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Immunological response to nitroglycerin-loaded shear-responsive liposomes *in vitro* and *in vivo*

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ABSTRACT

Liposomes formulated from the 1,3-diamidophospholipid Pad-PC-Pad are shear-responsive and thus promising nano-containers to specifically release a vasodilator at stenotic arteries. The recommended preclinical safety tests for therapeutic liposomes of nanometer size include the *in vitro* assessment of complement activation and the evaluation of the associated risk of complement activation-related pseudo-allergy (CARPA) *in vivo*. For this reason, we measured complement activation by Pad-PC-Pad formulations in human and porcine sera, along with the nanopharmaceutical-mediated cardiopulmonary responses in pigs. The evaluated formulations comprised of Pad-PC-Pad liposomes, with and without polyethylene glycol on the surface of the liposomes, and nitroglycerin as a model vasodilator. The nitroglycerin incorporation efficiency ranged from 25% to 50%. In human sera, liposome formulations with 20 mg/mL phospholipid gave rise to complement activation, mainly *via* the alternative pathway, as reflected by the rises in SC5b-9 and Bb protein complex concentrations. Formulations having a factor of ten lower phospholipid content did not result in measurable complement activation. The weak complement activation induced by Pad-PC-Pad liposomal formulations was confirmed by the results obtained by performing an *in vivo* study in a porcine model, where hemodynamic parameters were monitored continuously. Our study suggests that, compared to FDA-approved liposomal drugs, Pad-PC-Pad exhibits less or similar risks of CARPA.

1. Introduction

Over the last two decades, liposomal formulations, developed as containers for the targeted and controlled release of drugs, have been studied in detail [1–3]. Some of these drugs have been approved by the Food and Drug Administration (FDA) [4]. Controlled drug release over time in a stenotic artery due to specific liposome sensitivity on higher local wall shear stress, can potentially increase the therapeutic index of an encapsulated vasodilating drug [5]. Simultaneously, when the active drug, protected by encapsulation, is circulating in the human body, side effects observed after systemic administration are reduced as often described for oncological therapeutics [6]. For cardiovascular treatment agents, hypotension is a characteristic side effect which may be observed when vasodilating drugs are systemically administered in case of an acute ischemic event, in order to increase local perfusion pressure in a suffering organ such as the heart, the brain, or the kidneys [7].

The pathology of atherosclerosis is characterized by the narrowing and hardening of blood vessels as a result of atherosclerotic plaque formation [8]. This significant narrowing of the arterial blood vessels alters blood flow and causes changes in the average wall shear stress in comparison to physiological conditions [9]. It has been reported that an 80%-constricted artery gives rise to a wall shear stress increase of one order of magnitude [10]. Such an increase of shear stress has been

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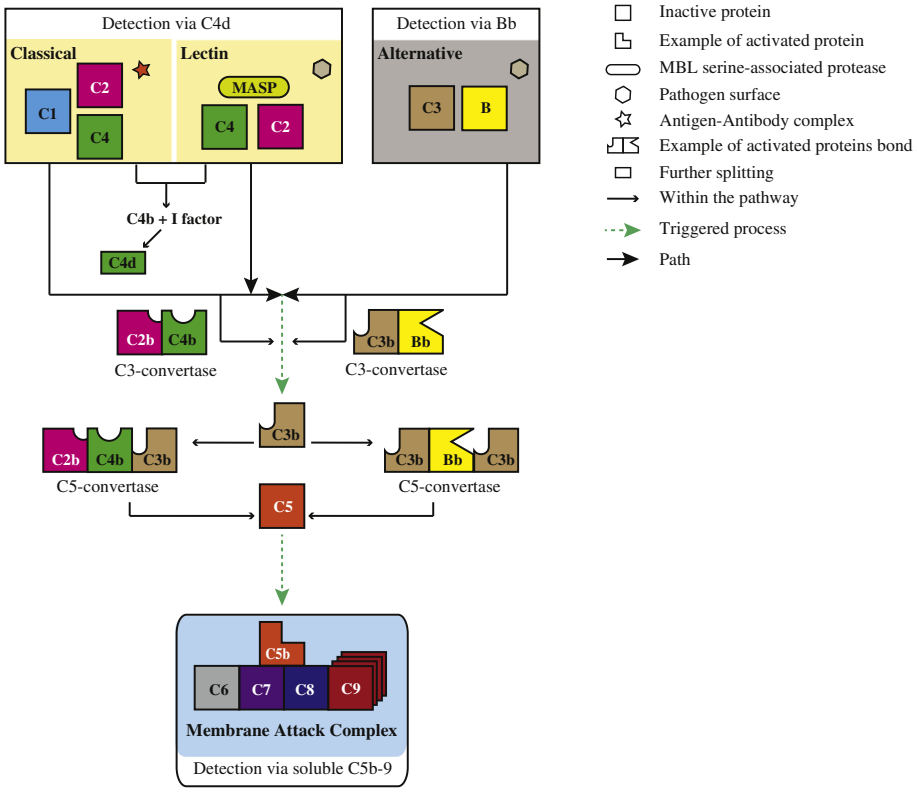

Fig. 1. Main steps of the complement activation cascade. The identification of the SC5b-9 complex (light blue-colored box) through the three pathways provides an insight into understanding the interaction between Pad-PC-Pad liposomes and the immune system. The classical pathway (yellow-colored box) is mediated by an antigen-antibody complex, whereas the lectin pathway (also a yellow-colored box) and the alternative pathway (gray-colored box) are initiated at the pathogen surfaces. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proposed as a trigger signal for targeted vasodilator delivery [5]. Nanometer-sized, lentil-shaped Pad-PC-Pad liposomes have shown the unique property of being able to release their cargo under mechanical stimuli [11]. Targeted drug delivery by these liposomes can be used to mitigate systemic side effects caused by the intravenous administration of vasodilators during cardiovascular emergencies. Based on these liposomes, we focus on the development of an infusion-based medication to be given to the patient at any location, for example at the site of a myocardial infarction. The medication should be active until the patient reaches the operation theater. As continuous infusion, it should not stay in the blood stream for prolonged residence time.

Nonetheless, liposomal drugs are often recognized as foreign particles and hence are attacked by the immune system leading to a biochemical cascade termed *complement activation*[12]. If there is any complement activation, it is helpful to understand the mechanism behind the interaction of liposomes with the immune system. In the serum, there are > 30 complement proteins which usually contribute as defenders. Some of them can split into fragments when stimulated. The complement protein cleavage (*cf.* Fig. 1) can be activated along three pathways, termed classical, lectin, and alternative [13]. The main differences among the three pathways are displayed in Fig. 1. In the classical pathway, which is mainly mediated by an antigen-antibody complex (star), the C1 complex (light blue-colored) initiates the activation of the C3 (light brown-colored) and C5 (orange-colored) proteins by triggering the proteins C2 (pink-colored) and C4 (green-colored), thereby giving rise to C3- and C5-convertases. In order to cleave the C5 protein, the immune system can also follow another route, namely the lectin pathway, which is mediated by the interaction between mannose-binding lectin (MBL) and the surface of a pathogen, leading to the cleavage of the C3 and C5 proteins. Cleaved C5 links the proteins C6 (gray-colored), C7 (purple-colored), C8 (dark blue-colored), and the polymeric C9 (red-colored), thereby producing the complex C5b-9, known as the *Membrane Attack Complex* (MAC) or the *Terminal Complement Complex* (TCC). The MAC induces the formation of a defect in the cell membrane that damages or destroys the suspected microorganism, and it can also form *via* the alternative pathway, due to interaction with the C3 protein, the B-factor fragment Bb (yellow), and the surface of a pathogen (hexagon). These interactions lead to the C5 protein and the MAC formation [14].

Released complement protein fragments such as C3a and C5a stimulate mast cells, granulocytes and macrophages, which can significantly contribute to cardiovascular distress [15–17]. The particle morphology can play a decisive role in preventing phagocytosis processes [18,19] like those ones mediated by pulmonary intravascular macrophages as it has been recently discussed [20].

The activation of the immune system may lead to an adverse immune phenomenon known as *complement activation-related pseudo-allergy* (CARPA) [21]. CARPA is used to describe acute hypersensitivity reactions (HSRs) to nanomedicines occurring in up to 45% of individuals [22]. In some individuals, these HSRs can be severe or even fatal [23]. It has been proposed that pigs can provide a sensitive, quantitative, and biologically relevant animal model of severe human CARPA [24–28]. The main manifestation of CARPA is cardiopulmonary distress, caused by pulmonary hypertension, which is critical for patients suffering from cardiac pathologies [23]. It leads to changes in pulmonary arterial pressure (PAP) and systemic arterial pressure (SAP) [26,29,30]. Due to this potential reactivity, the European Medicines Agency recommends *in vitro* and *in vivo* assessments of CARPA as a preclinical immune toxicity test in the development of liposomal drugs [31]. Detection and prevention of CARPA at an early stage of nanomedicine research is one of the strategies to successfully develop nanopharmaceutics. Liposomal drugs that cause CARPA have been reported previously [21,32]. Phospholipid bilayers can activate the complement system, and, among other factors, size, shape, lipid composition, and presence of surface charges are all sources for complement activation by liposomes *in vitro*[15,23,24]. Therefore, one step on the road to testing shear-responsive Pad-PC-Pad liposomes clinically is the evaluation and minimization of their risk for HSRs.

In the present communication, we hypothesize that Pad-PC-Pad liposomes, containing PEGylated lipids and nitroglycerin, hardly trigger CARPA.

Table 1
Liposomal suspensions tested *in vitro* and *in vivo*.

Label		Lipid composition (molar %)		Load
In vitro	*In vivo*	Pad-PC-Pad	DSPE-PEG2000	
L1, L1a[a]	L1′	95	5	Nitroglycerin
L2, L2a[a]	L2′	95	5	Bare
L3, L3a[a]	L3′	100	–	Nitroglycerin

[a] Liposomal suspension diluted ten times using saline solution.

We know that the activation of the complement system causes the formation of the lytic C5b-9 complex bound to the S protein. The concentration of the soluble C5b-9 (SC5b-9) protein complex was investigated *in vitro*, using an enzyme-linked immunosorbent assay (ELISA). Herein, human or animal sera were incubated together with the liposomal drug.

For the negative control, each donor's serum was treated with saline (liposome buffer) or nitroglycerin (liposome payload). For the comparison with established liposomal drugs and the positive control, each donor's serum was treated with Doxil, Abelcet, and zymosan. Doxil is widely used in cancer treatment and consists of PEGylated liposomes below 100 nm in size, encapsulating doxorubicin as the active drug [33]. Abelcet is one of the three commercially available lipid formulations containing amphotericin B, which is used to fight against systemic fungal infections. It consists of ribbon-like complexes 1 to 10 μm in size [34]. Zymosan, a well-known complement activator, is a bacterial glucan applied in experiments to quickly induce inflammation and complement activation [35]. Samples containing the human sera were subjected to SC5b-9 level detection. Furthermore, the three possible pathways, through which the MAC can be formed, were investigated individually through the detection of proteins within the complement cascade including C4d (classical and lectin pathways) and Bb (alternative pathway) protein concentrations. In porcine sera, complement activation was evaluated *via* C3 protein consumption. *In vivo,* the immune system response was investigated by intravenous injection of liposomes into pigs with continuous monitoring of the hemodynamic and hematological changes.

2. Materials and methods

2.1. Materials

A 0.1% commercially available nitroglycerin solution (Perlinganit, UCB-Pharma AG, Bulle, Switzerland, and Nitroglycerin Bioren 0.1%, Sintetica, Mendrisio, Switzerland) was used. PEGylated DSPE (DSPE-PEG2000) was a gift from Lipoid (Ludwigshafen, Germany). Zymosan, chloroform, and methanol were purchased from Merck p.a. (Darmstadt, Germany) and Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Doxil (Janssen-Cilag International NV, Beerse, Belgium) and Abelcet (Teva Pharmaceutical Works Ltd.) were used as purchased. Saline solution (0.9% NaCl, 308 mOsm/L) was purchased from Teva Pharmaceutical Works Ltd. (Budapest, Hungary) and Bichsel AG (Interlaken, Switzerland). All chemicals were of pharmaceutical grade and used without further purification.

Human serum samples of healthy volunteers were obtained according to the institutionally approved phlebotomy protocol at the Semmelweis University (Budapest, Hungary). Porcine sera were provided by the Institute of Animal Breeding and Nutrition Research Institute (Herceghalom, Hungary). All the sera were stored at a temperature of − 80 °C until use.

2.2. Lipid synthesis

Pad-PC-Pad (1,3-palmitoylamido-1,3-deoxy-*sn*-glycero-2-phosphatidylcholine) was prepared following the reported protocol [11,36]. Briefly, starting from 1,3-dichloropropan-2-ol, the protected phosphate head group was introduced by using phosphoryl chloride, *N*-Boc-aminoethanol, and ammonium hydroxide. Subsequent treatment with sodium azide led to the substitution of both chlorides. A Staudinger-type reduction of the resulting bis-azide yielded the free bis-amine.

Using a Schotten-Baumann-type protocol with palmitoyl chloride, fatty acyl chains were introduced. Finally, the head group was deprotected and quarternized, yielding Pad-PC-Pad. The product was purified sequentially by silica gel column chromatography (silica 63–200, 60 Å, Chemie Brunschwig AG, Basel, Switzerland), using a mixture of chloroform, methanol, and water (65:25:4 v/v/v), by precipitation in cold acetone, and by size exclusion chromatography on Sephadex LH-20 (GE Healthcare Biosciences, Uppsala, Sweden), using a mixture of chloroform, methanol, and water (65:25:4 v/v/v).

2.3. Liposome preparation

The phospholipids were dissolved in chloroform in molar ratios as described in Table 1: L1 and L2 contained Pad-PC-Pad and DSPE-PEG2000, while L3 was formulated from Pad-PC-Pad only. The three liposomal formulations were prepared *via* the standard thin film method [37,38], and they were either hydrated with saline solution, in the case of L2, or passively loaded with commercially available nitroglycerin, in the cases of L1 and L3. Each suspension was prepared in order to get a final lipid concentration of 20 mg/mL, the highest concentration achievable using the Pad-PC-Pad lipids. The suspensions were freeze-thawed in a twelve-step series: Frozen in liquid nitrogen and heated in a 60 °C water bath. In order to get a size distribution of around 100 nm in diameter, each liposomal suspension was downsized by multiple barrel extrusions using Liposofast LF-50 (Avestin Inc., Ottawa, Canada) through track-edged polycarbonate filter membranes (Whatman Nucleopore, Sigma-Aldrich, Buchs, Switzerland) of selected pore sizes: 400 nm (five times), 200 nm (five times), and 100 nm (15 times). The non-encapsulated nitroglycerin in the L1 and L3 suspensions was removed by buffer exchange, using PD-10 desalting columns with 5000 Da size exclusion limit, following the manufacturer's gravity protocol (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). In the *in vitro* experiments, the ten-fold diluted counterparts of L1, L2, and L3 were tested and labeled 'L1a', 'L2a', and 'L3a', respectively. The dilution was carried out using saline solution.

For the *in vivo* experiment, three batches of liposomes, labeled L1′, L2′, and L3′, were prepared using the same lipid composition as L1, L2, and L3, respectively (*cf.* Table 1), and by following the protocol described above. For the samples L1′ and L3′, Perlinganit was used as a drug, whereas the sample L2′ comprised drug-free liposomes. Lipid content was obtained by the phosphate test 2.0 [39]: 12 mg/mL for L1′, 16 mg/mL for L2′, and 11 mg/mL for L3′. All the liposomal suspensions were stored at room temperature until use.

2.4. Liposome characterization

Lipid content in the liposomal suspension was determined after purification by phosphate test 2.0 [39]. Briefly, liposome concentration was calculated from the measured phosphate content (phosphate moiety in the head group of the phospholipids): An aliquot (20 μL) of the liposome suspension was diluted with ultrapure water (180 μL, 18.2 MΩcm) in a 0.5 to 2 mL microwave vial (Biotage, Stockholm, Sweden). Then, 500 μL of a mixture (3/1 v/v) of nitric acid (65% Reag. ISO, Reg. Ph. Eur. grade, Honeywell, Morristown, USA) and sulfuric acid (95% Reag. ISO, Reg. Ph. Eur. grade, Honeywell, Morristown, USA) was added. The vial was sealed and heated in a microwave to a temperature of 180 °C for a period of 20 min (Biotage Initiator,

Stockholm, Sweden). The vial was allowed to cool down and the following solutions were added in sequence: Water (2.3 mL), ammonium metavanadate and ammonium heptamolybdate coloring agent (1 mL), and sodium hydroxide solution (10 M, 1 mL). The solution was allowed to rest for ten minutes. The solution was then pipetted into a 96-well plate (Nunc-Immuno plate F96 Polysorb, Roskilde, Denmark), using a multi-channel pipette (eight times 300 μL), and the absorbance was measured at a wavelength of 405 nm, using a plate reader (MultiskanFC 96 from Thermo Scientific, Waltham, USA).

Furthermore, the size of the liposomes, including the polydispersity index (PDI), was obtained by dynamic light scattering (DLS), using a Delsa Nano C (Beckman Coulter, Brea, USA). DLS measurements were performed at a temperature of 25 °C, using two laser diodes working at a wavelength of 658 nm. The scattering angle was set to 165 degrees.

The zeta (ζ) potential of the liposomal formulations was determined by laser Doppler micro-electrophoresis, using a Zetasizer Nano-ZS (Malvern Instruments Ltd., Malvern, UK). The measurements were carried out at a temperature of 25 °C, using a wavelength of 633 nm. The scattering angle was set to 173 degrees, using non-invasive back-scattered (NIBS) optics. The experimental data were treated according to the Smoluchowski equation.

The suspensions were diluted 30 times with saline prior to both DLS and ζ potential measurements.

Transmission electron microscopy (TEM) images of the three lipid formulations were obtained at cryogenic temperatures. Prior to the measurements, the three liposomal suspensions were diluted to a concentration of 5 mg/mL and were mounted on glow-discharged holey carbon grids, quickly frozen by a Cryoplunge CP3 system (Gatan Inc., Pleasanton, USA), and transferred to a JEM2200FS transmission electron microscope (JEOL Ltd., Tokyo, Japan) using a Gatan626 cryo-holder. Cryo-electron micrographs were recorded at an acceleration voltage of 200 kV at a magnification of 20,000, 4 to 8 μm under-focus, and a dose of 10 electrons/Å^2, using a F416 CMOS detector (TVIPS GmbH, Gauting, Germany).

The morphology of the liposomes exhibits deviations from the spherical shape towards ellipsoids, depending on the lipid composition. The local curvature C and the eccentricity ε of the liposomes were estimated from the cryo-TEM projections (*cf.* Fig. 2). Therefore, ellipses have been employed with a and b the major and minor semi-axes. The curvature of an ellipse can be written as $C = a\,b\,/\,(a^2 \sin^2(t) + b^2 \cos^2(t))^{3/2}$ being t the angle between a and a generic point lying on the ellipse. For sphere-like liposome $a = b = r$, the curvature is given by $C_{\text{circle}} = 1/r$, being r the outer radius of the fitted circle. In case of an elliptically shaped liposome, the local curvature C_{ellipse} ranges from b/a^2 to a/b^2. The eccentricity of an ellipse is given by $\varepsilon = \sqrt{1 - b^2/a^2}$ and ranges as $0 < \varepsilon < 1$; for circles it is zero.

The efficiency of nitroglycerin loading was estimated indirectly from the glucose content of the nitroglycerin solution measured by electrospray ionization mass spectrometry (ESI-MS) using a Bruker Daltonics esquire HCT ion trap mass spectrometry [40]. Here, a volume of 0.1 mL of each sample was diluted ten times with methanol and treated with 10 μL of trifluoroacetic acid. The ESI spectra were measured in the negative mode. The ESI-MS signal of the observed glucose-trifluoroacetic acid adduct at 292.92 amu was compared to the ESI-MS signal which an equally treated solution of nitroglycerin (Bioren 0.1% containing 1 mg/mL nitroglycerin as active pharmaceutical ingredient and 49 mg/mL glucose as excipient) yields. It was assumed that the ratio of glucose *versus* nitroglycerin, as found in the commercial nitroglycerin solution, stays constant during the liposome formulation and purification steps.

2.5. In vitro immunoassay in human sera via SC5b-9

Human sera from six donors were aliquoted and stored in Eppendorf tubes at a temperature of − 80 °C. Prior to use, they were thawed to 4 °C and kept in an ice bath. Each human serum was mixed with the three liposomal suspensions (L1, L2, and L3) and their ten-times diluted counterparts (L1a, L2a, L3a). Each serum was treated with saline and nitroglycerin solution being the negative controls. For the comparison with FDA-approved liposomal drugs and as positive control, mixtures of each serum with Doxil (2 mg/mL doxorubicin, 12.7 mg/mL phospholipids), Abelcet (5 mg/mL amphotericin B, 4.9 mg/mL phospholipids), and zymosan (1.5 mg/mL) were used. Each mixture was incubated at a temperature of 37 °C. The reaction was terminated after 5, 10, 20 and 40 min by adding 10 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich Kft, Budapest, Hungary). The level of SC5b-9 was

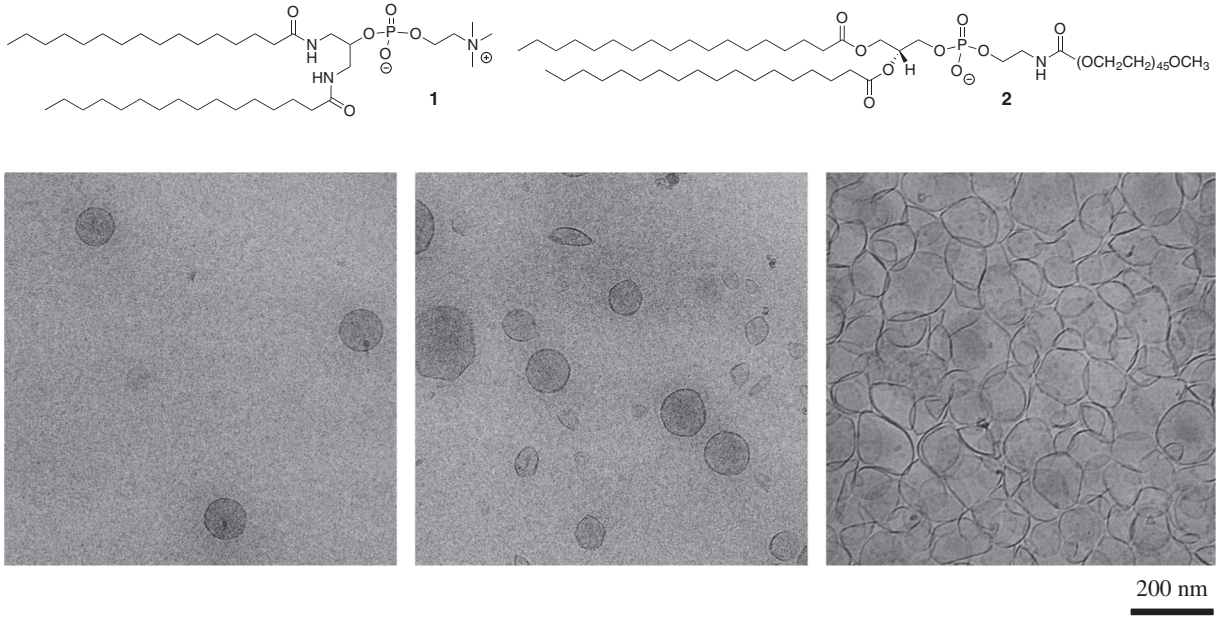

Fig. 2. Structure of the two phospholipids used for the liposome preparations: Pad-PC-Pad (1) and DSPE-PEG2000 (2). Cryo-TEM micrographs of L1 Pad-PC-Pad/DSPE-PEG2000/nitroglycerin (left), L2 Pad-PC-Pad/DSPE-PEG2000/saline (middle), and L3 Pad-PC-Pad/nitroglycerin (right) liposomes. The pure Pad-PC-Pad liposomal suspension contains many aggregated clusters, possibly because the stiff membranes lack repulsive undulation movement. The curvature C of the liposomes was estimated: $(51\text{ nm})^{-1}$ to $(44\text{ nm})^{-1}$ for L1, $(130\text{ nm})^{-1}$ to $(10\text{ nm})^{-1}$ for L2, and $(100\text{ nm})^{-1}$ to $(5\text{ nm})^{-1}$ for L3.

quantified by a MicroVue SC5b-9 Plus ELISA kit (Quidel, San Diego, USA). The assay was carried out following the manufacturer's protocol. Classical and alternative pathways were tested using the MicroVue C4d Plus ELISA and MicroVue Bb Plus ELISA kits (Quidel, San Diego, USA), to detect the concentration of C4d and of Bb, respectively. The samples prepared for SC5b-9, C4d, and Bb assays were diluted 20 times using the specimen diluent provided by the SC5b-9 kit and additional 10 mM EDTA, as the minimum dilution required to detect the optical density. The lectin pathway was investigated using samples treated as described above and supplemented with EGTA/Mg^{2+} (Ca^{2+} chelation), in order to block selectively the classical pathway [41]. In this case, the MicroVue C4d Plus ELISA assay was used to detect C4d concentration. After termination of the reaction, the samples were diluted 30 times with the specimen diluent provided by the C4d kit and additional 10 mM EDTA. Each sample was subjected to a further dilution step before transferring it to the ELISA plate. The optical density was measured with a 96-well plate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) at a wavelength of 450 nm (in the cases of SC5b-9 and Bb) and at a wavelength 405 nm (in the case of C4d).

2.6. SC5b-9 detection in porcine sera via C3 consumption

In a previous study [42], we tested complement activation in porcine samples, using the MicroVue Pan-Specific C3 Reagent Kit (Quidel, San Diego, USA). This test, however, is rather qualitative for a study involving porcine sera, because validity has only been demonstrated for human sera. Therefore, we searched for an alternative kit to measure directly the concentration of the SC5b-9 complex in porcine sera. We identified the ELISA kits from MyBioSource Inc. (San Diego, USA) as being possibly suited to this purpose. The manufacturer states that the kit is a quantitative sandwich ELISA assay, to be used for determining the level of SC5b-9 in undiluted original porcine body fluids. Following the guidelines of the manufacturer concerning sample preparation and the assay procedure, we did not find any significant difference between the negative (phosphate-buffered saline) and positive (zymosan) controls. As there was no support available from the supplier, we have to state that the kit was inappropriate for our study. Thereafter, we decided to investigate the level of SC5b-9 in porcine sera through the qualitative detection of C3 consumption, using a MicroVue Pan-Specific C3 Reagent Kit from Quidel Corp., which, to the best of our knowledge, is the only possibility currently available on market.

Each donor's serum was treated with the liposomes, the positive and negative controls as done with the human ones, see description in Section 2.5. As stated above, complement activation in porcine samples was assessed using the Quidel MicroVue Pan-specific C3 reagent kit. This kit allowed for converting the C3 activity in animal samples to the human SC5b-9 protein complex, which could be detected using the SC5b-9 Plus ELISA kit. The optical density was measured with a 96-well plate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) at a wavelength of 450 nm.

2.7. Statistical analysis

The statistical analysis was carried out using GraphPad Prism 6 (GraphPad Software Inc., San Diego, USA). All samples, except those incubated with zymosan, were compared with saline after 40 min of incubation, using ordinary one-way ANOVA, followed by Dunnett's multiple comparison test. *P*-values lower than 0.05 were considered as statistically significant.

2.8. CARPA in the porcine model

Six domestic male, mixed breed Yorkshire-Hungarian landrace pigs, weighing from 19 to 24 kg, were treated according to the protocol approved by the local Animal Subject Review Committees. Animals were pre-anesthetized intramuscularly with Calypsol/Xilazine 2–3/1.5–2 mL/kg injections in the stalls, to avoid stress, and further anesthesia was maintained with 2 to 3% isoflurane in O_2. Intubation was performed with endotracheal tubes to maintain open airways and to enable controlled ventilation. Spontaneous ventilation was followed by a pulse-oximeter fixed on the tail to monitor oxygen saturation. Temperature was measured rectally and monitored by an Innocare-P anesthesia monitor. A capnograph was connected to the tracheal tube to monitor end tidal CO_2 and respiratory rate (CAP10 Medlab, Medizinische Diagnosegeräte GmbH, Karlsruhe, Germany). Surgery was done after povidone iodine (10%) sterilization of the skin. In order to measure the PAP, a Swan-Ganz catheter (AI-07124, 5 Fr. 110 cm, Arrow International Inc., Reading, USA) was introduced into the pulmonary artery *via* the right external jugular vein, while the SAP was measured in the femoral artery. Blood samples were taken from the left femoral vein, before and after the injection of liposomes (time points after 1, 3, 5, 10, 20, and 30 min), and were collected in hirudin- and K_2-EDTA tubes. Liposome suspensions and zymosan were injected into the animals as bolus within a period of < 10 s *via* the left external jugular vein. Hemodynamic changes (PAP, SAP, and heart rate (HR)) were continuously monitored and either expressed in absolute or in relative terms compared to the baseline throughout this study.

3. Results

3.1. Liposome characterization

The total lipid concentration was measured after purification and found to be (20.40 ± 0.16) mg/mL for L1, (16.52 ± 0.10) mg/mL for L2, and (18.72 ± 0.03) mg/mL for L3. Table 2 lists the related DLS and ζ potential results. Within the estimated errors, the results for the liposomes with and without PEGylated lipids were comparable. The measured eccentricity ε of the three liposomal formulations ranged from zero to 0.95 (Table 2); the curvature C was estimated (Fig. 2, caption). Preliminary data of the nitroglycerin incorporation efficiency measured by ESI-MS showed 49% for L1 and 27% for L3. These results indicated a beneficial effect of the presence of PEG network for retaining nitroglycerin inside a liposome.

Fig. 2 displays cryo-TEM images representing the morphology of the liposomes. Pure Pad-PC-Pad liposomes formed aggregates (Fig. 2, right), probably due to limited membrane flexibility. Membrane undulation typically contributes to liposome-liposome repulsive forces [43], but this aggregation of Pad-PC-Pad liposomes can be prevented by adding a steric barrier in the form of 5% PEGylated lipids, as indicated in the other two cryo-TEM images (Fig. 2, left and middle). Cryo-TEM data further indicated that the liposome suspensions, containing the PEGylated lipids, were less faceted compared to the liposomes made

Table 2
Characteristics of the liposomal formulations tested *in vitro*.

Label	Lipid composition	Lipid content (mg/mL)	Mean diameter (nm)	PDI	Eccentricity		ζ potential (mV)
					ε_{min}	ε_{max}	
L1	Pad-PC-Pad/DSPE-PEG2000 (nitroglycerin)	20.40 ± 0.16	122 ± 2	0.09 ± 0.01	0	0	− 0.78 ± 0.18
L2	Pad-PC-Pad/DSPE-PEG2000 (bare)	16.52 ± 0.10	151 ± 2	0.21 ± 0.03	0.40 ± 0.01	0.91 ± 0.01	− 1.78 ± 0.14
L3	Pad-PC-Pad (nitroglycerin)	18.72 ± 0.03	166 ± 9	0.19 ± 0.03	0.47 ± 0.01	0.95 ± 0.01	− 0.60 ± 0.15

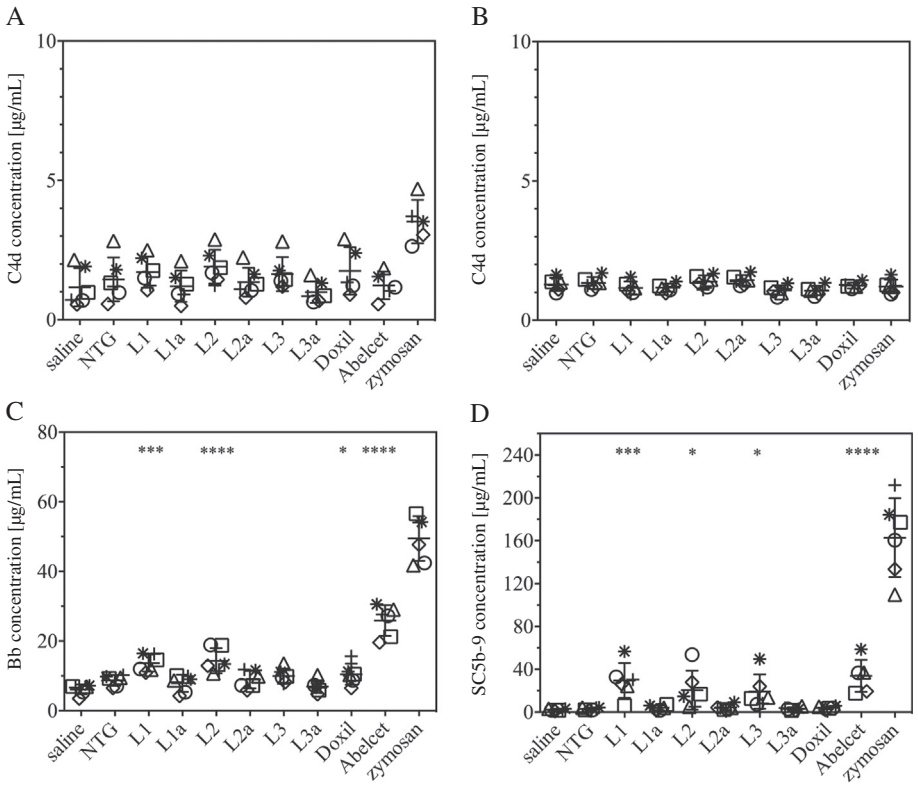

Fig. 3. The level of C4d (A), of C4d supplemented by EGTA/Mg^{2+} (B), of Bb (C), and of SC5b-9 (D) from human sera of six donors incubated for a period of 40 min at a temperature of 37 °C with saline, nitroglycerin (NTG), liposomes containing low (L1a, L2a, L3a) and high (L1, L2, L3) lipid contents, Doxil, Abelcet, and zymosan. The data are presented as mean values, including error bars derived from the standard deviation among the sera. Each symbol (triangle, square, circle, star, cross, rhombus) represents data from one of the six donors. The values were adjusted to the liposome concentrations of 2 and 20 mg/mL, respectively. For the statistical analysis, zymosan was excluded.

from pure Pad-PC-Pad, and the addition of nitroglycerin reduced the faceting of the liposomes, *cf.* Fig. 2. The diameters of liposomes derived from the cryo-TEM images were in agreement with those from the DLS data.

3.2. In vitro immunoassay in human sera

Fig. 3 shows the concentration of the protein C4d, without (A) and with (B) the addition of EGTA/Mg^{2+}, Bb (C), and the terminal complex SC5b-9 (D) tested in human sera from six donors. The sera were incubated with the liposomes or the appropriate controls for a period of 40 min. As the experimentally found lipid contents of the three liposomal formulations corresponded to (20.40 ± 0.16) mg/mL for L1, (16.52 ± 0.10) mg/mL for L2, and (18.72 ± 0.03) mg/mL for L3, the obtained data were adjusted to the liposome concentration of 20 mg/mL, in order to mitigate the effect of the lipid concentration on the complement protein levels. Thus, the data were multiplied by the factor 0.98 for L1, 1.21 for L2, and 1.07 for L3. The phosphate test and the DLS results enabled us to estimate the average liposome-entrapped volume, which was 61.7 μL per mL suspension for L1 and 91.4 μL per mL suspension for L3. According to the ESI-MS results, being the encapsulation efficiency 49% for L1 and 27% for L3, the total amount of nitroglycerin per mL suspension was 30.4 μg for L1 and 24.6 μg for L3.

The diagrams in Fig. 3A and B provide the results of the classical and lectin pathways, respectively. For the lectin pathway, the C4d level of the sample containing Abelcet was not available. The C4d concentration of the samples did not show any significant difference compared to saline. The only exception was zymosan, where a slight increase in C4d concentration was observed, as illustrated in the diagram of Fig. 3A.

Bb concentrations, see Fig. 3C, in the samples containing Abelcet and zymosan increased four- and eight-fold with respect to saline. For the liposomal suspensions L1 and L2, Bb concentration was found comparable to Doxil and more than twice that of saline. Liposome suspensions at a lower phospholipid content (L1a, L2a, and L3a), as well as L3, did not show any significant increase in Bb level with respect to saline.

The three pathways can lead to the formation of the terminal complement complex detected by soluble C5b-9 concentration in the serum. Fig. 3D displays the results with respect to the SC5b-9 concentration. It was found that zymosan activated the formation of the SC5b-9 complex within only five minutes of incubation, *cf.* Supplementary information, Fig. S1, and increased up to 80 times after 40 min of incubation as compared to saline. Abelcet increased SC5b-9 concentration > 15 times compared to saline. SC5b-9 concentration in the liposomal suspensions L2 and L3 increased five-fold compared to Doxil and ten-fold compared to the negative control, whereas L1 showed more than seven times and 15 times higher SC5b-9 levels compared to saline and Doxil, respectively. At a lower phospholipid concentration, *i.e.* L1a, L2a, and L3a, the level of SC5b-9 increased less than twice the amount of saline and, within the standard deviation, the results were comparable to Doxil.

The controls and the liposomes were also incubated with each human serum for 5, 10, and 20 min. These data at the selected time points show a trend summarized in Supplementary information, Fig. S1.

3.3. Validation of the in vitro human sera study

The results of the *in vitro* study using human sera were underpinned by the *in vitro* assessment of complement activation with porcine sera from six animals, and the *in vivo* experiment with continuous monitoring of the hemodynamic behavior of six Yorkshire pigs after the injection of Pad-PC-Pad liposomes.

3.3.1. In vitro immunoassay in porcine sera

The level of complement activation in porcine sera from six animals, tested through C3 consumption, is displayed in Fig. 4. The sample containing saline was used as a baseline. The level of C3 consumption in Abelcet increased up to 72% over the baseline and was twice that of zymosan. Doxil led up to a 20% increase, showing similar complement

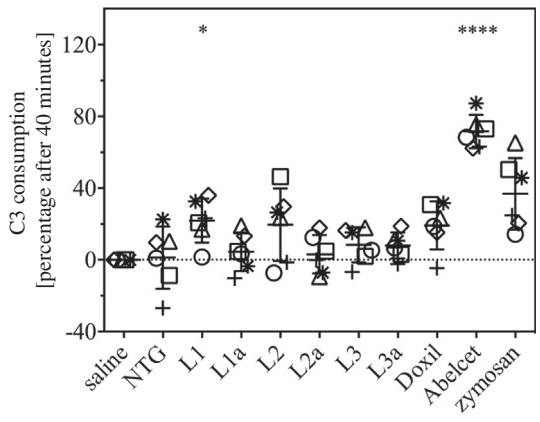

Fig. 4. Complement activation through C3 protein consumption relative to saline in porcine sera from six different animals incubated for a period of 40 min at a temperature of 37 °C with saline and nitroglycerin (NTG), liposomes containing low (L1a, L2a, L3a) and high (L1, L2, L3) lipid contents, Doxil, Abelcet, and zymosan. The data are shown as mean values, including standard deviation among animals. Each symbol (triangle, square, circle, star, cross, rhombus) represents data from one porcine serum. The values were adjusted to liposome concentrations of 2 and 20 mg/mL, respectively. For the statistical analysis, zymosan was excluded.

activity as in L1 and L2 with 22% and 20% rises over saline, respectively. Lower levels of C3 consumption were found for L3 and L3a - an increase of < 10% – and even less for L1a and L2a with increases over the baseline of 4% and 3%, respectively. According to the statistics, significant differences with respect to saline were only found for the undiluted sample containing Pad-PC-Pad/DSPE-PEG2000 loaded with nitroglycerin (L1) and for Abelcet.

3.3.2. *In vivo study in the porcine model*

Six pigs were used to explore in each the hemodynamic changes caused by sequential intravenous administrations of selected liposome boluses, as specified in Tables 3 and 4. L1′ was tested in three doses. L2′ and L3′ were injected using a pre-defined dose in the six animals. At the end of each experiment, zymosan was injected as positive control. The sequences, with which the liposomal formulations were injected, are represented in Supplementary information, see Table S2.

The human therapeutic dose (HTD) of intravenous nitroglycerin solution ranges from 13 to 133 μg in one minute [44,45]. The liposome doses were adjusted in order to obtain 0.5 mg Pad-PC-Pad per kg of pig and flushed into the circulation with 5 mL saline solution. Therefore, the total amounts of phospholipids for the samples containing PEGylated lipids (L1′, L2′) were 0.6 mg/kg (0.5 mg/kg Pad-PC-Pad plus 0.1 mg/kg DSPE-PEG2000) for HTD. Using the same lipid formulation as in L1′, another two nitroglycerin dosages were prepared: 0.33 × HTD and 3 × HTD with a total concentration of 0.2 mg/kg (0.17 mg/kg Pad-PC-Pad plus 0.03 mg/kg DSPE-PEG2000) and 1.8 mg/kg (1.5 mg/kg Pad-PC-Pad plus 0.3 mg/kg DSPE-PEG2000), respectively. According to ESI-MS measurements the dose of nitroglycerin was 94 μg per minute for L1′ and 79 μg per minute for L3′, in agreement with the HTD. As the injection time of the liposomal suspension was about 10 s, the nitroglycerin payload was 0.8 μg per kg pig for L1′ and 0.7 μg per kg pig for L3′.

As expected, the injection of liposomal drugs led to HR modification, a PAP increase, and a change of SAP. In one of the six pigs, the L1′ injection led to a slight increase of HR, which returned to baseline level within minutes. All other samples did not cause any visible change of the HR, besides the gradual, slow increase of the HR during the course of the experiment, owing to anesthesia. Moreover, no cardiac arrhythmia in the form of tachycardia or bradycardia, or extrasystoles, was observed.

Changes in PAP and SAP observed upon injecting the liposomes were calculated as relative changes in percentage terms of the baseline value at pre-injection time. An overview of the recorded PAP, SAP, and HR graphs upon injection of Perlinganit-loaded and drug-free liposomes is represented in the Supplementary information, see Figs. S2 to S7.

The zymosan injection always led to an immediate PAP increase of up to 180%. The injection of 70 μg nitroglycerin, however, caused a decrease in the PAP of maximal 25%. These results set the stage for assessing the CARPA caused by the liposomal suspensions.

The measured maximal changes in PAP upon injecting the corresponding liposomal formulations are listed in Table 3. In all cases, sample L1′ with 1.5 mg/kg of Pad-PC-Pad showed an increase of the PAP of up to 76%, thus indicating mild complement activation. This increase is similar to the hemodynamic changes that occurred following an injection of Doxebo liposomes [25]. Compared to other liposomal drugs, such as Ambisome, where the PAP increases by 200% for an injection of 0.01 mg/kg [27], or Doxil, with an increase up to 400% of the baseline [25] for 0.06 mg/kg phospholipid, the observed increase in PAP with liposomes encapsulating nitroglycerin also had a minimal effect of about a 33% increase in PAP, most likely caused by the aggregation of liposomes [42]. Furthermore, it should be noted that it is only the response to the first injection where cumulative effects can be excluded. In fact, self-induced tolerance, *i.e.*, tachyphylaxis has been known in case of certain liposomes. However, the fact that we saw reactions to the second and third boluses of L1′ argued against full tachyphylaxis after the first injection, enabling us to test L2′ and L3′ at the same doses as L1′ was first injected (0.5 mg/kg). The presence of very similar, small reactions to L2′ and L3′ cannot be explained by tachyphylaxis, as tachyphylaxis prevents follow-up reactions to identical doses, and L1′ was applied three times at much higher doses, which would fully eliminate any further reaction in the presence of tachyphylaxis. Partial tachyphylaxis cannot be ruled out, but overall, these pig data showed very mild changes, which are most likely biologically irrelevant. However, the mere presence of even small hemodynamic changes point to a risk of CARPA in case of substantial overdosing of test liposomes.

Table 3
Relative maximal changes in pulmonary arterial pressure. Bolus injections were separated by at least 30-minute recovery periods. Lipid concentrations correspond to the amounts of Pad-PC-Pad per kg (of pig). The sequence of liposomal injection is given in Supplementary information (Table S2).

Pig ID	L1′ 0.5 mg/kg	1/3 × L1′ 0.17 mg/kg	3 × L1′ 1.5 mg/kg	L2′ 0.5 mg/kg	L3′ 0.5 mg/kg
	PAP (%)				
1	14.9	17.7	16.9	4.7	3.5
2	NA	3.5	30.4	8.1	0.0
3	1.9	NA	75.9	2.9	0.0
4	0.0	NA	19.4	8.5	1.5
5	6.3	NA	7.2	NA	4.2
6	6.0	4.8	32.7	1.7	10.1
Mean ± SD	5.8 ± 5.7	8.7 ± 7.9	30.4 ± 24.2	5.2 ± 3.0	3.2 ± 3.8

3.3.3. *Tachyphylaxis in pigs after Pad-PC-Pad injection*

PAP usually increased after the first liposome injection. The reaction to a subsequent injection showed possible tachyphylaxis. An example of the PAP response is represented in Fig. 5 regarding the injection regimes, with the injection sequence being modified for samples L1′ through L3′. The upper panel on Fig. 5 displays PAP changes in a selected animal, induced by injecting the L1′ samples in order of increasing concentration, starting with the lowest lipid concentration of 0.17 mg/kg and finishing with the highest one of 1.5 mg/kg. The PAP always reached the same level. Changes in PAP monitored in another animal are shown in the lower panel in Fig. 5. When the highest phospholipid concentration was injected first, partial tachyphylaxis was observed.

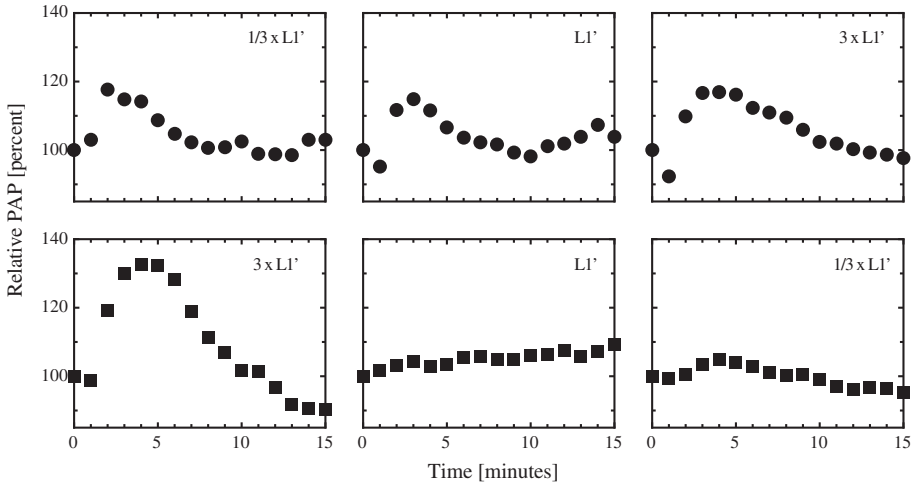

Fig. 5. Injection of PEGylated nitroglycerin-loaded Pad-PC-Pad liposomes in two selected animals. The results for the first animal are represented using filled black circles (three diagrams on the upper row) and the ones for the second animal are given by filled black squares (three diagrams on the lower row). Each row represents a consecutive injection protocol in one animal. The bolus injections were separated by a recovery time of at least 30 min.

Table 4
Relative maximal changes in systemic arterial pressure. Lipid concentrations correspond to the amounts of Pad-PC-Pad per kg (of pig). The sequence of liposomal injection is given in Supplementary information (Table S2).

Pig ID	L1 0.5 mg/kg	1/3 × L1′ 0.17 mg/kg	3 × L1′ 1.5 mg/kg	L2′ 0.5 mg/kg	L3′ 0.5 mg/kg
	SAP (%)				
1	0.0	0.8	9.2	4.9	1.0
2	NA	− 0.4	0.4	2.1	1.5
3	0.0	NA	23.5	0.7	3.5
4	0.0	NA	7.6	2.6	3.3
5	3.6	NA	7.4	NA	2.0
6	1.7	− 0.2	5.7	4.9	4.0
Mean ± SD	1.1 ± 1.6	0.1 ± 0.6	9.0 ± 7.7	3.0 ± 1.8	2.6 ± 1.2

3.3.4. Changes in systemic arterial pressure (SAP)

The extrema of the systemic arterial pressure measurements are listed in Table 4. Contrary to the consistent rise in PAP, SAP showed an immediate increase or decrease of up to 10% for the 0.1 mg/kg zymosan injection, with the type of reaction depending on the individual pig. The injection of 70 μg nitroglycerin caused a maximal decrease of 10% in SAP level. The injection of sample L1′ with 3 × HTD resulted in a significant change in SAP, albeit only for Pig 3.

4. Discussion

Liposome preparation through the extrusion technique is one of the common methods used to generate uniform liposome suspensions [37]. The mean sizes of the liposomes, being 20% to 70% larger than the smallest membrane pore size of the filters used, agrees with previous findings [46]. The liposomes of L2 and L3 exhibit a mean size that reflects their stiffness and degree of faceting. These liposomes were extruded above the membrane main phase transition temperature to facilitate sizing. Nonetheless, parts of the rather stiff membranes of L2 and L3 passed the extrusion filter pores without being sheared, possibly by adopting an orientation along the flow direction.

The ζ potential measurements characterize liposome surfaces and the stability of suspensions, both of which are essential for the efficacy of drug delivery [47]. It has been reported that the presence of PEGylated lipids affects ζ-potential, which depends on PEG size and anchor [27,48]. Our results show ζ-potential values close to zero, regardless of PEGylation (Table 2). This behavior can be explained by Pad-PC-Pad resulting in a neutral, zwitter-ionic amphiphile (Fig. 2, (1)). The addition of negatively charged DSPE-PEG2000 causes the expected marginal decrease in the ζ-potential, as the comparison between L1 and L3 indicates. Encapsulated nitroglycerin has a detectable impact, because the liposomes prepared in saline (L2) show a ζ-potential which is more than twice as high as the value of the formulations with nitroglycerin, *i.e.* L1 and L3.

Due to its simplicity, the passive loading of a drug into a liposome *via* thin lipid film hydration and freeze-thaw steps is widely used. The presence of a PEG network inside and outside of the liposome membrane in the formulations L1 and L2 was solely chosen to prevent the formation of aggregates, which increase the immune system response [42]. Furthermore, the addition of PEGylated lipids seemed to be beneficial to nitroglycerin retention and suggests a noncovalent interaction between the two molecules.

A semi-quantitative ESI-MS assay, carried out for the liposomal formulation L1 and L3, showed a high loading of 27% to 49% nitroglycerin into Pad-PC-Pad liposomes, respectively. The passive loading of nitroglycerin is higher than expected from literature data of passive loading of other drug molecules [49], possibly because of the low nitroglycerin molecular weight being 227.1 Da.

The present study identified the pathway, through which Pad-PC-Pad-based liposomes can trigger the complement system. Although the *in vitro* results of the immunoassay in human sera clearly indicate no activation of the lectin pathway, the result has to be treated with care, because for the lectin pathway test the sample containing Abelcet was unavailable. The classical pathway, however, can definitely be ruled out, as zymosan exhibited the expected rise. The predominant role of the alternative pathway with respect to its classical and lectin counterparts in the presence of PEGylated lipids (L1 and L2 *vs.* L3) agrees with information available in the literature [27,48].

Samples containing Abelcet activated the complement system *via* the alternative pathway stronger than Doxil and Pad-PC-Pad-based liposomes at both high and low phospholipid contents. This enhanced complement activation is possibly associated with the micrometer size of the lipid complexes loaded with the amphotericin B drug. A previous communication [23] reported that larger liposomes increase the risk of severe immune reactions.

It is not surprising that Pad-PC-Pad-based liposome suspensions L1a, L2a, and L3a show a SC5b-9 level similar to the Doxil sample. This behavior is supported by the fact that these liposomes exhibit a size of about hundred nanometers. These formulations caused mild complement activation. In particular, the L1 formulation, comprising Pad-PC-Pad, PEGylated lipid, and the nitroglycerin payload, could trigger the immune system. This fact is interpreted as being the result of amplification caused by combining PEGylated lipids with nitroglycerin, which is in agreement with our results. The same behavior is known for Doxil, as doxorubicin and PEGylated lipids cause a higher response than each component alone [24]. In addition, it has been reported [23] that the

presence of drugs in the liposome's surface and in the medium surrounding the liposomes could increase complement activation.

A previous *in vitro* study on Pad-PC-Pad liposomes, where the inner cavity was loaded with phosphate-buffered saline, showed negligible complement activation [42]. The nitroglycerin-loaded Pad-PC-Pad liposomes (L1a, L2a, and L3a) yielded a SC5b-9 level, well comparable to the FDA-approved Doxil, for all tested formulations. Furthermore, C3 protein consumption measured in the sample containing Doxil was comparable to the formulations L1, L2, and L3, a fact explained by the higher sensitivity of the immune system in the porcine model with respect to humans.

In the animal experiments, the administration of drug-free (L2′) and nitroglycerin-loaded (L1′ and L3′) liposomes with dosage up to 3 × HTD highlighted no significant changes in the monitored parameters, a result which agrees with previous data from liposomes of the same composition containing phosphate-buffered saline [42].

In addition to the complement activation by L1 formulation, which can be interpreted as being the result of an amplification caused by combining PEGylated lipids with nitroglycerin, the non-faceted shape of the liposomes has to be considered [13,20]. The cryo-TEM micrographs show that the liposomes prepared with or without the addition of PEGylated lipid and nitroglycerin payload do not only exhibit a size distribution but significantly differ in shape. As the curvature is size- and shape-dependent, it is suitable to characterize the liposome's anisotropy by the eccentricity – an approach, which accounts for the recent interpretation on adverse injection reactions to disk-like and spherical nanoparticles through shape modification [20].

According to the cryo-TEM images, the L3 formulation, where the highest eccentricity was found (*cf.* Table 2), caused the lowest complement activation. On the contrary, the L1 formulation comprising Pad-PC-Pad, PEGylated lipids, and the nitroglycerin payload with zero-eccentricity triggered a mild complement activation. The formulation L2 comprising Pad-PC-Pad, PEGylated lipids and loaded with saline showed eccentricity and complement activation values between the formulations L1 and L3. Therefore, the shape could be a key parameter regulating the acute adverse reactions.

Although the identity of the proteins and receptors, that cause the adverse reactions, remains unknown [20], one may speculate that the angles offered to the protein adsorption by the non-spherical liposomes gain access to the underlying phenomena, as found for nanopyramids influencing inflammatory reactions more than a decade ago [50,51]. Hence, the presence of hydroxyapatite crystallites in bone and the advantage of nanometer-scale roughness on lead-bearing implants is understood. In this context, studies on the three-dimensional structure of Pad-PC-Pad liposomes will follow as a prerequisite for a detailed understanding of acute adverse reactions.

One limitation of the Pad-PC-Pad liposomes for applications inside the human body lies in the main phase transition, which is very close to body temperature. Above 37 °C, the Pad-PC-Pad liposomes release 5(6)-carboxyfluorescein completely [11]. Consequently, for clinical use, one should search for a slightly more stable mechano-responsive container than the proposed Pad-PC-Pad liposomes.

5. Conclusion

Nanometer-sized Pad-PC-Pad liposomes with a concentration of 20 mg/mL initiate the complement cascade *via* the alternative pathway, concluded from an increase in the level of Bb protein. This finding is confirmed by significant differences with respect to saline in the final stage of complement activation, represented by the SC5b-9 concentration, which has been detected as significantly higher than for saline in the final stage of complement activation. Reducing the lipid content of Pad-PC-Pad to 2 mg/mL, the complement activation was negligible, a result derived from the *in vitro* experiments with human and porcine sera as well as an animal study. Therefore, the liposomes developed in this context are promising mechano-responsive container candidates for targeted drug delivery.

Despite the progress in understanding complex processes involving the immune system defenders, the shape of the particles may play a basic role in triggering an immune reaction. To this end, the structure of the liposomes should be better characterized and optimized towards slightly higher transition temperatures.

Future efforts will focus on the release kinetics of these mechano-responsive liposomes of 100 nm diameter for a detailed comparison with currently used non-encapsulated administration methods of nitroglycerin in emergency situations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2017.08.010.

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