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Hemocompatibility of liposomes loaded with lipophilic prodrugs of methotrexate and melphalan in the lipid bilayer

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ABSTRACT

A panel of in vitro tests intended for evaluation of the nano-sized drug delivery systems' compliance with human blood was applied to liposomal formulations of anticancer lipophilic prodrugs incorporated into the lipid bilayer. Liposomes on the basis of natural phosphatidylcholine (PC) and phosphatidylinositol (PI), 8:1 (mol) were loaded with 10 mol% of either methotrexate or melphalan 1,2-dioleoylglyceride esters (MTX-DOG and Mlph-DOG respectively) and either decorated with 2 mol% of sialyl Lewis X/A (SiaLe^{X/A}) tetrasaccharide ligand or not. Hemolysis rate, red blood cells and platelets integrity and size distribution, complement (C) activation, and coagulation cascade functioning were analyzed upon the material incubation with whole blood. Both formulations were negatively charged with the zeta potential value being higher in the case of MTX-DOG liposomes, which also were larger than Mlph-DOG liposomes and more prone to aggregation. Accordingly, in hemocompatibility tests Mlph-DOG liposomes did not provoke any undesirable effects, while MTX-DOG liposomes induced significant C activation and abnormal coagulation times in a concentration-dependent manner. Reactivity of the liposome surface was not affected by the presence of SiaLe^{X/A} or PI. Decrease in liposome loading with MTX-DOG from 10 to 2.5% resulted in lower surface charge density, smaller liposome size and considerably reduced impact on C activation and coagulation cascades.

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1. Introduction

Liposomes are an established and one of the most advanced drug delivery systems [1]. The inherent properties of the nano-scale liposomes increase patient tolerability toward the encapsulated drug [2], improve the drug therapeutic index [3–5], and provide pharmaceutical formulations for those drugs that cannot be administered by conventional means [6].

The growing number of studies on the hemocompatibility of nano-formulations [7–13] reflect the clinical relevance of evaluating the impact of systemic administration of nanoparticles on blood components.

Being composed of phospholipids, liposomes are biodegradable and have been traditionally considered biocompatible. However,

even the finest details in spatial organization of chemical groups on the surface of nanomaterial may be crucial for its hemoreactivity. The most notable example is the use of PEGylation to shield the surface of liposomes and other nanoparticles from interactions with plasma-circulating proteins [14]. Despite the general belief that PEG lacks toxicity and immunogenicity, PEGylated liposomes, including Doxil®, were found to induce a hypersensitivity syndrome called complement activation-related pseudoallergy [15–18]. On the contrary, vesicles of similar size distribution and bilayer composition but lacking the phospholipid-methoxyPEG conjugate, or bearing one methylated at the phosphate oxygen, did not produce elevated SC5b-9 (terminal complex of complement cascade) levels upon incubation with plasma, neither did monomers or micelles of the initial anionic phospholipid-methoxyPEG molecule [19]. Thus, phosphate methylation is believed to prevent complement activation by liposomes presumably due to interference of the methyl group with binding of phospholipid antibody and C1q of the classical complement activation pathway [19]. These observations allow to anticipate that the complex supramolecular structure of liposomes characterized by a specific spatial organization of functional groups on the bilayer surface can substantially affect biological cascades.

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The object of the current study is a liposomal system specifically designed for incorporation and transport of lipophilic prodrugs of chemotherapeutics of two distinct varieties, namely, an alkylating agent melphalan (Mlph) and an antimetabolite methotrexate (MTX) [20]. In brief, 1,2-dioleoylglyceride ester derivatives of the drugs (Mlph-DOG and MTX-DOG) are synthesized and loaded into liposomal lipid bilayer composed of natural phosphatidylcholine (PC) and phosphatidylinositol (PI) at the stage of the lipid film preparation. Besides, active targeting may be achieved through grafting carbohydrates of sialyl Lewis (SiaLe) family onto the surface of liposomes (Fig. 1). These tetrasaccharides are known to be ligands of selectins, generally a group of cell adhesion molecules expressed on activated leukocytes, platelets, and endothelium cells at inflammatory sites [21] also involved in tumor progression [22]. Earlier, liposome formulation of sarcosine (D,L-melphalan) lipophilic prodrug bearing tetrasaccharide SiaLe^X has been proven to be advantageous in preclinical trials [23].

Up to now, there is no general agreement on the conditions under which blood is to be subjected to the test material to provide the most relevant in vitro data in order to predict its in vivo reactivity [24–27]. The panel of hemocompatibility tests reported here has been carefully chosen for the characterization of nanoparticulate materials from the point of view of their primary effects toward principal blood components. The results of these tests, if positive, should draw attention to the issues which might be problematic in further in vivo exploitation of the formulations. On the other hand, they immediately suggest possible ways of interaction between nanoparticles and blood components, while the exact mechanisms need more thorough study.

Thus, in the present work we aimed to investigate blood compatibility of liposomes loaded with either Mlph-DOG or MTX-DOG through control of the following parameters: hemolysis, red blood cell (RBC) morphology, counting, and size distribution, platelet counting and size distribution, as well as C activation and coagulation cascade functioning. MTX-DOG liposomes, proven reactive, were studied at two MTX-DOG concentrations in the bilayer. We also checked if the reactivity of the liposomes was influenced by the presence and structure (SiaLe^X or SiaLe^A) of a carbohydrate ligand on the liposome surface. Finally, we introduced a sample devoid of phosphatidylinositol to evaluate the shielding properties of the PI hydrated head groups in the examined reactions. Physicochemical properties of the liposomes, in particular their mean size, polydispersity, stability and electrokinetic potential were controlled and analyzed in connection with the blood reactivity of the samples.

2. Materials and methods

2.1. Reagents and chemicals

Phosphatidylcholine (PC) from egg yolk and phosphatidylinositol (PI) from *S. cerevisiae* were obtained from Reakhim (Russia). 3-

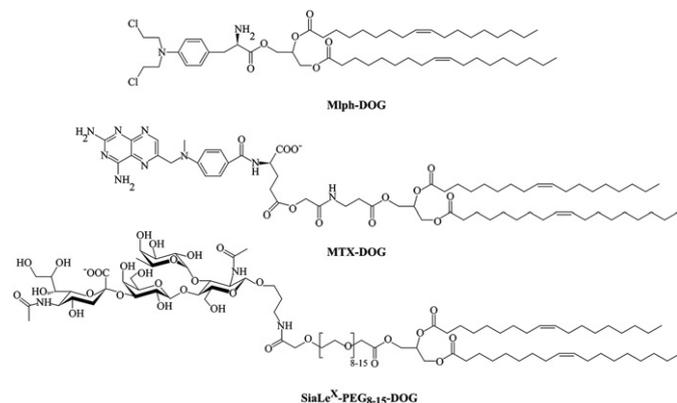


Fig. 1. Molecular structures of lipophilic prodrugs of melphalan (Mlph-DOG) and methotrexate (MTX-DOG), as well as of lipophilic conjugate of SiaLe^X (SiaLe^X-PEG-DOG).

aminopropyl glycosides of Sialyl Lewis X (SiaLe^X, Neu5Ac α 2-3Gal β 1-4(Fuc α 1-3)-GlcNAc β) and Sialyl Lewis A (SiaLe^A, Neu5Ac α 2-3Gal β 1-3(Fuc α 1-4)-GlcNAc β) were synthesized as previously described [28]. SiaLe^{X/A} conjugates (SiaLe^{X/A}-PEG-DOG) were synthesized from 3-aminopropyl glycosides and *rac*-1,2-dioleoyl-3-carboxymethylene [poly(9–16)oxyethylene]oxyacetyl glycerol as described for other glycoconjugates [29]. The lipophilic prodrugs, 1,2-dioleoylglyceride esters of melphalan (Mlph-DOG) [30] and methotrexate (MTX-DOG) [31], were synthesized as previously reported.

2.2. Liposome preparation procedure

Liposomes composed of PC, PI, and MTX- or Mlph-DOG, either equipped with glycoconjugate vectors SiaLe^{X/A}-PEG₈₋₁₅-DOG or not, were prepared as described earlier [20]. Briefly, lipid films were hydrated in PBS (1.5 mM KH₂PO₄, 1.1 mM NaH₂PO₄·2H₂O, 6.3 mM Na₂HPO₄, 2.7 mM KCl, and 136.8 mM NaCl, pH 7.2) containing 1 mM EDTA and subjected to 6–10 cycles of freezing/thawing (liquid nitrogen/+40 °C). The suspension was then extruded through two stacked polycarbonate membrane filters of 200 and 100 nm (Nucleopore, USA) progressively, 10 times through each pair, on a Mini-extruder (Avanti Polar Lipids, USA). MTX control solution was prepared in PBS supplemented with the minimum amounts of 0.1 M NaOH and used within 1 week of storage at +4 °C. See Table 1 for detailed sample description.

2.3. Liposome physical characteristics

Liposome size upon preparation was controlled in diluted suspensions (50 μ g total lipids/ml PBS) by photon correlation spectroscopy (PCS) using a Photocor equipment (helium–neon laser, 633 nm, 20 mW; 90° angle) and a BI9000 Brookhaven autocorrelator in at least three runs per sample. Prodrug concentration was controlled by UV spectrophotometry after liposome disruption with 5 volumes of ethanol (MTX-DG: λ_{\max} 302 nm, ϵ 25,000 M⁻¹ cm⁻¹; Mlph-DG: λ_{\max} 258 nm, ϵ 19,700 M⁻¹ cm⁻¹).

Zeta potential values were obtained using a Zetasizer Nano ZS equipment (Malvern Instruments Ltd, UK). Zeta potential analysis of the liposomes in the same PBS as used for liposome preparation was unsuccessful due to electrode blackening caused presumably by high conductivity of the buffer. Optimal conditions for reliable zeta potential determination were found to be the following: 0.9–1.0 mg/ml liposome suspensions prepared in 10 mM KCl solution buffered with 1 mM potassium phosphate buffer, pH 7.4, filtered through 200-nm membrane. Samples were equilibrated 3 min in the pre-rinsed disposable cuvettes before a minimum of 5 measurements per sample was performed at 25 °C under the voltage of 150 V and current intensity of 4 mA.

2.4. General conditions of blood sampling

In vitro hemocompatibility tests of the liposomal formulations were performed according to ISO standards (10993–4). Normal human blood from healthy volunteer donors was collected in Terumo Venosafe citrated tubes (Terumo Europe N. V., Belgium). Experiments were done within 2 h after blood collection. All tests were performed with the agreement of the local ethical committee of the Medicine Faculty of the University of Liège. To obtain blood samples, each formulation, either diluted 10 \times with PBS or not, was mixed (in an Eppendorf tube of 0.2 or 1.5 ml) with whole blood 1:9 to achieve final concentrations of 0.04 mM or 0.4 mM prodrug respectively. The mixture was then incubated for 15 min at 37 °C under lateral agitation at 250 rpm.

Table 1
Sample composition and description.

Sample ID	Sample description	Composition, mol%				
		PC	PI	Mlph-DOG	MTX-DOG	SiaLe ^{A/X} -PEG-DOG
PC/PI	Drug-free placebo liposomes	90	10			
Mlph-L	Liposomes, 4 mM Mlph-DOG	80	10	10	–	–
Mlph-L-SiaLe ^{A/X}	Targeted liposomes, 4 mM Mlph-DOG	80	10	10	–	2
Mlph-L-SiaLe ^A /PI	Targeted liposomes, 4 mM Mlph-DOG, without PI	80	–	10	–	2
MTX-L	Liposomes, 4 mM MTX-DOG	80	10	–	10	–
MTX-L-SiaLe ^A	Targeted liposomes, 4 mM MTX-DOG	80	10	–	10	2
MTX-L-10	Liposomes, 1 mM MTX-DOG	80	10	–	10	2
MTX-L-2.5	Liposomes, 1 mM MTX-DOG	80	10	–	2.5	2
MTX control solution	4 mM MTX solution in PBS, pH ~8	–	–	–	–	–
PBS control	1 mM EDTA in PBS	–	–	–	–	–

2.5. Red blood cell (RBC) and platelet counting and size distribution

An aliquot of each blood sample was diluted 40,020 times in isotonic medium for RBC counting. Platelets were assayed in a platelet-rich fraction obtained by 90 s centrifugation at 850 g of blood sample aliquots diluted 24-fold in isotonic solution. For measurement, 1 ml of each supernatant was further diluted in 40 ml isotonic solution and analyzed with a Coulter Multisizer II counter (Beckman Coulter Inc, USA) equipped with an orifice tube of 70 μ m. RBCs were counted between 3.7 and 8.0 μ m and platelets, between 1.0 and 3.7 μ m. Measurements were performed in duplicate.

Immediately after incubation, blood smears were prepared by spreading 5- μ l aliquots of samples on glass slides and viewed with Olympus Provis microscope at 50 \times magnification in transmission mode.

2.6. Hemolysis assay

Hemolysis assay was performed according to the standard practice [32]. In brief, an aliquot of each blood sample was centrifuged at 600 g for 5 min. Twenty five-microliter plasma aliquot was diluted with 225 μ l Drabkin's reagent (Sigma) in a 96-well plate and mixed for 2 min under lateral agitation (300 rpm). After 10 min equilibration at room temperature, optical density at 540 nm was recorded with an Anthos HTIII multiplate reader (Anthos Mikrosysteme GmbH, Germany). Whole blood hemoglobin was determined by reading the absorbance of 100-fold dilution of whole blood in Drabkin's reagent at 540 nm. Saponin (2 mg/ml final blood concentration) was tested as positive control and PBS, as negative. A sample of plasma without additives was considered basal conditions. Calibration curve was obtained with standard solutions containing 0.072 to 3.6 mg/ml bovine hemoglobin (Sigma) treated with Drabkin's reagent. The results are presented as percent hemolysis, i. e. free plasma hemoglobin (mg/ml) released as a result of contact with the test material divided by the total blood hemoglobin (mg/ml) multiplied by 100. Measurements were performed in duplicate.

2.7. Activated partial thromboplastin time (aPTT) and prothrombin time (PT)

An aliquot of each blood sample was centrifuged at 2000 g for 5 min. Clotting times of the collected plasma samples were measured on a Behring Coagulation Timer (Dade Behring Holdings Inc, US) using commercial reagents (Thromborel® S, Dade Behring/Siemens, for PT determination and C.K. PREST kit, Roche Diagnostics, France, for aPTT). Prior to analysis, a modified aPTT test without kaolin addition was performed in test plasma samples in order to verify that surface of the liposomes under study as such did not trigger coagulation cascade. Standard human plasma (Dade Behring/Siemens) was used to calibrate the equipment.

2.8. C3a ELISA

When incubation was over, further C3 conversion in blood samples was blocked by the addition of EDTA (10 mM final in blood). Plasma samples were isolated by 5 min centrifugation at 2000 g and when necessary frozen and stored at -70°C until analysis. To compare C activation in whole blood and serum, some liposome samples were also assayed in serum. C3a was determined with a Human C3a ELISA kit for quantification of Human C3a-desArg (Beckton Dickinson). The sample C3a concentrations were calculated from standard calibration curve. Zymosan (Sigma) at final blood concentration 2 mg/ml was used as positive control and plasma, without additives, as negative control. Measurements were performed in duplicate.

2.9. SC5b-9 ELISA

S-protein-bound (soluble) C terminal complex, formed as a consequence of C activation, was determined as described earlier [15], using the SC5b-9 kit from Quidel Corp. (San Diego, CA, ref: A029). Liposomes were incubated with normal human sera for 20 min at 37 $^{\circ}\text{C}$, and the reaction was terminated by the addition of 20 volumes of the sample diluent of the SC5b-9 kit, supplemented with 10 mM EDTA.

2.10. Measurement of complement consumption by the sheep red blood cell hemolytic (CH50) assay

Liposomes were incubated with human serum (1:3 volume ratio) and C consumption was measured using a modified version of the sheep red blood cell (SRBC) hemolysis assay [33]. Serum incubations with liposomes and the SRBC hemolysis were performed for 45 and 10 min at 37 $^{\circ}\text{C}$, respectively. Hemoglobin level was measured by a BMG FLUOstar Omega Plate Reader at 541 nm.

3. Results

3.1. Liposome characteristics

Table 2 summarizes the physicochemical characteristics of liposomes used in this study, along with their effects in the various hemocompatibility tests.

The liposome diameter values (Table 2) adhere to those previously established [34] taking into account differences in the equipment and data processing software. Higher SD values for MTX-bearing liposomes at 4 mM MTX-DOG reflect higher inter-batch variability as well as wider size distribution of the formulations. During the two-week storage at 4 $^{\circ}\text{C}$, mean diameter of Mlph-based liposomes increased by <5%, whereas in the case of MTX-DOG formulations, both targeted and not, the value increased by up to 12% within 10 days (Fig. 2A). This should be attributed to the cation-mediated aggregation of the MTX-DOG

liposomes due to the surface-exposed negatively charged carboxyl groups of MTX. In this connection, the presence of EDTA in the buffer for preparation of MTX liposomes was found to be crucial. Still, the stability of EDTA-Meⁿ⁺ complexes allows for the equilibrium cation exchange during the long-term storage.

Zeta potential profiles of the principal formulations, that is, Mlph-L, MTX-L (MTX-L-10), and MTX-L-2.5, are presented in Fig. 2B. As expected, all formulations were found to be negatively charged which is due to the presence of relatively high PI content (10 mol%) in the bilayer. Slight differences between the values obtained for the three liposomal formulations of the prodrugs are in agreement with the fact that melphalan moiety of Mlph-DOG is positively charged at pH 7.4 due to protonation of the primary amino group while the free carboxyl group of the methotrexate derivative provides MTX-DOG with negative charge.

3.2. RBC integrity and cell counts and size distribution in the presence of liposomal formulations

The liposomes under study did not affect RBC and platelet cell counts or size distribution profile (see Appendix A). As a confirmation to RBC counting, hemolysis rates determined in the presence of the formulations did not exceed the negative control value by more than 2% (see Fig. 3) and therefore the liposomes may be considered non-hemolytic [32]. Microscopic observations (Fig. 3, inset, and supplementary materials of the Appendix) demonstrating no morphology changes after blood contact with the samples also support the data of hemolysis tests and cell counting. Therefore, we may conclude that the liposomal formulations are inert toward the blood major cellular components.

3.3. Effect of liposomes on coagulation

Two conventional complementary tests are typically adopted in clinics to assess functioning of the coagulation system, namely, activated partial thromboplastin time (aPTT) and prothrombin time (PT). The former one is a measure of the intrinsic activation pathway and the latter one, of the extrinsic. In both tests plasma samples are first recalcified to reverse the effect of an anticoagulant used during blood collection and then supplied with the activators of coagulation (cephalin, a platelet substitute, plus kaolin suspension for the intrinsic pathway initiation and human placental thromboplastin, for the extrinsic one). Then, the time of clot formation is measured. Clotting ability of the standard plasma is assumed to be 100%. The longer it takes plasma to clot, the lower is its clotting ability, and the lower is the resulting test value expressed in percent to the standard plasma. It is also worth mentioning that these two coagulation pathways are intrinsically linked. For example, tissue factor-factor VIIa complex initiating the extrinsic pathway is also capable of activation of factor IX of the intrinsic pathway; in turn, the intrinsic tenase complex influences the tissue factor-dependent pathway [35].

Table 2

Physicochemical characteristics of the liposomes under study and their effects in hemocompatibility tests.

Liposomal formulation	Mean diameter, nm	RBC and platelet count and cell distribution	Clotting times aPTT/PT	Hemolysis	C3a ELISA	SC5b-9 ELISA	SRBC hemolysis
Mlph-L	85 ± 2 ^a	-	-	-	-	-	-
Mlph-L-SiaLe ^A	82 ± 1 ^b	-	-	-	-	NA	NA
Mlph-L-SiaLe ^X	90 ± 3 ^b	-	-	-	-	-	-
Mlph-L-SiaLe ^A \PI	92 ± 1 ^b	-	-	-	-	NA	NA
MTX-L	115 ± 9 ^a	-	+++	-	++	-	++
MTX-L-SiaLe ^A	119 ± 16 ^a	-	+++	-	++	-	++
MTX-L-10	98 ± 3 ^b	-	+++	-	++	NA	NA
MTX-L-2.5	85 ± 1 ^b	-	+/+	-	+	-	-
MTX control solution		-	-	-	-	NA	NA

Notes: -, no or weak effect, +, moderate effect, ++, strong interference, NA, not analyzed.

^a Two batch evaluation mean ± SD.

^b Range indicates measurement error.

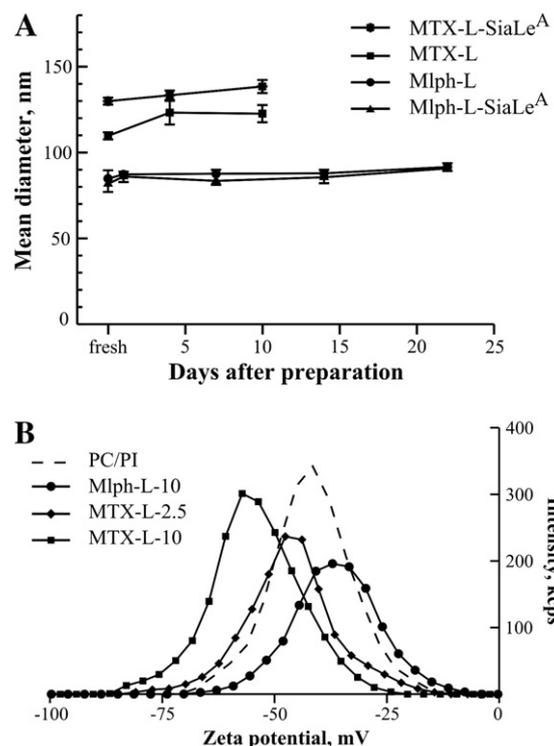


Fig. 2. (A) Changes of liposome size upon storage at 4 °C. Bars are means ± SE. (B) Representative zeta potential distribution profiles of liposome samples.

The results of the assays are presented in Table 3. aPTT was significantly prolonged in the presence of MTX-DOG containing liposomes, both targeted and ligand-free. Samples MTX-L-SiaLe^A and MTX-L induced ~55 and 15% reduction in the plasma clotting ability at 0.4 mM and 0.04 mM MTX-DOG final concentration in blood, respectively. Also, these liposomes demonstrated similar yet less considerable reduction of the plasma clotting ability in PT, by 33 and 7% respectively.

In view to better understand the origin of this hemostasis reaction, we also investigated two liposome samples, a replicate of MTX-L sample but prepared as four times less concentrated dispersion (MTX-L-10) and the other one, bearing four times fewer methotrexate entities on the bilayer surface (MTX-L-2.5) with the same total concentration of lipids as in sample MTX-L (see Table 1). As expected for the intermediate prodrug concentration (0.1 mM after dilution in blood), clotting time values of the two samples in both tests fell between the values produced by two different dilutions of MTX-L and MTX-L-SiaLe^A samples of the initial experiment. Between liposomes containing 1 mM MTX-DOG more pronounced inhibition of clotting was observed in the case of less concentrated by total lipids (producing lower total surface area) dispersion with higher surface density of MTX moieties. As reported recently [36,37], inhibition of intrinsic and extrinsic pathways of

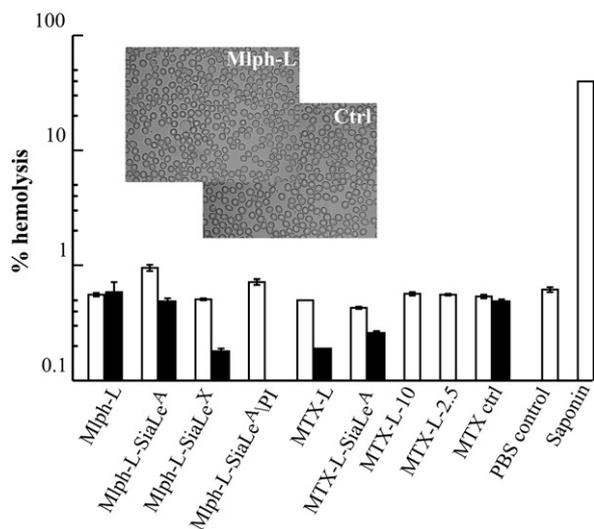


Fig. 3. Hemolysis induced by liposomes in whole blood, expressed as percent to whole blood hemoglobin content. Empty bars represent 0.4 mM final prodrug concentration in blood and shaded bars, 0.04 mM. Bars are mean \pm SE. In the inset, exemplary micrographs demonstrate the absence of action of Mlph-L liposomes (0.4 mM final prodrug concentration in blood) on RBC morphology by comparison to blood control.

coagulation can be explained by the non-specific adsorption of proteins involved in the coagulation cascades onto the surface of materials involved. Among these proteins, fibrinogen, factor IX, prothrombin, factor X, and antithrombin III deserve particular interest as key factors in the activation of all humoral blood reactions. Although it would be valuable to identify the exact nature of the factor(s) involved in the liposome-mediated coagulation inhibition, this mechanistic study was outside of the main focus of our work.

3.4. Effect of the liposomes on complement activation

Complement activation by liposomes in whole blood, plasma and serum were tested by three different assays: C3a and SC5b-9 ELISAs and a hemolytic assay of C consumption. These tests together provide information on anaphylatoxin production, i.e., likely anaphylactic activity, the formation of the terminal complex, implying activation of the whole cascade with consequent cell activations and/or cell membrane damage, and the C consuming activity of liposomes, a comprehensive measure of the activation of the whole C cascade. Fig. 4 shows that similarly to coagulation, Mlph-DOG liposomes exhibited no C activation (less than 150% C3a compared to plasma control; no change in C consumption), while the MTX-DOG formulations, with and without

Table 3
Results of clinical coagulation tests^a.

Liposomal formulation	aPTT extrinsic pathway		PT intrinsic pathway	
	Final drug concentration in blood			
	0.4 mM	0.04 mM	0.4 mM	0.04 mM
Mlph-L	100.0	96.7	86.2	81.3
Mlph-L-SiaLe ^A	100.0	100.0	89.8	95.4
Mlph-L-SiaLe ^X	100.0	100.0	89.8	100.9
Mlph-L-SiaLe ^A /PI	83.7	ND	93.5	NA
MTX-L	45.7	86.7	67.1	93.5
MTX-L-SiaLe ^A	42.6	83.1	67.1	93.5
MTX-L-10	52.5	NA	75.4	NA
MTX-L-2.5	68.0	NA	79.9	NA
MTX control solution	100.0	100.0	101.6	98.4
"-" control	100.0	100.0	99.1	97.2

NA, "not analyzed".

^a Reported is the clotting ability of the sample compared to the clotting ability of standard human plasma, %, as determined on a Dade Behring Coagulometer.

SiaLe^X, caused significant C3a liberation and C consumption (Fig. 4A, B). Here as well, decrease in liposome concentration led to decrease in C activation by factors of 1.2–1.5 (sample MTX-L-10; Fig. 4B). Even more so did the decrease in MTX-DOG loading from 10 to 2.5% (sample MTX-L-2.5). In both the coagulation tests and C3a ELISA assays, complement activation by the liposomes was not influenced by the introduction of SiaLe^{A/X} targeting moiety.

In contrast to the C3a and hemolytic C results, ELISA measurement of SC5b-9 elevation by the liposomes at 1 mM final prodrug concentration showed no significant elevation of this marker by either formulation (see Supplementary material), although the positive control (zymosan) did show major effect. At this time we do not have explanation for this discrepancy, which could result either from a blockage of SC5b-9 generation despite of complement activation, or binding of the terminal complex to liposomes instead of S protein, or decomposition of SC5b-9 under our assay conditions. This finding, if confirmed in further studies, would provide a rare (if not unprecedented) example for contradicting indication of C activation by C3a and SC5b-9 measurements. Based on the consistent presence of signs of C activation by the C3a and hemolytic assays, which is also in keeping with the coagulation data, despite the lack

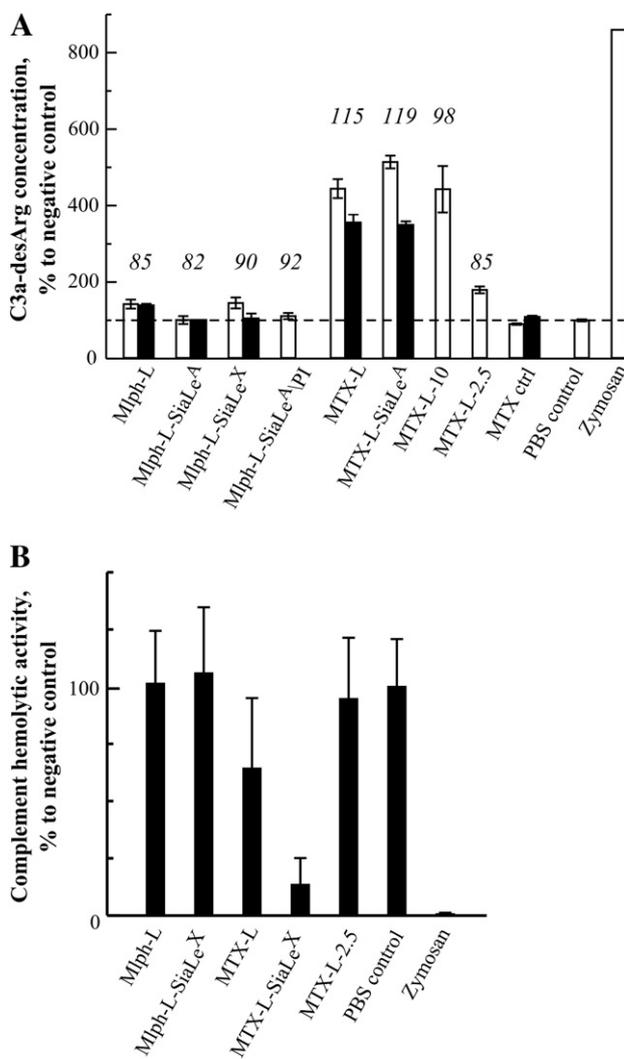


Fig. 4. (A) Rise of C3a-desArg following incubation with liposomes. Empty bars represent 0.4 mM final prodrug concentration in blood and shaded bars, 0.04 mM. Bars are mean \pm SE. Figures above bars indicate mean size (nm) of the corresponding liposome samples. (B) Complement consumption upon incubation of serum with liposomes. Data represent A540, percentage of baseline, and SEM for n = 3 different sera.

of SC5b-9 rise, our study provides strong indication for the C reactivity of MTX-DOG liposomes.

4. Discussion

Liposomal formulations were analyzed for hemocompatibility in frames of classical requirements considered in the ISO. They revealed good hemotolerance in relation to major cellular components of the blood. This observation is particularly valuable for platelets which are typically more reactive to foreign body surfaces, giving rise to their activation or aggregation. Moreover, the absence of platelet aggregation upon incubation with SiaLe^{A/X}-equipped liposomes indirectly testifies their potentialities as formulations targeted to the very participants of inflammatory response, as well as tumor dissemination development. Indeed, the adhesion of selectin ligand-decorated particles even to non-activated platelets could be apprehended. Also, Mlph-DOG containing liposomes were found to be tolerated regarding both the complement and coagulation cascades. Meanwhile, liposomes loaded with methotrexate prodrug displayed considerable levels of intervention with the two interlinked protective systems of complement activation and blood coagulation. Noteworthy, when tested *in vitro* under the same conditions as the liposomal formulations, MTX solution did not induce any undesirable response, neither in coagulation tests, nor in C3a assay (MTX control sample in Fig. 4 and Table 3). Thus, the main source of the hemoreactivity should be the nature of the drug moiety and, particularly, the effects it produces on the liposome surface. Indeed, recently it was reported that surface functionalization of phospholipid nanoparticles with certain lipid-anchored gadolinium chelates causes swift complement activation initiated by natural IgM antibodies and propagated via the classical pathway; the intensity of the response was shown to depend upon the chemical structure of the chelates and not zeta potential effects alone [38].

As for the MTX moiety, each prodrug molecule in the bilayer introduces a couple of aromatic amino groups together with a free alpha-carboxyl to the surface. Meanwhile, exposed amino (and hydroxyl) groups (and their arrangement) have been reported as a complement activation promoting factor which is attributed to their capability to induce nucleophilic attack on the internal thioester bond in the C3b thus accelerating alternative pathway turnover (see [39] and references therein).

Size of nanoparticles as such, has been found to be a critical factor influencing their reactivity in biological milieu [8,9,11,40] although there is no general rule on whether smaller (below 50 nm) or larger size is preferable for circulating nanoparticles in terms of their reactivity with complement. As for (conventional) liposomes, their size has been also shown to control C activation. Thus, phosphatidylglycerol-containing liposomes 350 nm in diameter and larger gave rise to complement-mediated pseudoallergic reaction while those of 190 nm appeared inert [41]. In principle, we observed similar tendencies among the studied liposomal formulations (Fig. 2A) with liposomes below 90 nm in diameter showing affordable levels of C3a, while those of 110 nm and more inducing considerable activation of the system. The mechanism of the unexpectedly fine regulation is to be clarified in our further studies.

The reason for MTX-DOG liposomes to interfere with the cascades is to be sought in the interactions between various protein factors with the liposome surface at the stage of sample pre-incubation with blood leading to lack of availability of the free and intact protein. Indeed, a commonplace truth is that immediately upon mixing with blood, either in the blood stream or in a tube, nanoparticles irrespective of the nature of their surface acquire a protein corona [42–44]. In the corona, proteins abundant in plasma, like serum albumin, IgG, and fibrinogen, are revealed in high amounts even if the affinity of these proteins to the specific surface of a nanoparticle is low [45,46]. Meanwhile, plasma concentrations of coagulation factors

are as low as 20, 10, and 200 nm for factors V, VII (in the activated plasma), and X to name a few, in contrast to 9 μ M for fibrinogen [47]. Thus, even slight decrease in plasma concentration caused by interaction with the particle surface may result in the shift of the cascade reactions' equilibrium. On the other hand, even non-specific deposition of C3 on some polymer surfaces upon conformational changes may result in initiating C3 convertase formation in the presence of factors B and D thus increasing the alternative pathway turnover [39].

Estimated values of the total surface area produced by equal concentrations of PC in the form of liposomes of 90 and 110 nm differ by less than 10% and by only 13%, for dispersions of 50 and 150-nm liposomes. Assuming that the reactivity of the surface is proportional to the number of available sites for protein interaction and, consequently, to the total surface area, this factor alone cannot be responsible for the observed sharp increase in the reactivity. The effects of other geometrical parameters deriving from the difference in size, such as curvature of the membrane or patterns, if any, of the precise spatial distribution of various functional groups of the matrix phospholipids and the prodrug, are difficult to estimate. The observed experimental data do not fit in the basic assumption that the smaller are the liposomes, the higher is the curvature radius and consequently the stored curvature elastic energy of the bilayer and reactivity at the surface. Indeed, as it has been demonstrated for several plasma proteins [40,48], secondary structure may be either preserved or destroyed upon interaction with the nanoparticulate surface of a specified curvature. Therefore, the whole ensemble of the proteins present in plasma, those involved in the hemostasis cascades, as well as not, dynamically interacts with the liposome surface, adapts conformation to the surface characteristics, and thus contributes to the effect of abnormal cascade functioning.

The charge is certainly another contributor to the effects the liposomes produce on blood humoral components. Increase in the mean zeta potential negative value from -34 mV for Mlph-liposomes to -40 – (-50) mV for MTX-liposomes of varying composition may promote binding to plasma proteins abundant in positively charged residues, e.g., coagulation factor XII and high molecular weight kininogen [49], or apolipoprotein H [50]. Differences in patterns of charge distribution over the lipid bilayer surfaces could affect their affinities to biomacromolecules of blood as well.

To conclude, the proposed hemocompatibility screening tests proved relatively good hemotolerance of the liposomes loaded with lipophilic prodrugs of melphalan and methotrexate. The interplay between all of the above-mentioned factors, namely, liposome diameter and size distribution, surface charge and patterns of the functional groups arrangement over the bilayer surface, determines the reactivity of the liposomes with the proteins of plasma coagulation and complement cascades. First and direct measure to overcome the adverse effects, as it is evidenced by samples MTX-L-10 and MTX-L-2.5, is to lower the concentration of the liposomal prodrug. This is possible since the lowest liposome concentration tested in the present work (40 μ M final concentration in blood) produced slight effect on blood components and biological cascades. It falls into the range of methotrexate low-dose therapy in leukemia treatment (20–60 mg/m² [51], which is approximately 15–35 μ M final concentration in blood for a 1.60–1.70-m tall 60–70-kg person with 5 L of blood). The dosage of liposomal formulation is yet to be seen however is expected to be not greater than that of the intact drug.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jconrel.2011.12.010.

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