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Reduction of the in vitro hemolytic activity of soybean lecithin liposomes by treatment with a block copolymer

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The in vitro hemolytic activity of liposomes made of soybean L-α-lecithin towards diluted (0.0086 v/v) human erythrocytes was used to investigate the effect of surface coating on the interaction of liposomes with cells. The increase in apparent volume of the block copolymer of ethylene glycol and propylene glycol, Pluronic F-127, in the presence of liposomes supports the hypothesis of either adsorption or penetration of the copolymer at the surface of the liposomes. When the liposomes are pre-incubated with Pluronic F-127, their lytic activity towards fresh erythrocytes is significantly reduced while it remains unchanged towards erythrocytes aged in vitro. It is also found that aging the liposomes has little effect on their lytic activity while aging of the erythrocytes makes them more fragile towards the liposomes. The results are discussed in terms of steric hindrance.

1. Introduction

Liposomes made of natural or synthetic phospholipids are used widely as models for biological membranes [1,2], drug vehicle [3,4] and to develop blood substitutes based on the encapsulation of hemoglobin [5–9]. The clinical use of liposomes is limited by their short shell-life and rapid clearance from the circulatory system [10,11]. It is known that the in vitro incubation of liposomes with erythrocytes leads to an exchange of membrane components [12–18] that might increase the fragility of the cells towards the liposomes. In vitro aging of erythrocytes is also known to increase their fragility [19–22].

It is important to understand how liposomes interact with cells and how these interactions can be controlled in order to improve the fate of liposomal material in vivo. In the present study, the hemolytic activity of liposomes made of soybean lecithin was used to investigate the interactions between liposomes and erythrocytes. The hemolytic activity of these liposomes is probably related to the presence of impurities in soybean lecithin which may be transferred to the erythrocytes by collisions or by diffusion through the aqueous space. Modifications at the surface of the liposomes should affect the mechanisms leading to hemolysis and this effect was investigated with liposomes pre-incubated with an amphiphilic triblock copolymer of ethylene glycol and propylene glycol, Pluronic F-127, which is expected to bind to the lipidic membranes of the liposomes through its hydrophobic block of polypropylene glycol.

2. Materials and methods

Soybean L-α-lecithin (Calbiochem, > 97%) and Pluronic F-127 (Polysciences, lot no. 0328-066)

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were used without further purifications. The water content of lecithin was lower than 1.5% (w/w) as determined by Karl Fisher titration. NaCl (0.85% w/w), NaN_3 (0.01% w/w) and Tris (0.12% w/w) were solubilized in deionized water and the solution was set to pH 7.4 with HCl. In the text, this solution will be referred to as the buffer.

Human erythrocytes were prepared from fresh blood with EDTA as an anticoagulant. The erythrocytes were washed three times with buffer and stored at 4°C. Prior to each incubation experiment, the erythrocytes were diluted with buffer to attain a hematocrit of about 0.0086 (v/v) which falls within the range of the linear concentration dependence (0–0.0090 v/v) of the light absorption of hemoglobin at 540 nm (Ultrospec II, LKB, 0.5 cm path length quartz cell). The average hematocrit (0.0086 ± 0.0002) of the diluted erythrocyte suspensions prepared corresponds to a concentration of membrane lipids [23] of about 50 μg cm^{-3}. The age of the erythrocytes is defined as the time elapsed between blood withdrawal and the end of the incubation experiments.

Liposomes made of soybean L-α-lecithin were prepared with the evaporation-hydration technique where a solution of lecithin in dichloromethane was evaporated in a flask and hydrated with the buffer under vortexing. The lecithin concentration of the mother suspension of liposomes was calculated with the weight of lecithin and the final weight of the suspension assuming a density of 1 g cm^{-3} for the buffer. The age of the liposomes is defined as the time elapsed between the preparation and the addition to the erythrocyte suspension.

For each incubation experiment, weighted amounts of the mother liposome suspension were added to 1.25 g of the diluted erythrocyte suspension (0.0086 v/v) to cover the desired range of lecithin concentration (C_L) expressed in μg cm^{-3} of the erythrocyte suspension assuming a density of 1 g cm^{-3} for the final suspensions. These suspensions were incubated for 20–24 h at room temperature on a rotatory agitator (27 rpm) followed by 30 s of centrifugation at 13000 × g (IEC Centra-M). The absorbance at 540 nm of the supernatant (A_{540}), corrected for the dilution of the erythrocytes by the addition of the mother suspension of liposomes, and the 100% hemolysis absorbance (A^{100}), obtained with one drop of 10% Triton x-100, were used to calculate the extent of hemolysis ($\%H = 100A_{540}/A^{100}$). The self-hemolysis ($\%H^0$) is defined as the extent of hemolysis of the erythrocyte suspension without added liposomes and subjected to the same experimental conditions.

Densities of the liposome (d_0) and copolymer/liposome (d) suspensions were determined at 25°C by flowing the agitated suspensions into a Sodev flow densimeter [24]. The apparent specific volume ($v_{p,\phi}$, cm^3 g^{-1}) of Pluronic F-127 in the liposome suspensions was calculated using:

$$v_{p,\phi} = \frac{1}{d} - \frac{(1 - W_p)(d - d_0)}{W_p(d - d_0)} \quad (1)$$

where W_p is the weight fraction of Pluronic F-127 in the suspensions.

3. Results and discussion

In the present work, the concentration of lecithin (C_L) accounts for all species present in the commercial soybean lecithin and this scale is only used for the comparison of independent curves of percentage of hemolysis vs lecithin concentration.

3.1. Hemolytic activity of lecithin liposomes

In order to compare different incubation experiments, we evaluated the reproducibility and the effect of aging the liposomes and erythrocytes on the trend of the percentage of hemolysis vs lecithin concentration.

The hemolytic activity of the lecithin liposomes was defined as the concentration of lecithin necessary to cause 50% hemolysis (C_{50}). The reproducibility of the curves of percentage of hemolysis vs lecithin concentration was evaluated by measuring the extent of hemolysis caused by three independent preparations of liposomes towards the same erythrocyte suspension of age 4 days. The results are reported in fig. 1 where the three curves agree very well with an average C_{50} of 39 ± 4 μg cm^{-3}. This dispersion in C_{50} should be regarded as the

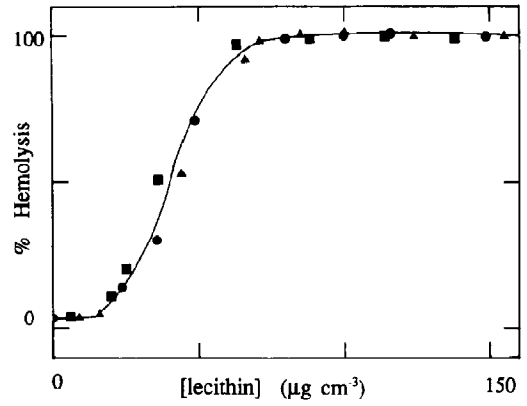

Fig. 1. Extent of hemolysis caused by three independent preparations of liposomes of age 1 day; (▲, ■, ●) incubated with erythrocytes of age 4 days.

maximum deviation due to the reproducibility of independent incubation experiments.

Aging the liposomes may produce hemolytic molecules due to the oxidation of the unsaturated alkyl chains (approx. 77% [25]) of soybean lecithin. This effect was checked and the curves of percentage of hemolysis vs lecithin concentration for fresh erythrocytes incubated with liposomes of age 5, 14 and 20 days are reported in fig. 2. The value of C_{50} decreases slowly upon aging the liposomes but the effect is relatively small (from 62 to 48 μg cm^{-3}) when compared to the reproducibility limit (± 4 μg cm^{-3}) of each incubation experiment.

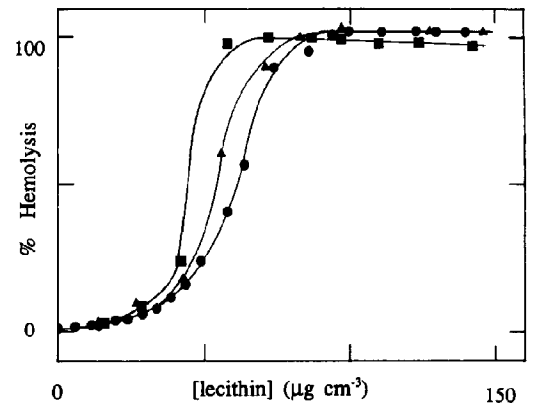

Fig. 2. Extent of hemolysis caused by liposomes of age: (●) 5, (▲) 14 and (■) 20 days; incubated with erythrocytes of age 1 day.

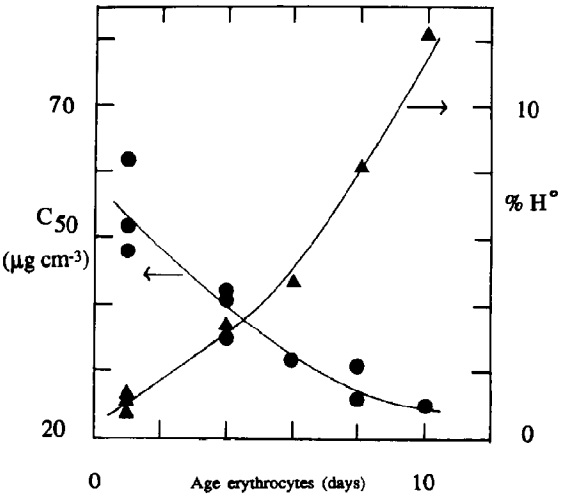

Fig. 3. Hemolytic activity (C_{50}, ●) of liposomes and self-hemolysis ($\%H^0$, ▲) of erythrocytes vs age of the erythrocytes.

The effect of aging the erythrocytes is presented in fig. 3 where the values of C_{50} and $\%H^0$, obtained from independent incubation experiments, are plotted against age of the erythrocytes. C_{50} decreases rapidly with age of the erythrocytes, suggesting that the erythrocytes become more fragile upon aging [19–22], in accordance with the increase in self-hemolysis ($\%H^0$) which is related to the fragility of the erythrocytes. The increased fragility of erythrocytes could be caused by surface modifications of the cells that would enhance the hemolytic activity of the liposomes.

These experiments show that the in vitro aging of the erythrocytes has a greater effect on the value of C_{50} than the in vitro aging of the liposomes. This shows that the investigation of the effect of a solute on the interaction between liposomes and erythrocytes requires that the erythrocyte suspensions used for the incubation experiments are of equal age.

3.2. Hemolytic activity of coated liposomes

The effect of surface coating on the hemolytic activity of lecithin liposomes was studied with an amphiphilic tri-block copolymer (Pluronic F-127, $\langle M_w \rangle = 12\,000$ g mol^{-1}). By itself, this copolymer had no hemolytic activity when incubated for 24 h

with fresh erythrocytes at concentrations up to 524 μg cm^{-3}. The central block of polypropylene glycol (30% w/w) is expected to bind the lipidic membrane of the liposomes by hydrophobic interactions while the two hydrophilic chains of polyethylene glycol (70% w/w) could remain in water, giving rise to steric hindrance at the surface of the liposomes.

It is known [26,27] that the apparent volume of a molecule is sensitive to its local environment in a medium. Usually, the transfer from water to a more hydrophobic medium leads to an increase in the apparent volume due to changes in the interactions and hydration of the molecule. This property was used to probe the environment of Pluronic F-127 in the presence of lecithin liposomes. For these experiments, lecithin was hydrated in saline (water + NaCl, 0.85% w/w) and the resulting liposome suspension was diluted with the saline to cover the range $0 < C_L < 10$ mg cm^{-3}. The suspensions were divided into two samples, to one of which Pluronic F-127 was added to achieve a concentration of 4.1 mg cm^{-3}. The difference between the density of the two samples was used to calculate the apparent volume of the copolymer in the mixture using eq. 1. At $C_L = 0$, the apparent volume of Pluronic F-127 reflects its environment in the saline and the trend of the apparent volume with C_L is related to changes in the local environment of the copolymer in the presence of liposomes.

Fig. 4 shows the trend of the apparent volume of Pluronic F-127 at a constant concentration of 4.1 mg cm^{-3} while C_L increases from 1 to 10 mg cm^{-3}, corresponding to a ratio of Pluronic F-127 to lecithin ranging from 4 to 0.4. At $C_L = 0$, the apparent volume of Pluronic F-127 is 0.860 cm^3 g^{-1} and increases rapidly to a constant value of 0.864 cm^3 g^{-1} in the presence of liposomes. The positive change in volume is significant within the experimental error and could be associated either with the hydrophobic dehydration of the copolymer upon its transfer from the saline to the liposomes or with increase in the local concentration of the copolymer resulting from its adsorption at the surface of the liposomes. Both cases support the hypothesis of surface coating of the liposomes by the copolymer molecules. The small

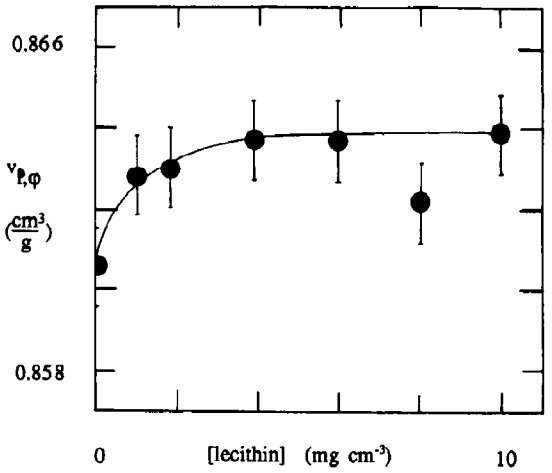

Fig. 4. Apparent specific volume ($v_{p,\varphi}$) of Pluronic F-127 at 4.1 mg cm^{-3} in the presence of liposomes at 25 °C.

change in volume could be a consequence of the transfer of only a fraction of the copolymer molecules. However, more experiments are required in order to reach a definite conclusion concerning the structure of soybean lecithin liposomes treated with Pluronic F-127.

For the incubation experiments, the liposomes were pre-incubated for 6 h with Pluronic F-127 at a weight ratio of copolymer to lecithin of 1.74 followed by incubation with the erythrocyte suspensions. Fig. 5 shows the curves of the percentage of hemolysis vs lecithin concentration for fresh and aged erythrocytes. With fresh erythrocytes, the two independent preparations of treated liposomes showed no hemolytic activity up to 140 μg cm^{-3} after 24 h of incubation as opposed to the corresponding untreated liposomes (dotted line). This effect could be related to increased hindrance caused by surface modifications of the liposomes by the copolymer molecules.

The situation is quite different for erythrocytes of age 10 days where the treated and untreated liposomes had comparable hemolytic activity (C_{50} = 23 and 25 μg cm^{-3}, respectively). It has been suggested that the aging of erythrocytes results in a change of cell surface carbohydrates [19] and more specifically glycophorin A [21] which is a bulky glycoprotein of red blood cells. One could speculate that the surface modifications of aged

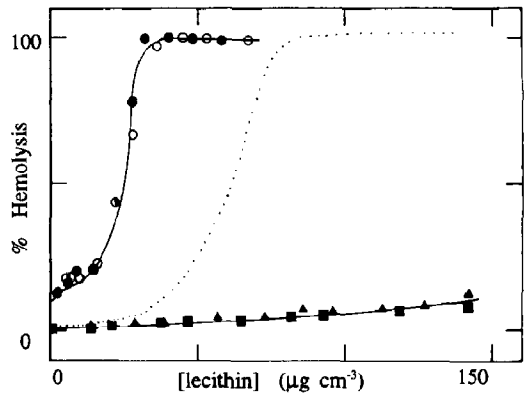

Fig. 5. Effect of Pluronic F-127 on the extent of hemolysis caused by liposomes. Treated liposomes (weight ratio of Pluronic F-127 to lecithin of 1.74) incubated with erythrocytes of age: (■, ▲) 1 day and (●) 10 days. Untreated liposomes incubated with erythrocytes of age: (○) 10 days and (· · · · · ·) 1 day [same curve as (●) in fig. 2].

erythrocytes cannot be compensated solely by surface modifications of the liposomes and that bulky molecules at the surface of erythrocytes are also required to prevent hemolysis by the liposomes.

The intrinsic lytic activity of soybean lecithin liposomes could be due to direct contact of the liposomes with the erythrocytes or to the release of lytic molecules that would diffuse from the aqueous space to the erythrocytes. In both cases, the presence of bulky molecules at the surface of the liposomes and erythrocytes could reduce the activity of either the lytic molecules or liposomes. In order to obtain qualitative information on the mechanism of hemolysis, we conducted an experiment where a suspension of liposomes was placed in a dialysis tube (molecular weight cut off of 12 000 g mol^{-1}) and immersed in a suspension of aged erythrocytes to give an equivalent lecithin concentration of 140 $\mu g\ cm^{-3}$. After 24 h of incubation, the extent of hemolysis ($\%H = 25\%$) was almost the same as that of the self-hemolysis ($\%H^0 = 26\%$) of the erythrocytes while the direct incubation of liposomes with the erythrocytes led to complete hemolysis ($\%H = 98\%$). The absence of lytic activity of the liposomes placed in the dialysis tubing supports the hypothesis of hemolysis by direct contact of the erythrocytes with liposomes or relatively large subunits that cannot diffuse through the tubing. However, more experiments are required in order to establish a definite mechanism for the in vitro hemolysis by soybean lecithin liposomes.

4. Conclusions

The apparent volumes and the incubation experiments both support the hypothesis of surface coating of the liposomes by Pluronic F-127 which would greatly reduce the hemolytic activity of soybean lecithin liposomes towards fresh erythrocytes. In vitro aging of the erythrocytes increases their fragility which might be the consequence of cell surface modifications that cannot be compensated by the steric hindrance at the surface of the liposomes.

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