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Effect of collagen on liposome permeability

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Summary

If liposomes are to be used as drug carriers, the encapsulating agents must not leak during storage. We showed previously that dispersion of liposomes (100% lecithin) in a collagen solution improves their chemical stability and decreases their permeability. This protective effect of collagen was studied further in a series of experiments: firstly, we tested the stability of various liposomes in the absence of collagen. When cholesterol or α-tocopherol was present in the bilayers of liposomes, both lipid peroxidation and liposome permeability decreased. Peroxidation was responsible for 25% of the release of carboxyfluorescein, under our experimental conditions (20°C, liposomes suspended in 300 mOsm/kg buffer); the release of the remaining 75% was probably due to simple passive diffusion of the molecules from the interior of the liposomes into the external media. Secondly, we examined the stability of liposomes in the presence of collagen. In liposomes containing 10 mol% of α-tocopherol, peroxidation was inhibited and no antioxidant effect of collagen was seen; however, permeability was decreased by 20% in presence of collagen. In liposomes containing 30 mol% cholesterol, the antioxidant effect of collagen and its effect on permeability were similar to that in liposomes consisting of 100% lecithin. Furthermore, we studied the effect of surface charge. In neutral and positively and negatively charged liposomes, the antioxidant effect of collagen was similar; however, the stabilizing effect of collagen on permeability was completely suppressed in positively charged vesicles but was 2-fold greater in negatively charged than in neutral liposomes. The antioxidant effect of collagen thus led to only 20% decrease in liposome permeability, and 80% of the decrease was due to another mechanism, which may involve the electrostatic forces between collagen and phospholipids. The interaction between collagen and liposomes may thus involve the positive charges of this protein.

Introduction

The use of liposomes as drug delivery systems is the subject of world-wide pharmaceutical research, particularly in relation to chemotherapy for cancer (Lopez-Berestein and Fidler, 1989; Treat et al., 1990) and fungal infections (Meunier et al., 1988; Collette et al., 1989). Liposomes are biocompatible systems for carrying various drugs, either in their internal aqueous space, in the lipid bilayers, or bound to the surface (Ostro, 1987). But certain difficulties have been encountered, in reproducibly preparing liposomes of defined size, and in maintaining their stability with respect to retention of entrapped solute(s) and their properties in vivo. With the development of extrusion

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techniques to narrow size distribution (Olson et al., 1979), liposomes of defined sizes can now be prepared reproducibly; however, instability due to both phospholipid oxidation and vesicle permeability remains a problem. Most of the phospholipid liposomal dispersions used contain unsaturated acyl chains as part of the molecular structure, and these chains are vulnerable to oxidative degradation (lipid peroxidation). The autoxidation of lipids is accelerated by metal ions, light (and other forms of radiation), some organic molecules and high pH (Kunimoto et al., 1981; Bose et al., 1989); however, autoxidation can be inhibited by metal chelators (EDTA) and antioxidants such as α-tocopherol and butylated hydroxytoluene (Holmann, 1954). With respect to liposome permeability several methods have been used to limit leakage of drugs from liposomes, including modification of membrane structure, using different lipid components or composition (Senior and Gregoriadis, 1984), and storage of liposomes in a freeze-dried state in the presence of cryoprotective agents (Ozer et al., 1988; Harrigan et al., 1990).

Although there is much evidence for an interaction between collagen and liposomes (Cohen and Barenholz, 1984; Weiner et al., 1985; Martinez del Pozo et al., 1988, 1989), the effects of collagen molecules on the stability of liposomes have not been studied. We described previously a dose-dependent decrease in both liposome permeability and lipid oxidation in the presence of collagen (Pajean et al., 1991). A parallel study with albumin and γ-globulin showed that the antioxidant effect of collagen cannot explain the decrease in liposome permeability entirely, and collagen molecules would appear to have an additional, specific stabilizing effect on vesicle permeability. The aim of the present study was to determine the effects on this property of collagen as a function of the lipid composition (cholesterol, α-tocopherol) and surface charge of liposomes.

Materials and Methods

Egg-yolk lecithin (> 90% phosphate) was obtained from Merck (Nogent-sur-Marne, France), and Nonidet P40, 5(6)-carboxyfluorescein (CF; approx. 99% pure by high-performance liquid chromatography: Ref. C-7153), stearylamine (approx. 90% pure: Ref. S-6755), L-α-phosphatidic acid (approx. 98% pure: Ref. P-9511), cholesterol (Ref. CH-K), and D-α-tocopherol (98–99.5% pure: Ref. T-3634) were purchased from Sigma (La Verpillère, France). Purified bovine type I collagen, with telopeptides removed chemically (Atelocollagen), was a gift from Coletica (France).

Collagen characterization

Atelocollagen was characterized by high-angle X-ray diffraction, amino acid composition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and rotary shadowing, as described previously (Ricard-Blum et al., 1985).

Preparation and characterization of liposomes

All lipids were conserved in solid form. As egg-yolk lecithins peroxidize with time, two batches of lecithin were used. The required amount of egg lecithin, with stearylamine, phosphatidic acid, cholesterol or α-tocopherol if required, was weighed into a flat-bottom flask and dissolved in a small volume of chloroform : methanol (9 : 1) solution. The solvent was removed under nitrogen, and the dried film was suspended in Tris-HCl buffer (10 mM) containing 5 mM CF, pH 7.4. The subsequent phospholipid concentration was about 4% (assayed by the method of Marshall-Stewart, 1980). Multilamellar liposomes were formed by mixing the suspension with a magnetic stirrer, followed by homogenization with an Ultra Turrax until a homogeneous suspension was obtained.

Liposomes can be separated from free CF by the mini-column centrifugation technique (Fry et al., 1978). Excess fluid was first removed from the Sephadex G-50 beads by centrifugation, and a mixture of liposomal entrapped and free CF was applied to the column bed. Centrifugation was repeated to force the liposomal material through the column into a test tube, while free CF was retained quantitatively in the Sephadex. Numerous samples can be processed simultaneously within minutes with no dilution of the liposomal preparation (lipid concentration, 3–4%).

Liposomes were characterized by electron microscopy using negative staining, as described previously (Pajean et al., 1991).

Leakage characterization

Purified liposomes were diluted in Tris-HCl buffer, 150 mM NaCl (300 mOsm/kg) or the same buffer containing purified type I collagen to obtain a final lipid concentration of 0.4%. These solutions were kept at 20°C, and release of CF from liposomes was recorded continuously for 1 week.

Lipid peroxidation

Peroxidation studies was studied in parallel with leakage characterization on the same liposome suspension using identical experimental conditions of storage, buffer, temperature and lipid concentration. The extent of lipid peroxidation was estimated by the 2-thiobarbituric acid method (Buege and Aust, 1978) and expressed as equivalents of malondialdehyde MDA per mg egg lecithin. A standard curve was drawn using tetraethoxypropane, a precursor of malondialdehyde.

We verified that CF molecules did not interfere with the thiobarbituric acid-malondialdehyde complex.

Results and Discussion

Egg-yolk lecithin contains an appreciable amount of unsaturated phospholipids (approx. 35% oleic acid and 20% linoleic acid), which may undergo oxidation. In the presence of collagen, dose-dependent decreases in both liposome permeability and lipid peroxidation were observed. The antioxidant effect of collagen cannot, however, explain entirely its stabilizing potential on liposome permeability, and, collagen molecules appear to have an additional, specific stabilizing effect (Pajean et al., 1991).

Incorporation of 10 mol% α-tocopherol into liposomes

α-Tocopherol (vitamin E) is known to be a potent anti-oxidant, protecting biological molecules and tissues from the toxicity of oxygen (Machlin, 1980). It has been assumed that the stabilizing effect of tocopherols is mediated via two main molecular mechanisms: (i) reaction with lipid peroxide radicals and quenching of singlet molecular oxygen (Fukuzawa et al., 1985); and (ii) ordering (i.e., restricting molecular mobility) the lipid bilayer. Erin et al. (1985) and Urano et al. (1990) demonstrated the existence of a hydrogen bond between the hydroxyl group of α-tocopherol and a carbonyl group of a fatty acid ester in the phospholipids of the liposome bilayer.

When we incorporated α-tocopherol into bilayers (Fig. 1A), the peroxidation of phospholipids was inhibited almost entirely. The permeability of the liposomes was reduced by 25% when α-tocopherol was incorporated into bilayers (Fig. 1B): 80% of CF was released from liposomes with 10 mol% α-tocopherol within 300 h, whereas 100% of the CF was released from liposomes consisting of 100% lecithin only 100 h after the beginning of the experiment.

These results suggest that peroxidation was responsible for 25% of the CF release under our experimental conditions (20°C, liposomes suspended in 300 mOsm/kg buffer). The release of the remainder was probably due to simple passive diffusion of CF from the interior of liposomes to the outside. Hunt and Tsang (1981) characterized the autoxidation process of phospholipids by an induction period of unpredictable duration followed by a rapid, autocatalytic oxidation phase, which in turn is followed by an increasing degree of liposome permeability. They observed that addition of 0.1 mol% α-tocopherol doubled the induction period, and the release of test molecules (sucrose) from the liposomes was entirely suppressed.

The antioxidant effect of collagen (0.8%) was completely suppressed (Fig. 1A) in liposomes containing α-tocopherol, since peroxidation was inhibited. The decreased permeability of liposomes containing 10 mol% tocopherol, was maintened in the presence of collagen: the permeability of 100% lecithin liposomes decreased by 38% and that of liposomes containing tocopherol by 30% (Fig. 1B).

At 20°C, the antioxidant effect of collagen was thus responsible for 20% of the decrease in per-

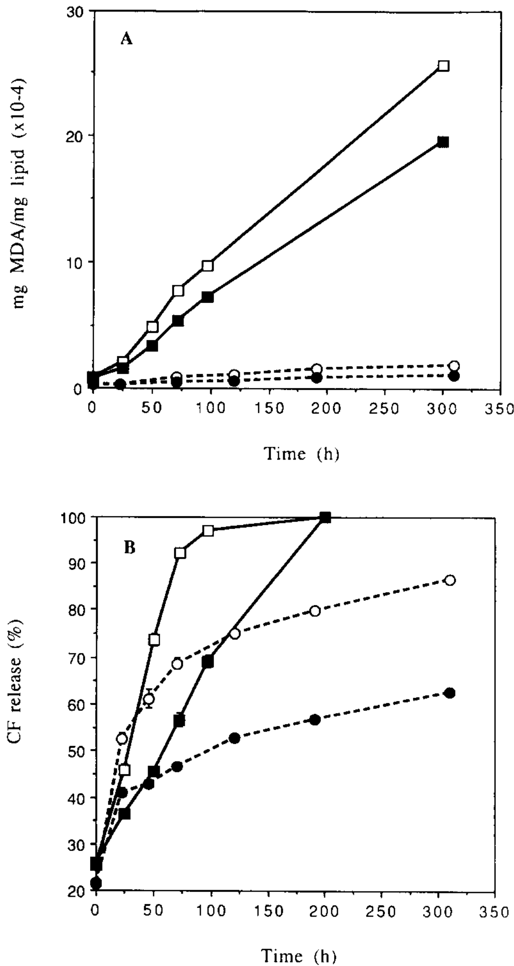

Fig. 1. Effect of α-tocopherol (incorporated at 10 mol% into liposomal membranes) on liposome peroxidation (A) and permeability (B) in the presence and absence of collagen. Lipid and protein concentrations were 0.4 and 0.8%, respectively. Each point is the mean ($\pm$SE) of three determinations. Liposome consisting of 100% lecithin (lot 1) (□); liposome consisting of 100% lecithin (lot 1)+collagen (■); liposome with 10 mol% tocopherol (○); liposome with 10 mol% tocopherol+collagen (●); CF, 5(6)-carboxyfluorescein; MDA, malondialdehyde.

meability of liposomes, and the other 80% must be due to another effect of collagen.

Incorporation of 30 mol% cholesterol into liposomes

Cholesterol is an important component of mammalian membranes, and its incorporation into liposome bilayers can cause major changes in their properties. Cholesterol does not itself form bilayer structures, but it can be incorporated into phospholipid membranes at very high concentrations (up to 1:1 or even 2:1 molar ratios of cholesterol to phospholipid). As it is amphipathic, cholesterol is inserted into the membrane with its hydroxyl group oriented towards the aqueous surface and the aliphatic chain aligned parallel to the acyl chains in the centre of the bilayer (Ladbrooke et al., 1968; New, 1990).

Liposome stability was significantly enhanced when cholesterol was incorporated into bilayers: oxidation of phospholipids was reduced by 20% (Fig. 2A); the rate of release of CF from liposomes with cholesterol was 52% less than from 100% lecithin liposomes at 53 h and 14% less at 98 h after the beginning of experiment (Fig. 2B). These results corroborate those of Nakazawa et al. (1990) and Hernandez-Caselles et al. (1990).

Ladbrooke et al. (1968) showed that when bilayers are in a fluid crystal state at a temperature above the phase-transition temperature of phospholipids, the presence of the rigid steroid nucleus of cholesterol alongside the carbons of the phospholipid chain has the effect of reducing the freedom of motion of these carbons. Furthermore, cholesterol is known to form a weak complex with phospholipids in the bilayer phase via H-bonding with the carbonyl oxygen of neighbouring phospholipids (Liu and Huang, 1989). The limited freedom of acyl chains causes the membranes to condense, with a reduction in area, closer packing and decreased fluidity (Takahashi et al., 1988). In our experiment, these changes in fluidity were paralleled by changes in the permeability of the membrane, and incorporation of cholesterol at 20°C into fluid crystal bilayers (membranes made from egg lecithin have a transition temperature of -15 to -7°C) decreased liposome permeability.

As oxidation of the fatty acids of phospholipids is accelerated by the propagation of free radicals (hydroxyl and superoxide radicals) in bilayers, the rigidification of membranes due to incorporation of cholesterol into liposome bilayers could lead to decreased propagation of free radicals and so diminish the peroxidation level. Contradictory re-

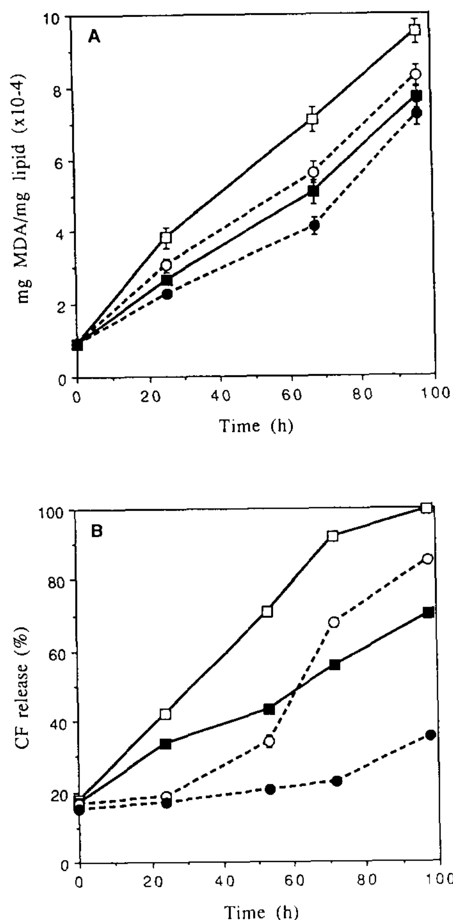

Fig. 2. Effect of cholesterol (incorporated at 30 mol% into liposomal membranes) on liposome peroxidation (A) and permeability (B) in the presence and absence of collagen. Lipid and protein concentrations were 0.4 and 0.8%, respectively. Each point is the mean (±SE) of three determinations. Liposome consisting of 100% lecithin (lot 1) (□); liposome consisting of 100% lecithin (lot 1)+collagen (■); liposome with 30 mol% cholesterol (○); liposome with 30 mol% cholesterol+collagen (●); CF, 5(6)-carboxyfluorescein; MDA, malondialdehyde.

sults have been reported from studies of liposome bilayers in the fluid liquid crystal state. Kunimoto et al. (1981) found no significant effect of cholesterol on the sensitivity of liposomes to peroxidation induced by ferrous ion and ascorbate. Nakazawa et al. (1990) found significant enhancement of lipid peroxidation induced by ^{60}Co gamma irradiation when cholesterol at 1–10 mol% was incorporated into bilayers. Montfoort et al. (1987) reported a concentration-dependent reduction effect of cholesterol in inhibiting peroxidation; low cholesterol : lecithin ratios were more effective: the greatest inhibition was found at a molar ratio of 0.25 and virtually no effect was found at molar ratios near 1.0.

Collagen had similar antioxidant properties whether cholesterol was incorporated into bilayers or not (Fig. 2A). The stabilizing effect of collagen on the permeability of liposomes enhanced that of cholesterol (Fig. 2B). The mechanism by which collagen stabilizes liposomes, other than antioxidant activity, is thus conserved, whether the bilayers are in a fluid crystal or solid state.

Incorporation of charged lipids into liposomes

Two charged lipids were incorporated into bilayers: phosphatidic acid charges the membrane negatively and stearylamine positively. After neutral and charged liposomes had been purified by chromatography, they encapsulated 20.5% (neutral), 24.1% (negatively charged) and 64.7% (positively charged) of the total CF used to make the liposomes. As demonstrated previously before (Yatvin and Lelkes, 1982), the presence of charged lipids in the bilayers caused adjacent lamellae to repel each other and so to increase the encapsulation efficiency of the liposomes. The increased encapsulation of CF by positively charged liposomes was probably due to the negative charges of CF at pH 7.4, the electrostatic interaction between drug and membrane resulting in tight binding to the liposome, increasing the concentration of encapsulated CF.

The rate of release of CF from liposomes with stearylamine was 22% less than that from liposomes consisting of 100% lecithin (electrostatic interaction between membranes and CF, leading to reduce the CF leakage) (Fig. 3B), but 8% more CF was released from liposomes with phosphatidic acid (electrostatic repulsion) (Fig. 4B).

We observed a decrease in peroxidation relative to that of liposomes consisting of 100% lecithin of 46% for negatively charged and 60% for positively charged liposomes (Figs 3A and 4A). Kunimoto et al. (1981) reported that lipid oxidation occurred independently of the charge

of liposomes when peroxidation was brought about by sonication, but when peroxidation of the liposomal membrane was induced by exogenous ferrous ion and ascorbate, neutral and negatively charged liposomes were sensitive to peroxidation, whereas positively charged liposomes were not. They considered that the different sensitivities of these liposomes depend either on electrostatic

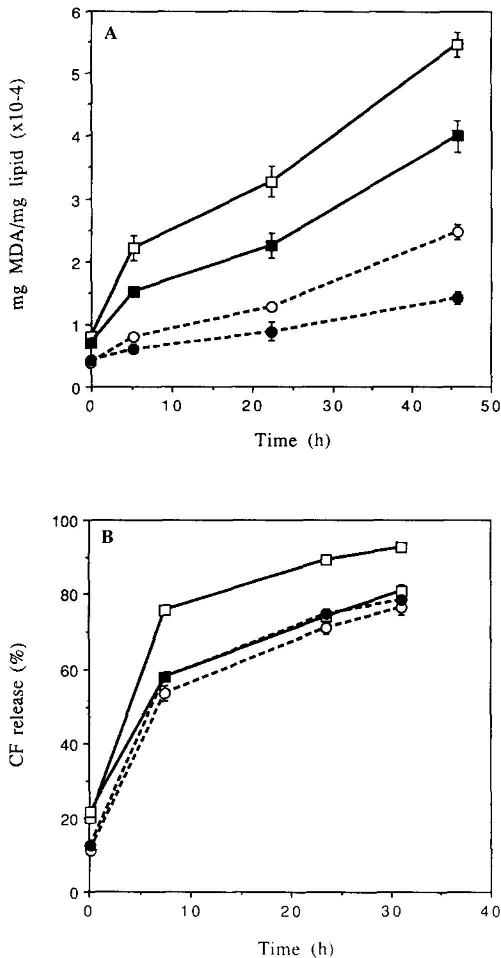

Fig. 3. Effect of positively charged phospholipids (incorporated at 20 mol% into liposomal membranes) on liposome peroxidation (A) and permeability (B) in the presence and absence of collagen. Lipid and protein concentrations were 0.4 and 0.8%, respectively. Each point is the mean (±SE) of three determinations. Liposome consisting of 100% lecithin (lot 2) (□); liposome consisting of 100% lecithin (lot 2)+ collagen (■); liposome with 20 mol% stearylamine (○); liposome with 20 mol% stearylamine+collagen (●); CF, 5(6)-carboxyfluorescein; MDA, malondialdehyde.

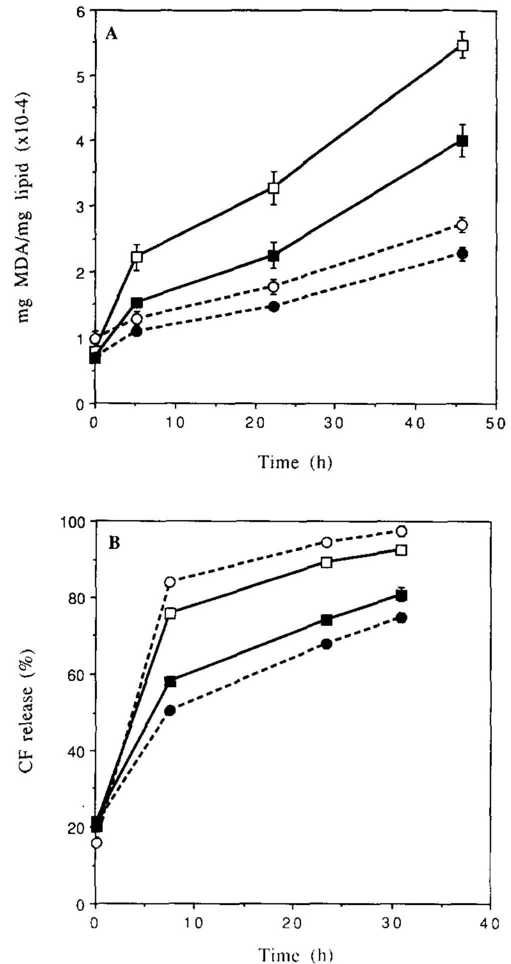

Fig. 4. Effect of negatively charged phospholipids (incorporated at 20 mol% into liposomal membranes) on liposome peroxidation (A) and permeability (B) in the presence and absence of collagen. Lipid and protein concentrations were 0.4 and 0.8%, respectively. Each point is the mean (±SE) of three determinations. Liposome consisting of 100% lecithin (lot 2) (□); liposome consisting of 100% lecithin (lot 2)+ collagen (■); liposome with 20 mol% phosphatidic acid (○); liposome with 20 mol% phosphatidic acid+collagen (●); CF, 5(6)-carboxyfluorescein; MDA, malondialdehyde.

binding or on differential accessibility of ferrous ions to the membrane surface.

The antioxidant effect of collagen was similar in liposomes containing 100% lecithin and 20 mol% stearylamine (Fig. 3A) but was reduced by 43% in negatively charged liposomes (Fig. 4A). The stabilizing effect of collagen on permeability,

however was completely suppressed in positively charged liposomes (Fig. 3B), but was 2-fold greater in negatively charged than in liposomes 100% lecithin (Fig. 4B).

In these experiments with charged liposomes, we could dissociate the antioxidant properties of collagen from its effect on permeability: its stabilizing effect is independent of its nonspecific (Pajean et al., 1991) antioxidant property and involves the electrostatic forces between collagen and phospholipid. Cohen and Barenholz (1984) demonstrated an interaction involving hydrogen bonding between the hydroxyproline and/or hydroxylysine residues of collagen like tail acetylcholinesterase and the interface region of the sphingolipid molecule. Martinez del Pozo et al. (1988) observed an interaction (simple surface binding) at pH 2.8 between the triple helix of type I collagen and phosphatidylcholine liposomes, resulting in relative immobilization of the phospholipid molecules around the protein and a consequent decrease in their fluidity. Martinez del Pozo et al. (1989) also demonstrated the involvement of the positive charges of the lysine residues of collagen (organized in fibrils) in protein-lipid (dimyristoylphosphatidylcholine) interactions at neutral pH. They found a greater protein-lipid interaction in dimyristoylphosphatidylglycerol liposomes (negatively charged) than in dimyristoylphosphatidylcholine (neutral).

In our experiments, the positive charges of collagen were involved in the protein-liposome interaction. When the bilayers were negatively charged, electrostatic interaction between collagen and liposomes led to a decrease in liposome permeability; when they were positively charged, electrostatic repulsion between proteins and liposomes led to an increase in permeability.

In conclusion, we found an improvement in the stability of liposomes (both peroxidation and permeability) in vitro when α-tocopherol and cholesterol were incorporated into bilayers. Type I collagen behaves like a peripheral protein in its interaction with phosphatidylcholine vesicles; it modified the environment of phospholipids, leading to a decrease in liposome permeability. In negatively charged liposomes, it decreased permeability, even further.

Although the interaction of collagen with specific cellular receptors could occur through another mechanism, our results show that collagen interacts with phospholipid vesicles in the absence of any other component.

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References

Bose, B., Agarwal, S. and Chatterjee, S.N., UV-A induced peroxidation in liposomal membrane. *Radiat. Environ. Biophys.*, 28 (1989) 59–65.

Buege, J.A. and Aust, S.D., Microsomal lipid peroxidation. *Methods Enzymol.*, 52 (1978) 302–310.

Cohen, R. and Barenholz, Y., Characterization of the association of electrophorus electricus acetylcholinesterase with sphingomyelin liposomes. Relevance to collagen-sphingomyelin interactions. *Biochim. Biophys. Acta*, 778 (1984) 94–104.

Collette, N., Van der Auwera, P., Pascual-Lopez, A., Heymans, C. and Meunier, F., Tissue concentrations and bioactivity of amphotericin B in cancer patients treated with amphotericin B-deoxycholate. *Antimicrob. Agents Chemother.*, 33 (1989) 362–368.

Crommelin, D.J.A. and Van Bommel, E.M.G., Stability of liposomes on storage: freeze dried, frozen or as an aqueous dispersion. *Pharm. Res.*, 1 (1984) 158–162.

Erin, A.N., Skrypin, V.V. and Kagan, V.E., Formation of α-tocopherol complexes with fatty acids. Nature of complexes. *Biochem. Biophys. Res. Commun.*, 815 (1985) 209–214.

Fry, D.W., Courtland White, J. and Goldman, J.D., Rapid separation of low molecular weight solutes from liposomes without dilution. *Anal. Biochem.*, 90 (1978) 809–815.

Fukuzawa, K., Takase, S. and Tsukatani, H., The effect of concentration on the antioxidant effectiveness of α-tocopherol in lipid peroxidation induced by superoxide free radical. *Arch. Biochem. Biophys.*, 240 (1985) 117–120.

Harrigan, P.R., Madden, T.D. and Cullis, P.R., Protection of liposomes during dehydration or freezing. *Chem. Phys. Lipids*, 52 (1990) 139–149.

Hernandez-Caselles, T., Villalain, J. and Gomez-Fernandez, J.C., Stability of liposomes on long term storage. *J. Pharm. Pharmacol.*, 42 (1990) 397–400.

Holmann, R.T., Autoxidation of fats and related substances. In Holmann, R.T., Ludberg, W.O. and Molkin, T. (Eds),

Progress in the Chemistry of Fats and Other Lipids, Vol. 2, Pergamon, London, 1954, pp. 51–98.

Hunt, C.A. and Tsang, S., α Tocopherol retards autoxidation and prolongs the shelf life of liposomes. Int. J. Pharm., 8 (1981) 101–110.

Kunimoto, M., Inoue, K. and Nojima, S., Effect of ferrous ion and ascorbate-induced lipid peroxidation on liposomal membranes. *Biochim. Biophys. Acta*, 646 (1981) 169–178.

Ladbrooke, B.D., Williams, R.M. and Chapman, D., Studies on lecithin-cholesterol-water interactions by differential scanning calorimetry and X-ray diffraction. *Biochem. Biophys. Acta*, 150 (1968) 333–340.

Liu, D. and Huang, L., Role of cholesterol in the stability of pH-sensitive, large unilamellar liposomes prepared by the detergent-dialysis method. *Biochem. Biophys. Acta*, 981 (1989) 254–260.

Lopez-Berestein, G. and Fidler, I.J., *Liposomes in the Therapy of Infectious Diseases and Cancer*, Liss, New York, 1989.

Machlin, L.J., *Vitamin E: A Comprehensive Treatise*, Vol. 1, Dekker, New York, 1980.

Marshall-Stewart, J.C., Calorimetric determination of phospholipids with ammonium ferrothiocyanate. *Anal. Biochem.*, 104 (1980) 10–14.

Martinez del Pozo, A., Onaderra, M., Laynez, J. and Gavilanes, J.G., Interaction of type I collagen with phosphatidylcholine vesicles. *Collagen Rel. Res.*, 8 (1988) 133–144.

Martinez del Pozo, A., Onaderra, M., Laynez, J. and Gavilanes, J.G., Interaction of type I collagen fibrils with phospholipid vesicles. *Matrix*, 9 (1989) 405–410.

Meunier, F., Scullier, J.P., Coune, A., Brassinne, C. and Laduron, C., Amphotericin B encapsulated in liposomes administered to cancer patients. *Ann. N.Y. Acad. Sci.*, 544 (1988) 598–610.

Montfoort, A., Bezstarosti, K., Groh, M.M.J. and Metsäketelä, T.J.A., The influence of the lipid composition on the degree of lipid peroxidation of liposomes. *Biochem. Int.*, 15 (1987) 525–543.

Nakazawa, T., Nagatsuka, S. and Yukawa, O., Effect of membrane stabilizing agents and radiation on liposomal membranes. *Drugs Exp. Clin. Res.*, 12 (1990) 831–835.

New, R.R.C., Introduction. In New, R.R.C. (Ed.), *Liposomes: A Practical Approach*, IRL, Oxford, 1990, pp. 1–31.

Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D., Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochim. Biophys. Acta, 557 (1979) 9–23.

Ostro, M.J., *Liposomes: From Biophysics to Therapeutics*, Vol. I, Dekker, New York, 1987.

Ozer, Y., Talsma, H., Crommelin, D.J.A. and Hincal, A.A., Influence of freezing and freeze-drying on the stability of liposomes dispersed in aqueous media. *Acta Pharmacol. Technol.*, 34 (1988) 129–139.

Pajean, M., Huc, A. and Herbage, D., Stabilization of liposomes with collagen. *Int. J. Pharm.*, 77 (1991) 31–40.

Ricard-Blum, S., Tiollier, J., Garrone, R. and Herbage, D., Further biochemical and physicochemical characterization of minor disulfide-bonded (type IX) collagen, extracted from foetal calf cartilage. *J. Cell. Biochem.*, 27 (1985) 147–158.

Senior, J. and Gregoriadis, G., Methodology in assessing liposomal stability in the presence of blood, clearance from the circulation of injected animals and uptake by tissue. In Gregoriadis, G. (Ed.), *Liposome Technology*, Vol. 3, CRC Press, Boca Raton, FL, 1984, pp. 263–282.

Takahashi, M., Tsuchiya, J., Niki, E. and Urano, S., Action of vitamin E in phospholipid liposomal membranes as studied by spin label technique. J. Nutr. Sci. Vitaminol. 34 (1988) 25–34.

Treat, J., Greenspan, A., Forst, D., Sanchez, J.A., Ferrans, V.J., Potkul, L.A., Wooley, P.V. and Rahman, A., Antitumor activity of liposome-encapsulated doxorubicin in advanced breast cancer phase II study. *J. Natl. Cancer Inst.*, 82 (1990) 1706–1710.

Urano, S., Kitahara, M., Kato, Y., Hasegawa, Y. and Matsuo, M., Membrane stabilizing effect of vitamin E: existence of a hydrogen bond between α-tocopherol and phospholipids in bilayer liposomes. *J. Nutr. Sci. Vitaminol.*, 36 (1990) 513–519.

Weiner, A.L., Carpenter Green, S.S., Soehngen, E.C., Lenh, R.P. and Popescu, M.C., Liposome-collagen gel matrix: a novel sustained drug delivery system. *J. Pharm. Sci.*, 74 (1985) 922–925.

Yatvin, M.S. and Lelkes, P.I., Clinical prospects for liposomes. *Med. Phys.* 9 (1982) 149–175.