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Localization of sterically stabilized liposomes in experimental rat *Klebsiella pneumoniae* pneumonia: dependence on circulation kinetics and presence of poly(ethylene)glycol coating

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Abstract

Preferential localization of liposomes at sites of infection or inflammation has been demonstrated in a variety of experimental models. Most studies report enhanced localization at the target site of poly(ethyelene) glycol (PEG)-coated liposomes as compared to conventional non-coated liposomes. It is generally accepted that the prolonged circulation time of PEG-coated liposomes increases target site exposure, which results in increased target localization. A quantitative relationship between circulation kinetics and localization at the pathological site has not been defined as yet. Besides, an effect of the PEG coating itself has been suggested, as theoretically the PEG coating may facilitate liposome extravasation. In the present study, in a rat model of an acute unilateral *Klebsiella pneumoniae* pneumonia, circulation kinetics of PEG-coated liposomes were manipulated by incorporation of different amounts of phosphatidylserine (PS) and variation of lipid dose, additionally allowing evaluation of the saturability of the localization process. In addition, this paper addresses the effect of the PEG coating, by comparing the circulation kinetics and target localization of long-circulating 'PEG-free' and PEG-coated liposomes. It is shown that the degree of liposome localization at the target site is positively linearly related to the area under the blood concentration time curve (AUC) of the liposome formulations, irrespective of PEG coating. This finding is discussed in relation to the equation of Kedem and Katchalsky, which describes protein influx into sites of infection or inflammation.

Keywords: Sterically stabilized liposome; Capillary permeability; Infection; Targeting; Circulation kinetics

1. Introduction

In a variety of experimental models of infection or inflammation, liposomes have been demonstrated to localize at the pathological site [1–7]. The localization is dependent on the inflammatory response as the localization at comparable anatomical sites in uninfected control animals is generally insignificant. As a result, liposomes have attracted considerable

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interest as targeted drug carriers for application in infection and inflammation. In order to maximize the degree of liposome localization at the target site, it is essential to identify which factors contribute to the passive targeting of liposomes to inflamed sites.

With respect to the inflamed target site, this area is characterized by an increased blood flow, and an increased capillary permeability causing edema. The fluid flux into the target site facilitates movement of the phagocytes and promotes plasma protein localization supporting the host defense. The influx of plasma proteins into the inflamed site has been mathematically described by Kedem and Katchalsky [8] as:

$$J_s = J_v\ (1-\sigma)\ C_s + P_c\ S_c\ \Delta C \qquad (1)$$

where J_s and J_v are the fluxes of the studied protein and of fluid, respectively, σ is the reflection coefficient (fraction of the protein that is stopped by the microvascular barrier), C_s is the average protein concentration in the capillary, $P_c\ S_c$ is the capillary permeability- capillary surface area product, and ΔC is the protein concentration difference across the endothelium. The first term of the equation describes the convective transport component (J_v (1-σ) C_s), whereas the second term describes the diffusion component ($P_c\ S_c\ \Delta C$). Assuming that liposome flux into the target site can also be described by this equation, two liposome-related factors would then determine the degree of liposome localization: the ability of the liposomes to cross the vascular endothelium (determined by σ) and the liposome concentration in the blood (C_s). The $P_c\ S_c\ \Delta C$ component of the equation is negligible due to the low diffusion coefficient of particulate systems. The fluid flux (J_v) is not related to the particulate system but to the inflammatory response. Therefore, only C_s and σ seem to be the main determining parameters in the liposome localization process.

Several studies have shown an improved target site localization of liposomes coated with poly(ethylene) glycol (PEG), also known as sterically stabilized liposomes (SSL), compared to conventional liposomes lacking the PEG coating [1–6]. It is generally accepted that the higher degree of localization is enabled by the prolonged circulation time of SSL. The increased average liposome concentration in the capillaries at the inflamed area, yields increased exposure of the target site to the liposomes, suggesting that the liposome concentration (or C_s) in time is important for target localization. Besides, permeability studies in tumor tissue suggest that the PEG coating itself can promote target localization [9]. A less interactive liposome surface, conferred by the PEG coating, which could also be interpreted as a reduced reflection coefficient (or σ), may facilitate their extravasation at sites of increased capillary permeability.

Up to now, a limited number of qualitative studies has appeared on the relationship between circulation kinetics (i.e. involvement of the C_s parameter) and degree of localization of SSL in the pathological target tissue, as has been discussed by Storm and Woodle [10]. In the present study, this relationship is explored in more detail. In a rat model of an acute unilateral *Klebsiella pneumoniae* pneumonia in rats, SSL circulation kinetics were manipulated by incorporation of different amounts of phosphatidylserine (PS) [11,12]. Circulation kinetics were also manipulated by variation of lipid dose, additionally allowing evaluation of the saturability of the localization process. A relationship between liposomal circulation time and target site localization was established on the basis of the collected data. In addition, this paper addresses the effect of the PEG coating itself (i.e. involvement of the σ parameter) by comparing the circulation kinetics and target localization of long-circulating 'PEG-free' liposomes and SSL.

2. Materials and methods

2.1. Liposome preparation and characterization

Liposomes were prepared as described previously [2] using appropriate amounts of the following lipids: partially hydrogenated egg phosphatidyl choline (PHEPC) (Asahi, Ibarakiken, Japan), cholesterol (Chol), L-α-phosphatidyl-L-serine (PS) (Sigma, St. Louis, MO), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[polyethylene glycol-2000] (PEG-DSPE), or distearoyl phosphatidyl choline (DSPC) (Avanti Polar Lipids, Alabaster, Alabama). In brief, lipids were dissolved in a mixture of chloroform and methanol. The solvent was evaporated and the lipids were dried, redissolved in 2-methyl-2-propanol (Sig-

ma, St. Louis, MO), frozen, and freeze-dried overnight. The resulting lipid film was hydrated in HEPES/NaCl buffer, pH 7.4 (10 mM *N*-[2-hydroxy ethyl]piperazine-*N*′-ethane sulfonic acid (HEPES) (Sigma, St. Louis, MO), 135 mM NaCl (Merck, Darmstadt, Germany), and 5 mM of the chelator deferoxamine mesylate (Desferal) (Novartis, Basle, Switzerland).

The liposome dispersion was sonicated for 8 min with an amplitude of 8 μm using a 9.5-mm probe in an MSE Soniprep 150 (Sanyo Gallenkamp PLC, Leicester, UK) to obtain liposomes with a mean particle size of 100 nm. Dynamic light scattering, detected at an angle of 90° to the laser beam on a Malvern 4700 System (Malvern Instruments, Malvern, UK), was measured to evaluate the particle size distribution. In addition to the mean particle size, the system reports a polydispersity index (a value between 0 and 1). A polydispersity index of 1 indicates large variations in particle size, a reported value of 0 means that size variation is absent. All liposome preparations used had a polydispersity index below 0.3. The DSPC:Chol preparation with a mean size of 80 nm was obtained by multiple extrusion at 60°C of the liposome dispersion through two stacked polycarbonate membranes (Nuclepore, Pleasanton, CA) with a final pore size of 50 nm. Mean particle size was 80 nm.

2.2. Radiolabeling of liposomes

Liposomes were radiolabeled with ^{67}Ga, as described previously [2]. In brief, non-encapsulated deferoxamine was removed by gel filtration. Liposomes were concentrated via ultracentrifugation. ^{67}Ga-citrate (1 mCi/ml) (Mallinckrodt Medical, Petten, The Netherlands) was diluted 1:10 in aqueous 5 mg/ml 8-hydroxyquinone (Sigma, St. Louis, Missouri) and incubated for 1 h at 52°C to yield ^{67}Ga-oxine. One milliliter of this solution was added per 1000 μmol total lipid (TL). ^{67}Ga-oxine can pass the liposomal membrane and form a complex with the entrapped chelator deferoxamine. The complex is an appropriate label for monitoring intact liposomes in the circulation as it is rapidly renally excreted once it is released from circulating liposomes [13]. Free label was removed by gel filtration and labeled liposomes were concentrated by ultracentrifugation. Resulting specific activities were between 1×10^4 and 2×10^5 cpm/μmol TL. Phosphate concentration was determined spectrophotometrically according to Bartlett [14].

2.3. Unilateral pneumonia

The animal experiments ethical committee of the Erasmus University Medical Center Rotterdam approved the experiments described in this study. Female albino RP/AEur/RijHsd strain albino rats, 18–25 weeks of age, body weight 185–225 g (Harlan, Horst, The Netherlands) with a specified pathogen-free status were used. A left-sided unilateral pneumonia was induced as described previously [15]. In brief, rats were anesthetized and the left primary bronchus was intubated. Through the tube, 0.02 ml of a saline suspension containing 10^6 *K. pneumoniae* (ATCC 43816, capsular serotype 2) was inoculated in the left lung lobe. Rats were housed individually with free access to water and SRMA chow (Hope Farms, Woerden, The Netherlands).

2.4. Blood clearance and biodistribution

Experimental groups consisted of six rats. At the indicated time points after bacterial inoculation of the left lung, liposomes were injected at the indicated dose in the tail vein. At indicated time points after injection, blood samples of approximately 0.3 ml were taken, by retro-orbital puncture, from alternate groups of three rats. After measuring sample volume, radioactivity was counted in a Minaxi autogamma 5000 gamma counter (Packard Instrument, Meriden, CT). To determine the tissue distribution of the liposomes, rats were sacrificed by CO_2 inhalation. Subsequently, organs were dissected, weighed and radioactivity was counted. Organ radioactivity was corrected for radioactivity present in the blood (see below).

2.5. Total blood volume and blood content of tissues

Total blood volume of infected rats was determined, in an independent experiment, as being 5.3% of the total body weight. Syngeneic erythrocytes labeled with ^{111}In-oxine according to Kurantsin–Mills were used [16]. Blood samples were taken

at 10 min after injection of the labeled erythrocytes assuming that all erythrocytes were still present in the circulation. The dilution factor of the radioactive label allowed calculation of the total blood volume. Sample volume and blood volume allowed calculation of total blood radioactivity content. This technique was also used to determine blood content of the tissues at appropriate time points after inoculation.

2.6. Statistical analysis

SSL display single first-order clearance rates, independent of dose [17,18]. Similarly, in our study, blood clearance profiles of individual animals could be well described by a linear relationship on a semilogarithmic plot ($0.88 < r < 1.00$), which allowed estimation of individual area under the blood concentration–time curve (AUC) values.

Liposome localization data were tested for homogeneity of variance using Levene's statistic. Data were log transformed in case of significant differences. Infected left lung and right lung localization was compared using the paired *t*-test. Comparisons between groups were made by one-way analysis of variance (ANOVA). The Bonferroni method was used to correct for multiple comparisons. All analyses were performed using SPSS for Windows software release 7.5.2 (Statistical Product and Service Solutions, Chicago, USA).

3. Results

3.1. Manipulation of AUC by incorporation of PS and effect on degree of infected left lung localization of SSL

Incorporation of PS was used to manipulate the circulation time of SSL, as PS is a strong recognition signal for macrophage uptake, which cannot be prevented by the presence of PEG-DSPE at the usually applied amount of 5 mol% [11,12]. The circulation kinetics and biodistribution profiles of SSL containing either 0, 1, or 10 mol% PS in the bilayer are shown in Fig. 1A,B, respectively. The AUC_{0-24h} of the 1 and 10 mol% PS-SSL was 2.0-fold and 2.6-fold lower, respectively, compared to that of the 0 mol% PS-SSL. Regarding the biodistribution profile at 24 h after injection, the three liposome preparations displayed an approximately 4-fold higher localization in the infected left lung compared to the uninfected right lung ($P < 0.001$, Fig. 1B). The degree of localization in the infected lung was 2.5-fold higher for the 0 mol% PS containing SSL compared to the 1 and 10 mol% PS containing SSL ($P < 0.001$). The differ-

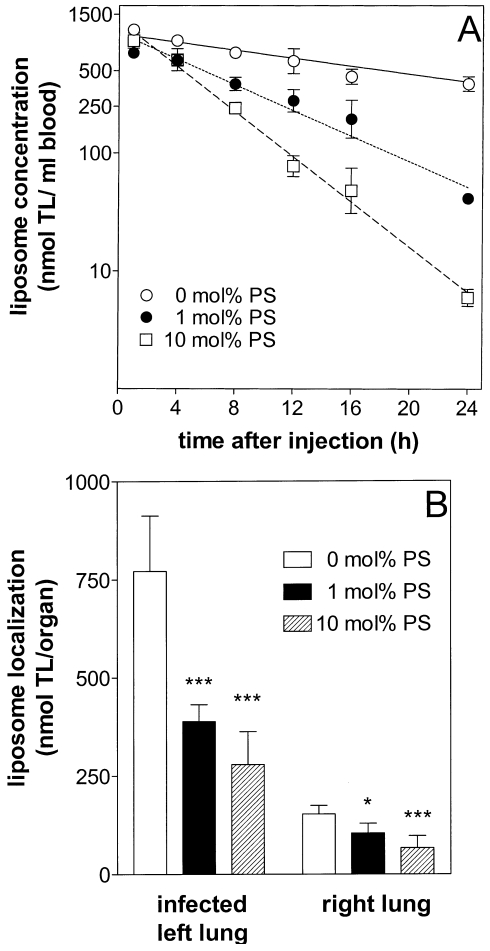

Fig. 1. Effect of incorporation of 0, 1, or 10 mol% PS on blood clearance and degree of localization of SSL in infected left lung and right lung tissue. ^{67}Ga-labeled liposomes were injected i.v. at 40 h after bacterial inoculation of the left lung at a dose of 75 μmol TL/kg. Lipid composition was PHEPC:Chol:PEG-DSPE:PS 1.85:1.00:0.15:0 mol:mol for the 0 mol% PS containing liposomes, 1.82:1.00:0.15:0.03 for the 1 mol% PS preparation, and 1.55:1.00:0.15:0.30 for the 10 mol% PS preparation. (A) nmol TL/ml blood was calculated from radioactivity recovered ($n = 3$, Mean ± S.D.). (B) nmol TL/lung at 24 h after injection was calculated from radioactivity recovered ($n = 6$, mean ± S.D.). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Significant differences versus the 0 mol% PS preparation are indicated.

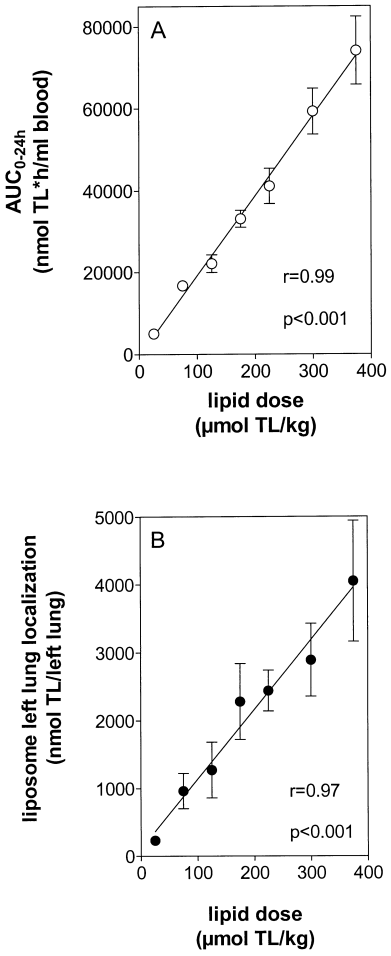

Fig. 2. Effect of lipid dose on AUC_{0-24h} (A) and degree of left lung localization (B) of ^{67}Ga-labeled SSL. Lipid composition was PHEPC:Chol:PEG-DSPE 1.85:1.00:0.15 mol:mol. See the legend of Fig. 1 for experimental conditions.

ences in localization in the right lung between the various SSL types showed a similar pattern ($P < 0.05$ and $P < 0.001$, respectively).

3.2. Manipulation of AUC by variation of lipid dose and effect on degree of infected left lung localization of SSL

SSL were injected i.v. at lipid doses from 25 up to 375 µmol TL/kg. The resulting values for AUC_{0-24h} and corresponding infected left lung localization at 24 h after injection are presented in Fig. 2A,B, respectively. The AUC_{0-24h} value as well as degree of left lung localization increased linearly with escalating dose. For all doses tested, the localization in the infected lung was approximately 3-fold higher than the localization in the right lung (data not shown, $P < 0.001$).

3.3. Effect of PEG coating on degree of infected left lung localization of liposomes

To assess whether the presence of a PEG coating has a facilitating effect on target site extravasation, two liposome types were studied: (1) long-circulating small rigid liposomes (LCL) without a PEG coating (DSPC:Chol, 2:1, mol:mol); and (2) SSL (PEG-DSPE:PHEPC:Chol 0.15:1.85:1.0 mol:mol). The

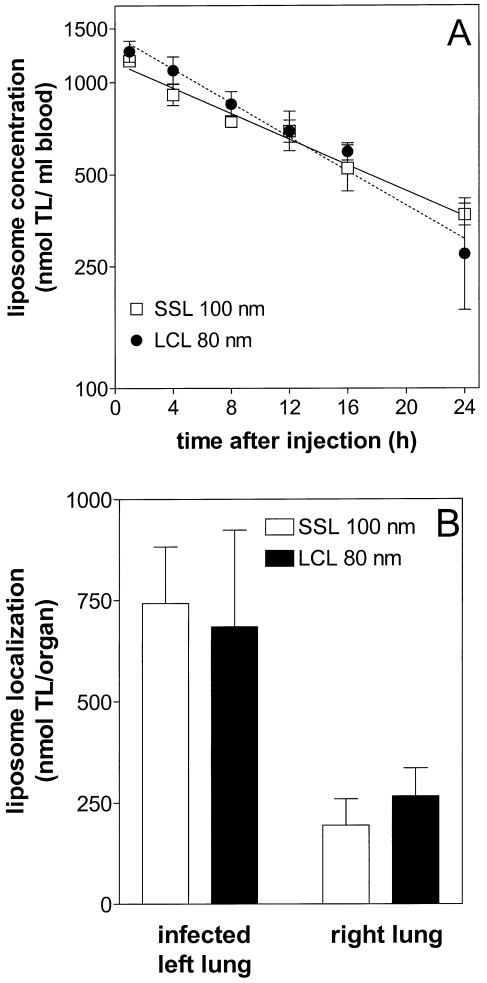

Fig. 3. Blood clearance (A) and degree of localization (B) in infected left lung and right lung tissue of ^{67}Ga-labeled SSL (lipid composition PHEPC:Chol:PEG-DSPE 1.85:1.00:0.15 mol:mol) and PEG-free small rigid long-circulating liposomes (LCL) (lipid composition DSPC:Chol 2:1 mol:mol). See the legend of Fig. 1 for experimental conditions.

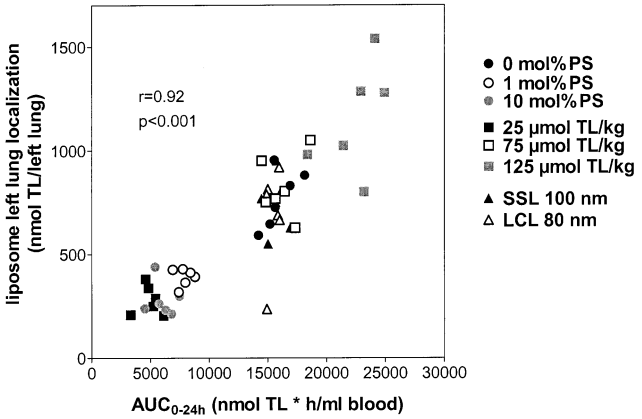

Fig. 4. Relationship between AUC_{0-24h} values and corresponding degree of left lung localization of individual animals for indicated liposome formulations.

circulation kinetics and biodistribution profiles are shown in Fig. 3A,B, respectively. The slow clearance profiles of the long-circulating PEG-free liposomes and the SSL were similar, as is reflected by approximately equal AUC_{0-24h} values. In line with these results, the degree of localization in the infected left lung was similar for both long-circulating liposome types. Both formulations demonstrated an approximately 3-fold higher degree of localization in the infected left lung compared to the degree of localization in the uninfected right lung ($P < 0.001$, Fig. 3B).

3.4. Correlation between AUC_{0-24h} and degree of infected left lung localization of liposomes

Collected data regarding AUC_{0-24h}-values and regarding degree of infected lung localization in individual animals taken from Figs. 1–3, are presented in Fig. 4. A positive linear correlation was obtained ($r = 0.92$, $P < 0.001$).

3.5. Relationship between infected left lung weight, AUC, and degree of infected left lung localization of SSL versus time after bacterial inoculation

An implication of liposome concentration as a driving force for target localization would be that the driving force gradually weakens in time as a result of reduced blood levels due to liposome clearance. Therefore, left lung weight (as a measure of fluid influx), liposomal AUC, and degree of infected left lung localization of SSL was determined at differ-

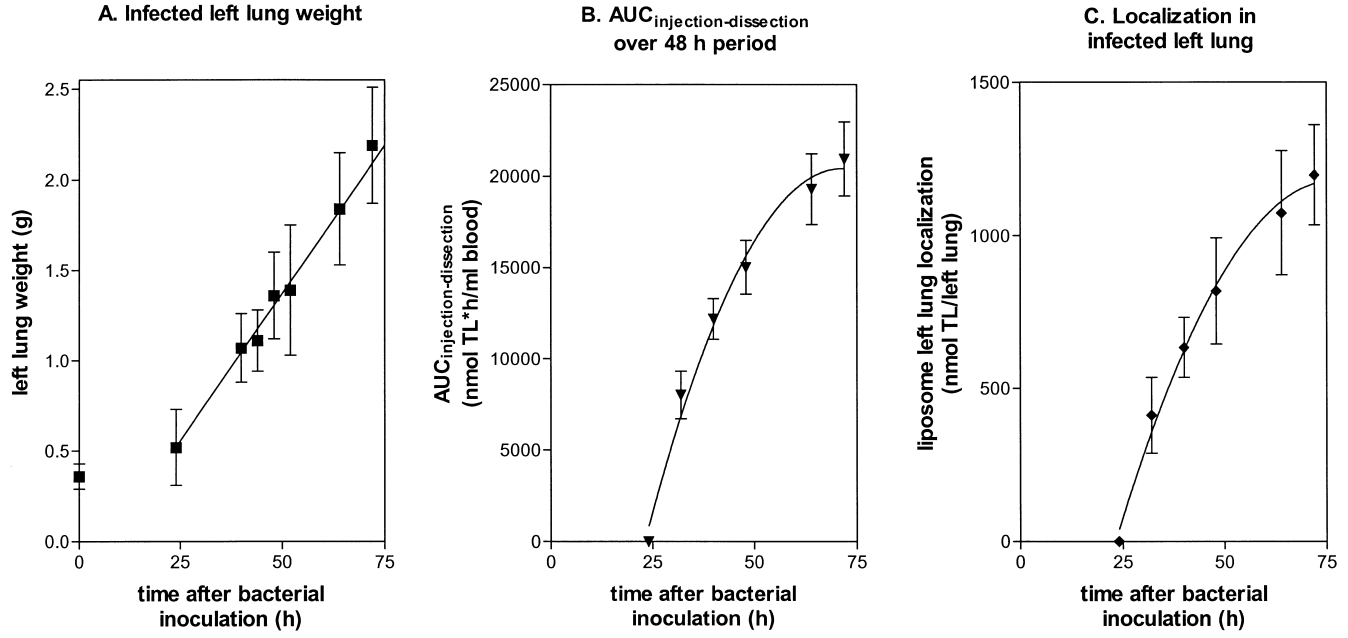

Fig. 5. Infected left lung weight (A), AUC value of SSL (B), and degree of infected left lung localization of SSL (C) at different time points after liposome injection. ^{67}Ga-labeled liposomes were injected i.v. at 24 h after bacterial inoculation of the left lung at a dose of 75 μmol TL/kg. Rats were dissected at the indicated time points and left lung weight, degree of SSL left lung localization, and SSL blood concentration were determined. The AUC values were calculated from individual blood clearance kinetics. $n = 3$–6 animals per time point. Mean ± S.D.

ent time points after liposome injection (Fig. 5A–C). To obtain a clear effect of liposome clearance from the bloodstream, of these long-circulating SSL, data had to be collected over a 48-h period. To be able to collect data over this time period, liposomes were injected at 24 h after bacterial inoculation as some animals died shortly after 72 h after inoculation. Fig. 5A shows that the increase in left lung weight is linear in time ($r = 0.92$, $P < 0.001$). Both liposomal AUC and degree of liposome left lung localization show a hyperbolic curve (Fig. 5B,C, respectively). Over 70% of the liposome AUC and degree of left lung localization is achieved in the first 24 h.

4. Discussion

Similar to plasma proteins, long-circulating SSL have been shown to localize preferentially at sites of infection or inflammation [1–7]. The equation of Kedem and Katchalsky (see Section 1) has been used to describe the influx of proteins into inflamed areas [8]. If the same equation also applies to liposome influx into inflammatory foci, then target localization would be dependent on the ability of liposomes to cross the vascular endothelium, determined by the reflection coefficient (σ), and liposome concentration in the bloodstream (C_s). SSL with prolonged circulation times generally show a higher degree of target site localization compared to non-coated liposomes with shorter circulation times, which points to the involvement of C_s [1–6]. In addition, the PEG coating of SSL may have a promoting effect on target site localization itself by conferring a less interactive surface to the liposome, which implies a reduction of σ [9]. At present, a systematic investigation of the effect of circulation time and PEG coating on the degree of liposome localization at inflamed areas is lacking. The approach taken in the present study to investigate the effect of circulation kinetics of SSL on target localization was to incorporate PS in SSL and to vary SSL dose. The effect of PEG coating was studied by comparing circulation kinetics and degree of target site localization values of long-circulating liposomes without PEG coating to those of SSL.

The rate of SSL removal from the blood increased with an increasing amount of PS in the liposomal bilayers. A similar PS effect has been observed by Boerman et al. [11]. Increased clearance from the circulation was paralleled by a diminished target site accumulation. The incorporation of PS could have an intrinsic effect on SSL biodistribution as a result of increased negative surface charge or induction of defects in the PEG coating. More likely, the reduced circulation time of PS-containing SSL reduces the number of liposomes able to interact with the target site over time (reduction in C_s), leading to a diminished target site localization. The results reveal that the reduction in AUC is directly proportional to the reduction in degree of target localization.

Liposomal AUC was also manipulated by variation of the lipid dose. The rise in AUC values was proportional to the increase in administered lipid dose, showing that the pharmacokinetics of SSL are independent of dose between 25 and 375 μmol TL/kg. Dose-independent pharmacokinetics of SSL over this dose range have also been observed earlier in healthy animals by Allen and Hansen [18]. Here we show that the degree of target site localization increases proportionally to the administered dose, which points out that saturation of target localization does not occur within the dose range of 25–375 μmol TL/kg and further supports the notion that the liposome concentration (or C_s) 'drives' the localization in the infected left lung.

It has been argued that the presence of a PEG coating facilitates liposome extravasation at sites of increased capillary permeability, which could be interpreted as a reduction of the reflection coefficient (σ). This issue was addressed by comparing the circulation kinetics and target site localization of long-circulating liposomes without PEG coating to SSL. Since the long-circulating liposome formulation lacking the PEG coating shows similar AUC and target site localization values as the SSL formulation, it appears that the presence of a PEG coating does not contribute significantly to the degree of liposome localization at the site of infection and that the value for σ is equal for both liposome types. Thus, the long-circulating property is the decisive parameter in this respect. Experiments, similar to those described by Waypa et al. [19], to measure the actual value of σ for liposomes in inflammatory conditions could yield important information to maximize the degree of liposome target localization.

The linear relationship obtained by plotting the AUC values of the liposome formulations tested and corresponding target site localization values in individual animals emphasizes that liposome concentration is the prime determinant of liposome extravasation at the target site and supports the usefulness of the Kedem and Katchalsky equation for describing liposome influx at the the site of infection.

Liposome concentration as a driving force for target localization implies that the liposome influx at the target site gradually slows down as a result of liposome clearance. Experimental support for this notion was obtained by determining lung weight, and degree of left lung localization of SSL at different time points after injection and calculation of the AUC values between liposome injection and tissue dissection. It is shown that the lung weight increase is linear in time, which indicates that fluid influx into the site of infection (J_v) occurs at an approximately constant rate ($r=0.92$, $P<0.001$). Both cumulative AUC and liposome localization show a hyperbolic curve, indicating that a gradual decrease in liposomal blood concentration results in a proportional decrease in target localization, which again confirms that AUC is the main factor driving the passive targeting effect.

A theoretical implication of the Kedem and Katachalsky is that, during fluid flux into the site of infection ($J_v>0$) and a liposomal reflection coefficient that allows passage through the vascular endothelium ($\sigma<1$), target localization of long-circulating liposomes will occur and will not be saturable. Therefore, the design of liposomes with a longer circulation time compared to that of the liposome formulations used in the present study can be expected to provide for a higher degree of target localization, indicating that there is still room for improvement. Our results deviate from those of Longman and coworkers who investigated the localization of i.v. administered liposomes with various circulation times in peritoneal fluid [20]. Localization of SSL in peritoneal fluid was not increased compared to conventional liposomes, despite significant differences in circulation times. Limited fluid transport (J_v) due to an intact microvascular barrier in the abdomen (possessing a high reflection coefficient (σ)) likely explains these results.

In conclusion, the present study clearly shows for the first time that the blood concentration is the primary factor driving localization of liposomes into the *K. pneumoniae*-infected target site. In addition, the PEG coating itself does not contribute significantly to the degree of liposome target localization. The design of liposomes with longer circulation times as compared to those of the SSL used in this study can be predicted to result in a higher degree of target localization of liposomes.

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