1. Introduction

Nanomedicines have been investigated and used intensively over the past decades for several biomedical applications. With numerous products already approved and a variety of preparations in clinical trials, liposomes are the most advanced and successful nanomedicine in clinic. Thereby, one of the main goals of liposomes is to protect the entrapped drug while also reducing its off-site toxicity, as shown for multiple formulations. To this end, increasing on-target tissue concentration is a key aspect which can be achieved both by nontargeted and targeted liposomes. In this regard, a crucial point is the prolongation of systemic circulation lifetime. It is well-known that upon injection the majority of conventional (plain) liposomes are cleared rapidly from the blood stream by cells of the mononuclear phagocyte system. To enhance circulation times, a variety of approaches to modify the surface of liposomes emerged yet ultimately polyethylene glycol (PEG) established itself as gold standard in the early 1990s aiming at decreasing opsonization. PEGylation not only reduces but also alternates the recognition of nanoparticles by complement factors and opsonins, resulting in a decreased nanoparticle recognition and clearance by macrophages. Manipulating opsonization, including the reduction of complement activation thus is one of the main goals of preventing rapid clearance.

Today, surface modifications of liposomes are mostly realized with amphiphilic lipid-polymer conjugates like 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-mPEG2k). Despite the benefits and being considered mostly nonimmunogenic, phospholipid-PEG conjugates have also demonstrated some drawbacks over the
last decade. Anti-PEG antibodies are a major concern regarding the clearance of intravenously administered liposomes modified with PEG,[36,27] frequently culminating in the rapid clearance of injected liposomes.[28,29] The occurrence of pre-existing anti-PEG antibodies even in healthy individuals has increased in everyday life due to exposure to PEG-containing products.[30] Besides this so-called accelerated blood clearance phenomenon, it transpired that both, the anchoring phospholipid and PEG itself are likely to cause specific and nonspecific[31] immune reactions. Such reactions are often caused by complement activation[32,33] and can lead to hypersensitivity reactions (HSRs) possibly triggering anaphylaxis.[34]

The negative charge at the phosphate group of DSPE-PEG derivatives is predominantly linked to this complement induction.[35,36]

Conclusively, the development of alternative surface modifications offers an interesting possibility to circumvent the above-mentioned limitations of PEG derivatives.[37–39] For this purpose, a multitude of hydrophilic materials has been proposed.[40–45]

In this work, we report the synthesis of the lipid-like amphiphile bisalkyl polysarcosine (BA-pSar) and its successful incorporation into the lipid bilayer of preformed liposomes. It was demonstrated earlier that pSar has properties similar to PEG, comprising high water-solubility, flexibility, and low immunogenicity in vitro,[46,47] making it a promising candidate for surface modification of liposomes.[48] The aim of this study was to demonstrate that surface modification using pSar-lipopolymers is a valuable strategy to increase circulation half-life of liposomes while also ensuring good biocompatibility (i.e., low complement activation). After synthesis, physicochemical properties of pSar-modified liposomes were compared with PEGylated and nonsurface-modified liposomes regarding their size, polydispersity, and zeta potential. To evaluate the extend of pSar-insertion into the bilayer, we determined the incorporation efficiency via high performance liquid chromatography (HPLC) with increasing amounts of BA-pSar added to preformed liposomes using the post-insertion method.

In order to study the pharmacokinetics of liposomes with different surface modifications, we selected the zebrafish model as a validated in vivo tool to assess circulation properties and macrophage clearance.[49]

Addressing the occurrence of HSR, we also investigated the complement activation potential of liposomes modified with BA-pSar and an optimized acetylated derivative of pSar (pSar$_{102}$Ac) with net-neutral charge.

### 2. Results and Discussion

#### 2.1. Synthesis of Bisalkylated Lipopolysarcosine

One part of the present work was the synthesis of a bisalkylated polysarcosine. The synthesis (Scheme 1) was adapted and modified from the literature.[45] The sarcosine-N-carboxyanhydride was synthesized and purified before the polymerization was started by the addition of the bisalkylamine. In contrast to monoalkylated pSar lipopolymers, solvent condition had to be adjusted due to the hydrophobicity of the initiator. Thus, polymerizations were performed in benzonitrile. After completion of the reaction, which was assured by Fourier-transform infrared spectroscopy, the polymer was precipitated in cold diethyl ether and purified via dialysis (Figure 1).

$^1$H NMR experiments demonstrate that the deviation between obtained and calculated degrees of polymerization is below 10% (Table 1 and Figure 1A). In line with MALDI-TOF MS (matrix-assisted laser desorption/ionization-time of flight mass spectrometry) $^1$H DOSY (diffusion-ordered spectroscopy) NMR shows the presence of only one diffusing species confirming the absence of free initiator and water-initiated polymer (Figure 1B). The discrepancy between the molecular weights determined by HFIP-GPC (hexafluoro-2-isopropanol-gel permeation chromatography) and NMR can be explained with the uncommonly high values of polymethylmethacrylate (PMMA) in HFIP.[56]

The reported method is a simple way to vary lipopolymer architectures in a one-step polymerization. Moreover, polysarcosine bears the potential for further end group modification, due to the terminal amine end group (Scheme 1). To maintain the nonionic nature of the polymer, pSar end groups were acetylated with acetic anhydride in a post-polymerization manner without any influence on the molecular weight distribution or chemical integrity of the polymer (Figure 2A,B). In addition, MALDI-ToF analysis of BA-pSar$_{102}$ was performed and is displayed in Figure 2C. Despite severe mass discrimination, the spectrum reveals the full incorporation of the bis-alkyl amine initiator (dioctadecylamine) without any detectable side products, i.e., initiation by traces of water or other impurities. Enlargement of the most intense peaks (Figure 2D) shows degrees of polymerization of $X_n = 19.20$ and sub-populations due to sodium and potassium ionization.

The second part of this work was focused on the incorporation of synthesized pSar derivatives into the lipid bilayer of

----

**Scheme 1.** Synthesis of BA-pSar$_{102}$ based on nucleophilic ring opening polymerization of sarcosine NCA and formation of BA-pSar$_{102}$Ac via end group modification (acetylation).
liposomes and the comparison with DSPE-mPEG2k. Therefore, BA-pSar102, BA-pSar102Ac, and DSPE-mPEG2k were used to modify the surface of preformed 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)/cholesterol (Chol) liposomes. The lipid ratios were chosen to be similar to the formulation of an approved PEGylated liposome formulation, namely, Doxil. Doxil is composed of hydrogenated soya phosphatidylcholine, Chol, and DSPE-mPEG2k at a molar ratio of 57:38:5.[50]

2.2. Characterization of Liposomes

The hydrodynamic diameter and polydispersity index (PDI) of different liposome formulations were determined before and after purification of liposomes from noninserted lipopolymer conjugates using dynamic light scattering (DLS). Zeta potential was measured after purification. Results for size and PDI before and after post-insertion of lipopolymers followed by purification are displayed in Table 2. Within the estimated errors, results obtained for size and PDI were comparable for all preparations and did not change over the process of post-insertion. Size was approximately 120 nm and PDI was ≤0.1, indicating homogenous size distributions for all formulations.

Figure 3 shows Cryo-TEM (cryogenic transmission electron microscopy) images displaying the morphology of representative liposomes which were selected for further in vitro and zebrafish experiments. Unmodified as well as surface-modified formulations show a narrow size distribution. Neither PEGylation nor modification with BA-pSar 102 induced significant changes in liposomal morphology or size distribution. Cryo-TEM images are in good agreement with size values obtained by DLS measurements.

2.3. Zeta Potential of Liposomes

To assess the surface charge shielding potential of the different surface modifications, an additional set of liposomal formulations was prepared containing the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, 10 mol%) (Figure 4A). Measurements of liposomes without DOTAP (Figure 4B) revealed only slight differences in zeta potential due to the neutral character of the formulation. Addition of DOTAP allowed to compare the influence on surface charge shielding of different formulations using zeta potential measurements. Notably, size characteristics for all preparations were comparable (Supporting Information, Figure S1). Unmodified liposomes showed a relatively high zeta potential of about +46 mV. In contrast, the zeta potential for liposomes modified with DSPE-mPEG2k and BA-pSar102 was reduced drastically, as already reported previously.[51] PEGylation resulted in a zeta potential of about −6 mV, whereas liposomes modified with pSar had a zeta potential around +9 mV, suggesting an effective shielding of charge. The slightly negative zeta potential for PEGylated liposomes on the one hand is probably due to the negative charge at the phosphate moiety of DSPE-mPEG2k. The slightly positive charge of BA-pSar102-modified liposomes on the other hand can be attributed to the terminal secondary amine of pSar, which is protonated at physiological pH. Measurements of liposomes modified with end-capped pSar (BA-pSar102Ac) displayed a zeta potential of around +2 mV. Since BA-pSar102Ac bears no charge, this suggests that an effective shielding of the positive charge introduced by DOTAP was achieved with this formulation.

2.4. Incorporation Efficiency

For all surface-modified liposomes, DSPE-mPEG2k, BA-pSar102, and BA-pSar102Ac incorporation efficiency into the liposomal bilayer was monitored via HPLC.
In preliminary tests, different amounts of BA-pSar$_{102}$ (1–15 mol%) were dried from stock solutions, hydrated with preformed liposomes, and post-insertion into the liposomal bilayer was analyzed. Thereby, incorporation of at least 4.5 mol% was achieved by hydrating 7.5 mol% dried BA-pSar$_{102}$ or BA-pSar$_{102}$Ac with preformed liposomes. Excess conjugate was removed by ultrafiltration. These results correspond to an incorporation efficiency of approximately 60% of added conjugate. All further experiments therefore were conducted using the same setup, adding preformed liposomes to 7.5 mol% of dried BA-pSar$_{102}$ or BA-pSar$_{102}$Ac, respectively. When hydrating 5 mol% of dried DSPE-mPEG$_{2k}$ in an analogous manner, complete incorporation was observed. Incorporation efficiency results obtained after purification are shown in Table 3.

In summary, BA-pSar$_{102}$ and BA-pSar$_{102}$Ac were successfully incorporated into the lipid bilayer, resulting in liposomes with a narrow size distribution. Liposomes modified with lipopoly-sarcosines also showed effective surface charge shielding, comparable with findings for PEGylated liposomes. To further evaluate the potential use of BA-pSar$_{102}$-modified liposomes, macrophages and circulation properties were investigated in vivo in the zebrafish model.

2.5. Assessment of Liposome Pharmacokinetics In Vivo

Fluorescently labeled liposomes with different surface modifications (i.e., DSPE-mPEG$_{2k}$ as positive control for stealth properties or BA-pSar$_{102}$) and unmodified (conventional) liposomes (negative control) were successfully incorporated into the lipid bilayer, resulting in liposomes with a narrow size distribution. Liposomes modified with lipopoly-sarcosines also showed effective surface charge shielding, comparable with findings for PEGylated liposomes. To further evaluate the potential use of BA-pSar$_{102}$-modified liposomes, macrophages and circulation properties were investigated in vivo in the zebrafish model.

Table 2. Physicochemical characterization of liposomal formulations after purification. Values are means ± SD, n = 3.

| Formulation             | Hydrodynamic diameter [nm] | PDI   |
|-------------------------|----------------------------|-------|
| DSPC/Chol/DiI           | 107 ± 10                   | 0.05 ± 0.02 |
| DSPC/Chol/DiI/DSPE-mPEG$_{2k}$ | 113 ± 9                   | 0.06 ± 0.02 |
| DSPC/Chol/DiI/BA-pSar$_{102}$ | 123 ± 10                  | 0.09 ± 0.02 |
| DSPC/Chol/DiI/BA-pSar$_{102}$Ac | 130 ± 2                   | 0.05 ± 0.02 |

including malformations or increased mortality were observed (data not shown), indicating the biocompatibility of all liposome formulations including both surface modifications.

At 3 h post injection (hpi), all liposome formulations demonstrated a diffusive fluorescence staining without accumulations in the posterior caudal vein (PCV) region (Figure 5). Recently, it has been shown that endothelial cells in the PCV express stabilin receptors which can scavenge liposomes and thereby prevent blood circulation.$^{[52]}$ However, an increased colocalization (yellow) of conventional liposomes (red) and macrophages (green) was already detected at this time point indicating macrophage clearance (white arrows, Figure 5).

A clear difference in circulation characteristics of different liposome formulations was observed 24 hpi (Figure 5). Unmodified DSPC/Chol liposomes demonstrated decreased circulation properties indicated by a dotted staining pattern (i.e., clearance by stabilin scavenger receptors). In contrast, both surface-modified liposomes (i.e., DSPE-mPEG$_{2k}$ and BA-pSar$_{102}$) still demonstrated a prolonged circulation lifetime.$^{[53]}$ Strikingly, macrophage clearance of unmodified liposomes increased significantly as compared to surface-modified liposomes (indicated by yellow color of colocalization).$^{[54]}$ Both PEG and pSar prevented macrophage clearance signifying their ability to confer a stealth effect to the liposomes (Figure 5). This can be explained by the advantageous properties of lipopolymer surface modification including decreased opsonization and steric stabilization. In addition, this prevents time-dependent formation of aggregates via steric stabilization preferentially sequestered by macrophages. Conclusively, BA-pSar$_{102}$-modified liposomes outperformed unmodified control liposomes (i.e., DSPC/Chol) with favorable pharmacokinetic characteristics—an improved blood circulation lifetime and a decreased macrophage clearance. Moreover, BA-pSar$_{102}$-modification of liposomes resulted in similar in vivo properties as DSPE-mPEG$_{2k}$ liposomes, which are considered as the gold standard for long circulating, stealth liposomes.

2.6. In Vitro Complement Activation

Figure 6 shows the elevated sC5b-9 levels after incubation of liposomes and controls with three individual serum samples.
As expected, there are variations in sC5b-9 levels in the sera of different donors, as can be seen for the negative control (HBS, HEPES buffered saline). Compared to HBS, PEGylated liposomes induced a 1.5- to 2.5-fold increase in complement activation (C activation) in all tested sera, which is in good accordance with values obtained from literature.\[36\] For BA-pSar102-modified liposomes, an increase in C activation was observed, resulting in 2.5- to 5-fold higher sC5b-9 levels as compared to HBS. Charged liposomes are known to elicit the complement system and cationic liposomes are known to do so via the alternative pathway.\[55\] Thus, C activation enhancement by BA-pSar102 compared to DSPE-mPEG2k comes as no surprise as the N-terminus of the polymeric chain bears a secondary amine, which is present in its ammonium salt form under physiological conditions. In a similar manner, the negative charge at the phosphate moiety of DSPE-PEG is well known to stimulate C activation.\[35\] In order to exploit the advantageous synthetic features of lipopolysarcosine, the optimized end-capped BA-pSar102Ac derivative was incorporated into liposomes. Interestingly, BA-pSar102Ac-modified liposomes demonstrated improved properties with decreased complement activation. Compared to HBS only a 2.5-fold increase in sC5b-9 levels in serum C, and only 1.5-fold increase in serum A and strikingly no increase in serum B was observed.

Our findings show that replacing the anchoring DSPE with dioctadecylamine and subsequent end-capping of pSar with a neutral acetyl moiety results in a significantly decreased C activation in two of the three tested sera when comparing DSPE-mPEG2k with BA-pSar102Ac. This most likely is a result of omitting the charges both at the anchor (hydrophilic–hydrophobic interface) and the polymeric chain, as it was previously demonstrated for free (nonliposomal incorporated) polymers.\[47\]

### 3. Conclusion

In this study, we present the one step synthesis of a lipopolysarcosine derivative enabling controlled modification of liposomes to enhance systemic circulation. The controlled polymerization conditions allow for an accurate degree of polymerization resulting in the precise tailoring of pSar chain length while keeping polydispersity low. Due to the reactive surface modification.

![Figure 3. Cryo-TEM images showing the morphology of different liposomal formulations. A) DSPC/Chol/Dil, B) DSPC/Chol/Dil/DSPE-mPEG2k, and C) DSPC/Chol/Dil/BA-pSar102.](image)

![Figure 4. Surface charge shielding of liposomes. Zeta potential of different liposomal formulations containing A) 10 mol% DOTAP or B) no DOTAP. ● nonsurface-modified liposomes, ■ PEGylated liposomes, ▼ liposomes modified with BA-pSar102Ac, and ▲ liposomes modified with BA-pSar102. Symbols depict measured values, dashed lines show mean values ± SD, n = 3.](image)

| Formulation                        | Incorporation efficiency [%] |
|------------------------------------|------------------------------|
| DSPC/Chol/Dil/BA-pSar102           | 63 ± 6                       |
| DSPC/Chol/Dil/BA-pSar102Ac         | 57 ± 4                       |
| DSPC/Chol/Dil/DSPE-mPEG2k          | 96 ± 6                       |
amino group, further diversification can be achieved in a simple post-polymerization modification step without altering the polydispersity of the precursor polymer. Using this approach, the positive net charge of BA-pSar102 was omitted by acetylation of the terminal amine. Subsequently, we were able to successfully incorporate BA-pSar102 and BA-pSar102Ac into the lipid bilayer of preformed liposomes using post-insertion technique. Liposome characterization including the determination of size and morphology revealed a monodisperse size distribution with mostly unilamellar vesicles. Zeta potential measurements of surface-modified liposomes demonstrated an effective shielding of surface charge. In contrary to modification with DSPE-mPEG2k and BA-pSar102, which still resulted in a slightly negative or positive zeta potential, BA-pSar102Ac-modified liposomes unveiled an almost net-neutral surface charge. Furthermore, the alternative hydrophobic anchor not only circumvents the negative charge that comes with the widely used DSPE-PEG derivatives but also provides sufficiently firm binding for the relatively large pSar chain into the bilayer. Moreover, BA-pSar-modified liposomes also featured prolonged circulation properties and decreased recognition by macrophages in the zebrafish model similar to PEGylated liposomes. Finally, liposome-mediated C activation could be reduced by modification of liposomes with an end-capped pSar derivative. All these results confirm that surface modification of liposomes with pSar or its derivatives is a promising alternative to well-established PEGylation strategies, especially in regard of reducing potential HSR. Equipped with an easily modifiable

Figure 5. Assessment of liposome pharmacokinetics in transgenic zebrafish. Conventional liposomes and liposomes surface-modified with DSPE-mPEG2k or BA-pSar102 were injected into the blood circulation of zebrafish (2 days post fertilization). Confocal images of tail region were acquired 3 and 24 hpi. White arrows indicate macrophage clearance of liposomes assessed by colocalization (yellow color) of green fluorescent macrophages (KAED) and fluorescently labeled liposomes (Dil, red). Quantitative analysis of macrophage clearance was performed using PCC. Values are means ± SD, n = 6. *p < 0.05 or **p < 0.01 (one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test) as compared to conventional liposomes composed of DSPC/Chol/Dil.
solid, free of phosgene and HCl, confirmed by testing against a silver
end-group, incorporation of lipopolysarcosine and further functionalization is also a promising option for the exploitation of active drug targeting or combination therapies.

4. Experimental Section

Materials: n-Hexane (Fischer Scientific, Waltham, USA) was distilled from Na/K. Benzonitrile was purchased from Sigma Aldrich (Steinheim, Germany), dried over CaH₂, and freshly distilled prior to use. Tetrahydrofuran (THF) and toluene were purchased from Fischer Scientific (Waltham, USA), dried over Na, and freshly distilled prior to use. HFIP was purchased from Fluorochem (Hadfield Derbyshire, UK). Tetrahydrofuran (THF) and toluene were purchased from Fischer (Steinheim, Germany), dried over CaH₂, and freshly distilled prior to use. HFIP was purchased from Fluorochem (Hadfield Derbyshire, UK). Diiodoacetylamine was purchased from Fluka (St. Gallen, Switzerland) and dried at 40 °C under vacuum (1 × 10⁻³ mbar) for 24 h. Dihydrosphene was purchased from Alfa Aesar (Ward Hill, MA, USA) and deuterated solvents from Deutero GmbH (Kastellaun, Germany).

DSPC and DSPE-mPEG2k were generously donated by Lipoid GmbH (Ludwigshafen, Germany). DOTAP was obtained from Merck (Darmstadt, Germany). 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Invitrogen (Thermo Fisher Scientific, Waltham, USA). Chol was purchased from Sigma Aldrich (Steinheim, Germany) and used as received unless stated otherwise. Human serum samples of ten healthy donors were obtained from the University Medical Center Freiburg. Sera were stored at a temperature of –80 °C until used.

Synthesis of Sarcosine N-Carboxyanhydride: A total of 14.92 g (167.4 mmol) sarcosine, dried under vacuum for 1 h, was weighed into a pre-dried, three-neck, round-bottom flask. A total of 300 mL of absolute THF was added under a steady flow of nitrogen, 16.2 mL (134 mmol) of dihydrosphene was added slowly via syringe, and the nitrogen stream was reduced. The colorless suspension was mildly refluxed for 3 h, yielding a clear solution. Afterward, a steady flow of dry nitrogen was led through the solution for another 3 h while the outlet was connected to two gas washing bottles filled with aqueous NaOH solution to neutralize phosgene. The solvent was evaporated under reduced pressure, yielding a brownish oil as a crude reaction product. The oil was dried under vacuum (1 × 10⁻³ mbar) for 2 h) to obtain an amorphous, free of phosgene and HCl, confirmed by testing against a silver nitrate solution. The crude product was redissolved in 40 mL of THF and precipitated with 300 mL of dry n-hexane. The solution was cooled to –18 °C and stored for 18 h to complete precipitation. The solid was filtered under dry nitrogen atmosphere and dried in a stream of dry nitrogen for 60–90 min and afterward under high vacuum for 2 h in the sublimation apparatus. The crude product was sublimated at 85 °C and 1 × 10⁻³ mbar. The product was collected from the sublimation apparatus in a glovebox on the same day. The purified product (110 mmol, 65% yield, colorless crystallites; melting point: 102–104 °C) was stored in a Schlenk tube at –80 °C.

1H NMR (300 MHz, CDCl₃): δ/ppm = 4.22 (2H, s, –CH₂–CO–), 2.86 (3H, s, –CH₃). Synthesis of Polysarcosine: Sarcosine NCA was transferred into a pre-dried Schlenk tube equipped with a stir bar under nitrogen counter flow and again flame-dried under high vacuum for 1 h. Subsequently, the NCA was dissolved in dry benzonitrile to yield a solution of 100 mg mL⁻¹ with respect to the NCA. 1/n equivalent of diiodoacetylamine was dispersed in pre-dried toluene and added to the NCA solution. The solution was stirred at room temperature and kept at a constant pressure of 1.25 bar of dry nitrogen via a Schlenk line to prevent impurities from entering the reaction vessel while allowing CO₂ to escape. Completion of the reaction was confirmed by Fourier transform infrared (FT-IR) spectroscopy (disappearance of the NCA related peaks (1853 and 1786 cm⁻¹)). After completion of the reaction, the polymer was precipitated in cold diethyl ether and centrifuged (4500 rcf at 4 °C for 15 min). After discarding the liquid fraction, new ether was added and the polymer was resuspended in a sonic bath. The suspension was centrifuged again and the procedure was repeated. The polymer was dissolved in water, dialyzed against MilliQ water and lyophilized, obtaining a colorless, stiff and porous solid. The yield after purification was 91% of the theoretically achievable mass of the polymer.

1H NMR (400 MHz; CDCl₃): δ/ppm = 5.1–3.80 (204H; br; (2n)–CO–CH₂–NMe₂–); 3.12–2.62 (306H; br; (3n)–N–CH₂–); 1.83–1.00 (64H; br; (CH₂–(CH₂)₆–CH₃); 0.87 (6H, t; (CH₂–(CH₂)₆–CH₃). Acetylation of Polysarcosine: 100 mg of polysarcosine (BA-pSar₁₀₂) was dissolved in 0.5 mL dimethylformamide (DMF), 10 equivalents (in respect to the calculated molecular mass of the polymer) of disopropylamine were added and the solution was stirred for 30 min at room temperature. Afterward, 5 equivalents of acetic acid anhydride were added and the solution was stirred overnight at room temperature. The polymer was precipitated in cold ether and centrifuged (4500 rcf at 4 °C for 15 min). After discarding the liquid fraction, fresh ether was added, the polymer was resuspended in a sonic bath and...
Table 4. Lipid compositions of prepared formulations.

| Formulation                        | Initial molar ratio | Ratio after purification |
|------------------------------------|---------------------|--------------------------|
| DSPC/Chol/Dil                      | 60:39:1             |                          |
| DSPC/Chol/Dil/DSPPE-mPEG2k         | 55:39:1.5           | 55:39:1:4.8              |
| DSPC/Chol/Dil/BA-pSar102           | 57:39:1:7.5         | 57:39:1:3.2              |
| DSPC/Chol/Dil/BA-pSar102Ac         | 57:39:1:7.5         | 57:39:1:3.4              |
| DSPC/Chol/DOTAP/                   | 50:40:10:0          |                          |
| DSPC-mPEG2k                        | 45:40:10:5          | 45:40:10:4.9             |
| DSPC/Chol/DOTAP/BA-pSar102         | 47:40:10:7.5        | 47:40:10:3.1             |
| DSPC/Chol/DOTAP/BA-pSar102Ac       | 47:40:10:7.5        | 47:40:10:3.3             |

centrifuged afterward. This procedure was dissolved in water, dialyzed against MilliQ water to remove excess of acetic acid and residual traces of DMF. After lyophilization, a colorless, stiff, and porous solid was obtained.

1H NMR (400 MHz; CDCl3); δ/ppm: 4.51–3.80 (204H; br; (2n)–CO–CH2–NH–); 3.12–2.62 (306H; br; (3n)–N–CH3–); 1.83–1.00 (64H; br; –CH2–(CH2)16–CH3); 0.87 (6H; t; –CH2–CH3).

1H NMR spectra were recorded on a Bruker (Billerica, MA, USA) AC 400 at a frequency of 400 MHz, respectively. 2D NMR spectra as 1H DOSY were recorded on a Bruker Avance III HD 400 at 400 MHz. All spectra were recorded at room temperature (25 °C) and calibrated using the solvent signals. Melting points were measured using a Mettler FP62 melting point apparatus at a heating rate of 2.5 °C min⁻¹. GPC was performed with HFIPI containing 3 g L⁻¹ potassium trifluoroacetate as the eluent at 40 °C with a flow rate of 0.8 mL min⁻¹. The columns were packed with modified silica (PFC column particle size: 7 µm, porosity: 100 and 1000 Å). PMMA standards (Polymer Standards Services GmbH, Mainz, Germany) were used for calibration and toluene was used as the internal standard. A refractive index detector (G1362A RID) and an UV-vis detector (at 230 nm unless otherwise stated; Jasco, Gross-Umstadt, Germany, UV-2075 Plus) were used for polymer detection.

Preparation of Liposomes: Liposomes were prepared using the thin-film hydration method. Forzebrafish experiments, 1 mol% Dil was added as a fluorescent label. Briefly, stock solutions of all components were prepared by dissolving dry powders in CHCl₃ and/or MeOH. Stock solutions were mixed in molar ratios according to Table 4. Organic membranes were hydrated with Lipid films were hydrated with

Table 5. RP-8 column gradient. A: MeOH; B: NH₄OAc buffer pH 4.0; C: ACN. A methanol/water/acetonic (7:2:1) gradient was used to separate PEGylated samples on a C8 reversed phase column. Solvents contained 4 mmol L⁻¹ ammonium acetate.

| Time  | Flow [mL] | %A | %B | %C | %D |
|-------|-----------|----|----|----|----|
| 0     | 0.75      | 70 | 20 | 10 |
| 5     | 0.75      | 70 | 20 | 10 |
| 15    | 0.75      | 90 | 0  | 10 |
| 25    | 0.75      | 90 | 0  | 10 |
| 30    | 0.75      | 70 | 20 | 10 |

Cryo-TEM: Liposomes were diluted with HBS to achieve a final concentration of 5 to 10 mmol L⁻¹ lipid. About 3 µL were applied on a 400 × 100 mesh Quantifoil S 7/2 hole carbon film on a copper grid (Quantifoil Micro Tools GmbH, Jena, Germany). Excess liquid on the grid was removed with filter paper. The sample then was flash frozen by injection into liquid ethane.

Table 6. HILIC column gradient. A: MeOH, B: H₂O, C: NH₄OAc buffer (100 mmol L⁻¹ ammonium acetate buffer at pH 5.0); D: ACN. A complex gradient containing 5 mmol L⁻¹ ammonium acetate buffer was used to separate samples containing lipopolysarcosines.

| Time  | Flow [mL] | %A | %B | %C | %D |
|-------|-----------|----|----|----|----|
| 0     | 1.0       | 0  | 5  | 95 |
| 4     | 1.0       | 0  | 5  | 95 |
| 7     | 1.0       | 10 | 3  | 82 |
| 10    | 1.0       | 10 | 3  | 82 |
| 14    | 1.0       | 40 | 25 | 5  | 30 |
| 17    | 1.0       | 40 | 25 | 5  | 30 |
| 19    | 1.0       | 40 | 50 | 5  | 5  |
| 21    | 1.0       | 40 | 50 | 5  | 5  |
| 22    | 1.0       | 0  | 5  | 95 |
| 25    | 1.0       | 0  | 5  | 95 |
of both polymer-conjugates was quantified with a Corona charged aerosol detector (Corona CAD ESA 542, Dionex, Sunnyvale, USA) after performing the post-insertion technique followed by a purification step. Data were analyzed with Empower 3.0 software (Waters, Milford, USA).

Incorporation efficiency was calculated using the formula shown in Equation (1).

\[ IE = \frac{c_{lp} \times c_{lp}}{c_{cp} \times 100\%} \]

Equation (1). Incorporation efficiency of polymer-conjugates

- \( c_{lp} \): polymer-conjugate concentration after purification
- \( c_{cp} \): theoretical polymer-conjugate concentration
- \( c_{lp} \): lipid concentration after purification
- \( c_{lp} \): theoretical lipid concentration

Zebrafish Experiments: Zebrafish larvae originating from adult Tg(mpeg1:Gal4;UAS:Kaedc) were raised at 28 °C in zebrafish culture media. Pigment cell formation was prevented by the addition of 1-phenyl-2-thiourea to the zebrafish culture media at 24 h post fertilization. All zebrafish experiments were performed in accordance with Swiss animal welfare regulations. Calibrated volumes of 1 nL at 5 mmol L\(^{-1}\) lipid concentration were injected directly into blood circulation via the duct of Cuvier. All injections were performed using a pneumatic PicoPump PV830 (WPI, Sarasota, Florida), and a Leica S8APO microscope (Leica, Wetzlar, Germany). Successfully injected zebrafish embryos (no yolk or heart injections) were kept at 28 °C and circulation via the duct of Cuvier. All injections were performed using a pneumatic PicoPump PV830 (WPI, Sarasota, Florida), and a Leica S8APO microscope (Leica, Wetzlar, Germany)

Complement Activation: Activation of the complement cascade was analyzed by determining SC5b-9 (Protein S-bound terminal complement complex c5b-9) levels using an immunoassay kit (MicroVue SC5b-9 Plus EIA, Quidel, Santa Clara, USA). In preliminary tests, liposome-mediated complement activation was investigated in freshly prepared human sera of 20 healthy donors. Sera of the three most sensitive individuals were chosen for further testing.

Applying the findings by Moghimi et al.,\(^{[94]}\) where concentrations of 3–4 mg lipid mL\(^{-1}\) serum were found to raise SC5b-9 levels the most, liposomes were diluted to 14.5 mmol L\(^{-1}\) media.\(^{[61]}\) In zebrafish culture c

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The research project was supported by the Swiss National Science Foundation grants no. 174975 (D.W.) and no. 173057 (J.H.).

Conflict of Interest

The authors declare no conflict of interest.
[32] A. Chanan-Khan, J. Szebeni, S. Savay, L. Liebes, N. M. Rafique, C. R. Alving, F. M. Muggia, *Ann. Oncol.* 2003, 14, 1430.

[33] J. Szebeni, *Toxicology* 2005, 216, 106.

[34] J. Szebeni, L. Baranyi, S. Savay, M. Bodó, J. Milosevits, C. R. Alving, R. Bünger, *Am. J. Physiol.: Heart Circ. Physiol.* 2006, 290, H1050.

[35] S. M. Moghimi, I. Hamad, T. L. Andresen, K. Jørgensen, J. Szebeni, *FASEB J.* 2006, 20, 2591.

[36] J. Szebeni, L. Baranyi, S. Savay, J. Milosevits, R. Bunger, P. Laverman, J. M. Metselaar, G. Storm, A. Chanan-Khan, L. Liebes, F. M. Muggia, R. Cohen, Y. Barenholz, C. R. Alving, *J. Liposome Res.* 2002, 12, 165.

[37] P. H. Kierstead, H. Okochi, V. J. Venditto, T. C. Chuong, S. Kivimae, J. M. J. Fréchet, F. C. Szoka, *J. Controlled Release* 2015, 213, 1.

[38] Y.-C. Hsieh, H.-E. Wang, W.-W. Lin, S. R. Roffler, T.-C. Cheng, Y.-C. Su, J.-J. Li, C.-C. Chen, C.-H. Huang, B.-M. Chen, J.-Y. Wang, T.-L. Cheng, F.-M. Chen, *Theranostics* 2018, 8, 3164.

[39] R. P. Garay, R. El-Gewely, J. K. Armstrong, G. Garratty, P. Richette, K. Knop, R. Hoogenboom, D. Fischer, U. S. Schubert, *Angew. Chem., Int. Ed.* 2010, 49, 6288.

[40] O. K. Nag, V. Awasthi, *Pharmaceutics* 2013, 5, 542.

[41] K. R. Whiteman, V. Subr, K. Ulbrich, V. P. Torchilin, *J. Liposome Res.* 2001, 11, 153.

[42] V. P. Torchilin, T. S. Levchenko, K. R. Whiteman, A. A. Yaroslavov, A. M. Tsatsakis, A. K. Rizos, E. V. Michailova, M. I. Shtilman, *Biomed. Materials* 2001, 22, 3035.

[43] J. M. Metselaar, P. Bruin, L. W. T. de Boer, T. de Vringer, S. Oussoren, M. H. M. Wauben, D. J. A. Crommelin, G. Storm, W. E. Hennink, *Bioconjugate Chem.* 2003, 14, 1156.

[44] M. Barz, R. Luxenhofer, R. Zentel, M. J. Vicent, *Polym. Chem.* 2011, 2, 1900.