOCH complex. To distinguish between these two possibilities, dynamic light scattering (DLS) experiments were carried out as now described.

Neutralization of the surface charge by oppositely charged ions (simple or multiple) is often accompanied by particle aggregation.[42-44] Enlargement of particles in the system thus reflects electrostatic particle-to-ion complexation. Figure 4 shows the average size of particles in the mixed SPB + EL/PS^{1-}/MOCH liposome system as determined by dynamic light scattering. Addition of liposomes to an SPB suspension at pH 7 led to formation of aggregates with a maximum size at Lip$_{total}$ = 0.5 mg/mL which is exactly where the EPM = 0 in Figure 3. No change in particle size was seen upon addition of liposomes to the same system at pH 5 (Figure 4, curve 2), indicating an absence of complexation and/or aggregation in acidic solution. The light scattering data are in agreement with the above described EPM results. Curve 3 in Figure 4 shows the sizes of SPB/ternary liposome complexes prepared in a pH 7 solution and then acidified down to pH 5. Acidification did not lead to decrease in size of complexes (cf. curve 1 and curve 3), obviously suggesting negligible removal of the ternary liposomes from the SPB surface upon acidification once the liposomes are already attached. In other words, the liposomes do not desorb under acidic conditions, their cationic charge notwithstanding.

How could the behavior of the ternary liposomes be rationalized? Recently we have examined adsorption of liposomes, composed of anionic PS^{1-} and zwitterionic lipid, onto the polycationic brushes using differential scanning calorimetry.[45] The complexation was accompanied by lateral lipid segregation in the liposomal membrane and formation of two separate types of microphases: one composed of zwitterionic lipids and the other composed of anionic PS^{1-} molecules electrostatically associated with cationic groups on the brush surface. At a 10 mol% PS^{1-} content (that is equal to the PS^{1-} content in the membrane of the EL/PS^{1-}/MOCH ternary liposomes) the vast majority of PS^{1-} molecules in the complex resides in that particular portion of the liposome contacting the brushes.

It is reasonable to expect similar structural rearrangements in the membrane of the EL/PS^{1-}/MOCH ternary liposomes bound to the SPBs at pH 7: clustering of PS^{1-} molecules in the areas of immediate contact with the brush surface while electroneutral EL and MOCH molecules are exposed to the external water. When pH is decreased down to 5, MOCH amino groups are protonated and generate a positive charge in the outer portion of adsorbed liposomes. However, liposomes remain held on the SPB surface due to strong pre-existing electrostatic interaction between the PS^{1-} clusters and grafted polycation chains. Since neutralization of the cationic SPB charge by the anionic charge of the PS^{1-} clusters minimizes repulsion between the now positive MOCH molecules and the SPB surface, liposomal complexation persists. Clustering of PS^{1-} molecules, experimentally detected earlier with adsorbed mixed liposomes,[36] seems to be a deciding factor that ensures the stability of SPB/ternary liposome complexes in both

pH 7 solutions and after acidification down to pH 5. In other words, once the liposomes are bound to the SPB particles, the PS^{1-} domains keep them there.

The number of the ternary liposomes bound to SPBs was estimated as follows. A suspension of SPBs in a pH 7 buffer was prepared and mixed with a pH 7 suspension of fluorescent-labeled liposomes. After 5 min SPB/liposome complex was separated by centrifugation. The fluorescence intensity in the supernatant provided the concentration of unbound liposomes using a corresponding calibration curve (see details in ESI S3). A dependence of unbound liposome vs. total liposome concentration is given in Figure 5. It is seen that all added liposomes were complexed with SPBs up to 0.7 mg/mL saturation point; at higher concentrations free (unbound) liposomes resided in the supernatant and were detected by fluorescence and DLS measurements.

The data from Figure 5 allowed the calculation of an ultimate liposome number capable of complexing with a single SPB particle as:

$$N = (C_{lip} \times S_1 \times Na \times D^3 \times \rho) / (6C_{brush} \times d^2 \times M) \quad (1)$$ [34]

where Clip is a lipid concentration at saturation, C_{brush} is a SPB concentration, D is a diameter of polystyrene core, 100 nm, and ρ is its density,[46] d is a mean liposome diameter, 40 nm, S_1 is a mean surface area per one lipid molecule, 0.7 nm^2,[44] M is a mean molecular weight of the lipid,[44] and Na is an Avogadro's number. The calculations based on Eq. 1 gave an N = 40. Since SPB/ternary liposome complex remained stable (did not dissociate) when decreasing pH down to 5, all 40 liposomes were held on the SPB surface in the saturated complex at pH 5.

An ability of the EL/PS^{1-}/MOCH ternary liposomes to encapsulate a small substance at pH 7 and to release it at pH 5 was examined by conductometry. Suspensions of the ternary liposomes loaded with a 1 M NaCl solution were prepared as described in the Experimental section. Release of NaCl from liposomes into surrounding solution was accompanied by an increase the suspension conductivity. The result was compared with the conductivity of a suspension from NaCl-loaded liposomes completely destroyed in the presence of a Triton X-100 surfactant excess and taken as 100% activity.

In a control experiment, the conductivity was shown not to change within 2 hours upon complexation of the NaCl-loaded EL/PS^{1-}/MOCH ternary liposomes and SPBs in a pH 7 solution. In other words, the ternary liposomes retained their integrity while bound to SPBs in a pH 7 solution. Further acidification of the surrounding solution down to pH 5 led to a fast leakage of NaCl solution from inside liposomes: up to 50% within first few seconds, with a maximum (60% release) 40 min after pH adjustment. Much slower NaCl release was observed for liposomes in the absence SPBs: only 10% within first 10 minutes after the acidification. Thus, the liposomes with an embedded conformational switch (fliposomes) release an encapsulated compound in response to change in solution pH. Interestingly, the rate of escape from SPB-complexed liposomes significantly exceeds the escape rate from the identical but non-complexed liposomes. This result demonstrates improved release properties for the EL/PS^{1-}/MOCH ternary liposomes complexed to SPBs compared with the liposomes modified by pH-senssitive polymers.[35,45]

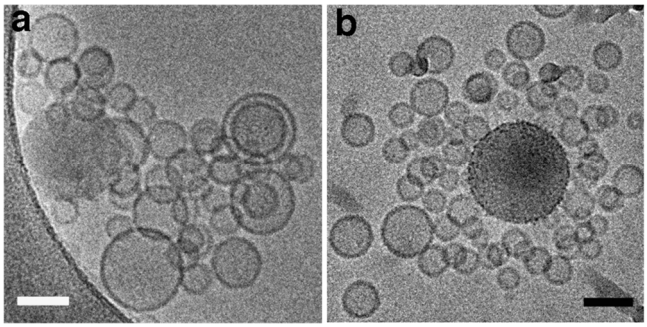

Fig.6. Cryogenic transmission electron microscopy images of mixed SPB+ EL/PS^{1-}/MOCH liposome suspension at pH 7 (a), and after its acidification down to pH 5 (b). [SPB+] = 8×10^{-5} M; 1 mg/mL lipid concentration. Bars correspond to 50 nm.

Two questions arise from the conductometric data: (a) Why do the pH-sensitive liposomes complexed with SPBs not release all the encapsulated NaCl in a pH 5 solution? (b) Why do the pH-sensitive liposomes show faster NaCl release when being complexed with SPBs? The membrane of the EL/PS^{1-}/MOCH ternary liposome is in the liquid crystalline state in which lipid and MOCH molecules are able to move along each leaflet of the membrane bilayer (lateral diffusion) as well as migrate between the outer and inner leaflets (flip-flop; it should not be confused with the conformational flip). Reasonably, the NaCl solution release occurs via permanent defects in the "liquid" liposomal membrane arising from conformational reorganization of MOCH molecules when the pH of solution changes from 7 to 5. After all the MOCH alkyl tails rapidly acquire a new conformation, the resulting membrane defects are "healed" owing to lateral and transmembrane migration of lipid molecules. If this is true, the amount of NaCl release is determined by the membrane healing rate relative to the rate of NaCl escape. In our case, the rate balance allows 50-60% of the encapsulated salt to escape from the SPB-complexed liposomes.

As to a faster NaCl release from the SPB-bound liposomes, this may be related to the ability of SPB polycationic chains to weaken the liposomal membrane by attracting the electronegative PS^{1-} molecules toward the contact areas, and/or to stabilize the "conducting" state of the liposomal membrane. Thus, partial SPB incorporation into the defects might impede the spontaneous healing of the defects and, consequently, accelerate the NaCl release.

The release of salt from the EL/PS^{1-}/MOCH ternary liposomes was visualized by cryogenic transmission electron microscopy (cryo-TEM). Sonicated EL/PS^{1-}/MOCH liposomes, 50-80 nm in diameter, loaded by a 1 M CsI solution, were prepared and mixed with a SPS suspension in a pH 7 solution. Figure 6a displays a typical cryo-TEM micrograph for the resulting SPB-liposome complexes. In the micrograph there are SPBs (black disks) surrounded by undisrupted spherical liposomes (black circles). The cryo-TEM data thus corroborate the integrity of the ternary liposomes after their binding to SPBs at pH 7, as shown above in

the conductivity experiments. In the micrograph taken after adjustment of pH down to 5 (Figure 6b) we see again SPBs surrounded by the undisrupted liposomes and additionally small black spots over SPB disks. The latter might reflect a release of CsI from liposomes, partial oxidation of I^- ions to I_2 by dissolved oxygen and formation of I_3^- ions[47] that finally condensed in small nanoparticles on the polystyrene core surface due to their electrostatic interaction with grafted cationic chains.

Experimental procedures

Small unilamellar anionic liposomes with mean diameter 40 nm and polydispersity index 0.256 were prepared by the standard sonication procedure (see details in ESI S2).[34] Appropriate amounts of EL, PS^{1-} and MOCH solutions in chloroform were mixed in a flask, after which the solvent was evaporated under vacuum. A thin lipid film was dispersed in a pH 7 10^{-2} M TRIS buffer or a pH 5 acetate buffer for 400s with a 4700 Cole-Parmer ultrasonic homogenizer. Liposome samples were separated from titanium dust by centrifugation for 5 min at 10,000 rpm and used within one day. Liposomes with a molar fraction of anionic PS^{1-}, designated as ν(a) where $\nu(a) = [PS^{1-}]/([PS^{1-}] + [EL] + [MOCH]) = 0.1$, and a molar fraction of MOCH, designated as ν(s) where $\nu(s) = [MOCH]/([PS^{1-}] + [EL] + [MOCH]) = 0.3$, were thus obtained. Additionally, PS^{1-}/EL binary liposomes with a molar fraction of anionic PS^{1-} equal to 0.1 were prepared and used as a control. Liposomes with a fluorescent dye, incorporated into the membrane, were made following by the sonication procedure described above except that 0.1 wt.% of PE* was added to the lipid mixture solution before chloroform evaporation. The fluorescence intensity of liposome suspensions with incorporated PE* was measured at λ_{em}=595 nm (λ_{ex}=565 nm) using a F-4000 Hitachi fluorescence spectrofluorimeter.

The mean hydrodynamic diameters of SPBs, liposomes, and SPB/liposome complexes 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. The electrophoretic mobility of SPBs, liposomes and SPB/liposome complexes was measured by laser microelectrophoresis in a thermostatic cell using a Brookhaven Zeta Plus system with the corresponding software.

Permeability of the liposomal membranes towards a simple salt was investigated by measuring the conductivity of NaCl-loaded vesicle suspensions with a CDM83 conductometer (Radiometer) as described in Ref. 34.

Vitrified specimens for cryogenic transmission electron microscopy (cryo-TEM) were prepared in a controlled environment vitrification system (CEVS), where a desirable temperature and humidity were maintained. Briefly, a drop of the liposome suspension, or SPB suspension, or mixed SPB/liposome suspension, was placed on a perforated carbon film-coated copper grid, blotted with a filter paper, and plunged into liquid ethane at its freezing point. The vitrified specimens were transferred to an Gatan 626 cryo cooling- holder, and observed in either a Philips CM120 or an FEI T12 transmission electron microscope at about -180 °C in the low-dose imaging mode to minimize electron-beam radiation damage. Images were digitally recorded with a Gatan 791 MultiScan cooled-CCD camera (CM120) or by Gatan US1000 high-resolution cooled-CCD camera (T12). More details may be found elsewhere.[48]

All the solutions were prepared with double-distilled water that was additionally treated by a Milli-Q Millipore system composed of ion-exchange and adsorption columns as well as a filter to remove large particles. All experiments were done at 20 ºC.

Conclusions

We have found that the ternary liposomes, composed of conventional anionic and zwitter-ionic lipids and a synthetic conformationally-triggered amphiphilic compound (fliposomes), are electrostatically complexed with SPB in a pH 7 solution and retain their size and encapsulating power. Decrease in pH down to 5 induces a conformation switch in the hydrocarbon tails of the amphiphilic molecules that leads to a fast release of the liposome content to surrounding solution. Fliposomes complexed to SPB release their cargo upon acidification much faster than the free liposomes. Such complexes, that contain approximately 40 pH-sensitive fliposomes per SPB particle, seem to be promising multiliposomal carriers for biologically active compounds in contrast to related publications.

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Notes and references

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