CHAPTER 8

COMPLEMENT ACTIVATION - RELATED HYPERSENSITIVITY REACTIONS CAUSED BY PEGYLATED LIPOSOMES
SEARCH FOR AN ALTERNATIVE LONG-CIRCULATING LIPOSOME FORMULATION WITHOUT COMPLEMENT ACTIVATION

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ABSTRACT

PEGylated long-circulating liposomes have been reported to cause immediate hypersensitivity reactions in 5-10% of the patients treated. These reactions may be explained by liposome-induced complement activation. The aim of this study was to design a long-circulating liposome formulation that does not induce complement activation. We monitored the formation of complement terminal complex SC5b-9 *in vitro* in human serum samples as well as hemodynamic changes in pigs upon i.v. injection of several PEGylated and non-PEGylated liposome formulations.

It was found that Doxil® (PEG-liposomal doxorubicin), similar PEG-liposomes without drug as well as size-matched (90 nm) empty non-PEG-liposomes, all caused significant *in vitro* complement activation as well as hypersensitivity reactions in pigs, indicating that PEG-PE and/or its negative charge cannot be the sole underlying cause. Smaller (<70 nm) non-PEG-liposomes composed of DSPC and cholesterol caused no complement activation and completely lacked the induction of hemodynamic changes in pigs, suggesting that size is an additional major factor in complement activation responses.

To evaluate the usefulness of these small non-PEG-PEGylated DSPC-cholesterol liposomes for passive targeting purposes, dexamethasone phosphate was encapsulated and this liposomal formulation was given to rats with experimental arthritis. Interestingly, our data show that these liposomes can circulate at least as long as PEG-liposomes. The therapeutic activity of the liposomally encapsulated drug was similar for the PEG-liposomes and non-PEG-liposomes in this study, indicating that small-sized (<70 nm) non-PEGylated liposomes may be preferred over PEG-liposomes as carrier for passive drug targeting purposes, as they appear not to induce complement activation.

INTRODUCTION

PEG-liposomes have extensively been studied as potential carriers for targeted drug delivery to tumors, infections and sites of inflammation, as they have been shown to selectively accumulate at these sites (1-4). The success of PEG-liposomes in the field of oncology has led to a growing use of this formulation in the clinic. One preparation is currently on the market for the treatment of solid tumors: Doxil® (Caelyx® in Europe), a PEGylated long-circulating liposome (LCL) formulation of doxorubicin, while others are in clinical trials.

The therapeutic value of PEG-liposomes in infection has been shown in a range of preclinical studies (5). Evaluation in experimental models of infection revealed that the therapeutic activity of antibacterial drugs can be improved by encapsulation in PEG-liposomes. In rat and murine models of experimental arthritis encapsulation in PEG-liposomes strongly improved the anti-inflammatory activity of glucocorticoids (see Chapter 3 and 4). Such promising results warrant clinical studies in patients in a short term.

However, PEG-liposomal formulations such as Doxil® can cause immediate allergic reactions in patients. These reactions have been observed in a significant proportion of patients. The symptoms occur upon the first infusion and include dyspnea, tachypnea, facial swelling, headache, chills, hypo- and/or hypertension, chest pain and back pain (6-9). They are generally mild and quickly resolve after interruption and resumption of the infusion at a slower rate. Most patients can receive further infusions without any complications. The reactions have been dubbed "pseudoallergic", as no prior sensitization is needed to induce them. Clinical studies performed with radioactively labeled PEG-liposomes without drug for diagnostic purposes also revealed pseudoallergic reactions, supporting the suggestion that the observed effects are due to the liposomal carrier rather than the drug (10).

In recent reports it was hypothesized that PEGylated liposome formulations induced these phenomena as a result of complement activation (11,12). A key argument for this hypothesis came from experiments in a porcine model of liposome-induced cardiopulmonary distress (13,14), which showed that the PEG-liposome-induced hemodynamic reactions in pigs are a consequence of complement activation (15). A relationship between complement activation and pseudoallergic reactions was recently confirmed in a clinical study in patients treated with Doxil® (16). Possibly the use of PEG-phosphatidyl ethanolamine (PEG-PE) is a key factor in the induction of complement activation.

The ultimate aim of this study was to design LCL, which do not induce complement activation. To this end, different types of PEGylated and non-PEGylated liposomes were evaluated regarding complement activation in human serum, pseudoallergic reactions in pigs, and long-circulating properties and therapeutic benefit in a rat model of experimental arthritis. For the two latter purposes, the anti-inflammatory glucocorticoid dexamethasone phosphate (DXP) was encapsulated as a model drug, as a previous study revealed that encapsulation of glucocorticoids in PEG-liposomes, resulted in a strong increase of the therapeutic benefit of the glucocorticoid in question by selective targeting to inflamed joints (Chapter 3).

MATERIALS AND METHODS

Liposomes

Liposomes were prepared by the film-extrusion method (17). Briefly, a lipid solution was prepared in ethanol, containing the phospholipid dipalmitoyl phosphatidylcholine (DPPC) or distearoyl phosphatidylcholine (DSPC) (Lipoid GmbH, Ludwigshafen, Germany) and cholesterol (Sigma Chemical Co., Poole, UK) in a molar ratio of 2:1. For PEG-liposomes, 7.5 mole % of the amount of total lipid in the mixture of poly(ethylene glycol)2000 coupled to distearoyl phosphatidylethanolamine (PEG-DSPE) (Avanti Polar Lipids, Alabaster, AL, USA) was added. The lipid solution was transferred to a round-bottom flask and a lipid film was created by rotary evaporation. The film was hydrated with 0.9% saline buffered with 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH: 7.4) in sterile water. DXP was encapsulated by dissolving 100 mg/ml in the hydration buffer before hydration. The resulting lipid dispersion was sized to the desired diameter by multiple extrusions through polycarbonate filter membranes. Non-encapsulated DXP was removed by dialysis with Slyde-A-Lyzer dialysis cassettes with a molecular weight cut-off of 10,000 (Pierce, Rockford, IL, USA). Mean particle size was determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd., Malvern, UK). Zeta potentials were measured with a Zetasizer (Malvern Ltd., Malvern, UK). Phospholipid content of the organic phase after extraction of liposomal preparations with chloroform was determined with a phosphate assay (18). DXP was determined by a reversed phase HPLC assay in the aqueous fraction after extraction.

In vitro complement activation assay in human serum samples

Blood samples from healthy volunteers were collected and serum was prepared and stored (at –20 °C) as described earlier (19). Complement activation was assessed by ELISA kits (Quidel Co., San Diego, CA, USA) measuring protein S (vitronectin)-bound C terminal complex (SC5b-9). The assay has been used in clinical studies to quantify complement activation with high sensitivity and specificity (20). Liposome dispersions containing 60 mM total lipid were diluted 5-fold in human serum and were incubated for 30 min at 37 °C with shaking at 80 rpm. After incubation the samples were diluted 20-fold in the "sample diluent" of the kit and 100 μl aliquots from this mixture were applied into the wells of the ELISA plate, usually in duplicate. The assay was validated by repetitive measurement of randomly selected samples at different times with >1 year separation, using different batches of the SC5b-9 kit, and blinding the assayer(s) with regard to the identity of samples. In agreement with the manufacturer's specification of the SC5b-9 kit, these experiments revealed approximately 10-20% variation of SC5b-9 readings (16).

All liposome formulations were tested in serum samples of 4 to 5 different subjects. For each subject the % increase of SC5b-9 concentration was calculated and compared to the person's own baseline, referred to as "PBS control". Complement activation is presented as the mean % increase observed in the 4 – 5 subjects in which the formulations were tested.

In vivo porcine model of complement-related pseudoallergy

Experiments were performed in accordance with guidelines of the Committee on Animal Care of the Uniformed Services University of the Health Sciences. Castrated male Yorkshire swine (48-215 LB) were sedated with intramuscular ketamine, anesthetized with isoflurane and instrumented as described previously (13). In brief, a catheter was advanced via the right internal jugular vein into the pulmonary artery to measure pulmonary artery pressure (PAP), central venous pressure (CVP) and cardiac output (CO); another catheter was advanced through the left femoral artery into the proximal aorta to measure systemic arterial pressure (SAP). Systemic vascular resistance (SVR), left ventricular end-diastolic pressure (LEVDP) and pulmonary vascular resistance (PVR) were calculated from SAP, PAP, CO, and CVP by standard formulas. Cerebral blood flow, pCO_2 and ECG were recorded continually.

Liposomes were diluted in 1 ml PBS and injected into the pulmonary artery of pigs, via the pulmonary arterial catheter. Liposomes were flushed into the circulation with 10 ml PBS. Based on our previous finding that the hemodynamic effects of small liposome boluses were non-tachyphylactic and quantitatively reproducible in the same animal, we injected increasing amounts of the same type of liposomes in each pig until a reaction developed, or, in the absence of reaction, until a certain predetermined top dose was tested. The doses applied were in the 0.15-1.5 µmol lipid/kg (pig) range except for Doxil®, which, because of the severity of the reactions, had to be given at an almost 10-fold lower dose. The responses were graded as follows: none, no significant alteration in ECG or any hemodynamic parameters; mild, transient (< 2 min) <50 % changes in at least one of the following parameters: heart rate, ECG, SAP, PAP, pCO2; severe, up to 10 min and >50% changes in at least one of the above parameters plus bradyarrhythmia; lethal, circulatory collapse within 2 min requiring epinephrine and cardiac massage for resuscitation. Typically mean SAP falls from 110 to <40 mm Hg, mean PAP rises from 18 to a maximum (60 mm Hg), pCO_2 in expired gas drops from 32 to <20 mm Hg, tachycardia is followed by severe bradycardia with arrhythmia, leading to cardiac arrest and death.

Pharmacokinetics and therapeutic activity non-PEG-liposomes vs. PEG-liposomes

For comparative assessment of the pharmacokinetics of PEG-liposomes versus non-PEG liposomal formulations, DXP was encapsulated in liposomes composed of PEG-DSPE, DPPC and cholesterol (90 nm) and in two liposome types composed of DSPC and cholesterol (90 nm and 65 nm in size).

DXP can easily be detected in plasma and has the attractive property of being almost immediately and fully converted into dexamethasone when free in the circulation. As in a previous study liposomal glucocorticoid was shown to not leak encapsulated drug in the circulation, plasma levels of DXP could be regarded as a measure of liposome-associated drug (see Chapter 3). For assessment of DXP in plasma samples, plasma was extracted according to a method reported by Derendorf et al. and assayed with a reversed-phase HPLC method, using UV-absorption detection at 254 nm (21).

Rat experimental model of adjuvant arthritis

The Dutch Committee of Animal Experiments approved these animal studies. Male inbred Lewis rats between 7 and 9 weeks of age (170-200 g) were obtained from Maastricht University, Maastricht, The Netherlands. Adjuvant arthritis was induced according to Koga and Pearson (22). Briefly, incomplete Freund's adjuvant containing heat-inactivated Mycobacterium tuberculosis was intracutaneously injected at the base of the tail. Paw inflammation started around day 10 after the immunization, reached maximal severity around day 20, after which the inflammation process gradually resolved. The rats were scored daily for the visual signs of inflammation. The severity of the joint inflammation was graded by assigning a score to each paw from 0 to 4, based on erythema, swelling and deformation of the joints. All rats were treated on day 15 post-induction with DXP in free form or DXP encapsulated in PEG-liposomes or DSPC-cholesterol liposomes (90 or 65 nm), when the average sum score of all paws of the rats in the experiment was around 7. The effect of treatment on clinical scores and body weight was monitored up to 4 weeks post-treatment.

Statistical Analysis

SC5b-9 values were expressed as mean ± SD of 4 – 5 subjects. One-sample t tests were used to determine whether the mean was significantly different from 100% (PBS value). Scores in the *in vivo* pig model were statistically evaluated with the nonparametric Wilcoxon/Kruskal-Wallis test (rank sums). For evaluation of pharmacokinetics one-way analysis of variance was used, whereas for evaluation of the therapeutic activity also the Wilcoxon/Kruskal-Wallis test was applied. P values of less than 0.05 were considered significant.

RESULTS

Liposome characteristics

Table 1 shows the characteristics of the different liposome types used in this study. In general, the size of the liposomes ranged between 80 and 100 nm except for the DSPC-cholesterol liposomes of 65 nm. Polydispersity was always lower than 0.1 except in the case of Daunoxome®, which had a polydispersity of >0.2. The zeta-potential of PEG-liposomes was negative. Non-PEG-liposomes proved to be around neutral. Each liposome formulation contained around 40 µmol phospholipid/ml. The formulations prepared with DXP contained between 5 and 10 mg DXP/ml.

Table 1. Liposome composition and characteristics

Composition	Molar ratio	Mean diameter (nm)	Polydispersity index	Zeta potential (mV)
DPPC PEG-DSPE Chol	1.85 : 0.15 : 1.0	88	<0.1	-37
DSPC PEG-DSPE Chol	1.85 : 0.15 : 1.0	87	<0.1	-28
DSPC Chol	2.0 : 1.0	90	<0.1	-1
DSPC Chol	2.0 : 1.0	66	<0.1	-1
DSPC PEG-DSPE Chol + doxorubicin (Doxil®)	1.85 : 0.15 : 1.0	85	<0.1	-43
DSPC Chol + daunorubicin (Daunoxome®)	2.0 : 1.0	74	>0.2	-2

In vitro complement activation in human plasma

Fig 1 shows the % increase of the human serum SC5-b9 concentration after addition of liposomal formulations as compared to the baseline concentration. All liposome formulations induced a significant increase of SC5-b9 levels except for 65 nm DSPC-cholesterol liposomes.

In vivo porcine model of complement-related pseudoallergy

Table 2 shows that 5 out of the 6 formulations tested caused pseudoallergic reactions in the pigs. Liposomes without drug roughly induced reactions in half of the pigs that were tested. Both Doxil and Daunoxome caused severe to lethal reactions in the majority of the pigs in which they were tested. Especially Doxil proved to be a strong inductor of pseudoallergy, as the reactogenic dose range was an order of magnitude lower than that of other liposomes. The only formulation, which did not induce complement activation and pseudoallergy, was the DSPC-cholesterol liposome formulation of 65 nm.
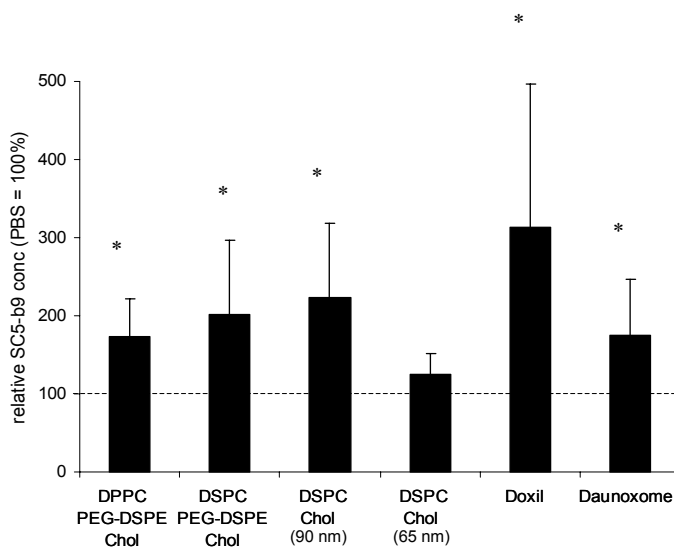
Figure 1. *In vitro* complement activation in human serum as measured by % increase of SC5-b9 levels as a result of addition of liposomes as compared to baseline SC5-b9 levels (addition of PBS). * = significant complement activation (p <0.05, single sample t test). Data represent means of 4 – 5 subjects + SD.

**Table 2.** Hypersensitivity reactions in porcine model

liposomes	lipid dose (µmol/kg)	frequency of reactions	severity of reactions
DPPC PEG-DSPE Chol	0.17-1.39	4/6	None (2), Mild (1), Severe (1), Lethal (2)
DSPC PEG-DSPE Chol	0.16-1.97	2/4	None (2), Mild (0), Severe (1), Lethal (1)
DSPC Chol (90 nm)	0.16-1.85	5/11	None (6), Mild (3), Severe (2), Lethal (0)
DSPC Chol (65 nm)	0.16-1.54	0/8	None (8), Mild (0), Severe (0), Lethal (0)
Doxil®	0.02-0.27	12/14	None (2), Mild (3), Severe (8), Lethal (1)
Daunoxome®	0.18-0.73	7/8	None (1), Mild (2), Severe (1), Lethal (4)

Circulation kinetics: non-PEG-liposomes vs. PEG-liposomes

Fig 2 shows that non-PEG-liposomes composed of DSPC and cholesterol can behave as long-circulating as PEG-liposomes. DSPC-cholesterol liposomes of <70 nm had an even longer circulation half-life (appr. 36 hrs) as compared to PEG-liposomes (22 hrs).
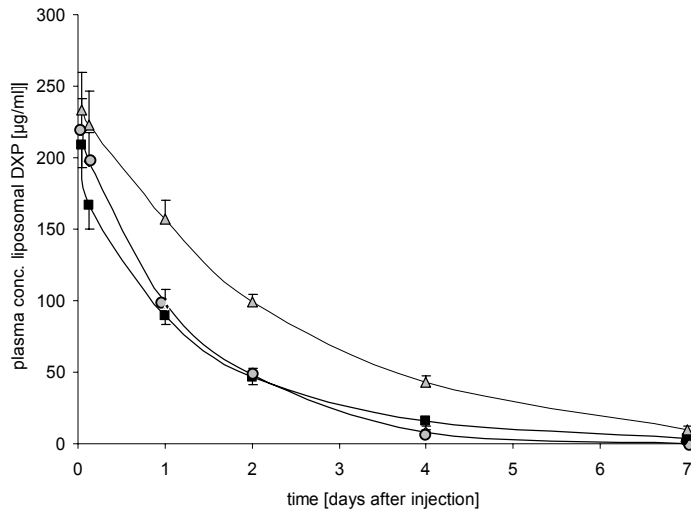
Figure 2. Plasma concentration-time curves of liposomal DXP after injection of 10 mg/kg DXP incorporated in DPPC-PEGDSPE-cholesterol liposomes (closed squares), DSPC-cholesterol liposomes of 90 nm (gray circles) and DSPC-cholesterol liposomes of <70 nm (gray triangles) in healthy rats. Data represent means +/- SD of groups of 4 rats.

Therapeutic activity of DXP-liposomes in rat adjuvant arthritis

Figure 3 A shows the effect of liposomal encapsulation of DXP on the therapeutic activity of the drug. Non-encapsulated DXP at 2 mg/kg reduced inflammation scores during one day, after which paw inflammation intensified again. In contrast, an equal dose of DXP encapsulated in PEG-liposomes led to complete remission of paw inflammation up to one week post-treatment. In Figure 3 B the effect of 1 mg/kg DXP in 90 nm and 65 nm DSPC-cholesterol liposomes without PEG is compared to an equal dose DXP-PEG-liposomes. Clearly, no difference in therapeutic activity is observed.
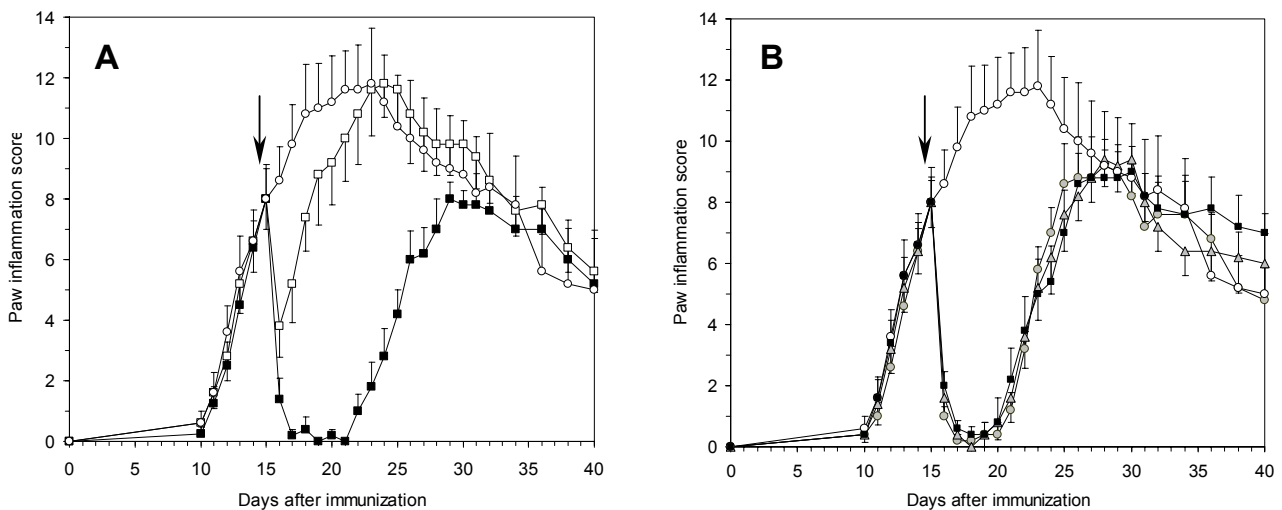
Figure 3. Therapeutic effect in rat adjuvant arthritis. (A) 2 mg/kg i.v. DXP in free form (open squares) given at day 15 (arrow), and 2 mg/kg DXP in PEG-liposomes (closed squares), both given as a single injection, and (B) 1 mg/kg DXP encapsulated in PEG-liposomes (closed squares), DSPC-cholesterol liposomes of 90 nm (gray circles) and DSPC-cholesterol liposomes of <70 nm (gray triangles) in adjuvant arthritis rats. Saline treated rats are used as controls (A and B) (open circles). Data represent means +/- SEM of groups of 5 rats.

DISCUSSION

Complement activation-related pseudoallergy in patients has been reported to occur with several lipid formulations (23). The most extensively studied liposome formulation in this respect is PEGylated liposomal doxorubicin (Doxil®, Caelyx®) (6-9). Of the formulations tested in the present study Doxil® indeed appeared to be the strongest inductor of complement activation. On the average, Doxil® caused a more than 3-fold increase of SC5-b9 in serum samples as compared to baseline values. Also, in the *in vivo* pig model, Doxil® caused reactions in 12 out of 14 pigs at 5- to 10-fold lower doses as compared to the other formulations tested.

This study confirms earlier observations that PEG-liposomes, also when doxorubicin is not encapsulated, can activate complement and cause hypersensitivity reactions in pigs (24). It is also in line with a report by Brouwers et al. on hypersensitivity reactions to ^{99}Tc-labeled empty PEG-liposomes in 3 out of 9 patients with Crohn's disease (10). Both the results of the *in vitro* SC5-b9 assay and the *in vivo* pig data reveal a response in roughly 50% of the test cases with PEG-liposomes. Remarkably, quite similar results were obtained with liposomes without PEG, indicating that PEG-PE and/or its negative charge cannot be the sole underlying cause of complement activation and subsequent hypersensitivity reactions.

In our studies, only the smallest size (mean diameter: 65 nm) DSPC-cholesterol liposome formulation was completely lacking complement-activating properties. Liposomes with the same composition but a somewhat larger mean diameter (90 nm) did show complement-activating activity, which indicates that size is an important factor in complement activation. Nevertheless, the presence of PEG-PE and/or its negative charge, and a relatively large size (i.e. ≥90 nm) may not represent the full list of risk factors for induction of complement activation. We found that Daunoxome®, a neutral, non-PEGylated DSPC-cholesterol formulation of daunorubicin, in our study showing a mean diameter of 74 nm, caused responses both *in vitro* and *in vivo* with a relatively high frequency. This observation is in line with the finding that Daunoxome® caused hypersensitivity reactions in 4 out of 15 of patients involved in a clinical phase II trial (25). Considering that Daunoxome® differed from the reaction-free empty control (DSPC-cholesterol) liposomes only in that it had substantially greater polydispersity index (see Table I), these observations suggest that the relatively large size distribution, probably caused by the presence of a fraction of larger liposomes or liposome aggregates among the small ones, is an additional major risk factor.

We have previously shown that Doxil® is a more effective complement activator than the corresponding empty PEGylated liposomes (24). Taken together with the present observations with Daunoxome®, it seems likely that the encapsulated doxorubicin and daunorubicin do play a role in C activation, despite the fact that both drugs are located within the liposome particles, apparently shielded from plasma. It has been observed that aggregates are present within these formulations (unpublished observations). Liposome aggregates, might be superior complement activators even in negligible quantities.

As small DSPC-cholesterol liposomes appeared to lack complement activating properties, it was of interest to evaluate whether these non-PEG-liposomes could also be used for passive drug targeting purposes. Therefore, we assessed the circulation time of DXP encapsulated in these liposomes in healthy rats. In addition, we evaluated the therapeutic activity of these liposomes in rat experimental arthritis. Our data showed that non-PEGylated DSPC-cholesterol liposomes circulated at least as long as PEG-liposomes. Interestingly, decreasing the size of DSPC-cholesterol liposomes from 90 nm to <70 nm extended the half-life to approximately 36 hrs in rats. In addition the therapeutic performance of these small non-PEGylated liposomes was similar to that of the PEG-liposomes.

In conclusion, the results presented in this study show that complement-related hypersensitivity reactions can occur either with or without the incorporation of PEG-PE in the liposomal formulation, indicating that PEG and/or a net negative surface charge may not be the (sole) key factor in the activation of complement by liposomes. Avoidance of complement activation may be achieved by sizing liposomes down to <70 nm with minimal polydispersity. The small, neutral DSPC-cholesterol liposome formulation, which in this study was completely lacking complement activation and hypersensitivity reactions, proved to be as valuable for targeting glucocorticoids to inflamed areas as 90 nm PEG-liposomes, which we used in most of the experimental work presented in this thesis. Therefore, the small non-PEGylated liposome formulation may be preferred over PEG-liposomes as carrier for passive drug targeting purposes.

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