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Interaction between human serum albumin and liposomes: a monolayer and liposome study

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Summary

The penetration ability of human serum albumin in neutral monolayers of cholesterol, dipalmitoylphosphatidylcholine and mixtures at the same molar ratio, has been studied. The effect of the protein on unilamellar liposomes of cholesterol–dipalmitoylphosphatidylcholine has been determined by measurement of the amount of carboxyfluorescein released from the liposomes. There is a relation between the penetration ability in such monolayers and the leakage effect on the liposomes.

Introduction

It is well established that liposomes interact with serum components, especially with lipoproteins and proteins (Zborowski et al., 1977; Weinstein et al., 1981; Agarwal et al., 1986; Shiffer et al., 1988). Such interaction can modify the release of the liposomal content or can even produce the leakage of liposomes.

From a pharmaceutical view-point, it is important to know about liposomal behaviour in biological fluids, since the effectiveness of liposomes as drug delivery vehicles will depend on their exposure to the blood and plasma components and also on protein–lipid interactions. When liposomes are used to introduce substances into cells they lose their stability when brought into contact with complex systems such as blood or culture media and thus release part of their content.

The interaction of hydrophobic proteins with some monolayers is governed by the surface activity (hydrophobicity) of the macromolecules. Hydrophobicity does not guarantee interaction of a protein with lipids in dispersion, but it favors penetration of proteins into monolayers at the air–water interface (Phillips et al., 1975). The situation is not so clear for highly water-soluble, globular proteins where complete penetration does not occur, although at least a part of the protein must be embedded in the lipid film. With such proteins, the adsorption could be affected by the electrostatic interaction between lipid and protein, but the hydrophobic interaction also plays an important role, since the surface activity and lipid-binding capabilities of proteins are thought to be mediated by the amphipatic α-helices within the protein (Krebs and Phillips, 1984).

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The stability of liposomes in the presence of serum proteins can be enhanced by changing the lipid composition of the liposomes. The partial replacement of phosphatidylcholine by cholesterol causes a slight decrease of leakage. Modifications in the phospholipid structure can also prevent the lysis of liposomes in serum. In this way, a structural change, consisting of the replacement of the C-2 ester bond in phosphatidylcholine by an ether linkage, enhanced the liposome stability (Agarwal, 1986).

This present work attempts to determine whether the interaction of uncharged unilamellar liposomes with albumin involves an increase in the permeability of liposomes to cholesterol and dipalmitoylphosphatidylcholine obtained by an extrusion procedure.

For this purpose we employed on one hand the monolayer technique to check the primary interaction between albumin and lipids. Monolayers are a convenient experimental model system for the study of protein–lipid interactions because they provide a stable interface with a lipid composition identical to liposomes. On the other hand, the effect of albumin on the integrity of membranes is conveniently measured in lipid vesicles with the water-soluble fluorescent marker, carboxyfluorescein. Its fluorescence is self-quenched during containment in liposomes and leakage into the surrounding medium can be continuously and sensitively monitored as an increase in fluorescence due to dilution of the marker.

Materials and Methods

Human serum albumin (HSA), L-α-dipalmitoylphosphatidylcholine (DPPC), specified as 99% pure, and cholesterol (CHOL), type 99 + % pure, were purchased from Sigma (U.S.A.). Carboxyfluorescein (CF) was purchased from Eastman Kodak (U.S.A.) and, since it contains both hydrophilic and hydrophobic impurities, a further purification was necessary which involves two steps: (1) recrystallization; and (2) gel filtration chromatography on a Sephadex LH-20 column (Pharmacia, Sweden) preswollen in distilled water. Organic solvents (chloroform, methanol and ethanol) obtained from Merck (F.R.G.) were twice distilled before use to avoid the presence of surface-active impurities. Water was double-distilled, the last time in the presence of potassium permanganate in borosilicate apparatus, and then purified through a Milli-Q system (Millipore, U.S.A.).

Monolayer technique

Films of DPPC and CHOL at several proportions (100 : 0, 50 : 50 and 0 : 100, molar ratio) were spread over the subphase of a teflon trough filled with Tris buffer solution, pH 7.4. HSA, at 40 μg/ml concentration in Tris buffer solution, was injected onto the subphase and the increase in surface pressure was measured. Other characteristics about the technique and apparatus have been described elsewhere (Hernández et al., 1989).

Liposome preparation

Liposomes of CHOL and DPPC in a molar ratio of 50 : 50 and encapsulating CF were obtained by extrusion through polycarbonate membrane filters of 0.1 μm pore size (Nucleopore, U.S.A.). Encapsulation efficiency was determined from fluorescence measurements with dilute vesicle suspensions employing a Perkin Elmer model 204 fluorometric spectrophotometer (Hernández et al., 1987).

Results and Discussion

Measurements of surface activity

Initially we determined the free protein adsorption to a surface without a lipid monolayer. As shown in Fig. 1, the increase in surface pressure presents a plateau (steady state) at concentrations above 80 μg/ml with a value of approx. 16–17 mN/m. At the protein concentration chosen to be injected into the subphase, 40 μg/ml, an increase of 14.60 mN/m was obtained.

Figs. 2–4 show the influence of the composition of the monolayer on the lipid–protein interaction at initial surface pressures of 20, 30 and 40 mN/m. In the surface activity experiments, an increase in surface pressure ($\Delta\pi$) of a lipid monolayer is taken to indicate an interaction of al-

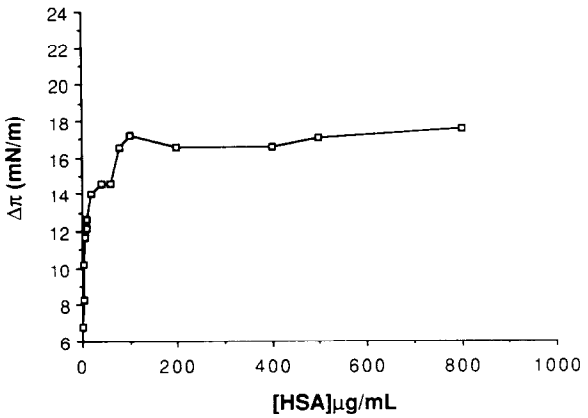

Fig. 1. Steady state surface pressure increase (mN/m) as a function of the concentration of human serum albumin for adsorption to a surface without lipid monolayer. Subphase buffer: 0.1 KCl–10 mM Tris-HCl, pH 7.4.

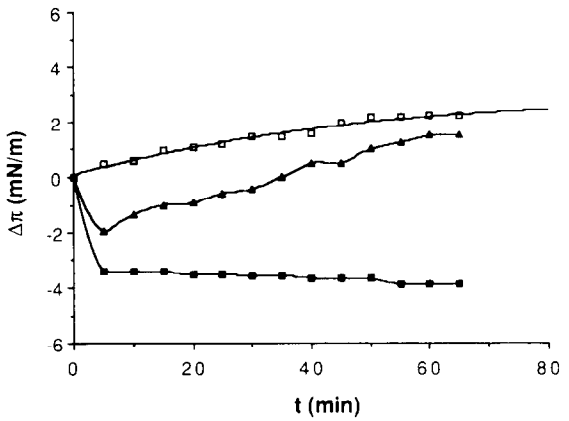

Fig. 3. Variation in surface pressure for the interaction of human serum albumin with cholesterol–dipalmitoylphosphatidylcholine (50:50, molar ratio) monolayer (for conditions, see legend of Fig. 2).

bumin from the subphase with the lipid film. Fig. 2 shows data from cholesterol monolayers plotted as $\Delta\pi$ vs. time for 1 h. Figs. 3 and 4 show similar plots with a mixed monolayer (CHOL : DPPC, 50 : 50) and a DPPC monolayer. Fig. 5 summarizes the variation in surface pressure at 60 min. From these results we concluded that an initial surface pressure of 20 mN/m is concomitant with an increase in surface pressure and consequently with a protein–lipid interaction, while at 40 mN/m initial pressure, a negative variation was obtained. Working at 30 mN/m initial pressure, the interaction depends on the monolayer composition.

The influence of the composition of monolayer films was determined by plotting $\Delta\pi$ (at 1 h) vs. π_i. The π_i intercept of a least-squares linear fit of these data with the π_i axis indicates the critical value above which no adsorption occurs. The π intercept reflects the ability of the protein to adsorb to an infinitely dilute lipid monolayer (practically speaking, a clear air/water interface). The

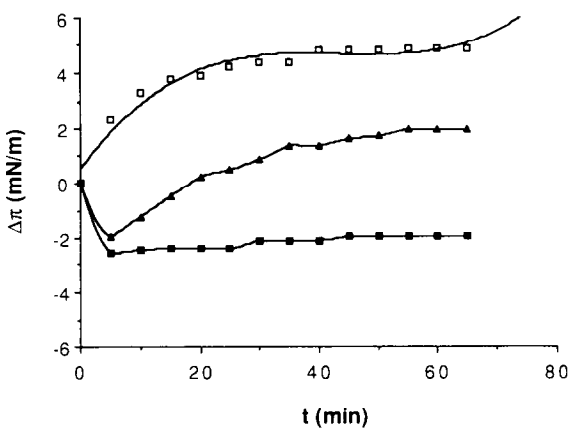

Fig. 2. Variation in surface pressure for the interaction of human serum albumin with cholesterol monolayer. Subphase buffer: 0.1 KCl–10 mM Tris-HCl, pH 7.4. The initial pressures of the film were 20 mN/m (□), 30 mN/m (▲) and 40 mN/m (■).

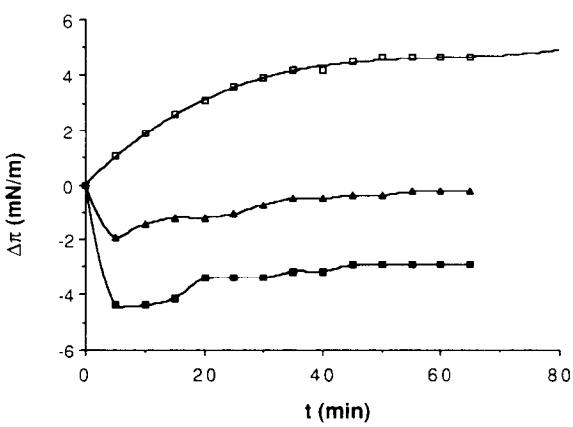

Fig. 4. Variation in surface pressure for the interaction of human serum albumin with a dipalmitoylphosphatidylcholine monolayer (for conditions, see legend Fig. 2).

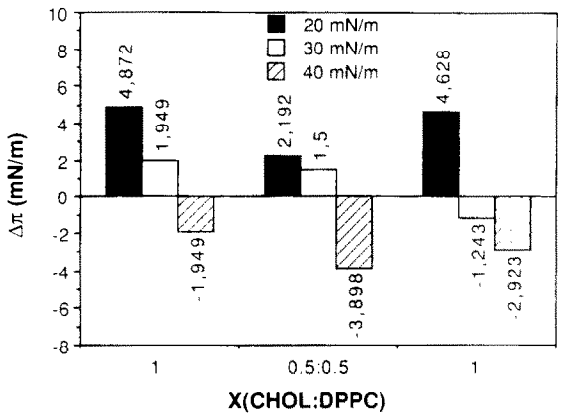

Fig. 5. Variation of $\Delta\pi$ for human serum albumin (concentration in the bulk: 40 μg/ml) adsorbing to three lipid monolayers at initial surface pressures of 20, 30 and 40 mN/m.

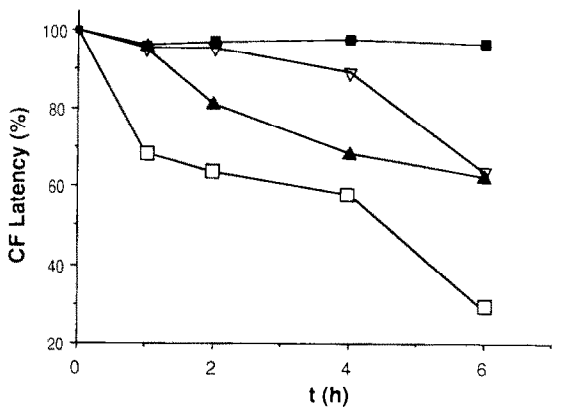

Fig. 6. Effect of human serum albumin on carboxyfluorescein leakage from liposomes of dipalmitoylphosphatidylcholine and cholesterol (50:50, molar ratio) at the following concentrations: 4 mg/ml (□), 400 μg/ml (▲), 40 μg/ml (▽), and 4 μg/ml (■). Fluorescence obtained after disruption of the liposomes with Triton X-100 (0.1%) was taken as 0% latency, whereas the residual fluorescence of the liposomes was taken as 100% latency.

values given in Table 1 demonstrate that DPPC and CHOL appear to interact similarly with HSA. However, the $\Delta\pi$ intercept for DPPC : CHOL was lower than for both pure CHOL and DPPC monolayers.

Measurements of released carboxyfluorescein

As far as the effect of HSA on liposomes is concerned, Fig. 6 shows the fluorescence retained by liposomes at different times of incubation. The residual fluorescence of the liposomes was taken as 100% latency. Maximal fluorescence, obtained after disruption of the liposomes with Triton X-100 (0.1%) was taken as 0% latency. As shown, the rate of efflux of CF is concentration-dependent. Liposomes rapidly lose entrapped CF when incubated in the presence of HSA solutions with concentrations higher than 40 μg/ml.

At a concentration of 4 μg/ml only a slight effect during the period 0 min to 24 h after injection was observed.

Based upon the above results, we can conclude that the values at the air/water interface indicate an interaction between monolayer films and HSA. Two mechanisms seem to be involved in the interaction: a process of 'solubilization' (at 30 mN/m initial surface pressure for DPPC monolayers and at 40 mN/m for whatever type of monolayer studied) and a 'penetration' in the rest. In this case, the penetration did not seem to be of an electrostatic nature, given that the lipids used are neutral. A Raman spectroscopic study (Lis et al., 1976) suggested that phosphatidylcholine undergoes a conformational change and that the hydrocarbon chain environment is more polar in the presence of albumin.

Beyond that, a equimolecular lipid ratio of CHOL and DPPC slightly reduced the interaction. The study of the lipid mixture in the liposome composition is therefore an aspect to bear in mind.

Finally, the correlation between monolayer and liposomes experiences has been evidenced.

TABLE 1

Monolayer	π_i Intercept (mN/m)	$\Delta\pi$ Intercept (mN/m)	Slope	r
DPPC	30.4	11.5	−0.378	0.95
DPPC : CHOL	29.8	9.1	−0.341	0.91
CHOL	34.8	11.9	−0.341	0.99

Data obtained from $\Delta\pi$ at 1 h vs. π_i plots of monolayers of cholesterol (CHOL), dipalmitoylphosphatidylcholine : cholesterol (50:50, molar ratio) (DPPC : CHOL) and dipalmitoylphosphatidylcholine (DPPC) at three initial surface pressures: 20, 30 and 40 mN/m.

References

Agarwal, K., Bali, A. and Gupta, C.M., Influence of the phospholipid structure on the stability of liposomes in serum. *Biochim. Biophys. Acta*, 856 (1986) 36–40.

Hernández, J., Estelrich, J. and Pouplana, R., Determination of the encapsulation efficiency in liposomes obtained by the "extruder method". *J. Microencapsulation*, 4 (1987) 315–320.

Hernández, J., Estelrich, J., Montero, M.T. and Valls, O., The action of indomethacin on neutral and positively charged monolayers. *Colloid Polym. Sci.*, 267 (1989) 622–626.

Krebs, K.E. and Phillips, M.C., The contribution of α-helices to the surface activities of proteins. *FEBS Lett.*, 175 (1984) 263–266.

Lis, L.J., Kaufmann, J.W. and Shriver, D.F., Raman spectroscopy detection and examination of the interaction of amino acids, polypeptides and proteins with the phosphatidylcholine lamellar structure. *Biochim. Biophys. Acta*, 436 (1976) 513–522.

Phillips, M.C., Evans, M.T.A. and Hauser, H., Interaction of proteins with phospholipid monolayers. In Goddard, E.D. (ed.), Advances in Chemistry Series, Vol. 144, American Chemical Society, New York, 1975, pp. 217–230.

Shiffer, K.A., Goerke, J., Düzgüneş, N., Fedor, J. and Shohet, S.B., Interaction of erythrocyte protein 4.1 with phospholipids. A monolayer and liposome study. *Biochim. Biophys. Acta*, 937 (1988) 269–280.

Weinstein, J.N., Klausner, R.D., Innerarity, T., Ralston, E. and Blumenthal, R., Phase transition release, a new approach to the interaction of proteins with lipid vesicles. Application to lipoproteins. *Biochim. Biophys. Acta*, 647 (1981) 270–284.

Zborowski, J., Roerdink, F. and Scherphof, G., Leakage of sucrose from phosphatidylcholine liposomes induced by interaction with serum albumin. *Biochim. Biophys. Acta*, 497 (1977) 183–191.