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Biophysical Studies on Collagen-Lipid Interaction

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The potential use of liposomes as a delivery system is still limited by the poor understanding of the interaction mechanisms of liposomes underlying with biological media. Interaction between liposomes and protein is important for the structure and function of cells. In the present work, the interaction between collagen and dipalmitoyl phosphatidylcholine (DPPC) liposomes was studied by solubilization using a nonionic detergent, octylglucoside (OG), as well as a monolayer technique. The solubilization of the liposomal membrane was found to proceed in three stages of transition from the vesicular form to the mixed micellar form. Moreover, the amount of detergent needed to completely solubilize the liposomal membrane was increased after the incubation of liposomes with collagen, indicating an increased membrane resistance to the detergent and hence, a change in the natural membrane permeation properties. The addition of collagen in the subphase of different monolayer films induced a considerable shift towards a larger area/molecule in the compression-isotherm curves. This is either due to the insertion of collagen into the monolayer via its hydrophobic residues or to adsorption causing a protein layer to be located parallel to the lipid monolayer. It was concluded that collagen significantly altered the physical state of the liposome membrane, which may be attributed to collagen interaction with the liposomal surface and/or to its incorporation within the bilayer membrane.

[**Key words:** collagen, liposome, dipalmitoyl phosphatidylcholine, solubilization, monolayer]

Collagen is a protein with various industrial and medical applications (1). It is a potentially useful biomaterial because it is a major structural component of many tissues such as those of the skin, bone, cartilage, tendons, and basement membranes. Several methods have been developed to improve the ability of collagen to stabilize liposomes (2–4). There are numerous observations on associations between collagen fibres and lipids in both normal and pathological tissues (5). Cohen and Barenholz found a strong interaction between collagen and sphingomyelin liposomes *in vitro* (6). Lipids and collagen have been used to prepare a gel matrix, which was reported as an effective delivery system (7). Rathman and Sun (8) described the interaction of collagen with a lipid monolayer. Shi *et al.* (9) found that collagen increases the stability of liposome vesicles. Fonseca *et al.* (10) found that coating of liposomes with collagen results in both a higher stability *in vitro* and a selective and almost immediate accumulation of vesicles in liver Kupffer cells; therefore, these liposomes might be effective in the treatment of some infectious diseases located within macrophages.

Lipid bilayers are conventionally accepted to be the simplest model that approximates some properties of biological membranes. Besides their structural resemblance, they have physical properties similar to those of biomembranes, including thickness, water permeability, bending rigidity, surface tension, and viscosity. Furthermore, artificial lipid membranes are well-defined systems and are readily prepared. Thus, they provide a unique opportunity to investigate certain physiological functions and processes in biological membranes.

In the present work, we deal with the interaction of collagen with different liposomal formulations solubilized with a nonionic detergent octylglucoside (OG), as well as using a monolayer of phospholipids at the air/water interface, which provides a suitable experimental system for investigating molecular interactions occurring in an oriented molecular array.

MATERIALS AND METHODS

Materials l-α-Dipalmitoyl phosphatidylcholine (DPPC) specified 99% pure, cholesterol (CHOL) 99% pure, type-I collagen from bovine, and nonionic detergent octylglucoside (OG) were purchased from Sigma (St. Louis, MO, USA). Distearoylphosphatidylethanolamine derivatized at the amino position with a 2000-molecular-weight segment of poly (-ethylene glycol) (PEG-PE) was obtained from Calbiochem (La Jolla, CA, USA). Organic solvents (chloroform and ethanol) were of analytical grade and obtained from Merck. Water used was double distilled in a glass apparatus (final distillation over alkaline $KMnO_4$).

Preparation of liposomes The liposomes used in this work were prepared from DPPC, DPPC/CHOL (5 : 1 mole ratio) and DPPC/CHOL/PEG-PE (5 : 1 : 0.2 mole ratio) using the reverse-phase evaporation technique (11). The lipids must first be dissolved and mixed in chloroform/ethanol (5 : 1, v/v) to ensure a homogeneous mixture of lipids. The organic solvent should be removed by rotary

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evaporation to obtain a thin lipid film formed on the sides of a round bottom flask. The lipid film is thoroughly dried to remove residual organic solvent by placing the flask on a vacuum pump overnight. Hydration of the dry lipid film is accomplished simply by adding an aqueous solution to the container of the dry lipid film and agitating at a temperature above the phase transition temperature of the lipid.

Membrane solubilization The solubilization of DPPC multilamellar liposomes of a certain concentration was followed by continuous addition of the nonionic detergent octylglucoside (OG), and monitoring by measuring the optical density (OD) of liposomes at 400 nm using a UV/visible spectrophotometer (Jenway 6405; Barloworld Scientific, Essex, UK). The OG solution was continuously added at a constant rate to the cuvette equipped with a paddle stirrer and containing 2 ml of liposomes solution. During OG injection, OD of the mixed solution was measured. From the data obtained, OG concentration was calculated using the formula of Paternostre *et al.* (12). Consequently, OD profiles of solubilized liposomes were plotted as a function of total detergent concentration. The initial concentration of lipid was 0.2 mM and collagen concentration was 9 nM.

Monolayer experiments Films of pure DPPC, DPPC/CHOL (5:1 mole ratio) and DPPC/CHOL/PEG-PE (5:1:0.2 mole ratio) dissolved in chloroform/ethanol (5:1, v/v) were spread in a subphase of double distilled water in a Teflon trough. Compression isotherms were measured on the basis the Wilhelmy method using an electromicrobalance (Sartorius A-120-S; C I Electronics, Salisbury, Wiltshire, UK) coupled to a chart recorder that gives a continous reading of the force on the dipping plate (Langmuir system was fabricated by Barry Wright Corp., UK). The dimensions of the trough were $28.5 \times 16.2 \times 2.5\ cm^3$. Surface pressure was measured with an accuracy of 0.1 mN/m. Compression was started at least 35 min after film spreading to ensure the complete evaporation of the solvent at a rate of 5 A^2/molecule/min. Measurements were repeated at least three times and reproduced after expansion and recompression. Temperature was maintained at 25°C. For each experiment, collagen (dissolved in 0.5 M acetic acid) was injected in the subphase using a Hamilton microsyringe. The final collagen concentration in the subphase was 9 nM.

RESULTS AND DISCUSSION

Membrane solubilization The clarification of phase behavior of mixtures of phospholipids and detergents in aqueous solutions is important basic importance for understanding the solubilization and reconstitution of biological membranes. Figures 1a–c show the changes in the turbidity of different liposomal formulations as a function of detergent concentration before and after adding collagen. The solubilization of liposomal membrane by the nonionic detergent octylglucoside (OG) was proceed in three stages of transition from the vesicular form to mixed micellar form, namely, stages I, II and III. It was also noted that the detergent concentration needed to achieve the complete solubilization of the membrane depended on the lipid-collagen interaction. Liposomal membrane solubilization results confirmed the need for a higher detergent concentration to solubilize the liposomal membrane with collagen than to solubilize that without collagen. As can be noted from Fig. 1a, detergent molecules started to solubilize lipids in stage I until point A. As detergent concentration increased, the detergent molecules began to be incorporated within the membrane bilayer. The increase in detergent concentration caused a

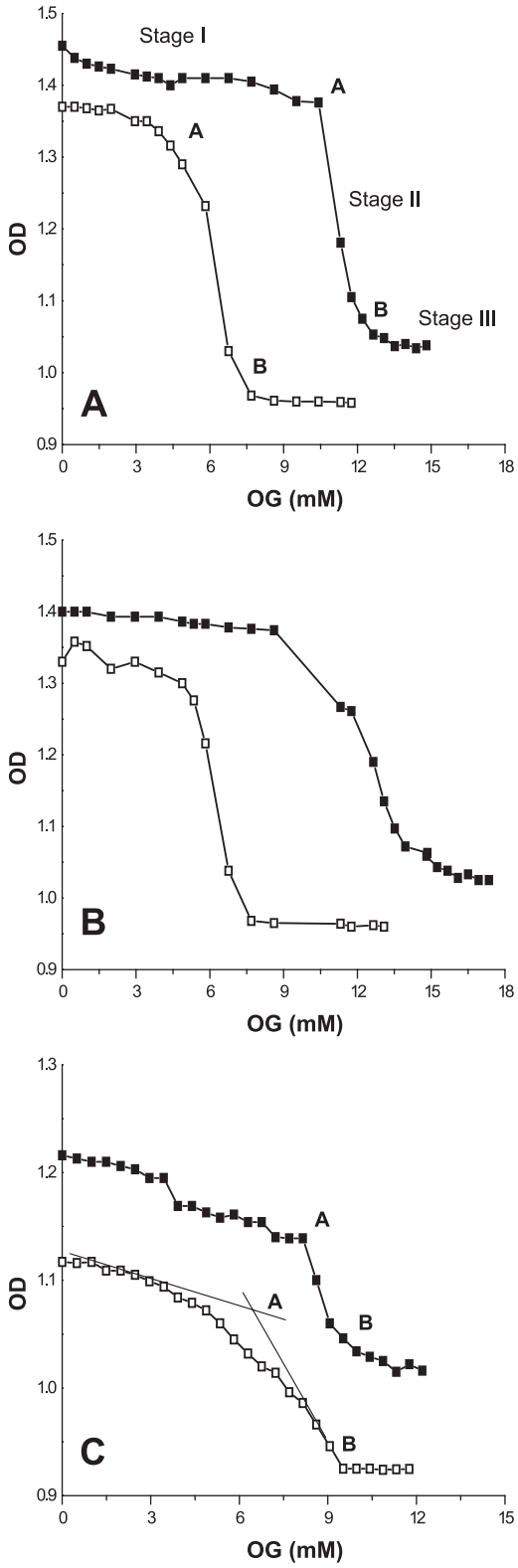

FIG. 1. Changes in optical density (OD) of different liposomal formulations. (A) Pure DPPC (open squares) and DPPC/collagen (closed squares); (B) DPPC/CHOL (open squares) and DPPC/CHOL/collagen (closed squares); (C) DPPC/CHOL/PEG (open squares) and DPPC/CHOL/PEG (closed squares) as a function of OG detergent concentration (mM). A and B in panels A and C mean how can you calculate transition points in Table 1.

TABLE 1. Break transition points A and B for different liposomal formulations

Sample	Transition point	
	A (mM)	B (mM)
DPPC	4.3	6.97
DPPC+collagen	8.15	8.92
DPPC+CHOL	4.96	6.68
DPPC+CHOL+collagen	7.5	9.3
DPPC+CHOL+PEG	6.46	9.5
DPPC+CHOL+PEG+collagen	8.15	9.9

corresponding increase of detergent molecules incorporated within the bilayer leading to the complete solubilization of the membrane (stage II until point B) and formation of mixed micelles. Stage III started after point B. The coexistence phase, that is, the vesicular/mixed micellar phase, shifted towards higher detergent concentrations after incubation with collagen (Table 1), indicating increasing membrane resistance to the detergent. The data indicate that the collagen-lipid interaction makes the liposomal membrane more rigid and less soluble in the detergent for the same formulation without collagen.

The results herein presented clearly demonstrate the existence of an interaction between monomeric type I collagen and phosphtidylcholine vesicles. This interaction results in the formation of a protein-phospholipid complex. We considered the potential contribution of this hydrophobic domain to the interaction of collagen with phospholipids vesicles. The phospholipid vesicle-collagen complex was proposed to be maintained by electrostatic interaction between the zwitterionic polar heads of phosphatidylcholine, the phospholipids used for such a study, and the amino acid side chains of the protein. The technique shows an adequate sensitivity in detecting membrane stability in liposomal formulation after incubation with collagen, and solubilization technique has a potential as a biophysical marker of lipid-protein interaction.

The addition of CHOL or PEG-PE to DPPC had a stabilizing effect against solubilization and minimized the interaction of collagen molecules with pure DPPC. For the DPPC monolayer (at a temperature below the phase transition temperature), cholesterol enhances the motion of the polar head group of DPPC owing to its liquefying effect (13). This minimized shift is partially attributable to the addition of PEG-PE to pure DPPC, which decreases the motion of the choline methyl group, predominantly through columbic and hydrophobic interaction forces (14).

Monolayer technique The changes in the compression isotherm of pure DPPC at different collagen concentrations are shown in Fig. 2. It is clear that the molecular area of DPPC in the protein-lipid monolayer is markedly larger than that of the pure DPPC monolayer at the same surface pressure, which is due to collagen-lipid interaction. Collagen molecules were expected to penetrate into the lipid monolayer in a parallel orientation and interact with the alkyl chains of DPPC with the hydrophobic residues on the collagen molecule surface. This larger molecular area was obtained at surface pressures from 5 mN/m to 50 mN/m after increasing collagen concentration in the subphase from 1.8 nM to 9 nM. For example, at a surface pressure of 30

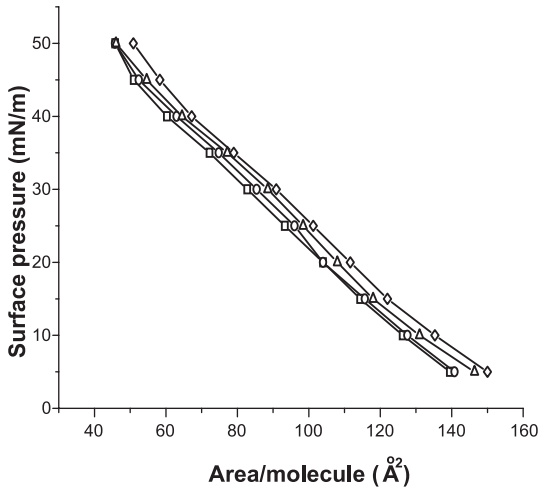

FIG. 2. Compression isotherms of pure DPPC monolayer spread at different concentrations of collagen. Symbols: squares, Pure DPPC; circles, DPPC+1.8 nM collagen; triangles, DPPC+3.6 nM collagen; and diamonds, DPPC+9 nM.

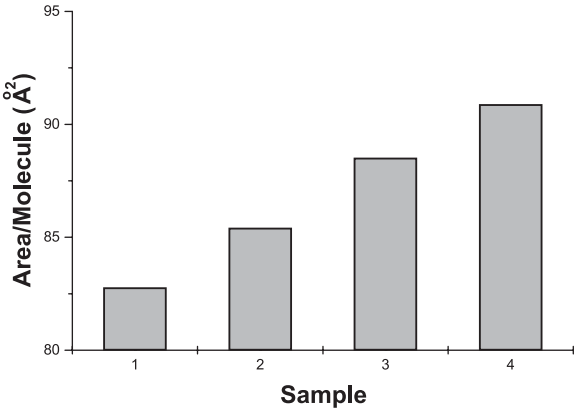

FIG. 3. Increase in area/molecule for pure DPPC monolayer spread at different concentrations of collagen at surface pressure of 30 mN/m. Samples: 1, pure DPPC; 2, DPPC+1.8 nM collagen; 3, DPPC+3.6 nM collagen; and 4, DPPC+9 nM collagen. The lipid concentration was 1 mM.

mN/m, there was a shift of 3.2% towards a larger area/molecule after the addition of 1.8 nM collagen in the subphase (Fig. 3). The shift was 6.9% at a collagen concentration of 3.6 nM. At 9 nM collagen concentration, there was a maximum shift 9.81% towards larger area/molecule at different surface pressures. Thus, this concentration was chosen to study the interaction between collagen and the lipid monolayer. To study the interactions between collagen and PC lipids and to explore the mechanism by which collagen increases the stability of lipsome vesicles, collagen was added in the subphase after the floating DPPC monolayer was compressed to reach the liquid expanded phase. Compression isotherms were measured after 30 min of collagen injection to ensure complete spreading of collagen (15).

The percentage increases in area/molecule for different DPPC films spread on collagen solution at a surface pressure of 30 mN/m are shown in Fig. 4. Collagen was added in the subphase in the region of the liquid expanded state (sur-

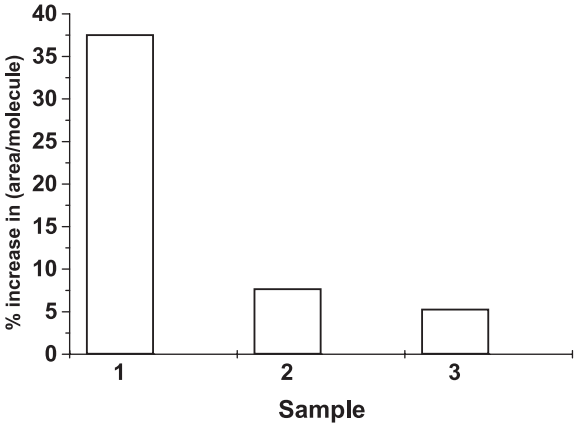

FIG. 4. Percentage increase in area/molecule for different liposomal compositions after addition of 9 nM collagen at surface pressure of 30 mN/m. Samples: 1, pure DPPC; 2, DPPC/CHOL; 3, DPPC/CHOL/PEG-PE. The lipid concentration was 1 mM.

face pressures, 5–10 mN/m), in which the monolayer is sensitive to any interaction (16). It is clear that the molecular area of DPPC in the protein-lipid monolayer was markedly larger than that of the pure DPPC monolayer at the same surface pressure, which is due to the collagen-lipid interaction. There is a shift of 37.55% towards a larger area/molecule after the addition of 9 nM collagen in the subphase. The less-ordered package of PC molecules provided for collagen sufficient space to be incorporated between lipid molecules, resulting in a marked shift towards a larger occupation area in the compression isotherm.

The incorporation of collagen within the lipid monolayer was monitored on the basis of the increase in the surface area of the lipid monolayer at a constant surface pressure (17). These results are in good agreement with those reported elsewhere (15, 17). The protein-lipid interaction is either due to the insertion of protein into the monolayer via its hydrophobic residues or to adsorption causing a protein layer to be located parallel to the lipid monolayer (1). Cohen and Barenholz 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 (6). Del Pozo *et al.* (18) observed an interaction (simple surface binding) between the triple helix of type-I collagen and phosphatidylcholine liposomes, resulting in the immobilization of phospholipid molecules around the protein causing a decrease in their fluidity. The effect of CHOL and PEG-PE on the rigidity of the DPPC monolayer is evident from the minimized shift to a larger area/molecule (Fig. 4). It is clear from Fig. 4 that the increase in area/molecule was minimized to a greater extent after the addition of CHOL (the increase in area/molecule was 7.7%) and PEG-PE (the increase in area/molecule was 5.3%) to the lipid monolayer. For the DPPC monolayer (at a temperature below the phase transition temperature), cholesterol increases the motion of the polar head group of DPPC owing to its liquefying effect (13). The shift to a larger area/molecule is minimized in the case of adding CHOL and PEG-PE to the lipid monolayer, which agrees with the findings of Gaber and co-workers (19, 20). The minimized shift is partially due to the addition of PEG-PE to the pure DPPC monolayer, which decreases the motion of the choline methyl group, predominantly through columbic and hydrophobic interaction forces (14).

We can conclude that collagen was proved to interact with all investigated lipid compositions in this study. This interaction may indicate an improvement in the stability of lipid vesicles composed of any of these compositions. These results with respect to the effect of collagen on the stability of lipid vesicles imply that the collagen-induced stability may be explained by collagen-lipid monolayer interaction and solubilization. The co-self-assembly mechanism for collagen and lipids in a film will be investigated, which shed light on the mechanism by which collagens will interact with lipids either in natural tissues or in artificial drug delivery systems. These results indicate that a lipid-collagen complex may be used either in cosmetology or pharmacology as an effective drug delivery system.

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