Influence of stabilizing components on the integrity of antitumor liposomes loaded with lipophilic prodrug in the bilayer

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A B S T R A C T
Previously, we proposed a liposomal formulation of melphalan (Mlph)—a chemotherapeutic alkylation agent—incorporated in a fluid lipid bilayer in the form of dioleoylglyceride ester. In this work, we compared the stabilizing effect of different amphiphiles included in the Mlph-liposomes, such as phosphatidylinositol (PI), ganglioside GM1, a conjugate of N-carboxymethyl-modified oligoglycine with dioleoylphosphatidylethanolamine (acidic lipopeptide), and polyethylene glycol (2000 Da) conjugated with dipalmitoylphosphatidylethanolamine (PEG-lipid), upon incubation in human serum. Mean hydrodynamic diameter values (86–90 nm) were similar among different liposome samples, while zeta potential values considerably varied. The formulations were incubated in human serum at 37 °C for different time intervals up to 24 h. Liposome integrity was evaluated by changes in fluorescence upon leakage of calcein or disruption of Förster resonance energy transfer between donor and acceptor fluorescent lipid probes in the bilayer. The best stabilization of liposomes was achieved upon the addition of ganglioside GM1, or the acidic lipopeptide. Inclusion of 10 mol% Pl improved liposome stability only for the first 4 h of incubation. Pegylated liposomal formulations of melphalan lipophilic prodrug with fluid phase bilayer were the least stable, which is probably due to the propensity of the PEG-lipid to exit liposome membranes. Cholesterol-containing bilayers of liquid ordered phase, supplemented with sufficient amounts of the PEG-lipid, showed good stability in serum.

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1. Introduction

Liposomes had been among the first drug delivery systems introduced back in late 1980s. Owing to their exceptional bio- and hemocompatibility among nanosized vehicles, they still attract pharmaceutical scientists, especially in the field of anticancer therapy [1]. To prevent premature withdrawal of liposomes from circulation by cells of reticuloendothelial system, polyethylene glycol (PEG) chains were proposed to be grafted on the surface of liposomes [2–4]. Today, commercial preparations of pegylated liposomes loaded with antifungal and cytostatic drugs have long been used in clinics (reviewed in [1]). The most successful among these is a liposomal formulation of doxorubicin DoxIL®, a widely used FDA-approved drug in treatment of ovarian and breast cancers [5].

Remote loading has been introduced as a technique to encapsulate water-soluble drugs into the inner aqueous phase of nanosized liposomes. However, it can only be applied to weak amphipathic acids or bases, e.g. for anthracycline antibiotics [6]. As for the passive encapsulation, the inner volume of a nanosized liposome is very small and the quantity of encapsulated solute is too low to provide for the sufficiently high concentration of a drug in target tissue (calculated loading capacity of a 100-nm liposome, even for a highly water-soluble drug, does not exceed 2–3 mol% to total lipids).

Another option to load a small molecule drug in nanosized liposomes is to design a lipophilic prodrug for incorporation in the lipid bilayer. The approach simplifies liposome production and provides for sufficiently high loading capacity of the carrier (reviewed in [7]). Previously, we developed a liposomal formulation of a chemotherapeutic agent melphalan incorporated in a fluid lipid bilayer in the form of a tailored lipophilic conjugate, i.e. a dioleoylglyceride ester (MlphDG, Fig. 1) [8]. We expect that in the cell MlphDG

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should be easily hydrolyzed by low-specificity esterases, present in most tissues in abundance, releasing melphalan [9]. Melphalan is a cell-cycle non-specific alkylating cytotoxic drug of the nitrogen mustard family. It is poorly soluble at physiological pH and therefore is administered in large doses causing multiple side effects. Encapsulation of melphalan in a nanocarrier should alleviate its high systemic toxicity and improve efficacy. However, numerous attempts to encapsulate intact melphalan in a nanoparticulate carrier failed (reviewed in [10]). Our liposomal formulation of MlphDG contains therapeutically relevant concentration of the active agent (10 mol%) [8]. Presumably, another advantage of this design is the increased stability of melphalan moiety to hydrolysis due to location of its alkylating groups in lipid environment, and not the bulk aqueous phase.

To minimize the disruption of the lipid bilayer packing when loading prodrug molecules, we use natural phospholipids forming a fluid bilayer, or the so-called liquid disordered phase. Another important and specific reason for this choice is the possibility of liposome production without heating, which is the advantage from the perspective of not only the technology of liposome production, but also stability to hydrolysis of alkylating chloroethyl groups. Finally, fluid lipid bilayers more easily fuse with membranes of tumor cells, which facilitates unloading of liposomes. Bilayer matrix of the formulation developed in our group is formed by the easily available egg phosphatidylcholine (ePC). Phosphatidylinositol (PI) is present in the bilayer in the amount of about 10 mol% (thus the final composition is ePC–PI–MlphDG, 8:1:1), which had been shown to prolong circulation half-lives of nanosized liposomes [11]. The use of PI instead of a PEG-conjugated lipid aims to avoid hypersensitivity reactions [12,13] and other anti-PEG immunity-associated issues [14] observed in response to intravenous administration of pegylated liposomes. Indeed, in a panel of in vitro tests MlphDG-loaded liposomes exhibited the overall good hemocompatibility and induced no complement activation [15]. In vivo the ePC–PI–MlphDG formulation was more effective than intact melphalan in inhibiting tumor growth in the Lewis lung carcinoma model [16]. However, primary pharmacokinetics data for a sibling formulation of methotrexate lipophilic prodrug (ePC–PI–prodrug, 8:1:1) demonstrated rather rapid distribution of the prodrug in organs and tissues (t_{1/2α} = 3.6 min) and elimination from plasma (t_{1/2β}~40 min) [17]. Therefore, we are looking for ways to further improve our liposome performance retaining the high fluidity of the matrix bilayer, which eliminates the need for heating during production of the formulation.

The goal of this study was to explore the effect of different amphiphiles introduced in the membrane of MlphDG-loaded ePC-based liposomes on the liposome stability in human serum. Other lipid compositions were used as reference ones and to expand our understanding of the behavior of the amphiphiles in the bilayer. Integrity of liposomes was evaluated by calcein release and Förster resonance energy transfer efficiency assays supported by other instrumental data.
2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (ePC) was obtained from Lipoid GmbH (Heidelberg, Germany); raw soybean phosphatidylinositol (PI) was a kind gift from Lipoid, it was further purified by column chromatography; distearoylphosphatidylcholine (DSPC), cholesterol (Chol), and dipalmitoylphosphatidylethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-PE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA); ganglioside GM1 was extracted from bovine brain and purified; dioleoylglycerol ester melphalan conjugate (MlphDG) [18], 1,3,5,7-tetramethyl-BODIPY-labeled phosphatidylcholine (TMB-PC) [19], and bis-cyclohexyl-BODIPY-labeled phosphatidylcholine (BCHB-PC) [20] were synthesized as previously reported; 3-perylenoyl derivative of the lipophilic melphalan conjugate (Per-MlphDG) was synthesized as previously reported for the merphalan (DL-melphalan) analogue [21]; lipopeptide CMG-PE was prepared from the precursor conjugate synthesized as reported in [22] by acetylation with five-fold excess of Ac2O and pyridine in aqueous medium, followed by freeze-drying. Calcein (tetrasyodium salt) was purchased from Serva; Sephadex G-50 was obtained from Pharmacia.

Normal human blood from healthy volunteer donors was collected in Vacutest clot activator tubes (Vacutest Kima, Italy), allowed to stay for 1 h at room temperature, and then centrifuged at 2000g for 10 min. Serum was transferred from vacuum tubes to Eppendorf tubes. Experiments were performed within 3 days upon blood collection; serum was stored at +4 °C.

2.2. Liposome preparation

Liposomes (large unilamellar vesicles) were prepared as described earlier [15]. Briefly, lipid films were obtained by co-evaporation of aliquots of stock solutions in chloroform–methanol (2:1) in round-bottom flask on a rotary evaporator, with subsequent drying for 45 min at 5 Pa. The resulting compositions were ePC (DSPC)–MlphDG, 9:1; ePC–MlphDG–PI, 8:1:1; ePC (DSPC)–MlphDG–PEG–PE, 8:1:1 or 8:8:1:0.2; ePC–MlphDG–GM1, 8:1:1 or 8:8:1:0.2; ePC–MlphDG–CMG–PE, 8:1:1 or 8:8:1:0.2; and DSPC–Chol–MlphDG–PEG–PE, 5:3:1:1 or 5:8:3:1:0.2 (by mol). Then lipid films were hydrated in phosphate buffered saline (PBS, pH 7.4), subjected to seven cycles of freezing/thawing (liquid nitrogen/+40 °C), and extruded 20 times through polycarbonate membrane filters (Nucleopore, USA) with a pore size of 100 nm on a Mini-extruder by Avanti. DSPC-containing samples were extruded on a water bath with heating up to +55 °C (the thawing step was also performed at 55 °C). Phospholipid concentrations in liposome dispersions were measured by the colorimetric assay [23]. Prodrug concentrations were controlled by UV spectrophotometry after liposome disruption with ethanol (λmax MlphDG 260 nm, ε 16100 M–1 cm–1). For FRET experiments, 0.5 mol% TMB-PC and 1.5 mol% BCHB-PC were added at the stage of lipid film formation.

To prepare liposomes with self-quenched calcein, lipid films were hydrated in PBS with 80 mM calcein and processed as described above. After extrusion, non-encapsulated calcein was removed by size exclusion chromatography on a Sephadex G-50 column (1.3 × 18 cm) equilibrated in PBS: 200-μl aliquots of calcein-containing liposome dispersions were applied onto the column, the void volume (4.5 ml) was discarded, and then 150–200 μL fractions were collected. Three or four peak liposome fractions were combined. Prodrug and calcein concentrations were evaluated by measuring UV spectra of decomposed liposomal dispersions in ethanol (λmax Calcein 504 nm, ε ~74000 M–1 cm–1).

Liposome dispersions were stored at +4 °C and used for experiments within 7 days.

2.3. Hydrodynamic diameter and total liposome concentration

Number-weighted hydrodynamic diameters and liposome concentration in samples were measured with Nanoparticle Tracking Analysis (NTA) technique using the Nanosight LM10-HSBF instrument (Nanosight, UK) equipped with a 405 nm, 65 mM laser and a high-sensitivity EMCCD (Andor Luca) camera. All measurements were performed according to the ASTM E2834-12 standard [24]. For detailed video acquisition and processing settings see Supplementary material, Nanoparticle Tracking Analysis settings.

2.4. Zeta potential protocol

For reliable measurements, liposome samples were prepared as described earlier [15]. Briefly, lipid films were hydrated in 10 mM KCl, 1 mM KH2PO4, 1 mM K2HPO4 solution (pH 7.35), and after freezing–thawing procedure they were extruded 20 times through 200 nm polycarbonate membrane filters to gain liposome diameter of 200 nm. Zeta potential values were obtained using ZetaPALS analyzer (Brookhaven Instruments Corp., Holtsville, NY; provided by the CoreFacility of the Institute of Gene Biology, Russian Academy of Sciences). Samples of liposomes (1.5 ml, 1 mg/ml total lipids) were equilibrated for 1 min in cuvettes before 10 runs of 25 cycles per sample were performed at 25 °C. Zeta potential values were calculated using Smoluchowski approximation.

2.5. Fluorescence measurements

An aliquot of liposome sample (5–7 μl for calcein-containing samples and 25 μl for samples with FRET probe pairs) was diluted in warm (37 °C) PBS or 80% serum to adjust the absorbance of the fluorescent dye (TMB-PC or calcein) to less than 0.05 a.u. to avoid the inner filter effect. The final concentration of total liposome lipids was in the range of 10−4–10−5 M. This concentration approximates liposome concentration in plasma that is expected upon intravenous administration of liposomes [25]. Then diluted samples were incubated in water bath at 37 °C for appointed time intervals (maximum of 24 h), new aliquot used for each incubation time. Fluorescence intensity of TMB-PC or released calcein was measured using the F-4000 (Hitachi, Japan) fluorescence spectrophotometer before and after liposome lysis. To lyse liposomes, 10 μl of 20% Triton X-100 was added to each sample of 200 μl. Each sample was immediately after dilution, emission intensity at different time points, λmax was measured with an error of ±0.1 nm.

Increase of BODIPY (TMB-PC) fluorescence (Ft) was calculated as

\[ \text{CR} = \left( \frac{l_t - l_0}{l_{\text{max}} - l_0} \right) \times \left[ \frac{l_{\text{max}}}{l_{\text{max}}(t) - l_0} \right], \]

where

- \( l_0 \) is intensity without incubation, measured immediately after dilution,
- \( l_t \) is intensity at different time points,
- \( l_{\text{max}} \) is absolute value of intensities may not be true at low calcein release percent. In our experiment, relative error of measurements in serum for different samples of the same liposome species was up to 10% and that in PBS, 18%. Taking into account the variation of absolute values of fluorescence intensities for liposome samples within a single series, the degree of calcein release (CR) was calculated according to the formula:

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where $I_{\text{max}}$ is the intensity after Triton X-100 addition, performed immediately after dilution, $I_{\text{max}(t)}$ is the intensity after Triton X-100 addition at different time points. CR values thus obtained were used to plot relative dequenching of calcein fluorescence in function of time.

Detailed consideration of the formula used to calculate CR from raw fluorescence intensities is given in the Supplementary material. The formula is readily reduced to the common one in case $I_{\text{max}(t)}/I_{\text{max},0} \approx 1$, when the measurement error is small enough.

To explore retention of the prodrg in the bilayer, an aliquot of liposome sample labeled with 1 mol% Per-MlphDG (added at the stage of lipid film formation) was diluted in warm PBS or 80% serum as described in the beginning of the section. After incubation for appointed time intervals, an aliquot of 200 µL of the mixture was applied onto a Sepharose CL-4B column (~1.1 × 19 cm; $V_g = 6$ ml) and eluted with PBS; fractions of 200 µL were collected and analyzed for the fluorescence intensity at $\lambda_{\text{ex}}$ 450, Xem, 520 nm. For each new time point, aliquots were taken from the same incubation mixture and applied onto the same column. A sample of 80% serum (200 µL) was used as a control to detect the elution profile of plasma proteins applied onto the column; protein content was determined in each fraction of 200 µL as described earlier.[30,31]

Experiments were partially carried out using the equipment provided by the IBCH core facility (CKPIBCH, supported by the Ministry of Education and Science of the Russian Federation, grant no. RFMEFI62117X0018).

3. Results and discussion

3.1. Characteristics of MlphDG liposomal formulations

We prepared egg phosphatidylcholine-based liposomal formulations of melphanal lipophilic prodrug with additives of four amphiphilic molecules that are assumed to be able to stabilize membrane of liposomes in blood plasma (Fig. 1). The inclusion of phosphatidylinositol (PI) in the bilayer has been shown to decrease liposome uptake by cells of reticuloendothelial system [11], which may be due to the negative charge of the lipid with a relatively bulky head group, along with the steric hindrances caused by highly hydrated inositol moieties on surface of liposome.[32]. The increase of circulation time by ganglioside GM1 additives has been shown to be even more pronounced than in the case of PI due to the voluminous and rigid negatively charged pentasaccharide residue [11,33]. Grafting of polyethylene glycol (PEG) chains on the surface of liposomes in the form of PEG2000-conjugated phosphatidylethanolamine is a proven method of their stabilization [1,3,5]. A new molecule that we chose to test for the ability to protect lipid bilayer surface, is a conjugate of an acidic oligopeptide CMG with dioleoylphosphatidylethanolamine (CMG-PE). The CMG peptide has previously been tested as part of peptide-CMG-PE conjugates used for screening of plasma proteins specifically binding the peptides [34]. When the conjugates were inserted in red blood cell membranes, we could not detect any non-target plasma protein–peptide interactions; therefore, we expect that CMG peptide could provide for the stealth effect of the liposomes.

Description of samples and their physicochemical characteristics are presented in Table 1. All liposomes had similar mean hydrodynamic diameter of 86–90 nm regardless of their composition. We suppose that outer diameter of the liposomes is defined by the pore size of membrane filter during extrusion.

NTA was also used to measure the total particle concentrations in all samples. Although there are reports that the absolute values of NTA concentrations could be biased for weakly scattering particles (e.g., [35]), in our experiments measured liposome concentrations were well within the predicted ranges (see Supplementary material, Predicted liposome concentrations, Table 15).

Results of zeta-potential measurements by photon correlation spectroscopy are consistent with the fact that melphanal moiety of MlphDG is positively charged at pH 7.4 (PBS) due to protonation of the primary amino group (phosphatidylcholine-based liposomes without any stabilizing additives (L) were positively charged (+18 mV; see Table 1). As expected, inclusion of 2 mol% PEG-PE, containing a phosphate group, or monosialoganglioside GM1 led to partial neutralization of the positive potential of the vesicles (down to +9 and +5 mV, respectively). A further increase in the content of these compounds to 10 mol% produced negatively charged liposomes. Similar result was obtained upon inclusion of 10 mol% PI. Liposomes containing the CMG-lipid showed the strongest negative charge, even at minimal concentration of this additive, because of the four COOH-groups (per mole) exposed over the surface of the lipid bilayer.

Additional information on liposome structure could be revealed by the analysis of single particle scattering intensities (Table 1) and number of lipids per liposome (see Supplementary material, Fig. 15). All samples, except 10mol% PEG-PE, are indistinguishable based on these two parameters. (We can only note a barely noticeable increase in scattering for samples L-PI and L-CMG, reflecting the increased surface density of the lipid bilayer due to inositol or pentasaccharide residues and/or tendency of the ganglioside molecules to cluster.) Highly pegylated liposomes show a significantly lower number of lipid per liposome and a lower scattering intensity. We propose that while having the same outer hydrodynamic diameter as their non-pegylated counterparts, pegylated liposomes have smaller diameter of lipid sphere and lower number of lipids per liposome due to bulkiness of PEG2000. The latter result, together with the low refractive index contrast of highly hydrated PEG2000 residue, leads to the lower scattering intensity of such liposomes. Notably, the effect is not observed even for 10 mol% CMG-PE additive. This fact could be explained by the shrinkage of oligopeptide chain due to shielding of charges at rather high ionic strengths (in PBS, $I = 163$ mM). The effect of conformational changes and shrinking of polyelectrolytes at high ionic strength is well described and directly measured for brushes of short ($n = 40$) polycrylic acids [36]. We also presume, that CMG chains are not

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Sample composition, mol</th>
<th>Mean size, nm</th>
<th>Mean scattering intensity, AU</th>
<th>Zeta-potential, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>ePC–MlphDG, 9:1</td>
<td>90 ± 2</td>
<td>3.6 ± 0.1</td>
<td>+18</td>
</tr>
<tr>
<td>L-PI</td>
<td>ePC–MlphDG–PI, 8:1:1</td>
<td>90 ± 2</td>
<td>3.7 ± 0.1</td>
<td>-25</td>
</tr>
<tr>
<td>L-GM1</td>
<td>ePC–MlphDG–GM1, 9:1:0.2</td>
<td>88 ± 2</td>
<td>3.6 ± 0.1</td>
<td>+5</td>
</tr>
<tr>
<td>L-GM1-10</td>
<td>ePC–MlphDG–GM1, 8:1:1</td>
<td>89 ± 2</td>
<td>3.7 ± 0.1</td>
<td>-30</td>
</tr>
<tr>
<td>L-CMG,2</td>
<td>ePC–MlphDG–CMG-PE, 8:1:2</td>
<td>86 ± 2</td>
<td>3.6 ± 0.1</td>
<td>-31</td>
</tr>
<tr>
<td>L-CMG,10</td>
<td>ePC–MlphDG–CMG-PE, 9:1:0:2</td>
<td>87 ± 1</td>
<td>3.6 ± 0.1</td>
<td>-66</td>
</tr>
<tr>
<td>L-PEG,2</td>
<td>ePC–MlphDG–PEG-PE, 8:1:1</td>
<td>88 ± 2</td>
<td>3.5 ± 0.2</td>
<td>+9</td>
</tr>
<tr>
<td>L-PEG,10</td>
<td>ePC–MlphDG–PEG-PE, 8:1:1</td>
<td>88 ± 1</td>
<td>3.4 ± 0.1</td>
<td>-10</td>
</tr>
</tbody>
</table>

* Errors reported as confidence intervals ($p = 95$).

** As assessed by photon correlation spectroscopy; data for 200-nm liposomes.
In the absence of any stabilizing components, formulation L showed very slow ongoing release of calcein (the degree of calcein release (CR) was approximately 0.08 after 24 h of incubation; Fig. 2a). We attribute this release to diffusion of calcein through the fluid lipid bilayer. The ability of calcein to permeate through liposomal membrane without its disruption, presumably through water channels therein, and dependence of the calcein release rate on the fluidity of the bilayer has been thoroughly proven by Maherani et al. [28].

When liposomes L were incubated in serum, a somewhat more intense, yet gradual calcein release was observed, with CR reaching 0.19 by the end of incubation. This result may be a consequence of progressive degradation of the fluid lipid bilayer under the action of enzymes in serum.

When we replaced ePC with distearoylphosphatidylcholine (L-DSPC), CR value barely reached 0.03 upon a 24-h incubation in PBS and calcein release was completely blocked in serum (Fig. 2a). The sharp decrease of calcein release from DSPC-based liposomes in PBS agrees with the mechanism of calcein diffusion through the hydrocarbon portion of the bilayer [28]. Indeed, diffusion through a fluid bilayer (sample L) should be much easier than through the tight packing of steroyl chains (sample L-DSPC). The presence of MlphDG in the bilayer imparts them with a positive charge, which should lead to a dense protein corona [37].

Interestingly, in PBS, calcein leakage from L-PI liposomes was practically the same as from the positively charged L liposomes. According to differential scanning calorimetry data [see Supplementary material, Fig. 25], bilayer matrix prepared of ePC–PI, 9:1, is characterized by the phase transition temperature $T_{m} = -9^\circ C$. Incorporation of increasing amounts of MlphDG into the bilayer leads to gradual decrease of $T_{m}$ and enthalpy of the transition, implying slight disturbance introduced by the prodruk molecules in the bilayer, yet the bilayer of L-PI represents a fluid, or liquid disordered, phase ($T_{m} \sim -16^\circ C$). The essential negative charge of L-PI liposomes (zeta potential $-25 \text{ mV}$) in principle could affect the rate of outflow of the negatively charged dye, yet it was not the case. This suggests that bilayer phase has greater effect on the dye leakage than charge of the liposomes.

In serum, 10 mol% of PI in ePC-based bilayer successfully blocked calcein release in the first 4 h (sample L-PI; Fig. 2b). After 24 h of incubation, the release of calcein was the same as for liposomes without PI (CR 0.20). In our previous studies on binding of plasma proteins by the liposomes using Western blotting [30,31], we observed minute amounts of ApoA1 among proteins associated with ePC–PI–MlphDG liposomes upon 15 min incubation with human plasma. As for ApoA1, it demonstrated significant binding and concentrated on liposome surface. Both apolipoproteins have been shown to mediate phospholipid efflux from model DMPC vesicles [38] and thus could contribute to bilayer deformations and gradual calcein leakage through water channels thus formed. Yet, the explanation is questionable, because amphiphatic helical domains of ApoA1 were shown to hardly penetrate fluid lipid bilayers built from phospholipids with unsaturated acyl chains [39]. Other plasma proteins associated with MlphDG-loaded liposomes include C4b-binding protein and factor H. However, we could not find any reports on how exactly these proteins interact with lipid bilayers to conform or disprove their possible contribution to water channel widening and/or facilitated penetration of calcein across the membrane.

The addition of 2 mol% GM1 to the basic formulation resulted in the most prominent stabilizing effect on the lipid bilayer: the L-GM1,2 liposomes practically did not release calcein for 24 h, demonstrating nearly identical patterns in buffer and serum (CR 0.06–0.07, Fig. 3a). Obviously, such a small concentration of negatively charged and rigid pentasaccharide residue of ganglioside molecule on the positively charged surface of the liquid matrix tightly bound to positively charged melphanal moieties due to the same reason of charge shielding at high ionic strengths; rather they “stick” out of the membrane surface.
(ePC/MphDG) provides a near-optimal shielding from perturbing effects of serum proteins on the bilayer and prevents diffusion of the dye through it. On the contrary, inclusion of 2 mol% CMG-PE (L-CMG.2 liposomes) resulted in a sharp initial release of calcein from liposomes in serum (up to CR 0.17, Fig. 3a) followed by a plateau until the end of incubation. Most likely, this effect is due to electro-static repulsion between the negatively charged CMG-chains and plasma proteins that interact with portions of the lipidosome surface unoccupied by the oligopeptide, which leads to rapid lateral diffusion of lipids causing cracks in the membrane presumably through widening of preexisting/inherent water channels. The difference in the effect of low concentrations of GM1 and CMG-PE in the matrix bilayer on its permeability in serum agrees with projection of the oligopeptide chain over the membrane surface (see Section 3.1), as well as with the values of zeta potentials: +5 mV against −31 mV.

The L-PI liposomes are only slightly less negatively charged than L-CMG.2, however inositol head groups are located in the plane of the lipid bilayer surface and the plausible binding of plasma proteins does not disturb packing of the amphiphilic molecules in the membrane.

In an increase in the concentration of GM1 in the matrix bilayer to 10 mol% led to a sharp initial leakage of calcein up to CR 0.60 (Fig. 3b, circles). In this case, an array of bulky and rigid oligosaccharide residues still leaves a space for plasma proteins on the surface of liposomes, however the proteins seem to have to push apart negatively charged glycans and therefore cause disturbance in the bilayer and formation of cracks therein (see above). When we included 10 mol% CMG-PE, the initial leakage of calcein in serum increased even to CR0.80 (Fig. 3b, squares). This result also does not contradict our assumptions on widening of water channels in fluid bilayer matrix under the action of proteins, since CMG chains are more exposed to aqueous milieu and have higher negative charge than polar head groups of GM1 molecules (L-CMG.10 liposomes have the largest negative charge, −66 mV).

To confirm our reasoning on the formation of cracks in liquid matrix bilayer containing amphiphilic with bulky negatively charged polar residues (GM1 and CMG-PE) under the effect of plasma proteins, we replaced ePC with DSPC. The presence of 10 mol% of either additive led to an almost immediate cracking of the vesicles upon contact with the serum (Fig. 3b, empty badges). In the absence of these additives, liposomes loaded with MphDG in the gel phase of the lipid bilayer (L-DSPC), maintained integrity during the whole incubation period (Fig. 2a).

Finally, we evaluated how selected formulations retained the MphDG produrg upon incubation in serum for 0, 1, and 4 h using the fluorescently labeled analogue Per-MphDG. Liposomes were separated from unbound proteins using gel chromatography on a calibrated sepharose column (see Supplementary material, Figs. 3S, 4S, and 5S). The integral fluorescence intensity of liposome-containing fractions (peak eluted with the void volume V0) slowly decreased with time, however we did not observe any fluorescence signal in subsequent fractions where smaller protein–lipid complexes, free proteins, and lipid micelles were eluted (Fig. 5S). We ascribe the decrease of total liposome fluorescence to quenching of the perylenoyl group emission by the proteins associated with the bilayer. Indeed, quantum yield of perylenoyl group within the probe molecule is already depressed by a factor of 1.7 under the influence of melphalan moiety, which is similar to that of phenylalanine [21]; also, we have previously observed quenching of perylenoyl fluorescence upon membrane interaction with proteins, in part due to increased membrane permeability to water (unpublished results)). Moreover, it seems that the degree of fluorescence quenching is inversely proportional to the degree of liposome surface shielding. Indeed, the decrease of fluorescence for samples L-PI (Fig. 5Sa), L-GM.2 (Fig. 5Sb), and L-CMG.10 (Fig. 5Sc) was 30.6, 13.7, and 11.3% after 90-min incubation, and 40.7, 31.9, and 30.2% after 4-h incubation, respectively. These results are in good agreement with our data and interpretations on the leakage of calcein.

But when cracks are formed in the lipid bilayer, do fluid phase liposomes remain the initial vesicles owing to their elasticity? Or are they broken into micellar complexes with proteins, which retain part of self-quenched calcein? Our attempts to monitor liposome fate using ultracentrifugation in salt density gradient were not successful. Incubation of liposomes labeled with the TMB-PC lipid probe in the bilayer in plasma for 1 h resulted in approximately 70% of fluorescence in the layer of low-density lipoproteins. When the liposomes were incubated in lipoprotein-deficient plasma, they also distributed to the layer corresponding to low-density lipoproteins (unpublished results). To assess liposome integrity upon contact with plasma, we turned to an approach based on the Förster resonance energy transfer (FRET).

3.3. Studies using FRET between donor and acceptor fluorescent lipid probes in the bilayer

Recently we developed a new donor–acceptor pair of fluorescent lipid probes with almost identical structures (TMB-PC and BCBH-PC, Fig. 1), which allows to study protein–bilayer interactions using Förster resonance energy transfer (FRET) analysis [20,40]. Both probes—the donor (TMB-PC, the reporter molecule) and the acceptor (BCBH-PC)—were incorporated into the membrane of liposomes (samples L-PI, L-GM.10, and L-CMG.10). The overall concentration of probes (2 mol%) was kept reasonably low, so as not to introduce perturbations in the lipid matrix. Particularly, we found that the presence of 1.5 mol% TMB-PC and 0.5 mol% BCBH-PC in membrane provides for the optimal quenching of the donor within the intact membrane. Any changes in the membrane structure (curvature, integrity, etc.) would affect the average donor–acceptor distance. Changes in the distance, in turn, affect the efficiency of energy transfer either increasing or decreasing the fluorescence intensity thereby. Among the events that can change the distance between probes in a FRET pair are embedding of proteins in the lipid bilayer, escape of the probe(s) from the bilayer, including in complex with proteins, and change of the membrane curvature under the effect of proteins.

When liposomes were incubated in PBS, the fluorescence intensity of donor TMB-PC remained almost unchanged during the first 5 h in the case of L-GM.10 and L-CMG.10 liposomes (Fig. 4). Sample L-PI showed a very slight build-up of fluorescence in the first 30 min up to a plateau that lasted till the end of incubation. This build-up pattern reflects a rapid establishment of a steady-state arrangement of probes in the system “lipid bilayer–aqueous phase.” In the case of the L-GM.10 liposomes, the progressive yet very slight increase of the fluorescence signal by the end of incubation is most likely caused by the gradual transfer of GM1 into aqueous phase with the formation of micelles capturing (with CMC of ∼10−5 M, GM1 is prone to form micelles). In serum, all three types of liposomes showed significant increase in fluorescent signal, which had been developing during the first 1–4 h (Fig. 4). The most rapid and most intensive build-up of fluorescence (up to 80% of maximum) was observed for L-PI liposomes. Taking into account that during the first 4–5 h these liposomes retained calcein very well (Fig. 2b), the only interpretation of such a flare-up can be the formation of dense protein corona on membrane surface. Obviously, the proteins can get partly embedded in the outer leaflet of the bilayer or the probes can move to bind to hydrophobic sites of proteins, both of which leads to an increase in the distance between probes in the FRET pair and decreases quenching of the TMB-PC donor fluorescence. However, the liposomes maintain their vesicular structure.
Following the same logic, we assume that L-GM\textsubscript{1,10} and L-CMG\textsubscript{1,10} liposomes (Fig. 4) were not destroyed in serum either. Moreover, they were better shielded from proteins than in the case of L-PI liposomes, since they demonstrated lower degree of fluorescence build-up. The CMG-PE lipopeptide seems to provide the best protection: by the end of incubation L-CMG\textsubscript{1,10} liposomes showed 50% of the maximal fluorescence intensity against almost 65% in case of the L-GM\textsubscript{1,10} sample. This does not contradict our reasoning on the emergence/appearance of cracks and argues in favor of the formation of the most loose protein corona in the case of the L-CMG\textsubscript{1,10} liposomes. Stabilization of fluorescence signals of all liposomal samples at the same level by the end of incubation may result from gradual transfer of probe molecules to protein corona due to hydrophobic interactions between the proteins and fluorophores.

3.4. The effect of PEG-conjugated lipid on the stability of a fluid-phase lipid bilayer

Studies of calcein release from liposomes bearing 2 mol% PEG-PE (L-PEG\textsubscript{2}) produced results that can only be interpreted as the evidence of release of the PEG-lipid from the membrane into aqueous phase (Fig. 5a). Indeed, even in PBS, fluorescence signal began to rise 1 h after the start of incubation.

Previously, it has been established that PEG-modified phospholipids can be transferred out of the liposome membrane, presumably, by monomer diffusion or via micellar intermediates [41]. Among the factors that affect the rate of PEG-lipid “desorption” is the strength of hydrophobic interactions responsible for their association with the membrane: the longer is the acyl chain, the stronger are the hydrophobic interactions [42]. It was found that PEG2000 conjugated through carbamoyl bond to dipalmitoylphosphatidylethanolamine (PEG-PE; C16:0 acyl chains) or dioleoylphosphatidylethanolamine (C16:1) were transferred out of liposomes composed of DSPC–cholesterol–PEG-lipid, 50:45:5 (mol), within several hours of incubation at 37 °C in the presence of acceptor membranes; PEG2000–DSPE (C18:0) conjugate showed a much longer time of transfer out of the liposomes [43]. In the absence of multimellar vesicles mimicking cell membranes, escape of various PEG-lipids out of DSPC–cholesterol liposomes was negligible [44].

We assume that in our experiments PEG-PE could leave the bilayer of the L-PEG\textsubscript{2} fluid-phase liposomes in the form of micelles, which can carry calcein, together with other components of liposomal membrane. In serum, calcein release proceeds through several cycles of fluorescence build-up and decay (Fig. 5a). Such shape of the calcein release curve could be due to the complexity of the system, where PEG-lipid, other liposome lipids, proteins, calcein, and Triton X-100 can form a variety of (micellar) phases. These phases and PEG-lipid exchange between them cannot be resolved using the method applied. In any case, it is clear that the bilayer of the L-PEG\textsubscript{2} liposomes is unstable.

On the contrary, addition of 10 mol% PEG-PE conferred good stability in PBS upon liposomes; they also demonstrated gradual release of calcein during the first 2 h of incubation in serum followed by an additional slow-phase release up to 40% of the maximum by the end of incubation (Fig. 5b). The better stability of the L-PEG\textsubscript{10} liposomes can be attributed to the denser PEG coating. At 10 mol% PEG2000-lipid in the bilayer, the polymer should be in brush conformation (as opposed to mushroom conformation at concentrations below 5–6 mol% [45]), which prevents both fast exit of PEG-lipid and interactions of proteins with the lipids.

Let us note that L-PEG\textsubscript{2} and L-PEG\textsubscript{10} samples are liposomes made of liquid disordered (or fluid) bilayers. To bridge our findings on poor stabilization of ePC-based bilayers in our experimental settings with the literature data on PEG conjugate behavior in liquid ordered (LO) and gel (solid ordered, SO) phases, we explored calcein release from liposomes composed of DSPC–MphDG–PEG-PE (SO phase) or DSPC–Chol–MphDG–PEG–PE (LO phase).

When MphDG-loaded DSPC-liposomes with 2 mol% PEG-PE were incubated in PBS they retained calcein only during the first 4–5 h; in serum they ruptured within 1 h (Fig. 6a). The increase in PEG-lipid concentration to 10 mol% gave the same moderate effect on the bilayer stabilization in serum as in the case of fluid-phase liposomes L-PEG\textsubscript{10} (compare Figs. 5b and 6a). In PBS, the course of calcein leakage from DSPC-liposomes coated with different quantities of PEG was almost identical. When DSPC matrix was supplemented with cholesterol (45 mol%), the patterns of calcein release changed dramatically, both in PBS and in serum (Fig. 6b). Except for the biphasic leakage of calcein from liposomes with 2 mol% PEG-PE in serum (up to 38% of the maximum by the end of incubation), cholesterol-containing liposomes showed good stability. However, even most stable liposomes with 10 mol% PEG-PE showed approximately 0.1 CR value during 4-h incubation in serum (Fig. 6b). Studies of the prodrg retention in these liposomes using
Per-MlphDG analogue revealed not only decrease of the fluorescence intensity by 20.8 and 29.1% after 90-min and 4-h incubation, respectively, in the peak of liposomes (demonstrating the formation of protein corona and presumably with approximately the same density as in the case of liposomes L-GM\textsubscript{12} and L-CMG\textsubscript{10}) but also fluorescence growth in the course of elution of smaller complexes and micelles (see Supplementary material, Fig. 6Sa). The latter pattern was not observed in the case of incubation in PBS (ibid, Fig. 6Sb). Therefore, these liposomes lose a small part of the prodrug in serum with time.

To exist stably, liposomes must have a tightly packed and dehydrated (compressible) lipid bilayer. The LO phase, generated by cholesterol present in the bilayer at a concentration of not less than 33% \([46]\), produces the most condensed, tightly packed bilayer devoid of the phase transition. The stabilizing effect of high molecular weight PEG chains (2000 Da or more) is due to dehydration of the lipid bilayer \([47]\).

The authors \([45]\) showed that packing and compressibility of lipid bilayer reach the maximum values at 7±2 mol% PEG-DSPC independently of the membrane phase state of the nanosized unilamellar liposomes composed of the mixtures of matrix lipids—ePC or hydrogenated soy PC (HSPC, \(T_m\) 52 °C)—with cholesterol (40 mol%) and PEG-lipid. However, packing characteristics of the bilayer in HSPC-liposomes devoid of cholesterol, at any concentration of PEG-lipid investigated (0–30%), approached those of the micellar phase. Thus, PEG-containing HSPC-based liposomes should be the least stable. Similarly to DSPC, which is known to form gel-phase bilayers in a wide range of PEG2000-DSPC concentrations (20–60 mol%) \([48]\), HSPC-liposomes have SO bilayers. Our data obtained for pegylated liposomes support the conclusion that while LO phase supplemented with sufficient amount of PEG-lipid generates the most stable liposomes, SO phase-based pegylated liposomes are the least stable.

### 4. Conclusion

Our results show that the best stabilization of nanosized liposomes with fluid lipid bilayer is achieved by incorporation of ganglioside GM\textsubscript{1} or lipidic conjugate of oligopeptide, CMG-PE, in liposome membrane. Even small amounts (2 mol%) of these molecules can protect liposomes from the destructive action of blood plasma proteins for at least 24 h. When the content of these amphiphiles in the membrane increases (up to 10 mol%), water-soluble drugs encapsulated in the internal volume of liposomes can leak through widened water channels, or cracks, formed in the bilayer due to interactions of proteins with bulky negatively charged residues of amphiphiles protruding outwards. If the drug is in the form of a lipophilic conjugate anchored in the membrane of liposomes, it is unlikely that it will leave the matrix of the lipid bilayer. The inclusion of phosphatidylinositol (10 mol%) ensures stabilization of the liposomal formulation in serum only for the first 4 h, which, however, may be significant in terms of therapeutic efficacy. Among the three amphiphiles—CMG-PE, GM\textsubscript{1}, and PI—the conjugate of acidic oligopeptide shields the surface of the liposomes from the adsorption of plasma proteins the best (according to FRET analysis data). The pronounced positive effect of the inclusion of ganglioside GM\textsubscript{1}, a natural molecule, into liposomes has been already validated in vivo (for example, \([11,33]\)). In the case of lipopeptide CMG-PE, further biological experiments are required.

The presence of PEG-lipid conjugate in the membrane of fluid-phase liposomes promotes exit of the bilayer components, presumably through micelle formation. This can result in both loss of a water-soluble drug and a lipophilic (pro)drug. Pegylated gel-phase membranes are even less stable in serum as compared to the fluid-phase ones. In accordance with known data and argumentation, cholesterol-containing liquid ordered bilayers supplemented with sufficient amounts of PEG-lipid show good stability in serum. Nevertheless, the feasibility to apply such a composition of lipids when incorporating drugs in the lipid bilayer remains in question. It is not only that a small leak of active pharmaceutical ingredient can occur, which we observed on the example of a lipophilic produg. It is equally important that such tight packing of the lipid bilayer can prevent the inclusion of extraneous molecules and reduce the payload of liposomes. In the case of MlphDG this was not an obstacle due to the appropriate molecular structure of the produg.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.colsurfb.2018.02.061.

### References


Analysis of the effects of liposomal drug delivery systems on tumor microenvironment.


Abstract

The tumor microenvironment (TME) is a complex and heterogeneous environment that plays a critical role in the development and progression of cancer. Liposomal drug delivery systems have been widely studied for their potential to improve the therapeutic efficacy of anticancer drugs by targeting the TME. In this review, we summarize recent advances in the development of liposomal drug delivery systems that have the potential to modulate the TME and improve the therapeutic outcome of cancer treatment.

Keywords: Tumor microenvironment; Liposomal drug delivery systems; Cancer therapy; Antitumor effects; Therapeutic outcomes.