Platelet Distribution iri Rabbits Following Infusion of Liposomes

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Key words

Platelets – Liposomes - Platelet-endothetial interactions – Liposomc biodistribution – Radiolabelled platelet kinetics – Platclct-liposome interactions

Summary

This investigation determined the organ distribution of liposomes containing egg phosphatidylcholine and cholesterol with egg phosphatidylglycerol (PG liposomes) or without (PC liposomcs) and the effect of each liposomc **on** platelet distribution in rabbits. Eight minutes after ^{51}chromium-labelled platclets were given intravenously, either saline (n = 7), iodinated PG liposomes (n =5) or iodinated PC liposomes (n =5) were infused. Two minutes later the organ distribution of ^{51}Cr-platelets and ^{125}I-liposomcs werc compared. The PG liposomes produced a $41 \pm 5\%$ reduction in circulating platelet counts while PC liposomes did not. The PG liposomes decreased circulating ^{51}Cr-platelets by a factor of 2 and increased platelet recoveries in the liver and lungs. The increased platelet recovery in the liver was associated with a greater PG liposome recovery. When animals receiving PG liposomes were studied over 60 minutes, both the labelled and unlabelled platelet counts returned to control values by 30 minutes and the ^{51}Cr-platelet distribution between organs was similar to control values. These data indicate that platelets and PG liposomcs initially sequester together and that this platelet-liposome interaction is specific for PG liposomes. However, the platelet sequestration is transient and by 60 minutes the platelets were released and circulating.

Introduction

Liposomcs have been **used clinically as a vehicle** for delivery of drugs (1, 2) and they have potential importance in reducing the toxicity of therapeutic agents and in targeting the agent to a particular organ, tumor or inflammatory site (3-8). Previous studies have shown that multilamcllar vesicles composed of phosphatidylglycerol (PG liposomes) produced a transient reduc-.tion in circulating platelets when infused into rats (9). In contrast,

liposomes composed of egg phosphatidylcholine and cholesterol alone (PC liposomes) did not decrease circulating platelet counts. Both the mechanism of the transient thrombocytopenia induced by PG liposomes and the site of platelet sequestration have not been clarified. The possibility that platelet-liposome interactions may play a role in determining liposome biodistribution is of particular interest for understanding the therapeutic use of liposomes as a drug delivery system.

The purpose of this investigation was to determine the organ distribution of platelets in untreated rabbits and in rabbits treated with either PG or PC liposomes and to compare the distribution of platelets with that of each type of liposomc. These studies show that PG liposomes increased the recovery of platclcts in the lung and the liver and decreased the circulating platelet recovery after 2 minutes of circulation. In addition, the retention of platelets within regions of the lung was correlated with the local retention of liposomes. By 60 minutes, the circulating levels of both labelled and unlabelled platelets and the organ distribution of radiolabelled platelets returned to control values, and most of the recovered PC liposomes were in the liver. In contrast, after 2 minutes the PC liposomes decreased the pulmonary recovery of platelet but did not alter the liver or blood recoveries. This suggests that the platelets and PG liposomes initially sequester together by a reversible interaction and that the PG liposome-platelet interaction is different from that of PC liposomes.

Materials and Methods

Materials

Egg phosphatidylcholinc (PC) and egg phosphatidylglyccrol (PG) were obtained from Avanti Polar Lipids, Inc., Birmingham, AL, U.S.A. Cholesterol (Chol) was purchased from Sigma Chemicals Co., St. Louis, MO, U.S.A. All other chemicals were reagent grade.

Methods

A. Isolation and Radiolabelling of Platelets

Platelets were isolated from 46 ml rabbit blood that was collected into polypropylene tubes containing 14 ml acid-citrate-dextrose (ACD). The blood was centrifuged at 400 x g for 10 minutes at room temperature. The platelet-rich plasma was centrifuged at 800 x g for 10 minutes and the plasma was removed. The platelets could easily be separated from a small number of well-packed RBCs at the base of the platelet pellet by gently rcsuspcnding the platelets in buffer (8.1 mM $Na_2HPO_4 \cdot 7H_2O$ - 1.5 mM KH_2PO_4 -27 mM KCl-138 mM NaCl- 5.5 mM glucose [pH 7.4]) with 10% autologous rabbit plasma and transferring them to a clean tube. The platelets were resuspended in 10 ml buffer-10% plasma and labelled with 600 μCi "chromium for 60 minutes at 37" C. Buffer was added to a total volume of 30 ml and the platelets were ccnugcd at 800 x g for 10 minutes. The ^{51}chromium-labclled platelets (^{51}Cr-platclets) were resuspended in 15 ml buffer-l0% plasma. Following isolation and radiolabelling, the platelets were discoid and not aggregatcd. Aliquots were taken to determine the total number of platelets injected into   animal.

B. Isolation and Radiolabelling of RBCs

RBCs from 10 ml rabbit blood anticoagulated with ACD were isolated and radialabclld with 100 µCi ^{99m}technetium (^{99m}Tc-RBCs) using the method of Gutkowsky and Dworkin (10).

C. Preparation of Iodinated Liposomes

^{125}I-labelled dipalmitoyl-N-[3-(3-[^{125}I]iodo-4-hydroxybenzyl)-propionyl]phosphatidylethanolamine (^{125}I-DPPE) was synthesized according to the method of Schroit (11) and purified by thin-layer chromatography. All ^{125}I-DPPE employed contained less than 2% fatty acid.

Multilamellar vesicles were prepared by first dissolving the lipids in chloroform and then evaporating the solvent under high vacuum for 1 hour. The dried lipid film was hydrated in 0.9% NaCl, 25 mM HEPES buffer at pH 7.4 and the solution freeze-thawed five times to ensure uniform solute distribution (12). The total phospholipid content of the vesicles was 55 mole% with the remaining 45% composed of cholesterol. 125 I-labelling wasaccomplished by adding ^{125}I-DPPE (0.05 µCi per pmolc lipid) to the initial lipid-chloroform solution.

The liposomes composed of either PC: Chol (55:45 mole%, PC liposomes) or PG:PC:Chol (10:45:45 mole%, PG liposomes) were injected at a concentration of 50 mg total lipid/ml for a final administered dose of 25 mg total lipid/kg body weight.

D. Animal Preparation

Scvcntccn New Zealand white rabbits (3.5 ± 0.09 kg) were anesthctizcd with intramuscular kctamine hydrochloride (25-40 mg/kg) and acepromazine maleate (2-3 mg/kg). The skin over the ventral neck was shaved and 1 ml xylocaine was injected subcutaneously. A tracheostomy tube was inscrtcd and the animal breathed room air spontaneously throughout the experiment. A polyethylene catheter was inscrtcd into the aorta (Ao) through the carotid artery. Two intravenous lines were placed in the marginal car veins. Heparin (100 U/kg) was given following surgery.

E. Platelet Distribution 2 min after Liposome Injection

Immcdiatcly prior to radiolabelled platelet injection, blood was drawn for platclct counts and arterial blood gases. The cardiac output was dctcrmincd using the indicator dilution method with ^{99m}Tc-RBCs as the indicator (13, 14). The cells were injected as a bolus into the car vein and samples were collected at 0.5 second intervals from the arterial line. At time 0, the platelets were infused into the car vein. Additional ^{99m}Tc-RBCs were injected arterially to mark the blood volume. After 7 minutes, an arterial blood sample was drawn for platelet counts. At 8 minutes either PG liposomes (25 mg total lipid/kg body weight, n =5), PC liposomes (25 mg total lipid/kg body weight, n=5), or saline (controls, n = 7) were injected into the contralatcral car vein catheter. At 9 minutes ^{103}ruthcnium-labclld microspheres wcrc injected intravenously to mark the pulmonary blood flow. At 9.5 minutes arterial blood was drawn for platclct counts, arterial blood gases, and refcrcncc values for the blood volume, blood flow, circulating platelet and circulating liposome counts. At 10 minutes (2 minutes after liposomes were given), the animal's heart was stopped by arterial injection of saturated potassium chloride.

The chest and pcricardial sac were then rapidly opened and a tic was placed around the base of the heart to maintain the pulmonary blood volume. The lungs were fixed in situ by intratracheal instillation of 7.5% glutaraldchydc in sodium phosphate buffer at 20 cm H_2O pressure. The heart and lungs were rcmovccl en bloc and the lungs sectioned into 25 pieces and placed in prcweighed scintillation vials for gamma counting. The blood vessels supplying the liver were clamped and the liver removed without loss of blood. After sectioning, all liver parenchyma, spleen, I kidney and samples of blood were placed in scintillation vials.

Tissue and blood samples were counted in a Beckman 7000 gamma counter coupled to an Apple II computer with windows selected to maximize counts for each radioisotope while minimizing spillover into other channels. Corrections were made for overlap of radioisotope peaks using pure rcfcrence samples of each isotope. Corrections were made in all samples for radiodecay.

Table I Circulating platelet counts 2 minutes after liposome injection

| | Platelet counts (x10^9/l) | |
	Pre-injection	Post-injection
Controls*	361 ± 33	345 ± 31
PG liposomes"	367 ± 61	14.5 ± 20"
PC liposomes"	307 ± 25	233 ± 48

^{a} Animals were given intravenous injections of either saline (controls, n = 7), PG:PC:Chol multilamcllar vesicles (10:45:45 mole %, PG liposomes, n = 5), or PC: Chol multilamellar vesicles (55: 45 mole %, PC liposomes, n =5), at 25 mg total lipid/kg body weight 8 minutes after injection of radiolabelled, platelets. Blood counts were determined immediately prior to (prc-injection) and 2 minutes after (post-injection) liposome or saline infusion.
^{b} p <0.05 when compared to controls.

E. Platelet Distribution 60 min after Liposome Injection

After blood was sampled for platelet counts and arterial blood gases, ^{51}Cr-platclets were injected intravenously. After they had circulated for 8 minutes, blood was drawn for labelled and unlabelled platelet counts and iodinated PG liposomes (25 mg total lipid/kg) were injected into the ear vein (time 0). At 2, 5, 15, 30, 50 and 60 minutes, blood was taken to determine radiolabellcd platclct and liposome counts, as well as unlabelled platelet counts and arterial blood gases. At 50 minutes, ^{99m}Tc-RBCs were infused to determine blood volume. At 60 minutes, the animal's heart was stopped by intraarterial injection of saturated potassium chloride and the organs were processed as dcscribcd in section E.

G. Calculations

The regional blood volume and blood flow were measured using methods that have been described in detail elsewhere (13, 14). Briefly, in the animals studied 2 minutes after liposome injection, blood volume. was determined in each piece of lung by dividing the ^{99m}technetium counts in the sample by the counts/g in the reference blood sample. The regional blood flow was determined by dividing the ^{103}ruthenium microsphere counts by the sum of the ^{103}ruthenium counts in the lung and multiplying by the cardiac output. No ^{103}ruthenium counts were circulating at 10 minutes. The delivery of either ^{51}Cr-platclets or the ^{125}I-liposomcs to each piece of lung was calculated by multiplying the total number of counts injected by the fraction of the cardiac output that piece rcccivcd. The number of ^{51}Cr-platclets or ^{125}I-liposomes retained was the number of counts present in the piece after ten minutes. The ^{51}Cr-platclet IO minute % retention and the ^{125}I-liposome 2 minute % retention was computed by dividing the retained counts by the delivered counts.

The number of radiolabelled platelets or liposomcs present in the lungs and other organs were determined by summing the noncirculating counts (actual counts minus those present due to blood volume). The distribution of the labelled platelets or liposomei among the lungs, liver, spleen. and kidney was determined by dividing the number of noncirculating radiolabcllcd counts by the total counts of each infused. The circulating blood recovery of platelets was obtained by multiplying the counts/g blood by the rabbit's blood volume (58 ml/kg body weight) and then subtracting the counts that were free in the plasma. The liposome blood recovery was calculated using the total counts/g blood.

In the animals studied 2 minutes after liposome injection, linear regression lines were constructed to compare the relationship bctwccn the percent retention of platelets and liposomes in the 25 lung pieces of each rabbit. The correlation between the platelets and liposomes was evaluated using both parametric (correlation coefficients) and nonparametric (Spearman's rank correlation) tests since percentages are not normally distributed.

In the animals evaluated 60 minutes after PG liposomes were injected, the organ distribution of ^{51}Cr-platelets and liposomes was computed as described above. In addition, changes in the circulating radiolabcllcd and unlabcllcd platclct counts were compared over 60 minutes. The changes in circulating PG liposomc levels were expressed as % of total input.

All values are expressed as the mean and standard error. Variables were compared between the three groups using multivariant analysis. Diffcrcnccs were identified using univariant analysis or nonparametric tests when the group variances were unequal.
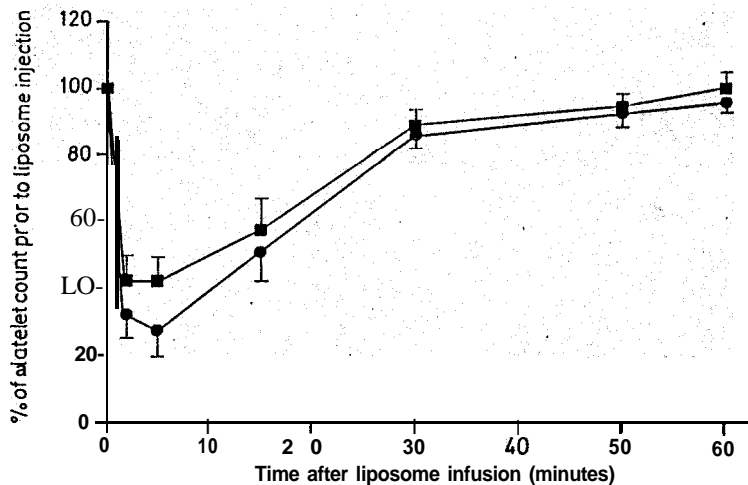
Fig. I **Changes** in circulating radiolabelled (filled circles) and unlabelled (filled squares) platelet counts after injection of PG liposomes (n = 5 in each group)

Table 2   Cardiac output and arterial blood gases

	Controls[a]	PG liposomes -a	PC liposomes-a
Cardiac output (ml/min)[b]	620 ± 74	580 ± 32	582 ± 36
Arterial blood gases[c]			
pO_2 (mm Hg)			
0 minutes	104 ± 3	95 ± 4	110 ± 4
IO minutes	101 ± 6	105 ± 7	118 ± 6
pCO_2 (mm Hg)			
0 minutes	34 ± 2	32 ± 2	35 ± 1
10 minutes	34 ± 2	29 f 1	33 ± 2
pH			
0 minutes	7.41 ± 0.02	7.41 ± 0.02	7.42 ± 0.01
10 minutes	7.40 ± 0.01	7.42 ± 0.01	7.43 ± 0.01

[a] Animals were injected with either saline (controls, **n = 7),**
PG : PC: Chol multilamellar vesicles (10: 45 : 45 mole %, PG liposomes,
n = S), or PC: Chol multilamellar vesicles (55: 45 mole %, PC liposomes,
n = 5) at 25 mg total lipid/kg body weight.
[b] The cardiac output was determined immediately before injection of radiolabelled platelets, prior to liposome injection.
[c] Arterial blood gases were obtained immediately before injection of radiolabelled platelets and at the end of the experiment after platelets had circulated for 10 minutes and liposomes had circulated for 2 minutes.

Table **3   Organ distribution of platelets-a (percent** rccovery)

	Controls[b]	PG liposomes[b] 2 minutes	60 minutes	P C liposomes[b] 2 minutes
Lungs	0.9 ± 0.4%	8.4 ± 1.7%[c]	0.6 ± 0.1%	0.05 ± 0.05%[c]
Blood	54.8 ± 2.2	22.7 ± 1.9[c]	53.8 ± 6.3	45.6 ± 5.5
Liver	14.4 ± 3.3	33.2 ± 2.4[c]	11.7 ± 1.6	21.1 ± 4.3
Spleen	5.4 ± 1.8	4.7 ± 0.6	5.5 ± 0.4	3.4 ± 0.5
Kidney	0.7 ± 0.1	0.8 ± 0.2	1.0 ± 0.5	0.9 ± 0.3
Total	76.2 ± 3.5	68.2 ± 5.7	72.5 ± 5.8	71.0 ± 3.9

[a] Blood recoveries were expressed as the percent of total radiolabelled platelets injected that were present in the circulating blood volume. In all other organs the radiolabelled platelet recovery was calculated by dividing the noncirculating platelets by the total number injected. The animals were studied 2 or 60 minutes after liposome injection.
[b] Animals were injected with either saline (controls, n = 7)
PG : PC: Chol multilamellar vesicles (10:45 : 45 mole %, PG liposomes, n = 5) or PC:Chol multilamellar vesicles (55:45 mole %, PC liposomes, n = 5) at 25 mg total lipid/kg body weight.

Table 4   Organ distribution of liposomes (percent recovery)

	PG liposomes[a] 2 minutes	60 minutes	PC liposomes[a] 2 minutes
Lung	3.8 ± 0.8%	1.3 ± 0.2%	6.9 ± 2.0%
Blood	26.8 ± 1.5	2.2 + 0.1	50.5 ± 6.3[c]
Liver	26.2 ± 2.8	30.0 ± 1.8	10.4 ± 2.5[c]
Spleen	2.1 ± 0.4	1.9 ± 0.1	1.3 ± 0.3
Kidney	0.3 ± 0.04	0.4 ± 0.02	0.4 ± 0.1
Total	58.4 ± 3.8%	35.8 ± 2.0	69.5 ± 5.4%

[a] **Blood recoveries were expressed as the percent of total radiolabelled liposomes injected that were present in the circulating blood volume. In all other organs the radiolabelled liposome recovery was calculated by dividing the noncirculating liposomes by the total number injected. The animals were studied 2 or 60 minutes after liposome injection.**
[b] Animals were injected with either PG:PC:Chol multilamellar vesicles (10:45:45 mole %, PG liposomes, n = S), or PC:Chol multilamellar vesicles (55:45 mole %, PC liposomes, n = S), at 25 mg total lipid/kg body weight.
[c] p <0.006 compared to PG liposomes.

Results

The platelet. counts in control animals that **did not receive liposomes were constant Table 1). Two minutes after administration** of PG liposomes, the platelet count decreased to 41.4 ± 4.9% of the initial value (p <0.05 when compared to the change in **control values, Table 1)** while platelet counts in the animals receiving PC liposomes showed no significant change. Fig. I shows the circulating unlabelled and radiolabelled platelet counts expressed as the change from baseline measurements prior to liposome injection. Both the labelled and unlabelled platelet counts showed a similar decrease that was significantly different from baseline values 2, 5 and 15 minutes following PG liposome **injection** (p <0.01). Both labelled and unlabelled platelets behaved similarly and returned to baseline levels by 30 minutes. In contrast, there were 13.3 ± 1.8% of the PG liposomes circulating at 2 minutes, 6.7 ± 0.5% at 5 minutes (p <0.005 when **compared to 2 minute value), 2.9 ± 0.2% at 15 minutes and no further change at 30, 50 or 60 minutes.**

The cardiac output measured before the infusion of liposomes in the animals studied at 2 minutes was not different between **groups** (Table 2). The arterial blood gases in the controls were stable throughout the experiment while the liposome-treated animals showed a trend toward slight hyperventilation (Table 2). No change in arterial blood **gases** was seen at 5 minutes or 60 minutes in the animals studied 60 minutes after injection of I'G liposomes.

The organ distribution of platelets is shown **in Table 3. At 2** minutes, the recovery of platelets in the lungs increased from **0.9 ±** 0.4% to 8.4 ± 1.7% with infusion of PG liposomes while the blood recovery decreased by half and the liver **recovery increased more than twice. By** 60 minutes, the organ distribution had returned to control values. In contrast, PC liposomes at 2 minutes resulted in a decreased platelet recovery to 0.05 ± **0.05%** in the lungs and no change in blood or liver recoveries **(Table** 3). Although PG liposomes caused a greater retention of **platelets in** the lung at 2 minutes than PC liposomes, the actual **amount** of each liposome species retained in the lung was similar (Table 4). The majority of the recovered PG liposome counts **were** evenly divided between **the blood and the liver while most of** the **PC** liposomes were circulating (Table 4). After 60 minutes, the majority of the recovered PG liposomes were in the liver (Table 4)

Animal	PG liposomes Correlation coefficient	Spearman rank correlation	PC liposomes Corrclation coefficient	Spearman rank correlation
1	0.74"	0.78[a]	0.03	0.03
2	0.55"	0.42	0.08	0.06
3	1.00"	0.95[a]	0.00	0.26
4	0.57[a]	0.75"	-0.17	-0.21
5	0.77"	0.25	0.01	0.07

[a] Significant correlation bctwcen the percent retention of platelets and liposomcs.

The relationship between the presence of platelets and liposomes in the pieces of lung from each rabbit 2 minutes after liposomc injection was cvaluatcd by comparing the correlation coefficient and the Spearman rank correlation for the linear regression describing platelet vs liposome % retention. The correlation coefficient between PG liposomes and platelets was significant in all 5 animals and the Spearman rank correlation was significant in 3 animals (Table 5). In contrast, the relationship between PC liposomes and platelets was not significant in any animal. The insignificant correlation'between PC liposomes and platelets reflected in part the very low number of platelets rccovcrcd in the lungs of this group.

Discussion

Previous work has shown that liposomes composed of PG: PC: Chol (PG liposomes) induced a transient reduction in circulating platelets in rats while liposomes containing PC: Chol (PC liposomes) did not (9). This study extends these observations by measuring both labelled and unlabelled platelet counts and correlating the biodistribution of labelled platelets with that of liposomes. The distribution of platelets within organs was altered 2 minutes after liposome infusion. Fewer PG 'liposomes than PC liposomcs were circulating after 2 minutes, and this lower cir-culating RC liposomcs was associated with a greater recovery of both platelets and PG liposomes in the liver as well as a correlation between platelet and liposome sequestration within regions of the lung. In contrast, many of the PC liposomcs were still circulating at 2 minutes. This suggests thal rcticulocndolhclial cell clearance of PG liposomes is associated with plateletaccumu-lation and that these liposomes differ in their ability to interact with platelets. Although the lung recovery of PG liposomes tendcd to be less than that of PC liposomes, the platelet recovery was increased only when PG liposomcs were infused.

To determine if the sequestration of platelets and PG liposomcs seen 2 minutes after injection was reversible, the organ distribution of each was examined 60 minutes after injection. The number of both circulating labelled and unlabelled platelets fell to comparable levels 2-15 minutes after liposome injection and returned to pre-treatment values at 30 minutes (Fig. 1). In addition, the recovery of platelets in the liver decreased from the value seen after 2 minutes to that of controls and the organ distribution of platelets was similar to controls (Table 3). These data indicate that the thrombocytopenia seen 2-15 minutes after PG liposome injection was reversible. Because both the labelled and unlabelled platelet counts recovered and the number of sequestered platelets in the liver decreased, the increase in circulating platelets to normal values by 30 minutes was due to the release of sequestered platelets and not due to release of new platelets from the bone marrow. In contrast, the number of circulating PG liposomcs fell rapidly and rcmaincd low, indicating

that the sequestered liposomes were not released. Most of the liposomes recovered after 60 minutes were in the liver although the total recovery was low, most likely due to breakdown of liposomes or to metabolism of the ^{125}I-DPPE lipid radiolabel employed.

The mechanism for platelet sequestration immediately after liposome injection has not been well characterized. Since the radiolabelled platelets showed similar changes to the unlabelled ones, the decreased platelet counts were not due to the formation of large platelet clumps that were still circulating after 2 minutes. However, since the PG liposomes and the platelets appear to sequester together within the lung as well as the liver, sequestra-tion could be due to adherence of platelets to liposomes within the large or small blood vessels. These liposome-platelet aggregates may be removed either by the reticuloendothelial system or by trapping within the microvasculature. In a previous paper (9) we have demonstrated that PG liposomes and platelets can exhibit a transient in vitro microaggregation in the presence of hcparinizcd plasma. It may therefore be suggested that the in vivo transient reduction in platelet count arises from trapping of these microag-gregates within the microvasculature of the lung and the liver. The subsequent dissociation of the liposome-platelet complexes would allow the platelet levels to return to normal levels.

The mechanism of the liposome-platelet association and subse-quent dissociation is clearly of interest. Specifically, the nega-tively-charged PG-containing liposomes may bind serum proteins more avidly than neutral PC liposomes, and these bound proteins may play a role in subsequent binding to platelets. This interac-tion may play a role in the clearance of liposomes.

In summary, this work shows that platelets and PG liposomes accumulated in the same organs 2 minutes after PG liposomc injection: This association occurred only when the liposomes contained PG, and the platelet sequestration was reversible. The correlation between platelet .and PG liposome retention within the lung suggests that they sequester together. It is suggested that platelets ma) be involved in the clearance of liposomes and the transient formation of platelet-liposome complexes may modulate liposome clearance. The interaction of platelets and liposomes is important for understanding the biodistribution and clearance of liposomes when used as a vehicle for drugs, as well as to investigate platelet interactions with negatively charged lipids.

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