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Interactions of functionalized polymeric liposomes having a porphinato-iron complex with some biological cells and components in vitro

Etsuo Hasegawa [a], Kiyoshi Eshima [a], Yoh-Ichi Matsushita [a], Hiroyuki Nishide [b] and Eishun Tsuchida [b]

[a] *Research Institute, Taiho Pharmaceutical Co., Ltd., Tokushima 771-01 (Japan) and* [b] *Department of Polymer Chemistry, Waseda University, Tokyo 160 (Japan)*

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The hemocompatibility of functionalized polymeric liposome particles (diameter: 20–32 nm), which have a synthetic porphinato-iron complex in their polymerized bilayers and can carry oxygen, was studied in vitro. The ultramicroparticles did not induce hemolysis, platelet aggregation and plasma coagulation directly and were stable against hydrolysis by phospholipases A_2 and D.

Introduction

Liposomes (vesicles) which are closed and spherical structures having an internal aqueous compartment and lipid bilayers are considered good models for biomembranes [2], but their low stability in the interaction with natural cell systems or of long-time storage makes their use for limited purposes.

As stable model membranes, polymeric liposomes, which are microcapsules prepared by polymerization of the lipid bilayers of liposomes composed of polymerizable lipids, have been developed [2–6]. Applications of the stable liposomes to controllable releasing of contents [7], enzyme fixation [8,9], rhodopsin fixation [10,11] and encapsulation of hemoglobin [12] have been reported. Recently our investigations of functionalized liposomes have demonstrated that stable oxygen carriers are prepared when a synthetic heme complex is embedded in the lipophilic region of the lipid bilayers of liposomes [13–16]. To improve the mechanical stability of the liposomes, we have synthesized a series of polymerizable phospholipids [17,18] to be used as carriers of heme. Thereby, a highly concentrated, physically and mechanically stable and storageble heme solution could be prepared [19].

The utilization of liposomes for intravenously injecting biological active materials requires that the microparticles enter the blood stream where they may interact with cells or plasma proteins. The interactions of polymeric liposomes with platelets [20] and their thrombogenicity [21] have been reported.

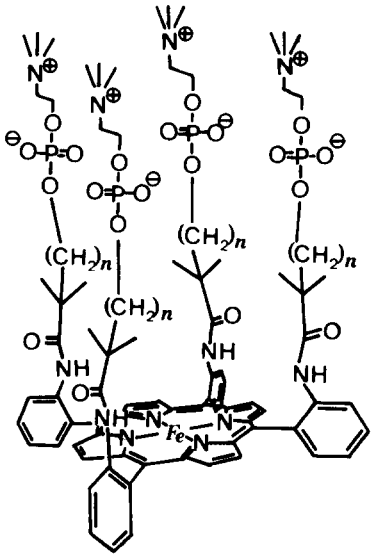
In this communication the reaction of the hemin-bearing polymeric liposomes [19] with some biological components and the effect on coagulation are preliminarily reported to elucidate the hemocompatibility of the functionalized microparticles in vitro.

Correspondence: Dr. E. Tsuchida, Department of Polymer Chemistry, Waseda University, Tokyo 160, Japan.

Materials and Methods

Materials

Hepes (Dojin Lab., Kumamoto, Japan), Reinecke salt (Nakarai Chemicals, Kyoto), protohemin (Sigma), phospholipase A_2 (Sigma, No. P8387), phospholipase D (Sigma, No. P0640), egg yolk phosphatidylcholine (Sigma, No. P2772) and bovine serum albumin (Sigma, No. A4503) were available commercially. Synthetic hemins 1 ($n = 1$, 10 and 18) as shown in Scheme I and 1-dodecylimidazole 2 were synthesized as described in previous papers [14,15,22]. Two polymerizable lipids (3a: 1-[9-(*p*-vinylbenzoyl)nonanoyl]-2-*O*-octadecyl-*rac*-glycero-3-phosphocholine [17] and 3b: 1,2-bis(octadeca-2,4-dienoyl)-*sn*-glycero-3-phosphocholine [23]) as illustrated in Scheme II were prepared. Isotonic buffered saline was freshly prepared on each day by diluting 40 ml of the stock solution ($NaCl/KCl/Na_2HPO_4 \cdot 12H_2O/KH_2PO_4$, 80 : 2 : 29 : 2 gram dissolved in 800 ml of distilled water) up to 500 ml by distilled water. The pH and the osmolarity were 7.0 and 275 mosM, respectively.



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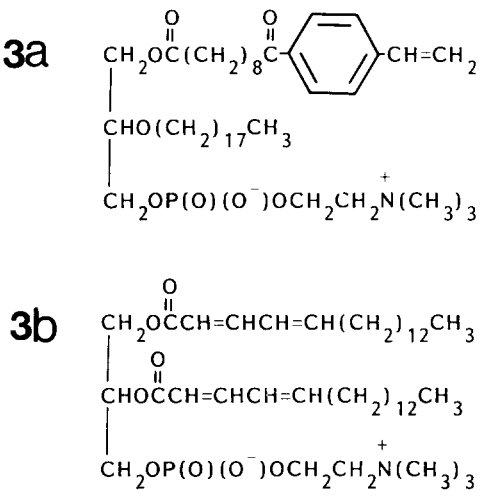
Scheme I. Illustration of the synthetic hemin 1 ($n = 1$, 10 and 18).



Scheme II. Structures of polymerizable phospholipids 3a and 3b.

Preparation of polymeric liposomes

The ultramicroparticles were prepared according to the previous paper [19] from 1 ($n = 18$), 2 and a polymerizable lipid 3a or 3b. The 1($n = 18$)/2/3 molar ratio was 1 : 3 : 50. The suspension obtained by photopolymerization was concentrated to 21.5 %w/v using a ultrafiltration system (Minitan System, Millipore Corp., MA, U.S.A.) and was finally passed through 0.45 μm filter (Millex-HA, Millipore). The physical properties of the solutions were similar to those of whole blood [19]. For example, pH, osmolarity and viscosity were 7.4, 330 mosM and 4.0 cP (37°C), respectively. The polymeric liposome microparticles derived from 3a and 3b are abbreviated as 4 and 5, respectively. The average diameters of 4 and 5 were determined by a laser particle analyzer (Coulter N4D, Coulter Electronics, Inc., FL, U.S.A.) to be 32 and 20 nm, respectively. The solutions were stored in a sterile glass bottle and kept in the dark at room temperature, where they were stable for a month.

Hemolysis of red blood cells

All experiments were performed on fresh and heparinized blood drawn from male Wister rats. The erythrocytes were freed of plasma by five washing with cold isotonic saline (0.9% NaCl) and sedimented by centrifugation at $3000 \times g$ for 5

min at 4°C. The pellets were diluted with the isotonic buffered saline to give 0.6 %v/v suspensions. They were incubated for 1–5 h with the polymeric liposome particles in a plastic vessel in a shaking bath at 3 Hz and 37°C. The final concentration of erythrocytes was 0.5 %v/v. The mixtures were then cooled and centrifuged at 3000 $\times g$ for 10 min at 4°C. The supernatant collected was diluted by 2 volumes of saline and 2.0 ml of the solution was applied to the column (2 × 40 cm) containing Sephacryl S-300 (Pharmacia Fine Chem., Uppsala, Sweden) and eluted with isotonic saline (0.9 %w/v NaCl, pH 7.0). The eluates were analyzed spectrophotometrically at 412 nm. 100% hemolysis values were taken by measuring the hemoglobin released after hypotonic dilution with distilled water followed by centrifugation at 10000 $\times g$ for 30 min at 4°C. Experiments were simultaneously performed without the polymeric liposomes for correcting percent hemolysis values (thus controlled experiments gave 0.8–2.5% hemolysis).

Hemolysis in a whole blood/isotonic buffered saline (1:1, v/v) mixture was examined and analyzed as described above.

Experiments on protohemin dissolved in 0.02 M NaOH and on the water soluble 1 ($n = 1$ and 10) were carried out as described above and the supernatant collected was directly analyzed without column separation. Percent hemolysis values were means of four or five different determinations.

Platelet aggregation

Fresh blood (9 ml) was drawn from a male Wister rat and mixed with 1 ml of 3 %w/v citrate. It was centrifuged at 1000 rpm for 10 min at room temperature and the upper layer (platelet rich plasma) was collected. The lower layer was further centrifuged at 3000 rpm for 10 min and the upper layer (platelet and plasma) was collected. Platelet rich plasma was adjusted to a concentration of $50 \cdot 10^4$ cells/μl by diluting with platelet poor plasma. Cell concentrations were determined by a cell counter (Celltac MEK-4500, Nippon Koden, Co., Tokyo). To platelet rich plasma (0.425 ml) in a siliconized glass vessel was added 0.05 ml of a polymeric liposome suspension (0.67 %w/v) and the aggregation process was monitored after adding 0.025 ml of a ADP solution at 37°C with a platelet aggregometer (HUSM System, Rikadenki Kogyo Co., Tokyo). Platelet rich plasma from a male rabbit was used for studying platelet aggregation induced by collagen (Collagenreagent Horm, Hormon-Chemie, Munich). The concentration was $44 \cdot 10^4$ cells/μl.

Coagulation

Citrated plasma was prepared as follows: Fresh blood (9.0 ml) was drawn a male beagle dog and mixed with 3.8 %w/v citrate (1.0 ml). This was centrifuged at 1500 rpm for 10 min and the upper layer was collected and used for coagulation tests.

Prothrombin time, PT: After a solution containing a polymeric liposome solution (0.02 ml) and plasma (0.08 ml) was preincubated at 37°C for 300 s, 0.2 ml of 'Simplastin Automated (Warner-Lambert Co.)' was added and then a coagulation process was monitored spectrophotometrically (Coagmaster, Sankyo Co., Tokyo). Activated partial thromboplastin time, APTT: After preincubation of plasma (0.08 ml), a polymeric liposome solution (0.02 ml) and 'Plasterin-plusactivator' (Warner-Lambert Co.) (0.1 ml) at 37°C for 300 s. 0.1 ml of 0.025 M $CaCl_2$ was added to initiate coagulation.

Times were mean values of three different determinations.

Hydrolysis by phospholipases

The enzymatic hydrolysis by phospholipase A_2 was followed by an automatic titration of the liberated fatty acid by 0.01 M or 0.02 M NaOH with a pH-statt titrator (RTS822 System, Radimeter, Copenhagen) equipped with a microburette [24,25]. All experiments were carried out at 25°C in saline (0.9 %w/v NaCl) having 10 mM $CaCl_2$ under the stream of CO_2-free N_2 presaturated with water. The pH of a 1.0 %w/v lipid suspension was adjusted to 8.00 with aqueous NaOH and the reaction was then allowed to start by adding 150 units of phospholipase A_2 suspended in the same solvent (pH 8.0, 0.15 ml). Normal liposomes of egg yolk phosphatidylcholine with or without 1 ($n = 18$) or 2 were prepared by ultrasonication followed by filtration through 0.45 μm membrane and then the suspensions were annealed [24,26]. The molar ratio 1/2/3 was the same as for the polymeric liposomes. In all experiments the initial

phospholipid was 125 μmol.

The results were shown as mean values of three different determinations.

The reaction with phospholipase D was carried out as follows [27,28]: liposomes (10 mg) were suspended in 1.0 ml of 1/15 M Hepes buffer (pH 5.6) having 10 mM $CaCl_2$. To the suspension were added phospholipase D (4800 units, 8.0 mg) and 1.0 ml of peroxide-free diethyl ether. The mixture was shaken at 25°C for 5 h. Ether was removed by a stream of N_2 and to the residual aqueous phase were added methanol (2.5 ml) and chloroform (1.25 ml). The mixture was shaken and then chloroform (1.25 ml) was further added. After shaking well, it was centrifuged and the upper layer was used for the choline analysis by the Reinecke salt method [29]. The choline salts were analyzed by measuring the absorbance at 526 nm. Three different determinations were carried out.

Results and Discussion

Hemolysis

Percent hemolysis was determined by measuring the amount of released hemoglobin spectrophotometrically at 412 or 540 nm. Because of the intense absorption due to the synthetic hemin 1 ($n = 18$), it was necessary to remove polymeric liposome microparticles from hemoglobin. It was achieved by the use of gel filtration chromatography as described in Methods. Typical elution patterns are shown in Fig. 1. Small particles (hemoglobin, Stokes radius: 0.25 nm [30]) could be isolated from large particles (liposomes, diameter: 20–30 nm). When hemin concentration was rather low as in the case of the water-soluble hemins (protohemin and 1 ($n = 1$ and 10)), it was possible to determine directly the hemoglobin content of the supernatant obtained by centrifugation. The results are summarized in Fig. 2 and Table I.

It is well-known that the natural hemin (protohemin) has strong hemolytic activity for washed red blood cells [31,32]. As shown in Fig. 2, protohemin showed high activity in the hemolysis of washed red blood cells. Under the conditions used here 50% hemolysis was produced by 9 μM protohemin. The water-soluble and amphiphilic hemin 1 ($n = 10$) had almost the same dose-responce as protohemin, while 1 ($n = 1$) showed very weak activity at the concentration up to 20 μM and rather lipophilic 1 ($n = 18$) embedded in the polymeric liposomes had very weak activity also. Many

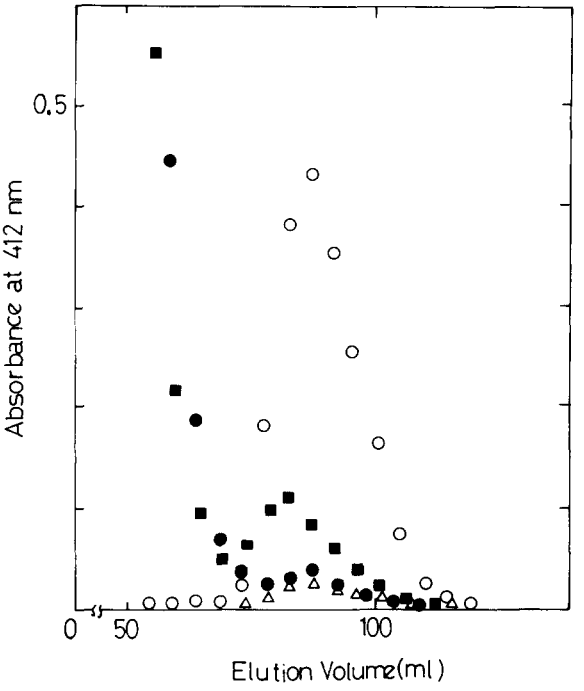


Fig. 1. Elution patterns of gel permeation chromatography on Sephacryl S-300 for analyzing released hemoglobin contents. Column: 2×40 cm; eluting medium: 0.9 %w/v NaCl (pH 7.0). Hemolysis of 0.5 %v/v red blood cells induced by 4 (1 ($n = 18$): 850 μM, 5 h) (●), 5 (1 ($n = 18$): 850 μM, 5 h) (■) and for control (△) and 100% hemolyzed as standard (○).

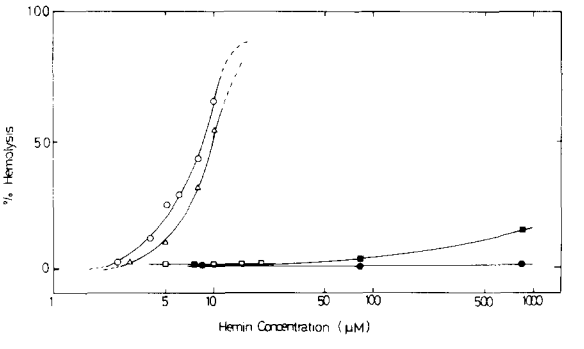


Fig. 2. Effect of hemin concentration on hemolysis of washed red blood cells at 37°C in isotonic buffered saline in the dark. red blood cells: 0.5 %v/v; Reaction time was 2.5 h for protohemin (○), synthetic hemins 1 ($n = 10$) (△) and 1 ($n = 1$) (□) and 5 h for the polymeric liposomes, 4 (●) and 5 (■).

TABLE I

HEMOLYSIS INDUCED BY VARIOUS HEMINS AND EFFECT OF ALBUMIN

Red blood cells (%v/v)		Hemins	[Hemin] (μM)	[Albumin] (μM)	Hemolysis (%) [a]		
					1 h	2.5 h	5 h
Washed RBS	(0.5)	protohemin	10	0		65.0 [b]	
				5		2.8	
				10		1.4	
				20		0.9	
				40		0.2	
		1 ($n = 10$)	10	0		54.0 [c]	
				5		1.0	
				20		0.2	
		4	85	0			−2.6
			850	0		1.3	1.2
		5	85	0			2.9
			850	0			16.5
				5			10.9
				50			−3.4
	(20.0)	4	850	0	1.1		2.0
		5	850	0	1.8		10.6
Whole blood	(20.0)	4	850	340	−0.2		1.6
		5	850	340	−0.4		−2.1

[a] S.D. was 0.3–0.6.
[b] S.D. was 3.5.
[c] S.D. was 3.2.

radical scavengers like dimethylurea, thiourea, L-tryptophan, sodium formate, D-mannitol and EDTA (each 5 mM) had no effect on the hemolysis induced by 1 ($n = 10$) (8.3 μm) or protohemin [32]. These and the fact that 1 ($n = 10$) can form aggregates (aggregation number: 40) in water while 1 ($n = 1$) disperses monomolecularly [22] may suggest that hemolysis by 1 ($n = 10$) is largely caused by the amphiphilic property of 1 ($n = 10$) as lysophosphatidylcholine [33] but not by radicals or peroxides and that protohemin triggers hemolysis by binding some specific site(s) on red blood cell membranes such as phosphatidylserine [34] but not through its amphiphilicity or peroxides.

The polymeric liposome particles containing 1 ($n = 18$) and 2 had very weak hemolytic activity. The polymeric liposome 4 induced no hemolysis for washed red blood cells and whole blood, while 5 showed very weak activity for washed red blood cells but not for whole blood. This may indicate that the structure of polymerizable lipids is responsible partly for hemolytic activity.

The result that 4 and 5 had apparently no hemolytic activity in the whole blood may be explained by the effect of serum proteins because in the presence of albumin 5 caused no hemolysis of washed cells (Table I). Albumin is known to protect erythrocytes from hemolysis induced by sulfhydryl blocking agents [35]. It was suggested that albumin of high concentration (25 %w/v, 3.5 mM) acted through its osmotic property [35]. On the other hand hemoglobin binds protohemin and removed it from the erythrocyte surfaces [36]. Albumin less than the equivalent moles of 1 ($n = 10$) or protohemin blocked completely the hemolysis induced by the hemins as shown in Table I, while even at high concentration of 1 ($n = 18$) embedded in the polymeric liposome particle (5) albumin of low concentration (less than 0.06 equiv./mol) effectively reduced the percent of hemolysis.

The fact that the polymeric liposome microparticles had no or very weak hemolytic activity in comparison with protohemin or 1 ($n = 10$) is ex-

plained by the fixation of 1 ($n = 18$) and 2 in the stable (polymerized) bilayers apparently reducing their hemolytic activity.

Platelet aggregation

It has been reported that unchanged small unilamellar liposomes of 1,2-diacyl-*sn*-glycero-3-phosphocholine did not induce aggregation in platelet rich plasma, nor did they affect the ability of ADP to mediate aggregation [20]. The polymeric liposome particles derived from 1,2-bis[12-(methacryloyloxy)dodecanoyl]-*sn*-glycero-3-phosphocholine did impair the thrombin-induced platelet aggregation at its high concentration (20 %w/v), but not at low concentration (4 %w/v) [20]. It is also reported that imidazole derivatives, especially 1-substituted ones, selectively inhibit human platelet thromboxane synthetase [37–39]. The IC_{50} value for 1-dodecylimidazole 2 is 3.2 μM [38].

The polymeric liposome particles, 4 and 5, showed no clear sign of directly causing platelet aggregation at concentration up to 3.3 %w/v.

The results on examining the effect of 4 and 5 on the ability of platelet to aggregate in response to ADP or collagen are summarized in Table II. Both particles slightly inhibited ADP-mediated platelet aggregation, while they did not affect the ability of collagen. The inhibitory effect of the polymeric liposomes on ADP-induced aggregation may be caused by the imidazole component 2, of which concentration employed was rather high (51 μM), although 2 is expected to be embedded in the polymerized bilayers.

TABLE II

EFFECT OF POLYMERIC LIPOSOME PARTICLES ON PLATELET AGGREGATION INDUCED BY ADP OR COLLAGEN AT 37°C

Liposomes	Aggregation intensity (%)		
	ADP [a]		Collagen [b] (5 μg/ml)
	86 μM	10 μM	
Control	95	79	57
4	69	60	57
5	67	61	–

[a] Rat platelet: $42 \cdot 10^4$ cells/μl.
[b] Rabbit platelet: $37 \cdot 10^4$ cells/μl.
4 or 5: 0.72 g/l (1 ($n = 18$) = 17 μM, 2 = 51 μM, 3 = 850 μM)

Coagulation

The polymeric liposome particles, 4 and 5, were non-thrombogenic at their concentration up to 3.3 %w/v.

The effect of 4 and 5 on coagulation of beagle dog plasma was then studied by the use of the well-known clinical methods. Prothrombin times for the extrinsic pathway [40] and activated thromboplastin times for the intrinsic pathway [41] were measured in vitro. The results are summarized in Table III. At higher concentration (0.67 %w/v) examined it seems that the polymeric liposomes prolonged the coagulation time. It was reported that an imidazole derivative shows an anti-thrombogenicity [41] and that hematin activates the factor XII-dependent pathway in human plasma [43] while hematoporphyrin inhibits the factor VIII [44]. The polymeric liposomes derived from 1,2-dipentacosanoyl-10,12-diyne-*sn*-glycero-3-phosphocholine was non-thrombogenic and did not affect the Stypven clotting times at the concentration of 0.1 %w/v [21]. The apparent prolongation of coagulation times may be explained by the complex summation of the effect of each components of the polymeric liposomes, 4 and 5.

Hydrolysis by phospholipases A_2 and D

The hydrolysis of various liposomes with average diameters ranging between 20 and 36 nm by phospholipase A_2 was followed by titrating the amounts of the liberated fatty acids by alkaline. The reaction processes and the results are shown in Fig. 3 and Table IV. It was found that (1) the

TABLE III

EFFECT OF POLYMERIC LIPOSOME PARTICLES ON COAGULATION OF BEAGLE DOG PLASMA AT 37°C

S.D. was 0.1 s and 0.2 s for prothrombin time (PT) and activated partial thromboplastin time (ATPP), respectively.

	Control	4 (%w/v)			5 (%w/v)		
		0.0067	0.067	0.67	0.0067	0.067	0.67
PT (s)	14.1	14.1	14.0	14.4	14.1	14.3	16.3
APTT (s)	21.8	22.0	22.1	24.4	22.0	22.3	23.5

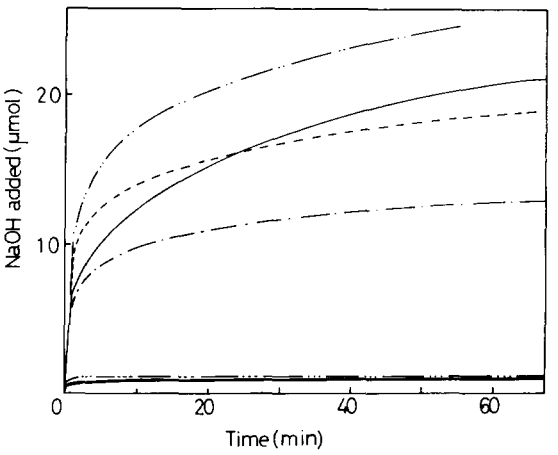


Fig. 3. The reaction processes for the hydrolysis of phospholipid liposomes by phospholipase A_2 (150 units) at 25°C in 0.9 %w/v NaCl having 10 mM $CaCl_2$ (pH 8.00). 4 (▬▬), 5 (— · · · —), egg yolk phosphatidylcholine (———) and egg yolk phosphatidylcholine having 1 ($n = 18$) (— · —), 2 (— · · —) and 1 ($n = 18$) and 2 (- - - - -). Lipid concentration: 1.0 %w/v.

polymeric liposome particles were not susceptible to the phospholipase A_2 hydrolysis; (2) the liposomes of egg yolk phosphatidylcholine were hydrolyzed and liberated fatty acids; (3) the presence of 1 ($n = 18$) or 2 in the bilayers of the egg yolk phosphatidylcholine liposomes reduced or enhanced the hydrolysis, respectively, and (4) the non-polymerized liposomes of 3b which is the derivative of *sn*-glycerol were hydrolyzed, but at a lower rate than egg yolk phosphatidylcholine liposomes. The fact that the average diameters of the polymeric liposomes did not differ before and after hydrolysis at 24 h and 37°C corresponds with the lack of fatty acid liberation.

The liposomes derived from egg yolk phosphatidylcholine were hydrolyzed with phospholipase D and liberated more than 44% of the original choline, while both of the polymeric liposomes liberated less than 2–3% of the original choline.

These results indicate that the polymeric liposome particles are stable against phospholipases because no hydrolysis was induced in the inner (lipophilic) and the outer (liophilic) regions of the polymerized bilayers.

The experiments of these functionalized polymeric particles with macrophages and their behaviour in the vascular system will be reported in a following paper using these polymeric particles as a model for red blood cells.

TABLE IV

HYDROLYSIS OF PHOSPHOLIPID LIPOSOMES BY PHOSPHOLIPASE A_2 AT 25°C

Figures are presented as means ± S.D.

Liposomes [a]	Average diameter (nm)	Fatty acids formed after 30 min (μmol) [b]	Average diameter (nm) after 24 h
EYPC	30 ± 8	14.6 ± 1.0	
EYPC/1 ($n = 18$)/2	36 ± 9	13.9 ± 1.0	
EYPC/1 ($n = 18$)	26 ± 3	9.8 ± 0.6	
EYPC/2	36 ± 8	18.4 ± 1.2	
3b/1 ($n = 18$)/2	20 ± 15	5.0 ± 0.5	
4	32 ± 9	< 1.0	33 ± 9
5	20 ± 5	< 1.0	20 ± 7

[a] EYPC, egg yolk phosphatidylcholine.
[b] Initial phospholipid: 125 μmol.

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