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Coating liposomes with collagen (M_r 50 000) increases uptake into liver

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Abstract

Collagen-coated small unilamellar liposomes were prepared by incubation of two hydrophobic derivatives of collagen (average M_r 50 000) with preformed vesicles. The introduction of hexyl and lauryl residues to the collagen molecule improved by 10-fold the ability of collagen to coat liposomes. In vitro stability of the different coated vesicles prepared, was studied by their ability to retain entrapped carboxyfluorescein as a function of the time. Coated vesicles were clearly more stable in vitro than control liposomes, except for those containing the lauryl derivative in a protein/phospholipid weight ratio higher than 10^{-3}. Vesicle clearance from circulation as well as tissue distribution were also determined. Pharmacokinetics (determined by both fluorescence and radioactive techniques) were highly dependent on the injected dose, phospholipids used and the content of collagen. Half-lives were maximum for liposomes composed of saturated phospholipids injected at a dose of 2 μmol phospholipid. Besides, blood elimination of collagen-containing vesicles was about 2-fold faster and liver uptake 1.5 to 2-fold higher than control liposomes.

Keywords: Liposome; Collagen; Collagen-coated liposome; Stability; Half-life; Tissue distribution; Liposome–protein interaction

1. Introduction

The problem of binding proteins to liposomes has been given increasing attention mainly because the attachment of some specific proteins to the surface of liposomes has proved useful in creating homing devices able to deliver drugs to a target group of cells or tissues [1].

Besides, it has been well established that the incorporation of certain molecules to the liposome membrane can alter their in vivo behavior, thus providing great versatility for liposome therapeutic applications. It is well known that liposomes are rapidly cleared from circulation mainly due to two processes: (a) lipid exchange with high-density lipoproteins (HDL) [2], leading to liposome disintegration, and (b) opsonization followed by macrophage uptake [3,4]. However, the presence of cholesterol [5,6], gangliosides [7] and – more recently – poly(ethylene glycol) [8–13] has proved to reduce the rates of these interactions, resulting in enhanced circulating times. On the contrary, other components, such as phosphatidylserine [14] or certain polysaccharides [15] have proved to promote liposome clearance from circulation.

It seems well established that the beneficial effect of PEG or gangliosides on prolonging half-life is due to the fact that they provide a hydrophilic shelter around the external layer of liposomes hindering the attachment of plasma proteins to its surface. Following this assumption we explored the possibility of using a widely spread protein (collagen) to provide the same sterical effect. In addition, as collagen plays an important role in the cell adhesion process it could also be possible that it facilitates the binding of liposomes to cells.

As a first approach, in this paper we report the results obtained after incorporation of hydrophobically derivatized collagen to the surface of liposomes as far as its stability and biodistribution is concerned.

2. Materials and methods

2.1. Materials

Collagen used in this study was kindly provided by Lipotec (Spain). The average molecular weight determined by SDS-PAGE electrophoresis [16] was 50 000. Hydro-

Abbreviations: Fmoc, 9-fluorenylmethoxycarbonyl; Gly, glycine; pfp, pentafluorophenyl; HOBt, 1-hydroxybenzotriazole.

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genated phospholipids (HPC), consisting in a mixture of hydrogenated egg phosphatides (type V) that contained more than 75% of phosphatidylcholine, were from Asahi. Natural phosphatidylcholine (PC) was supplied by Avanti. Cholesterol purchased from Sigma. Carboxyfluorescein (CF) (Eastman Kodak) was purified by column chromatography. Fmoc-Gly-Opfp and HOBt were supplied by Novabiochem. Diethylenetriamine pentaacetic acid distearylamide complex (DTPA-SA) was synthesized and [^{111}In]DTPA-SA prepared as reported by Kabalka [17].

2.2. Methods

2.2.1. Labeling procedure

In order to increase the affinity of the protein for liposome bilayers, hydrophobic derivatives of collagen containing hexyl or lauryl residues were synthesized as reported elsewhere [18]. Furthermore, native collagen (C) as well as the hexyl (C-H) and lauryl (C-L) derivatives were fluorescently labeled by chemical linkage of the 9-fluorenylmethoxycarbonyl (Fmoc) moiety. In brief, to a solution of 1 g of collagen or collagen derivative in 5 ml of distilled water, Fmoc-Gly-Opfp and HOBt (dissolved in *N,N*-dimethylformamide) were added in amounts equimolecular to the assumed number of free amino groups present in the protein, as estimated by reaction with 2,4,6-trinitrobenzenesulfonic acid. After 4 h of reaction at room temperature, the reacting mixture was lyophilized. The residue was washed with DMF and the absence of Fmoc amino acid and HOBt was controlled by TLC. Fluorescence intensity of the collagen derivatives was measured at excitation-emission wavelengths of 265–305 nm on a LS 50 Perkin Elmer spectrofluorimeter. In some experiments collagen was radiolabeled with ^{125}I by the IodoGen method (Rockford, IL).

2.2.2. Preparation of collagen-coated liposomes

2.2.2.1. Fluorescently labeled liposomes. Small unilamellar vesicles composed of HPC/Chol (1:1 molar ratio) were prepared in the presence of 50 mM carboxyfluorescein (CF). In brief, the dry lipid film on the walls of a flask was hydrated with a solution of the marker in acetate buffer (pH 7.4) at 55°C and subsequently sonicated and allowed to stand for 1–2 h at the same temperature. SUV were separated from free CF by column chromatography in Sephadex G-50 followed by dialysis. Liposomes thus prepared were incubated at room temperature for 6 h with a solution of one of the different collagens (C, C-H and C-L) in a weight ratio of 0.4 mg collagen per mg phospholipid. In the case of C-L weight relationships of 0.2 (C-$L_{0.2}$) and 0.1 (C-$L_{0.1}$) mg collagen per mg phospholipid were also assayed. Coated liposomes were isolated by gel permeation chromatography on Sepharose CL 6B and the effectiveness of the coating was controlled by fluorescence techniques. Fractions that were fluorescent both, at excitation-emission wavelengths of 490 and 520 nm, respectively (conditions for the detection of CF) and at 265–305 nm (detection of Fmoc group) were considered to contain collagen-coated liposomes. Mean diameter of all preparations were within the range 115–135 nm.

2.2.2.2. Radioactively-labeled liposomes. In this case liposomes of PC/Chol (1:1 molar ratio) composition were prepared by hydrating a thin lipid film containing a trace amount of [^{111}In]DTPA-SA with an isotonic solution of PBS (pH 7.4) for 1 h at room temperature. Then, liposomes were incubated overnight with a C-H solution with trace amount of [^{125}I]C-H in a phospholipid:protein weight ratio of 0.4:1. Excess of protein was eliminated by centrifugation for 30 min at $100\,000 \times g$. Thus obtained collagen-coated liposomes were then extruded through two stacked polycarbonate filters of 200 nm pore size. As reference non-coated liposomes were also prepared. The mean diameter, measured by dynamic laser light scattering, was 193 ± 62 nm for non-coated vesicles and 190 ± 32 for collagen-coated liposomes.

2.2.3. In vitro stability studies

Incubations of the different liposomal preparations based on hydrogenated phospholipids, viz., non coated liposomes and liposomes coated with native collagen or C-H or C-$L_{0.4}$, C-$L_{0.2}$, C-$L_{0.1}$ were carried out at 37°C. Samples were taken at different time intervals and fluorescence measured before and after Triton X-100 treatment (λ_{ex}: 490, λ_{em}: 520). Latent CF of the different liposomal preparations expressed as a function of the incubation time, was determined. Furthermore, liposomes used for the in vivo experiments (PC/CHOL, 1:1), namely, uncoated liposomes and liposomes coated with the hexyl derivative of collagen containing CF were incubated at 37°C in the presence of 5 volumes of prewarmed rat plasma so as to simulate dilution in blood after intravenous injection. At time intervals samples were assayed for latent CF and mean vesicle size was determined.

2.2.4. In vivo experiments

2.2.4.1. Administration of fluorescently-labeled liposomes. Female B6D2F mice weighing 20–30 g were injected intravenously with 0.1 ml of the liposomal preparations (lipid composition: HPC/Chol 1:1, molar ratio) that showed stable in the stability studies (dose of 0.2 μmol phospholipid). Five animals were used for each preparation. At time intervals after injection, 25 μl of blood was collected from the tail vein in calibrated heparinized glass micropipettes and rapidly mixed with 1 ml cold PBS. Immediately after, the diluted samples were spun at 1700 $\times g$ for 10 min and supernatants analyzed for CF in the presence of Triton X-100 (1% final concentration). Total CF in the blood was expressed as percent of the injected dose. Plasma concentration of CF, calculated by means of a calibration curve, versus time was individually fit to 1-, 2- or 3-compartment models with first-order elimination

by using least-squares linear regression on a ESTRIP program. According to correlation coefficients and the sum of squares, data were best fit to a two-compartment open model. Pharmacokinetic parameters of the different formulations were statistically compared by means of the non parametric Kruskall Wallis test. Differences were considered significant at $P < 0.05$.

2.2.4.2. Radioactively-labeled liposomes. ^{111}In-labelled non-coated liposomes and liposomes coated with the hexyl derivative of collagen (lipid composition: PC/Chol 1:1, molar ratio) were injected i.v. into female CD-1 mice weighing 20–25 g at a dose of 0.2 and 2 μmol phospholipid. At specified time intervals, mice were anaesthetized, bled by retroorbital puncture, and then sacrificed by cervical dislocation and dissected. Blood and major organs including spleen, liver, lung, heart and kidneys were collected and weighed. Three animals were used per sample and time point. Clearance from circulation and biodistribution of liposomes were determined by analysis of ^{111}In radioactivity in blood and each organ using a Beckman gamma-counter. Liposome levels in the blood were determined by assuming that the blood volume of the mouse is 7.3% of the total body [19].

For intrahepatic distribution studies male Wag/Rij rats (TNO, Rijswijk, The Netherlands), weighing 220–260 g were injected intravenously (penis vein) with 5 μmol of liposomal phospholipid in less than 1 ml of Hepes-buffered NaCl, pH 7.4. In this case liposomes were radiolabeled with a tracer amount of [^{3}H]cholesterol oleylether (Amersham), which was included in the lipid mixture during liposome preparation. 16 h after injection, the animal was anesthetized with diethylether, a blood sample was taken from the tail vein, the spleen was removed and homogenized and the liver was flushed in situ with saline to remove blood. Total uptake of liposomes by liver and spleen was determined by measuring radioactivity in the total liver cell suspension obtained during cell isolation or in the spleen homogenate, respectively. For hepatocyte and macrophage isolation the liver was perfused with collagenase or pronase and parenchymal and non-parenchymal cell fractions were isolated as described by Scherphof and co-workers [20,21]. The number of cells per fraction was determined microscopically and radioactivity of each fraction was determined. From these data the total amounts of radioactivity per cell population, after correction for % recovery, were calculated [20].

3. Results and discussion

3.1. Preparation of collagen-coated liposomes

Among the different methods described as useful for binding proteins or other polymers to liposomes, here we have chosen one of the simplest to incorporate collagenous fragments to liposome bilayers. For this purpose, two hydrophobic anchors, consisting of fatty acid chains of 6 and 12 carbon atoms, have been incorporated to a certain number of the protein primary amino groups. Subsequently, these collagen derivatives have been incubated with preformed small unilamellar vesicles. In collagenous fragments of average molecular weight 50000, about 10 lysines (2.5% of total aminoacids), in addition to the terminal amino groups, are expected per fragment. Therefore, the protein will, presumably, be anchored by several points to the vesicles membrane. Furthermore, as the attachment takes place after formation of the vesicles, the protein is expected to become grafted just in the outer half of the bilayer.

Table 1
Collagen content in the different coated liposomes

	$C_{(0.4)}$	$C\text{-}H_{(0.4)}$	$C\text{-}L_{(0.4)}$	$C\text{-}L_{(0.2)}$	$C\text{-}L_{(0.1)}$
μg/mg PL	0.46	5.75	4.91	1.88	0.76

Collagen, either in native state or derivatized with hexyl or lauryl residues was incubated with preformed unilamellar liposomes (HPC/Chol 1:1 molar ratio) prepared by sonication. Effectiveness of coating was determined by fluorescence techniques (λ_{ex}: 265 nm, λ_{em}: 305 nm) (* mg collagen per mg phospholipid during the incubation with liposomes).

Collagen-coated vesicles were isolated from excess of protein by gel-filtration chromatography. Similar elution diagrams were obtained for liposomes coated with native collagen or the hexyl or lauryl derivative. The content of collagen in the different protein-liposome systems was calculated by fluorescence techniques as described in Section 2. According to Table 1, the efficacy of collagen binding to preformed liposomes was increased about 10-fold by incorporating hydrophobic chains to the protein molecule. Thus, when native collagen was used 0.46 μg of protein were bound per mg phospholipid, while in the same conditions, about 5 μg of C-H and C-L per mg phospholipid remained associated to the vesicles. It is noteworthy that when liposomes composed of unsaturated phospholipids were incubated with the hexyl derivative of collagen a similar amount of protein became attached to the vesicles (4.5 μg/mg phospholipid). Torchilin et al. [22] obtained similar results ($4.7 \cdot 10^{-5}$ mol protein per mol lipid) in the binding of a hydrophobic derivative of α-chymotrypsin to liposomes (initial protein/lipid molar ratio 1:100). Table 1 also shows that although most of the protein did not take part in the coating process, increasing amounts of collagen in the incubation media lead to a higher content of protein in the final liposomal preparation.

3.2. In vitro stability studies of collagen-coated liposomes

The effect that coating the vesicles with native collagen or its hydrophobic derivatives had on their permeability was studied using carboxyfluorescein as a model entrapped solute. When liposomes become leaky, entrapped CF es-

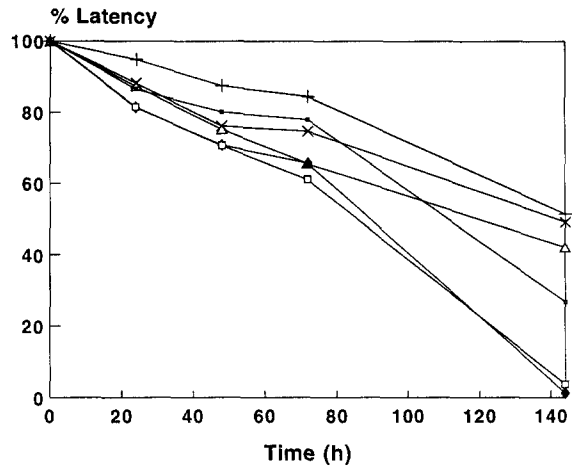

Fig. 1. In vitro stability at 37°C of control (-■-) and liposomes coated with native collagen (+), hexyl collagen (-×-) and C-$L_{0.4}$ (-□-), C-$L_{0.2}$ (-◆-) and C-$L_{0.1}$ (-△-). Lipid composition in all cases was HPC/Chol (1:1 molar ratio).

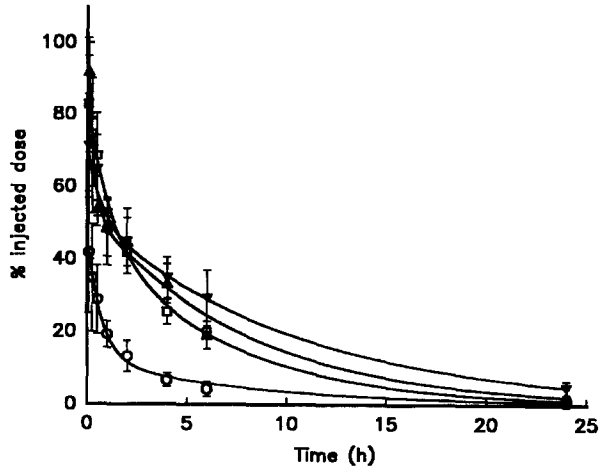

Fig. 2. Comparison of the clearance of collagen-coated and non-coated vesicles from the circulation of mice. SUV (~100 nm) of composition HPC/Chol (1:1 molar ratio) either coated with C (□), C-L (△), C-H (○), or unmodified (▼) were injected intravenously into mice (0.2 μmol phospholipid/25 g mouse). Total blood levels of CF (% dose ± S.D. five animals) based on an assumed blood volume of 7.3% of the total body are shown.

capes into the medium to attain concentrations which allow it to fluoresce, according to its ability to self-quench. In our present work, coated and non-coated liposomes containing quenched CF and composed of HPC/Chol (1:1 molar ratio) were incubated at 37°C and CF latency values after several time intervals were determined (Fig. 1). Lauryl collagen containing liposomes were clearly more unstable than non-coated liposomes, except for those whose protein/phospholipid ratio was lowest.

In a previous work [18] we found that the lauryl derivative of collagen showed at concentrations over 0.2 μM, both a surface activity and interaction with phospholipids (monolayers and bilayers) lower than expected according to its hydrophobicity, thus indicating the presence of highly stable internal aggregates or micelles. In the present experiment the concentration of the protein C-L derivative in the incubation media was in all cases lower than 0.05 μM; therefore, in this highly diluted media, the presence of aggregates can be discarded and it is reasonable that the more hydrophobic derivative (C-L) show at identical concentrations a stronger interaction with liposomes than the hexyl derivative (C-H), and in consequence induce an increased leakage of the entrapped marker.

In addition to liposomes coated with C-$L_{0.1}$, liposomes containing native collagen and C-H also showed a higher stability than those of reference, the former showing latency values twice as high as the latter after 144 h of incubation at 37°C. These results are in agreement with those reported by Pajean and Herbage, who suggested that the presence of collagen decreased liposomes CF leakage at low temperature [23]. Moreover, Sunamoto and coworkers also reported a decrease in the permeability of liposomes after coating their outer surface with some hydrophobic derivatives of polysaccharides [15].

3.3. Vesicle clearance from circulation

Fig. 2 shows that plasma concentration of liposomes coated with native collagen were similar to those observed for non-coated vesicles of the same lipid composition (HPC/Chol 1:1 molar ratio) and characteristics (size about 100 nm; dose of 0.2 μmol phospholipid). This may indicate that the protein is so weakly bound to the vesicles, that in contact with the biological fluids becomes detached.

Table 2
Effect of the different collagen coatings in the pharmacokinetic variables of liposomes

Variables	Collagen used for coating			
	ϕ	C	C-H	C-L
$t_{1/2\alpha}$(h)	0.458 (0.39)	0.784 (0.42)	0.818 (0.31)	0.374 (0.20)
$t_{1/2\beta}$(h)	7.99 (1.05)	7.42 (1.02)	9.46 (1.75)	6.97 (1.44)
K_{el} (h^{-1})	0.133 (0.01)	0.201 (0.03)	0.304 * (0.07)	0.220 * (0.01)
Cl (ml/min kg)	0.218 (0.06)	0.294 (0.02)	0.940 * (0.26)	0.309 (0.11)
V_D (l/kg)	0.097 (0.02)	0.087 (0.01)	0.194 * (0.07)	0.084 (0.03)

Liposomes containing CF (0.2 μmol phospholipid/mouse) with defined size and composed of HPC/Chol (1:1 molar ratio) were i.v. injected into mice. Blood samples (25 μl) were collected at different time intervals after injection and latent CF was determined (λ_{ex}: 490 nm and λ_{em}: 520 nm). Data represent average values (S.D.) of five mice.

Another possible explanation for this fact is that the amount of native collagen attained in the liposome surface (0.46 μg/mg phospholipid) is not enough to exert any type of effect on the in vivo behavior of the vesicles.

Liposomes coated with C-L also lead to plasma concentrations similar to those of the control liposomes. However, the curve corresponding to liposomes coated with C-H was remarkably different. In that case, a rapid decrease in the plasma levels was observed during the first minutes. Thus, 30 min after injection just 30% of the injected dose could be found in the blood, while at the same time, 60% of the injected control liposomes remained in circulation. We consider that the differences observed between the preparations containing the two hydrophobic derivatives are due to the different protein concentration in the liposome surface, 5.75 μg/mg phospholipid in the case of C-H and just 0.76 in C-L containing liposomes. A higher concentration of C-L was not used in the in vivo experiments, since it could induce to a destabilization of the vesicles, as observed in the in vitro stability studies.

Pharmacokinetic parameters of these data are given in Table 2. The elimination half-life of all preparations were not significantly different ($P > 0.05$). However, differences on other parameters also related to the clearance from circulation, such as the elimination rate constant (K_{el}) or the total systemic clearance (Cl) were significant ($P < 0.05$) between liposomes coated with C-H and the other preparations assayed, the higher values corresponding to the former. Hexyl-collagen coated liposomes also showed a greater volume of distribution, thus indicating that this kind of liposomes distribute in a greater extent to tissues.

Circulation time of liposomes composed of unsaturated phospholipids (PC/Chol, 1:1 molar ratio), either coated with hexyl-collagen or unmodified, were also studied. In that case, when the same dose as before was injected (0.2 μmol phospholipid), no significant differences were observed between the two preparations (Fig. 3), elimination half-lives being 0.91 ± 0.21 and 0.96 ± 0.27, respectively. However, when the dose of injection was increased 10-fold, again liposomes coated with C-H were more rapidly cleared from the blood than those of reference.

The results above described also give information about other parameters affecting circulation time of liposomes. In complete agreement with bibliographic data [24–26], our results indicate that liposomes remain longer in the blood when a higher dose of lipid is injected (Fig. 3). Most authors agree that this dose-dependence is a consequence of the saturation of the mononuclear phagocytic system, responsible for the uptake of most of circulating liposomes. This saturation of the MPS, especially Kupffer cells located in the liver, is evidenced in our own results by an increase in the vesicles plasma levels and a decrease in the percentage that accumulates in liver as will be

Table 3
Effect of the collagen coating in the distribution of liposomes

(A)	% Injected dose											
Time (h)	uncoated vesicles						vesicles coated with hexyl-collagen					
	blood	spleen	liver	lung	heart	kidneys	blood	spleen	liver	lung	heart	kidneys
0.08	62.36	2.38	16.71	2.09	1.70	2.72	63.09	3.36	32.78	1.52	1.05	1.84
	(6.07)	(0.19)	(0.58)	(0.41)	(0.34)	(0.52)	(5.24)	(0.30)	(6.55)	(0.21)	(0.52)	(0.36)
0.5	44.78	6.00	24.43	1.75	1.26	1.80	32.11	8.76	45.88	1.28	0.56	1.47
	(3.72)	(0.60)	(1.92)	(0.41)	(0.46)	(0.34)	(0.28)	(3.38)	(5.58)	(0.34)	(0.13)	(0.23)
1	34.99	8.25	29.25	1.27	1.09	1.67	14.65	10.98	50.22	0.57	0.27	0.95
	(1.63)	(0.52)	(3.48)	(0.27)	(0.07)	(0.17)	(5.78)	(1.75)	(4.45)	(0.23)	(0.07)	(0.35)
6	7.66	8.31	51.86	0.52	0.18	1.06	3.79	12.72	64.35	0.30	0.14	0.75
	(0.88)	(1.79)	(1.42)	(0.06)	(0.05)	(0.06)	(0.68)	(1.64)	(5.58)	(0.07)	(0.03)	(0.05)
(B)	% Injected dose											
Time (h)	uncoated vesicles						vesicles coated with hexyl-collagen					
	blood	spleen	liver	lung	heart	kidneys	blood	spleen	liver	lung	heart	kidneys
0.08	45.12	3.17	26.34	1.28	1.13	1.89	34.60	7.18	51.75	1.43	0.89	1.10
	(0.81)	(0.31)	(2.43)	(0.13)	(0.43)	0.05)	(1.59)	(1.93)	(4.31)	(0.17)	(0.51)	(0.09)
0.25	27.35	9.99	40.95	0.95	0.47	1.29	16.24	10.09	65.73	0.85	0.27	0.81
	(0.29)	(1.23)	(0.08)	(0.13)	(0.11)	(0.17)	(3.40)	(0.46)	(4.23)	(0.25)	(0.06)	(0.27)
0.5	19.33	10.72	41.06	0.70	0.49	1.11	12.93	14.75	65.37	0.80	0.23	0.67
	(4.58)	(3.44)	(4.98)	(0.27)	(0.12)	(0.48)	(4.73)	(3.22)	(4.24)	(0.31)	(0.09)	(0.31)
1	12.97	14.01	39.14	0.55	0.38	0.74	3.71	14.15	68.42	0.46	0.11	0.36
	(3.46)	(1.14)	(2.07)	(0.13)	(0.09)	(0.20)	(0.67)	(4.08)	(9.74)	(0.26)	(0.02)	(0.12)
3	2.97	9.99	48.02	0.24	0.12	0.43	1.58	12.76	77.73	0.25	0.06	0.27
	(1.40)	(2.56)	(2.74)	(0.19)	(0.05)	(0.08)	(0.65)	(3.81)	(4.11)	(0.05)	(0.02)	(0.07)

^{111}In-labeled liposomes with defined size and composed of PC/Chol liposomes were i.v. injected into mice. Phospholipid injected dose was 2 (A) or 0.2 (B) μmol. At different time points after injection mice were killed and the ^{111}In radioactivity in each organ was counted.

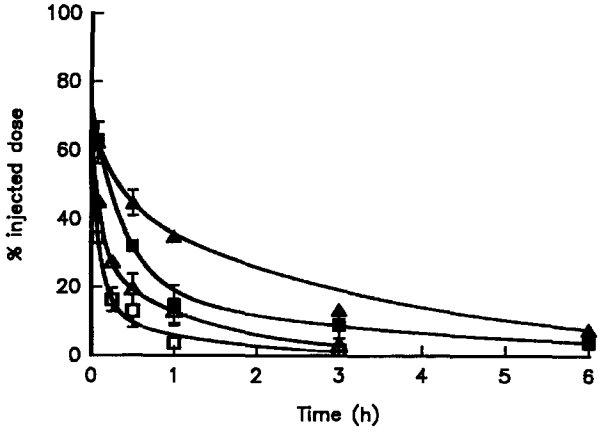

Fig. 3. Clearance from circulation of C-H coated (□) and control liposomes (△) (lipid composition: PC/Chol 1:1, molar ratio) after the injection of 2 (filled symbols) or 0.2 (hollow symbols) μmol phospholipid. Values are percentage ± S.D. of injected dose.

detailed in next section. This work also shows that liposomes of similar size and identical dose of injection remained longer in the blood when saturated phospholipids are used in the composition of liposomes. Thus, a half-life of 7 h was obtained for liposomes composed of HPC/Chol compared to about 1 h for PC/Chol liposomes. This fact has also been well-established by other authors [5,27–29].

3.4. Tissue distribution of collagen-coated vesicles

We then examined whether collagen attachment to liposomes affects their tissue distribution. Although carboxyfluorescein has proved to be a good marker for vesicle clearance [30], it is not appropriate to measure its distribution in tissues. For such a purpose, ^{111}In has often been used, either associated to an aqueous solute [30] or to a lipidic component of liposomes [31]. This marker has the advantages that is retained by tissues for several days, is unable to reach tissues significantly by itself, and can be conveniently measured in a gamma counter. For this reason, in this study ^{111}In has been used as a marker of the lipid phase of liposomes. Liposomes composed of PC/Chol (1:1 molar ratio) either coated with the hexyl derivative of collagen or uncoated were prepared by the extrusion method, since sonicated liposomes have been reported to be not homogenous enough for this kind of experiments [32]. These formulations were injected i.v. at a dose of 0.2 and 2 μmol phospholipid and the biodistribution was examined at different time intervals. A comparison of conventional liposomes or liposomes coated with C-H are given in Table 3A and B.

For both doses assayed, collagen coated liposomes had substantially higher levels in liver than control liposomes at all time points. On the other hand, in both cases the accumulation of liposomes in liver was lower when the higher dose of phospholipid was injected, probably due – as explained before – to the saturation of MPS. Thus, 1 h after injection of 2 and 0.2 μmol phospholipid of control liposomes, 29% and 39% of the injected dose was recovered in this organ, respectively. Likewise, 50% and 68% was found after administration of the same doses of liposomes coated with collagen.

The higher levels observed in liver for the collagen-coated vesicles, were accompanied by lower concentrations in blood and other organs, such as heart, lung and kidneys. With regard to the concentrations observed in spleen, no significant differences were detected between coated and non-coated vesicles.

To be sure that the higher accumulation in liver observed for collagen coated vesicles was not due merely to an increase in size of those liposomes in circulation, their stability in the presence of plasma was studied. For that purpose, liposomes with the same composition and size as those used for the biodistribution studies were prepared containing CF as an entrapped marker and were incubated at 37°C in the presence of rat plasma for 24 h. At different time intervals, CF latency values and distribution of vesicles size were determined. Almost no leakage was observed in any of the assayed preparations. After 24 h of incubation CF latency values of 94.2% ± 0.13 and 96.7% ± 2.01 were found for uncoated and C-H coated vesicles, respectively. On the other hand, although the size distribution was rather broad, the mean vesicle size of both preparations did not increase during the time of the experiment. Moreover, a slight tendency to a decrease in vesicles diameter was observed for both preparations.

There are other examples in the literature in which certain liposome formulations, such as liposomes composed of PS [14] or coated with *O*-palmitoylamylopectin or *O*-palmitoylpullulan [15], are rapidly removed from circulation and accumulate in liver and alveolar macrophages respectively. In the case of collagen-coated liposomes, the rapid removal from the blood vessels might be due to an opsonic effect, either directly induced by collagen or by some opsonic protein such as fibronectin. This glycoprotein can be found in extracellular matrices, on cell surfaces or circulating in blood [33] and it has been postulated to have opsonic effect on liposomes after i.v. administration [34]. On the other hand, it is known that collagen presents some binding sites for fibronectin and that the binding of the two proteins is the first step on the cell adhesion process [35]. Therefore, it is possible that circulating fibronectin binds to the collagen covering the vesicles, thus facilitating the uptake and phagocytosis by macrophages, mainly located in liver. In fact, Wolff et al. [36] demonstrated that fibronectin stimulated macrophages to engulf gelatin-coated erythrocytes.

On the other hand, the liposomes used in this study were not expected to be extensively taken up by hepatocytes, since their mean size (~ 200 nm) was substantially higher than the average diameter of the fenestrations in the liver sinusoidal endothelium (~ 100 nm). Nevertheless, to discard this possibility some preliminary experiments with

a limited number of animals were done to investigate the intrahepatic biodistribution in rats of collagen-coated liposomes. Supporting our first impression, 16 h after administration almost the total dose recovered in liver was found in Kupffer cells. Currently, further studies are being undertaken on the intrahepatic distribution of smaller collagen-coated liposomes, which have a higher opportunity to end up in hepatocytes.

In summary, it has been demonstrated that the coating of liposome external surface with collagen (average M_r 50 000) involves both, a higher stability in vitro and, in spite of the hydrophilic layer, a selective and almost immediate accumulation of the vesicles in liver Kupffer cells. Such a formulation might be, therefore, most useful in the treatment of some infectious diseases located within macrophages, as was demonstrated for PS containing liposomes against intracellular leishmanias [37]. Because of their intracellular location, such infections causes serious problems to the conventional antimicrobial therapy. Collagen-coated liposomes look promising to overcome most problems associated to parasitic diseases to MPS as well as for drug-mediated activation of macrophages into tumoricidal, microbicidal or virucidal state.

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