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Interactions of Liposomes Carrying Lipophilic Prodrugs in the Bilayer with Blood Plasma Proteins

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Abstract—Interactions of 100-nm liposomes prepared from egg yolk phosphatidylcholine and baker's yeast phosphatidylinositol carrying diglyceride ester conjugates of melphalan (Mlph-liposomes) and methotrexate (MTX-liposomes) in the bilayer with blood plasma proteins were studied with Western blotting. Earlier hemocompatibility tests have demonstrated that the liposomes did not affect main blood cells, but MTX-liposomes, and not Mlph-liposomes, induced complement (C) activation in vitro. Here, we show that introduction of polyethylene glycol conjugate (instead of phosphatidylinositol as a stabilizing lipid) or a targeting carbohydrate conjugate has little effect on interaction of Mlph-liposomes with major C components and apolipoproteins, as well as the total protein binding ability of the liposomes. Liposomes loaded with Mlph prodrug did not trigger fragmentation of C3 protein, the central component of the complement, while MTX-liposomes did so, which agrees with our previous findings. Analysis of MTX-liposome binding with C3 protein and its fragments, regulatory C proteins, and immunoglobulins allowed for the conclusion that MTX-liposomes activate complement via the alternative activation pathway. As shown earlier, the decrease in the prodrug concentration in the bilayer to the level corresponding to MTX low-dose treatment regimen allows avoiding C activation.

Keywords: nanomedicine, liposomes, melphalan, methotrexate, lipophilic prodrugs, blood plasma, protein corona, complement activation pathways

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INTRODUCTION

Liposomal drug delivery systems hold the leading position in the area of nanomedicine [1]. Due to their inherent low toxicity, they are suitable for intravenous injection, which is especially important for chemotherapy of metastasizing tumors. However, upon contact with blood liposomes, as well as other nanoparticles, are rapidly covered with a complex layer of proteins, lipids, and other plasma components [2]. According to the proteomic analysis data, functionalized silicon dioxide or polystyrene nanoparticles become covered with a specific "protein corona" already 30 s after the contact [3]. The corona modulates physicochemical properties at the surface of the nanocarrier and defines its behavior in bloodstream, that is, ultimately it determines pharmacokinetics and biodistribution of the encapsulated drug [4]. Therefore, the complex formed by the nanoparticle and the surface-bound proteins becomes the true drug delivery system.

The composition of the protein corona depends on such properties of the nanoparticle surface as curvature, charge distribution, and functional groups. The effect of the corona on the possibility of targeted delivery of nanoparticles, as well as the corona involvement in the body response to nanoparticle administration, is being intensively studied [5, 6]. Surface steric shielding with highly hydrated polymers, such as polyethylene glycol (PEG), has been shown not to prevent nonspecific protein binding; moreover, it may cause immunogenicity of nanoparticles [7–9]. Through opsonization of the carrier particle with the complement (C) proteins, protein corona promotes nanoparticle recognition by receptors of immunocompetent cells.

Nanoparticles resemble human pathogenic viruses not only in size, but also surface structure, since molecular templates and repeating elements can be formed thereon. Therefore, all nanoparticles, including those prepared of nonimmunogenic starting molecules, are expected to be targets of the innate immune system, primarily the complement [10, 11]. Hypersensitivity reactions of varying severity, including anaphylaxis, associated with C activation have been noted in

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Abbreviations: C, complement; DG, diglyceride; fH, complement factor H; HSA, human serum albumin; L, liposomes; Mlph, melphalan; MTX, methotrexate; P_B, protein binding; PBS, phosphate buffered saline, pH 7.4; ePC, egg phosphatidylcholine; PEGPE, PEG2000-dioleoylphosphatidylethanolamine; PI, phosphatidylinositol; $SiaLe^X$-DG, diglyceride conjugate of the $SiaLe^X$ tetrasaccharide.

many patients upon intravenous administration of liposomal drugs Doxil® (100-nm liposomes with doxorubicin encapsulated in the inner water volume stabilized with PEG chains covalently bound at the surface, the so-called Stealth® liposomes), Ambisome® (amphotericin B liposomes), Taxol® emulsion (paclitaxel-loaded micelles stabilized with a pharmacopoeial detergent Cremophor®EL) [12].

Earlier, we have elaborated liposomal formulations of an alkylating agent melphalan and a folic acid antimetabolite methotrexate [13, 14]—two drugs of utmost importance in oncology. The drugs are incorporated in a liquid lipid bilayer in the form of lipophilic prodrugs—diglyceride ester conjugates of melphalan (Mlph-DG) and methotrexate (MTX-DG) (Fig. 1)—to allow loading of therapeutically efficient quantities of drugs in the nanosized liposomes. The liposomal matrix is formed by egg phosphatidylcholine and baker's yeast phosphatidylinositol. For targeted delivery of the liposomes to angiogenic endothelium of tumors, liposomes can be equipped with a lipophilic conjugate of a carbohydrate selectin ligand $SiaLe^X$ ($SiaLe^X$-DG, Fig. 1). (Selectins are molecules of cell adhesion involved in tumor progression and metastasis considered to be a relevant drug target [15].)

We have demonstrated that upon intravenous injection to Lewis lung carcinoma model incorporation of a $SiaLe^X$ conjugate in liposomes with Mlph-DG provides their transport to tumor vessels and subsequent antivascular and antitumor effects, as well as the in vitro selective effect on activated endothelial cells of blood vessels [17].

In a panel of in vitro hemocompatibility tests, the liposomal formulations, including the targeted ones, did not affect human blood erythrocytes and platelets [18]. However, MTX-DG-bearing liposomes (MTX-liposomes) caused moderate impairment of the blood coagulation system and C activation: they induced C3a anaphylotoxin production and exhausted to a considerable extent C as a whole (judging by the blood serum residual hemolytic activity test results) [18]. We speculated that impaired functioning of coagulation and C cascades is due to interaction of the liposomes with the protein factors, as well as a lack of intact plasma proteins in plasma. For example, even an insignificant sorption of one of the coagulation factors on the surface of liposomes can lead to a shift of the equilibrium in the reactions of the cascade: plasma concentrations of coagulation factors V, VII (in activated plasma), and X are as little as 20, 10, and 200 nM, respectively, while concentration of fibrinogen, one of the major plasma proteins, is 9 μM [19].

Recently, we obtained primary data on differential binding of plasma proteins by liposomes loaded with Mlph-DG and MTX-DG: only MTX-liposomes bound C components, fragments of C3 factor, and factor H (fH) [20]. Total sorption capacity (the amount of bound protein, or protein binding, P_B) of the liposomes was rather high, which speaks for their rapid elimination from bloodstream [21, 22]. The current work expands our earlier findings.

We used liposomes loaded with Mlph-DG (Mlph-liposomes) to find out whether introduction of stabilizing components and the $SiaLe^X$ ligand influences total sorption capacity of the liposomes and binding with individual functionally important plasma proteins. Then, we studied binding of MTX-liposomes with the important C components as a function of plasma incubation conditions to elucidate how C is activated by the liposomes.

RESULTS AND DISCUSSION

To study the effect of lipid bilayer-stabilizing components and the target carbohydrate ligand on the total sorption capacity of liposomes loaded with Mlph-DG (Mlph-liposomes), samples of liposomes were prepared of egg phosphatidylcholine with phosphatidylinositol (PI), PEG-lipid, and $SiaLe^X$ conjugate (see Table 1). Earlier, incorporation of sufficient amount (at least 7 mol %) of PI in the lipid bilayer has been demonstrated to prolong liposome circulation in blood [23]. The decrease in liposome elimination by reticuloendothelial system cells is due to both the negative charge of the lipid with a bulky head group and steric shielding of the lipid bilayer with highly hydrated myoinositol residues at the surface [24]. Utilization of a natural phospholipid instead of PEG conjugate should minimize the adverse effects associated with immunogenicity of pegylated liposomes (see Introduction).

In our previous in vitro and in vivo studies, liposomal formulations contained 10 mol % PI [16–18]. According to the dynamic light scattering data (Table 1), all samples were liposomes of close diameters within the nanoscale range with low polydispersity. Therefore, the effect of particle size on interaction with plasma proteins can be excluded in practice.

To study plasma protein binding, Mlph-liposomes were incubated with 80% plasma for 15 min (in the same manner as in hemocompatibility tests having demonstrated the differences in the effects of Mlph- and MTX-liposomes on C and coagulation [18]). Then, liposome-containing fractions were isolated with gel permeation chromatography on Sepharose and total amount of protein was quantified with a modified Lowry technique. Typical elution profiles are presented in Figs. 2a and 2b. To control the efficiency of separation of liposome–protein complexes from the major fraction of unbound plasma proteins, plasma samples incubated with PBS were used as controls. The total amount of lipids in liposomes was determined by spectrophotometry measuring the concentration of Mlph-DG, the content of which in the bilayer is 10 mol % (molar ratio of starting lipid components is maintained in liposomes prepared by extru-

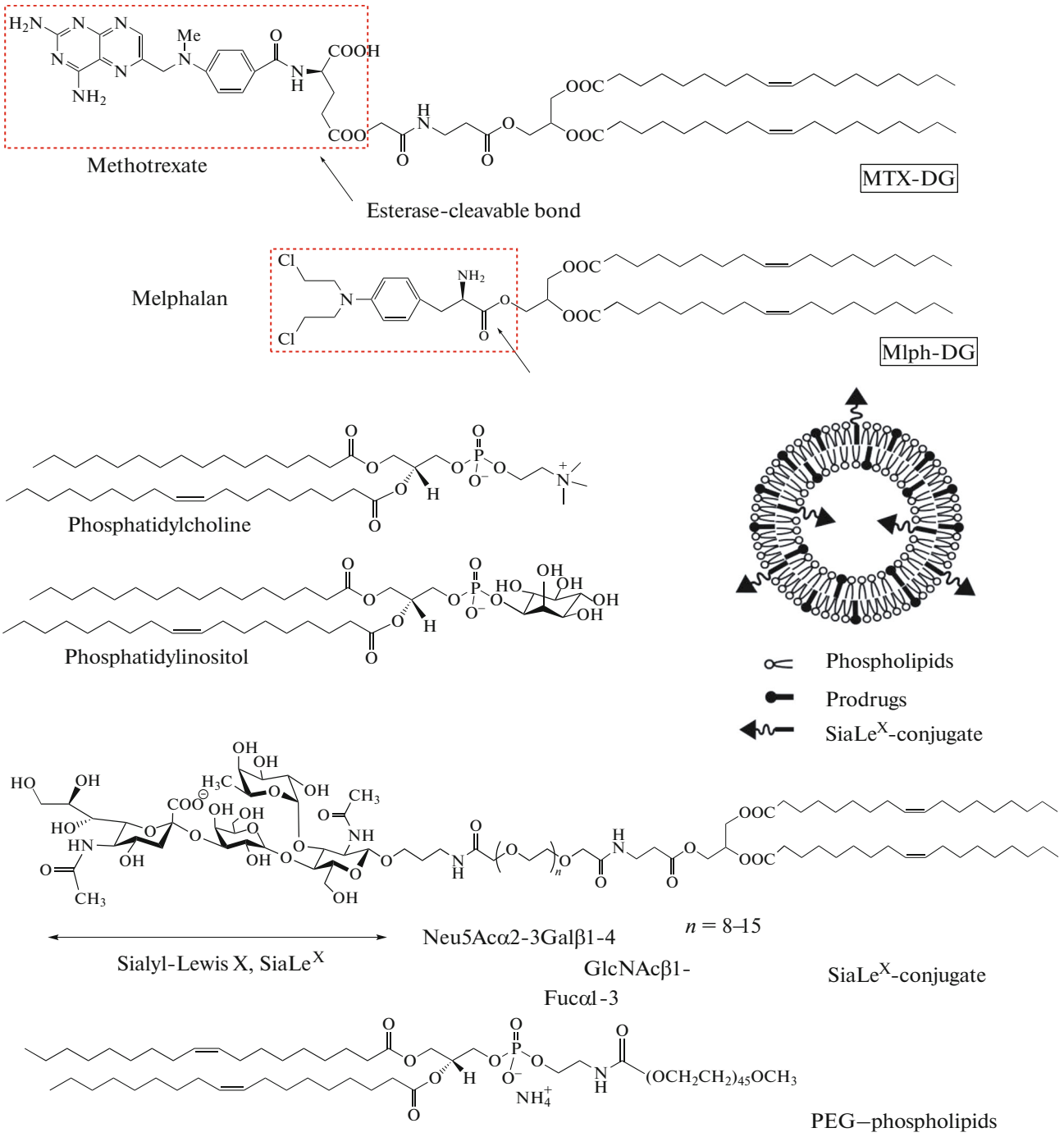

Fig. 1. Structures of lipophilic prodrugs Mlph-DG and MTX-DG, $SiaLe^X$ conjugate, PEG–phospholipid conjugate, and matrix phospholipids and a schematic representation of a liposome.

sion [13, 14]). Total sorption capacity P_B of liposomes was calculated as the ratio of total amount of bound protein to total amount of lipids in liposomes.

The P_B values thus obtained allow us to predict the rapid elimination of liposomes from the bloodstream, although the relevant data in the literature are ambiguous. As has been reported in an early review [21], the plasma half-life period of liposomes binding more than 50 g protein/mol lipids is only 2 min, while binding of less than 20 g protein/mol lipids results in a plasma half-life of 2 h. The former group of formulations comprises negatively charged liposomes containing up to 20% phosphatidic acid, phosphatidylserine, and cardiolipin, the latter group comprises neutral liposomes, for example, liposomes prepared from mixtures of phosphatidylcholine and cholesterol.

Table 1. Composition and size* of liposome samples with melphalan prodrug **Mlph-DG** (mlph-liposomes) and their total sorption capacity

Sample	Composition, mol/mol	D, nm	$H_{1/2}$, nm	PDI	P_B**
Mlph-L	ePC–PI–Mlph-DG, 8 : 1 : 1	140.6	37.7	0.072	59.9 ± 9.9
Mlph-L-SiaLe^X	ePC–PI–Mlph-DG–SiaLe^X-DG, 8 : 1 : 1 : 0.2	133.3	31.4	0.065	59.8 ± 1.9
Mlph-L-PEG\PI	ePC–Mlph-DG–PEG-PE, 9.8 : 1 : 0.2	116.5	26.6	0.052	67.2 ± 6.2
Mlph-L\PI	ePC–Mlph-DG, 9 : 1	126.3	31.3	0.061	59.4 ± 2.6

* Dynamic light scattering on a Brookhaven 90PLUS Particle Size Analyzer, Brookhaven Instruments: D, average diameter; $H_{1/2}$, half-height half-width of the peak; PDI, polydispersity index.

** Liposomes were incubated in 80% plasma for 15 min: P_B, bound plasma protein, g/lipids, mol. Average values of two independent experiments are reported.

Negatively charged liposomes containing phosphatidylglycerol and phosphatidylinositol are characterized by intermediate P_B values and circulation time. In our case, all liposomes except for the sample without PI (**Mlph-L\PI**; zeta-potential was +18 mV), are negatively charged because of PI presence in the bilayer (zeta-potential of **Mlph-L** sample was –26 mV). At the same time, P_B values of positively charged **Mlph-L\PI** and negatively charged liposomes with PI, **Mlph-L** and **Mlph-L-SiaLe^X** (charge of the latter liposomes is augmented with ionized COOH groups of terminal sialic acid residues), are almost the same (Table 1). Obviously, interaction of liposomes with plasma proteins is determined not only by liposome charge, but also structure of the liposome surface, which is mainly formed by polar head groups of lipids.

Our data evidence the insufficiency of knowledge of the tendencies of the liposome interaction with plasma proteins. There are methodological discrepancies between different works, for example, concerning the methods to determine the total amount of bound protein. Moreover, the prognostic value of P_B is rather relative. For example, recently, compositions of protein corona formed in murine and human blood sera were shown to differ considerably by the composition and amount of components [25], while in the above-cited review [21], pharmacokinetics data of liposomes of varying composition are compared upon intravenous administration to mice.

Pegylation (covering with PEG chains) is known to decrease the possibility of protein interaction with surface of nanoparticles, including liposomes (for example, [1, 6]). Nevertheless, liposomes containing PEG–lipid conjugate as a stabilizing agent instead of PI (**Mlph-L-PEG\PI**; Table 1) exhibited the highest level of plasma binding exceeding the average P_B value of other samples in the series by approximately 10% (Table 1). Fluid phase of the lipid bilayer, which is provided by the matrix egg phosphatidylinositol (ePC), can promote extraction of PEG–lipid conjugate into the aqueous phase under the incubation conditions. In the course of circulation, even liposome prepared from "solid-phase" lipids (distearoylphosphatidylcholine–cholesterol, 55 : 45) loose PEG2000-dioleoyl-

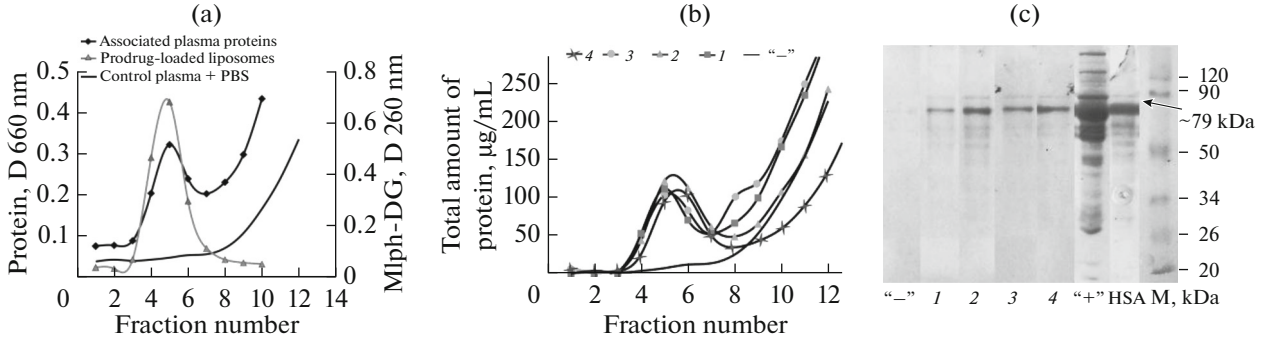

Fig. 2. (a) Elution profiles of liposomes loaded with Mlph-DG and proteins upon isolation via gel permeation chromatography (GPC) on a Sepharose CL-4B column after liposome incubation in 80% plasma for 15 min at 37°C; Mlph-DG was determined by UV spectrophotometry and protein, using modified Lowry procedure. (b) Comparison of protein elution profiles upon liposome incubation in plasma and those of control sample after plasma incubation with PBS. (c) Separation of proteins associated with liposomes with SDS-PAGE according to Laemmli (samples contained equal amounts of lipids until delipidization was performed prior to electrophoresis): "–", negative control, human blood plasma upon incubation with PBS, GPC, and delipidization, that is, treated the same way as liposomes; (*1*) **Mlph-L**; (*2*) **Mlph-L-SiaLe^X**; (*3*) **Mlph-L-PEG\PI**; (*4*) **Mlph-L\PI**; "+", positive control, plasma diluted 1/100; M, molecular weight marker. See Table 1 for sample description.

phosphatidylethanolamine (PEG-PE) faster than PEG2000-distearoylphosphatidylethanolamine [26]. Apparently, liposome surface shielding requires much more than 2 mol % of PEG-PE (Doxil liposomes contain 5 to 10 mol % of PEG2000-distearoylphosphatidylethanolamine). It is desirable that the PEG–lipid conjugate should contain saturated acyl chains.

To establish the differences in composition of protein coronas formed by different Mlph-liposomes (Table 1), standard denaturing PAGE was performed. The pattern produced by non-specific silver reagent staining is presented in Fig. 2c. Blood plasma incubated with PBS and subjected to gel filtration and delipidization, as well as plasma diluted 100 times with PBS immediately before PAGE (lanes "–" and "+", respectively, in Fig. 2c), were used as controls.

As expected, all samples are dominated by the band corresponding by molecular weight to HSA (~66 kDa, lane HSA). The protein is usually detected in large amounts in complexes with liposomes since it is the most abundant one in plasma (≥55% of total plasma protein) even if its affinity to liposome surface is not high [27] (with time, it can be forced out of the corona with proteins characterized by lower plasma concentration but higher affinity to liposomes surface—this phenomenon is known as the Vroman effect [28]). Also, the band at ~79 kDa is present in all samples at equal amounts; it may evidence binding of the β-subunit of the C3 complement component, the α-subunit of the C4b-binding protein, or one of the immunoglobulin M chains. Among bands in the range of 50–60 kDa, fibrinogen monomer (55 kDa) is likely to be present—it is another highly abundant protein that is subjected to the Vroman effect.

We were most interested in C components involved in liposome surface opsonization and elimination from blood stream, as well as components responsible for hypersensitivity reactions. The central C component is the C3 protein comprising two polypeptide chains of α- and β-subunits (113 and 75.5 kDa, respectively) [29] (Fig. 3). Activation of the C cascade via one of the three major pathways leads to formation of C3 convertases, which cleave the small C3a fragment (9 kDa, anaphylatoxin) from C3 yielding the C3b opsonin (α'- and β-chains, 104 and 76 kDa, respectively). The presence of C3 fragments indicates C activation. Normal C3 blood concentration is 0.8–1.8 mg/mL plasma (approximately 3 to 7 μM). Such level allows tracing changes of the protein with immunoblotting.

Plasma concentration of factor H (fH) of the alternative C activation pathway is also rather high, 100–500 μg/mL. This regulatory glycoprotein (150 kDa) acts together with factor I as a cofactor inactivating the C3b opsonin through cleaving fragments C3f, C3g, and C3d from the α-chain (Fig. 3), which inhibits C activation. Binding of fH to C3b, along with the protective effect of membrane-bound regulators, leads to inactivation of the C cascade on the surface of the organism host cells [30]. Therefore, maintenance of fH secretion (by liver, as well as epithelial and endothelial cells and platelets) is required for suppression of opsonization of the organism host cells caused by the presence of a certain amount of C3b in blood [31]. The iC3b2 fragment thus formed is not capable of Bb fragment binding (is not shown in Fig. 3) to form a C3 convertase (however, when bound on pathogen surfaces it becomes an active opsonizing agent).

Regulation (inhibition) of the classic C activation pathway occurs under the effect of C4b-binding protein (C4BP) homologous to factor H. The most abundant form of a multimer glycoprotein C4BP comprises seven α-subunits (75 kDa) and a single β-subunit (45 kDa) [29]; C4BP is secreted by the liver, its plasma concentration is 200 μg/mL. C4BP can act both individually and together with factor I causing dissociation of C3 convertase of the classical pathway or inhibiting its formation through C4b hydrolysis.

Immunoblotting with antibodies to C proteins C3, factor H, and C4BP revealed that all Mlph-liposomes bind the C3 component (Fig. 4a). However, C activation is not observed: α'-chains of C3b (103 kDa) and fragments of its further cleavage are absent from the blot (Fig. 3); only α- and β-subunits of uncleaved C3 protein (~118 and 76 kDa, respectively) can be identified. A small amount of bound fH inactivating the C cascade was also present in complexes with liposomes of the series (Fig. 4b).

Recently, in in vitro experiments factor H has been shown to efficiently prevent C activation initiated by the effect of micellar and liposomal drug formulations (such as rituximab, Cremophor®EL, and Ambisome®); this opens prospects of fH application in clinics to prevent the development of hypersensitivity reactions [32].

C4b-binding protein was identified by its heaviest subunit α (75 kDa). The C4BP regulatory protein was much more concentrated on surfaces without PEG–lipid or $SiaLe^X$ conjugates (Fig. 4c). Apparently, PEG chains and tetrasaccharide ligand residues, also containing an oligoethylene glycol spacer, shield liposomes from the protein binding. In the work [20], considerable binding of C4BP was established for both Mlph-liposomes (corresponding to **Mlph-L** of the current work) causing no C activation and liposomes loaded with MTX-DG triggering C activation in vitro [18]. One may suppose that this regulatory protein does not affect C functioning in the presence of these liposomes.

Apolipoproteins are potentially capable of affecting pharmacokinetics and biodistribution of lipid complexes. Results of immunoblotting with anti-ApoAI (28 kDa) antibodies are presented in Fig. 5a. A very weak band is observed for **Mlph-L** liposomes with phosphatidylinositol, while other samples bind ApoAI even less. This apolipoprotein is a major structural

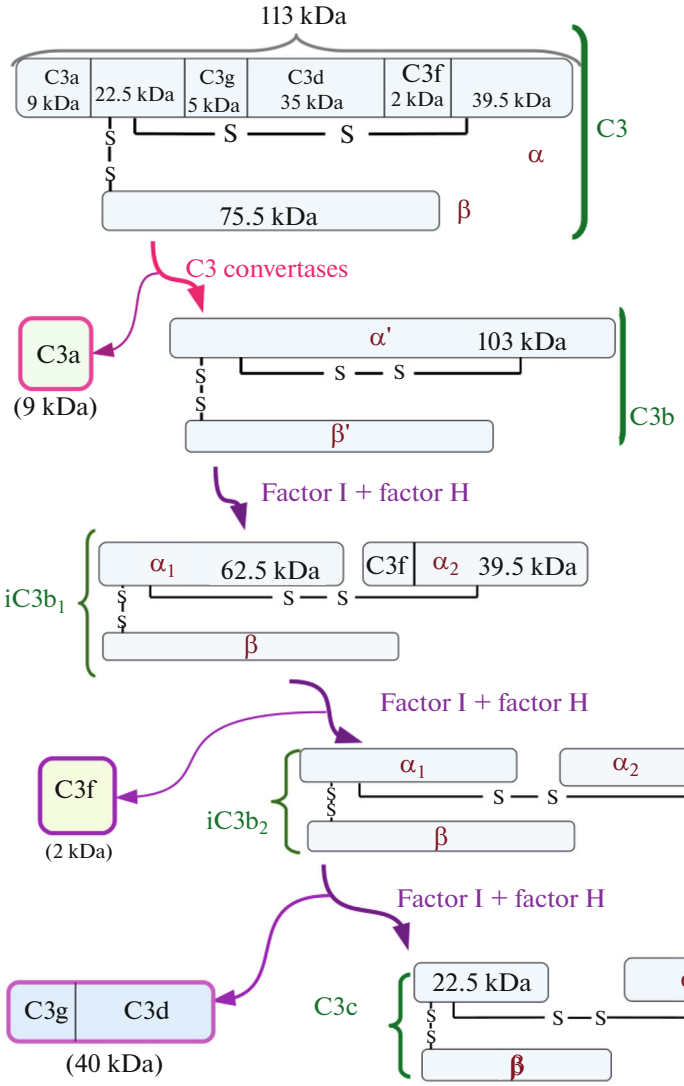

Fig. 3. Schematic representation of structure and transformations of the C3 component of complement.

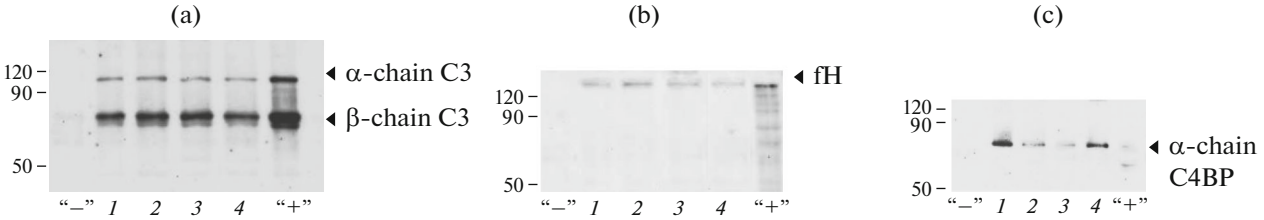

Fig. 4. Identification of proteins associated with Mlph-liposomes by immunoblotting using antibodies to the complement components C3 (a), factor H (b), and C4b-binding protein (c): "–", negative control, human blood plasma upon incubation with PBS, GPC, and delipidization; (*1*–*4*) liposome samples **Mlph-L** (*1*), **Mlph-L-SiaLe^X** (*2*), **Mlph-L-PEG\PI** (*3*), and **Mlph-L\PI** (*4*) after 15-min incubation with human blood plasma, isolation of liposome–protein complexes, and delipidization; "+", positive control, plasma diluted 1/500.

component of high-density lipoproteins; normally, its plasma concentration reaches 3 mg/mL. ApoAI activated lecitin-cholesterol acyltransferase, which governs formation of cholesterol esters providing for reverse transport of the lipid from peripheral tissues to liver. ApoAI is involved in destabilization of lipid

Fig. 5. Identification of proteins associated with Mlph-liposomes by immunoblotting using anti-ApoAI (a) and anti-ApoE (b) antibodies. For designations, see caption to Fig. 4.

buildups in the bloodstream, with amphipathic helical domains of the protein hardly penetrating fluid (with low temperature of phase transition) lipid bilayers made of phospholipids with unsaturated acyl residues [33]. The presence of cholesterol in liposomes promotes ApoAI binding with the lipid bilayer [34]. Since merely an insignificant concentrating of plasma ApoAI occurs on liposomes (Fig. 5a, lanes *1* and "+"), one may conclude that the protein is not involved in liposome elimination from the bloodstream.

In contrast to data on ApoAI, blot with anti-apolipoprotein E (ApoE) antibodies demonstrated binding of the protein with all samples of the Mlph-liposome series (Fig. 5b), although ApoE concentration is two orders of magnitude lower, approximately 40 μg/mL (this is why it was not detected in control plasma at 1/500 dilution, Fig. 5b, lane "+"; ApoE was detected in donor plasma at 1/100 dilution, data not shown).

A similar picture was observed for liposomes loaded with MTX-DG [20]. ApoE (34 kDa) is the major component of chylomicrons and intermediate-density lipoproteins. It has high affinity to low-density lipoprotein receptors and one may suppose that liposomes will be actively captured by tumor cells, which exhibit elevated expression of the receptors to consume structural lipids. However, according to the authors of [33], neither ApoE, nor the low-density lipoprotein receptors play a considerable role in the catabolism of liposomes made of phosphatidylcholine.

Therefore, the results agree with the data on the absence of C activation under the effect of liposomes loaded with Mlph-DG [18] and show a picture of interaction with C components in more detail than in the initial study [20]. Indeed, in the work [20] Mlph-L liposomes did not exhibit binding to the C3 component and factor H inhibiting C activation. Such a contradiction can be explained by methodological issues and sensitivity limits of blot analysis or individual properties of donor plasma. Our results also show that incorporation of stabilizing components (PI or PEG–lipid conjugate) or a targeting carbohydrate ligand ($SiaLe^X$ conjugate) in the bilayer insignificantly affects both interactions with the above-mentioned C components and apolipoproteins and total sorption capacity of liposomes. A pronounced effect was only observed with respect to the C4BP protein: PEG–lipid or $SiaLe^X$ conjugate shields liposomes from binding this regulatory protein, an inhibitor of the classic C activation pathway (Fig. 4c).

Our next goal was to study the interactions of liposomes loaded with a methotrexate prodrug MTX-DG (MTX-liposomes) with C components to elucidate the possible C activation pathways. To follow the dynamics of protein binding, liposomes were incubated in 80% plasma for 5 and 30 min, time periods sufficient for complete development of interactions with C proteins [35]. Another sample of MTX-liposomes was incubated for 30 min in plasma subjected to preliminary 10-min incubation with ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetate (EGTA; 10 mM) and Mg^{2+} (2.5 mM) ions as described in [35]. The chelating effect of EGTA is manifested mainly on calcium rather than magnesium ions. The presence of EGTA and magnesium in a system promotes inhibition of classical C activation pathway through binding of Ca^{2+} ions, which are present in physiological medium and are necessary for C1 protein functioning. EGTA not only completely blocks C activation via the classical pathway, but also decreases the efficiency of activation via the alternative pathway, which depends on magnesium ions Mg^{2+}. When Mg^{2+} ions are added to the chelating agent, the effect produced on the alternative pathway is negligible [36].

The pattern of nonspecific gel staining upon electrophoresis of proteins bound to MTX-liposomes is presented in Fig. 6a. Albumin is associated with the liposomes at large amounts, similar to liposomes loaded with Mlph-DG (Fig. 2c). In addition, in all samples of the second series there is a band corresponding to a molecular weight of ~79 kDa, which most likely represents the β-subunit of the C3 component. Intensity of the protein binding depended on the incubation time and the presence of EGTA/Mg^{2+}, which considerably inhibits C3 binding, in the medium (Fig. 6a, lanes *1–3*). Importantly, after a 5-min incubation C3 concentrated on the surface of MTX-lipo-

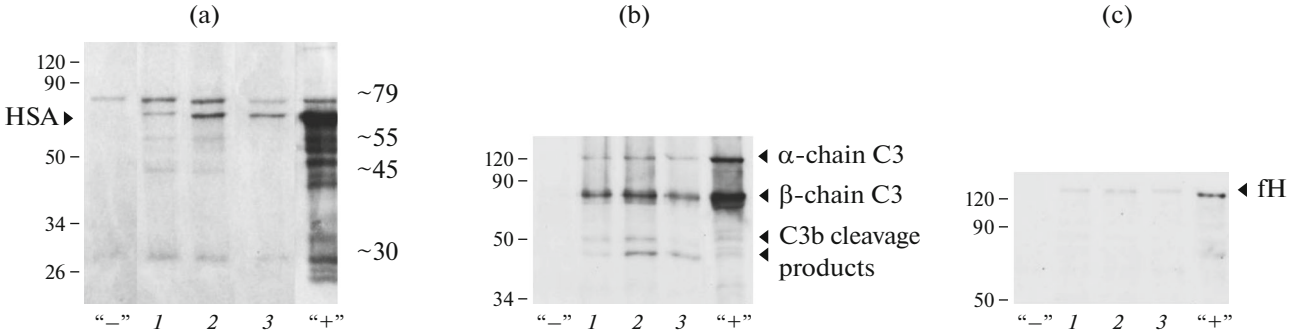

Fig. 6. (a) Separation of proteins associated with MTX-liposomes (MTX-L) using Laemmli electrophoresis: "–", negative control, human blood plasma upon incubation with PBS, GPC, and delipidization; MTX-L incubated with plasma for 5 (*1*) and 30 min (*2*); MTX-L incubated with plasma for 30 min after preincubation with the EGTA/Mg^{2+} inhibitory mixture for 30 min (*3*); "+", positive control, plasma diluted 1/500. After incubation with plasma, samples (*1–3*) were subjected to GPC to isolate liposome–protein complexes and delipidization. Identification of proteins using anti-C3 (b) and antifactor H (c) antibodies.

somes to a greater extent than in the case of a 15-min incubation with Mlph-liposomes, judging by relative intensities of C3 and albumin bands (Fig. 2c, lanes *1–4*). Among the bound proteins (Fig. 6a) there were proteins with weight corresponding to that of fibrinogen (approximately 55 kDa) and fragments of the α'-chain of the C3b component (approximately 45 and less than 30 kDa).

Immunoblotting results allowed us to conclude that with time MTX-liposomes adsorb more C3 protein represented by α- (118 kDa) and β-subunits (79 kDa) (Fig. 6b, lanes *2* and *3*). At the same time, prolonged incubation increased the number of fragments of this protein (~52 and ~46 kDa; the former fragment is presumably the α1 chain of C3b). The rise of the fragments is explained by degradation of C3b in the presence of factors I and H (Fig. 3), the latter being also detected in complexes with liposomes (Fig. 6c).

At the same time, concentration of the regulatory fH on the surface of liposomes compared to plasma is much less pronounced here than in the case of liposomes loaded with Mlph-DG (Fig. 4b) although greater electronegativity of the MTX liposome surface compared to Mlph-liposomes (zeta potential values are –53 and –34 mV, respectively [18]) should promote nonspecific binding with fH similar to polyanionic structures on the cell surface, such as sialic acid, heparan sulfates, and glycoseaminoglycans [30]. Consequently, the possibility of inhibition of the C activation signal via the alternative pathway under the effect of factor H adsorbed on surface of MTX-liposome decreases. Intensification of the band corresponding to ~46 kDa (Fig. 6b, lane *2*) evidences more complete cleavage of C3b according to the scheme presented in Fig. 3 and accumulation of degradation fragments with similar molecular weights: an α'-subunit fragment of 41.5–43 kDa; C3gd of 35–40 kDa; and α2 of 39.5–40 kDa, according to the data from various sources [29, 35]. Addition of the EGTA/Mg^{2+} pair inhibiting the classical pathway of C activation considerably decreases the amount of C3 protein associated with the liposomes and, which is more important, prevents its degradation (Fig. 6b, lane *3*).

Immunoblotting also demonstrated an increase in immunoglobulin G bound to MTX-liposomes with time and inhibition of the process in the presence of EGTA/Mg^{2+} (~63-kDa band, Fig. 7a). The result implies the presence of certain functional groups or sites on the liposome surface for which binding with IgG depends on the presence of calcium ions in the medium. Formation of complexes with IgG as such promotes C activation via the classical pathway, however, this class of immunoglobulins can cause activation via the alternative pathway upon binding of IgG α-chain with C3 protein [37].

Binding of immunoglobulins M to MTX-liposomes also increased upon increase of incubation time, however, the addition of the inhibitor pair EGTA/Mg^{2+} produced no effect on the process (Fig. 7b, lanes *1–3*). Therefore, formation of such immune complexes should not lead to C activation via the classical pathway, which requires Ca^{2+} ions. Along with the participation in C cascade triggering via one of the pathways, association with IgM can promote accelerated blood clearance (ABC) of liposomes typical of pegylated liposomes and other PEG-bearing nanoparticles [38].

The ABC phenomenon describes the change in the pharmacokinetics of the second dose of a drug: the first injection induces secretion of specific IgM antibodies in spleen which then bind PEG thus promoting C activation and elimination of the nanoparticles from bloodstream by liver macrophages. The extent of the phenomenon depends on the dose and properties of the carrier, including size and charge, as well as density and length of PEG chains; the ABC effect is partially alleviated within 2 weeks [39].

C activation by liposomes of different composition has been poorly studied. In earlier works, negatively

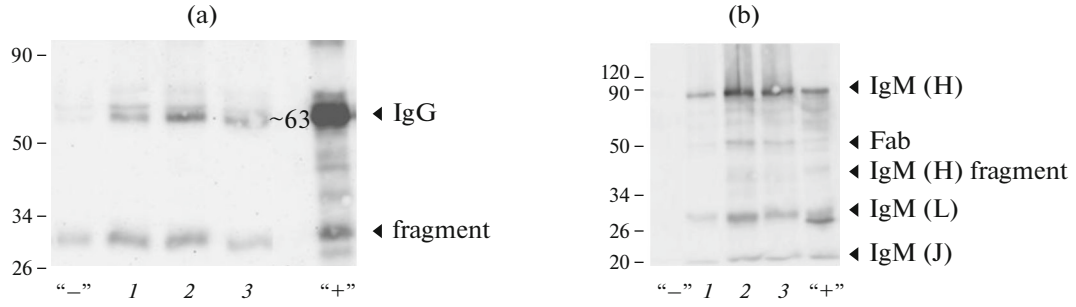

Fig. 7. Identification of proteins associated with MTX-liposomes using anti-IgG (a) and anti-IgM (b) antibodies. (a) The band at ~29 kDa may be referred to the IgG light chain, Fv-fragments, or reduced Fad fragment. (b) In addition to the heavy (H ~83 kDa), light (L ~31 kDa), and J (~20 kDa) chains of IgM, bands of Fab fragments (~52 kDa) and H chain fragments (~40, 44, and 48 kDa, according to [51]) are detected. For designations, see caption to Fig. 6.

charged liposomes have been shown to activate C via a classical pathway and through direct interactions of the C1q component with anionic lipids; on the contrary, positively charged liposomes activate C via the alternative pathway (see [40] for review). The reactivity of negatively charged MTX-liposomes is most likely due to deformations in liposome surface structure introduced with bulky methotrexate residues exposing a pair of exocyclic aromatic amino groups of the residues and a free α-COOH group. Amino (and hydroxy) groups arranged in a specific manner on the surface of liposomes can cause C activation through a nucleophilic attack on the inner thioester bond of the C3b fragment [41] leading to acceleration of spontaneous hydrolysis of the C3 component and activation of the C activation alternative pathway [42]. In general, our results evidence triggering of an alternative C activation pathway under the effect of MTX-liposomes: firstly, binding of factor H (inhibitor of the alternative pathway) to the liposomes decreased compared to Mlph-liposomes (which do not activate C); secondly, formation of complexes of liposomes with IgM did not depend on the presence of calcium ions in the medium; finally, as previously established [20], the level of C4BP (inhibitor of classic C activation pathway) binding was rather high for both MTX- and Mlph-liposomes. According to authors of a recent publication [43], an alternative C activation pathway plays the key role in recognition of nanosized complexes in human plasma.

EXPERIMENTAL

Materials. Diglyceride conjugates of melphalan [44], methotrexate [45], and tetrasaccharide $SiaLe^X$ (Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ) [46] were synthesized as described previously; $SiaLe^X$ 3-aminopropylglycoside was kindly provided by G.V. Pazynina (Institute of Bioorganic Chemistry, Russian Academy of Sciences). Egg yolk phosphatidylcholine (ePC) and phosphatidylinositol (PI) of *S. cerevisiae* were obtained from Reakhim (Russia), PEG2000-dioleoylphosphatidylethanolamine (Avanti Polar Lipids, United States), Sepharose CL-4B (Pharmacia, United States), ehtylenediaminetetraacetic acid (EDTA), ethyleneglycol-bis(2-ethylamino)tetraacetate (EGTA), and other reagents were from Sigma and Flow Laboratories (United States). Solvents were purified using standard protocols; solvents were evaporated under vacuum at temperatures below 40°C. Buffers were supplemented with 1 mM EDTA: PBS, pH 7.05, phosphate-buffered saline (KH_2PO_4, 0.2 g/L; $NaH_2PO_4 \cdot 2H_2O$, 0.15 g/L; Na_2HPO_4, 1.0 g/L; KCl, 0.2 g/L; NaCl, 8.0 g/L).

Preparation of liposomes. Liposomes composed of **ePC–PI–MTX-DG/Mlph-DG**, 8 : 1 : 1 (mol), were obtained by extrusion through calibrated polycarbonate nuclear filters as previously described [13, 14]. Mixtures of lipids and prodrugs at the relevant ratio in chloroform were evaporated in round-bottom tubes on a rotary evaporator. The mixtures contained 7.5 mg ePC, 1 mg PI, and 1 mg Mlph-DG for the **Mlph-L** samples; 0.5 mg $SiaLe^X$ conjugate was added in the **Mlph-L-SiaLe^X** sample. Liposomes without PI contained 1 mg Mlph-DG and 8 mg ePC (**Mlph-L\PI**) or 9 mg ePC and 0.7 mg PEGPE (**Mlph-L-PEG\PI**).

To prepare liposomes loaded with MTX-DG, mixture of 2.8 mg MTX-DG, 14.9 mg ePC, and 2.1 mg PI was prepared. Lipid films were dried at 7 Pa (INEI-4 freeze-drier by Institute for Biological Instrumentation, Russian Academy of Sciences, Russia) for 40 min, then hydrated for 2 h at room temperature in PBS with 1 mM EDTA: buffer volume for Mlph-DG-containing samples was 0.3 mL and for those with MTX-DG, 0.6 mL. The suspension was shaken, subjected to 10 cycles of freezing/thawing (liquid nitrogen/+40°C water bath), and passed through polycarbonate membrane filters (Nucleopore, United States) with 200- and 100-nm pores sequentially using a Mini-extruder (Avanti Polar Lipids, United States). The concentration of prodrugs in dispersions was determined after destruction of liposomes with 20-fold dilu-

tion in ethanol: UV spectra were registered and optical density at absorbance maximum (MTX-DG: λ_{max} 302 nm, ε ~25000; Mlph-DG: λ_{max} 258 nm, ε ~19700) was measured on an SF-256-UVI (Lomo Fotonika, Russia) double-beam spectrophotometer. The size of liposomes loaded with Mlph-DG was controlled by dynamic light scattering on a Brookhaven Particle Analyzer 90+ (Brookhaven Instruments Corp., United States; helium–neon laser, λ 633 nm, 90° scattering angle); the results are reported in Table 1. According to measurements on a DynaPro Titan TC (Wyatt Technologies, United States; helium–neon laser, λ 830 nm, 90° scattering angle), diameter of MTX-DG liposomes was in the range of 100–105 nm.

To determine the zeta-potential, samples of 200-nm liposomes loaded with Mlph-DG were prepared in 1 mM potassium–phosphate buffer containing 10 mM KCl and 1 mM EDTA with total lipid concentration of 1.0 mg/mL; measurements were performed on a ZetaPALS (Brookhaven Instruments Corp., United States) instrument.

Incubation of liposomes with plasma and isolation of liposome–protein complexes. Blood of four healthy donors was collected in Vacuette® EDTA tubes (Greiner Bio-One, Germany). Plasma was separated by centrifugation for 10 min at 2000 g (Jouan BR4i, Thermo Fisher Scientific, United States). Supernatants were moved to fresh tubes and remaining platelets and other cells were separated by centrifugation for 30 min at 2000 rpm at room temperature (Sorvall RT 7 Plus, Thermo Fisher Scientific, United States). The supernatants were pooled, plasma was stored at +4°C and used within 2 days of collection. Control aliquots of plasma for electrophoresis were frozen in liquid nitrogen and stored at –70°C. Liposomes (90 μL) were incubated with 360 μL of plasma at 37°C under weak stirring in 1.5-mL Eppendorf (Germany) tubes for 15 min, if not stated otherwise. A sample of plasma diluted with buffer 1 : 4 was used as a negative control. The mixture was applied to a column with CL-4B Sepharose (~1.1 × 19 cm) and eluted with buffer; fractions of ~400 μL were collected. To aliquots of the fractions (80 μL), 400 μL ethanol was added; the mixtures were centrifuged for 10 min at 10000 rpm and the prodrug content was determined in supernatants by spectrophotometry. In parallel, 100 μL of each fraction were collected to determine protein content. Liposome–protein complexes were isolated at least twice for each liposome sample.

Total protein was determined with a modified Lowry [47] technique. Reagent C was prepared immediately before use: to 100 parts of reagent A (2% Na_2CO_3 + 0.4% NaOH + 0.26% $NaKC_4H_4O_6$ + 1% SDS in bidistilled water), 1 part of reagent B (4% $CuSO_4$ solution in bidistilled water), by volume, was added. To 100 μL of analyzed sample, 300 μL reagent C was added, the solution was mixed, and after 10 min 30 μL Folin–Ciacalteu reagent diluted with distilled water 1 : 1 was added. After 60 min, the optical density was measured at 660 nm. The control sample contained 100 μL PBS.

Delipidization of joint protein fractions and Laemmli SDS-PAGE. Delipidization was performed according to [48]. To 100 μL of joint fractions of liposome–protein complexes, 400 μL cool MeOH was added, mixed, and centrifuged for 3 min at 9000 *g* (11000 rpm; Eppendorf 5415 centrifuge). To the solution, 200 μL $CHCl_3$ was added, actively mixed, and centrifuged for 3 min at 9000 *g*. After the addition of 300 μL H_2O, active mixing, and centrifugation for 4 min at 9000 *g*, phase separation was observed with protein concentrating at the interphase. Approximately 700 μL of the upper phase was removed. To the remaining solution 300 μL more MeOH was added, the solutions were mixed, and centrifuged for 4 min at 9000 *g*. Supernatant was decanted leaving ~30–50 μL in the tube, which was evaporated to dryness on a rotary evaporator. To samples, 36 μL two-fold reducing sample buffer (0.075 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue) was added, the solution was mixed and stirred on a water bath (90–95°C) for two min twice under active mixing. Laemmli PAGE [49] was performed in 6% concentrating and 12% separating gels using a VE-2M (Helicon, Russia) setup: preliminary electrophoresis, 6 min at 10 mA; concentrating, 30 min at 18 mA; separation, 55 min at 28 mA. Proteins were visualized by silver staining [50]. Electrophoregrams were analyzed using ImageJ software. To match molecular weights, SigmaMarker (Sigma) and Prestained Protein Molecular Weight Marker (Fermentas, Lithuania) were used.

Immunoblotting. Proteins were transferred onto an Immobilon-P (Merck Millipore, Germany) polyvinylidene fluoride membrane using the Semi-dry transfer equipment (Helicon) during 30–40 min at 35 V. When the transfer was over, the membrane was rinsed with bidistilled water, washed with TBS buffer (NaCl, 4.39 g; Tris, 3.03 g; H_2O_{dd}, 500 mL), pH 7.97, and then incubated in a 5% low-fat dry milk suspension in TBS supplemented with 0.1% Tween 20 (TBS/T) for 1 h at room temperature to prevent nonspecific sorption. Then, the membrane was washed with TBS/T (3 × 5 min) and incubated with primary antibodies to plasma proteins (sheep polyclonal antihuman C4b-binding protein antibodies, AbCam, United States; goat antihuman C3 and antihuman factor H antibodies, CopmlementTech, United States; rabbit polyclonal antihuman apolipoprotein AI, mouse monoclonal antihuman apolipoprotein E, goat polyclonal antihuman immunoglobulin G and antihuman immunoglobulin M antibodies, IMTEK, Russia) in 0.5% solution of bovine serum albumin overnight at 4°C. The membrane was washed with TBC/T buffer for 15 min and 3 × 5 min, incubated with secondary IgG antibodies conjugated with horseradish peroxidase (rabbit antisheep and antigoat IgG, Jackson ImmunoResearch, United States; goat antirabbit

and antimouse IgG, Sigma) and then the membrane was again washed with TBS/T for 15 min and 2 × 5 min and then once with TBS buffer for 5 min. Immunodetection was performed with a Clarity™ ECL Western Blotting Substrate (Bio-Rad, United States) reagent and VersaDoc 4000 (Bio-Rad) system.

CONCLUSIONS

Results of the present work confirm the previously obtained data on the inertness of liposomes loaded with a melphalan prodrug with respect to human complement cascade [18]. Introduction of stabilizing lipids (phosphatidylinositol or PEG–lipid conjugate) or a targeting carbohydrate conjugate had an insignificant effect on the interactions with major C components and apolipoproteins, as well as the total protein sorption capacity of the liposomes. Therefore, such modifications of the lipid bilayer should not cause undesirable effects in the bloodstream. Results of the experiments with MTX-liposomes allow for a conclusion that they activate the complement via the alternative pathway. Earlier, we demonstrated that upon a decrease in the liposome loading with MTX-DG to 2.5 mol % (corresponding to the low-dose methotrexate therapy regimen) no complement activation [18] or binding/fragmentation of the C3 component [20] was observed, which reflects the influence of liposome surface structure on their reactivity in plasma. Manifestations of the liposome–protein interactions in vivo are the subject of our further studies.

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