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SHORT COMMUNICATION

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Protective effect caused by the exopolymer excreted by *Pseudoalteromonas antarctica* NF_3 on liposomes against Triton X-100

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Abstract The capacity of the glycoprotein (GP) excreted by *Pseudoalteromonas antarctica* NF_3 to protect phosphatidylcholine (PC) liposomes against the action of Triton X-100 was studied in detail. Increasing amounts of GP assembled with liposomes resulted in a linear increase in the effective surfactant-to-PC molar ratios needed to produce the same alterations in liposomes and in a linear fall in the surfactant partitioning between the bilayer and the aqueous phase. Thus, the higher the proportion of GP assembled with liposomes the lower the surfactant ability to alter the permeability of vesicles and the lower its affinity with these bilayer structures. In addition, increasing GP proportions resulted in a progressive increase in the free surfactant concentration (S_W) for the same surfactant–liposome interaction step. The fact that S_W was always lower than the surfactant critical micelle concentration indicates that the interaction was mainly ruled by the action of surfactant monomers, regardless of the amount of GP assembled.

Key words *Pseudoalteromonas antarctica* NF_3 · Exopolymer of glycoproteic character · Phosphatidylcholine liposomes · Triton X-100 · Permeability alterations

Introduction

In the course of evolution prokaryotic organisms have developed a broad spectrum of cell envelope structures. Despite this diversity, two separate surface-enveloping structures can be distinguished: the plasma membrane and the associated cell wall proper [1–3].

The use of liposomes as vehicles for drug delivery is limited because of their short survival time in blood. The effect of poly(ethylene glycol) in the fusion of phospholipid vesicles and in the prolongation of their circulation time in blood has recently been studied [4, 5]. Liposomes have also been used as membrane models to study the solubilizing effect of surfactants [6–9].

In earlier papers we reported investigations of the ability of an exopolymer of glycoproteic character excreted by a new Gram-negative species, *Pseudoalteromonas antarctica* NF_3, to coat phosphatidylcholine (PC) liposomes and to protect these bilayers against the action of various surfactants [10–12]. We also investigated the interaction of a series of octylphenols, in particular Triton X-100 ($T_{X\text{-}100}$) and its mixtures with sodium dodecyl sulfate, with PC and stratum corneum lipid liposomes [13–17]. In the present work we seek to extend these investigations by studying in detail the overall interaction of $T_{X\text{-}100}$ with PC liposomes coated with increasing amounts of this exopolymer. To this end, we studied the variations in the effective molar ratio of surfactant to phospholipid (*Re*) and the partition coefficients (*K*) of surfactant between bilayers and water as a function of the proportion of the exopolymer present in the system. This information may be useful in order to establish a criterion for the evaluation of the protective effect caused by this exopolymer against the action of this surfactant, which has been demonstrated to be a good solubilization agent for PC liposomes [14, 16, 18].

Materials and methods

PC was purified from egg lecithin (Merck, Darmstadt, Germany) according to the method of Singleton et al. [19] and was shown to be pure by thin-layer chromatography (TLC). The nonionic surfactant $T_{X\text{-}100}$, octylphenol polyethoxylated with ten units of ethylene oxide and active matter of 100%, was purchased from Rohm and Haas (Lyon, France). Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) was obtained from Merck. PIPES buffer was prepared as 20 mM PIPES adjusted to pH 7.20 with NaOH, containing 110 mM Na_2SO_4. The starting material 5(6)-carboxyfluorescein (CF) was obtained from Eastman Kodak (Rochester, N.Y., USA) and was further purified by column chromatography [20]. The glycoprotein (GP) produced by *P. antarctica* NF_3 was excreted by this microorganism into the culture medium and, consequently, did not form part of the bacterial cell wall. The original isolate was obtained from a sludge sample collected at the bottom of a glacier in the region of Inlet Admiralty Bay (King George Island, South Shetland Islands) [21, 22]. The purified GP is at present available on a laboratory scale [23].

Preparation and characterization of GP/liposome systems

Unilamellar PC liposomes of about 200 nm were prepared by extrusion of large unilamellar vesicles obtained by reverse-phase evaporation in PIPES buffer [14, 15]. Liposomes were extruded through 800–200 nm polycarbonate membranes at 25 °C using a thermobarrel extruder equipped with a thermoregulated cell compartment (Lipex Biomembranes, Vancouver, Canada) to achieve a uniform size distribution. To determine the liposome permeability alterations, PIPES buffer was supplemented with 100 mM CF. Liposomes were combined with GP aqueous dispersions to obtain different GP/liposome mixtures (PC/GP weight ratios 9:1, 8:2 and 7:3) [11]. The resulting GP/liposome aggregates were freed of the GP nonassembled with liposomes. To this end, the aggregates were sedimented at 140 000 *g* at 25 °C for 2 h and then resuspended in PIPES buffer. No PC was detected by TLC coupled to an automated flame ionization detection (TLC-FID) [24] in any supernatant in spite of the opalescent nature due to the presence of free GP. The amount of GP assembled with liposomes (versus PC concentration) was determined as the difference between the amount of GP added and that remaining in the supernatant after sedimentation of the GP/liposome aggregates. This remaining amount (directly related to the opalescence of supernatants) was determined by measuring the static light scattering of the supernatants using a Shimadzu RF-540 spectrofluorophotometer at 25 °C with both monochromators adjusted to 500 nm [17].

To study the permeability changes, vesicles containing CF were freed of the unencapsulated CF by passage through Sephadex G-50 medium resin (Pharmacia, Uppsala, Sweden) by column chromatography [15, 16]. The PC concentration in the liposomes was determined by TLC-FID [24]. The vesicle size distribution after preparation was determined with a photon correlator spectrometer (Malvern Autosizer 4700c PS/MV) [18]. After preparation the size of the vesicles varied very little showing a similar value of about 200 nm (polydispersity index lower than 0.12), thereby indicating that the vesicle distribution was very homogeneous.

Parameters involved in the interaction of $T_{X\text{-}100}$ with coated liposomes

In the analysis of the equilibrium partition model proposed by Schurtenberger et al. [25] for bile salt/lecithin systems, Lichtenberg [26] and Almog et al. [27] have shown that for a mixture of lipids, at a concentration L (millimole), and surfactant, at a concentration S_T (millimole), in dilute aqueous media, the distribution of surfactant between lipid bilayers and aqueous media obeys a partition coefficient K given (in units of reciprocal millimole) by

$$K = S_B/[(L + S_B)S_W] \quad , \tag{1}$$

where S_B is the surfactant concentration in the bilayers (millimole) and S_W is that in the aqueous medium (millimole) [14]. For $L \gg S_B$, the definition of K, as given by Schurtenberger, applies:

$$K = S_B/(L\,S_W) = Re/S_W \quad , \tag{2}$$

where Re is the effective surfactant-to-lipid molar ratio in the bilayers ($Re = S_B/L$). Under any other conditions, Eq. (2) has to be employed to define K; this yields

$$K = Re/S_W[1 + Re] \quad . \tag{3}$$

The determination of Re, S_W and K was carried out on the basis of the linear dependence existing between the surfactant concentrations needed to reach the interaction steps studied and the PC concentration in liposomes, which can be described by the equation

$$S_T = S_W + Re\,L \quad , \tag{4}$$

where, in each curve, Re and S_W are, the slope and the ordinate at the origin (zero PC concentration) respectively. Permeability changes caused by $T_{X\text{-}100}$ in liposomes coated with increasing amounts of GP were determined by monitoring the rise in the fluorescence intensity of liposomes due to the CF released from the interior of vesicles into the bulk aqueous phase. Fluorescence measurements were made with a Shimadzu RF-540 spectrofluorophotometer [16].

Results and discussion

We previously reported the ability of the GP excreted by *P. antarctica* NF_3 to coat PC liposomes [10–12]. In the present study, we first determined the proportion of GP assembled with these liposomes for different amounts of GP added to the system (PC concentration 5.0 mM). The percentages of GP remaining in the supernatants after sedimentation of the GP/liposome aggregates (determined by static light scattering) were 6, 9 and 17% for the systems formed by the PC/GP weight ratios 9:1, 8:2 and 7:3, respectively. As a consequence, the weight percentages of assembled GP (with respect to the PC) were 9.4, 18.2 and 24.9%, respectively.

Interaction of $T_{X\text{-}100}$ with coated PC liposomes

It is known that in surfactant/lipid systems complete equilibrium may take several hours [8, 27]; however, in subsolubilizing interactions a substantial part of the surfactant effect takes place within approximately 30 min after the addition of surfactant to the liposomes [28]. In order to determine the time in which the leakage ceased, a kinetics study of the interaction of $T_{X\text{-}100}$ with coated liposomes containing CF was carried out (PC concentration ranging from 0.5 to 5.0 mM). Coated vesicles were treated with $T_{X\text{-}100}$ at subsolubilizing concentrations and subsequent changes in CF release

were studied as a function of time. The CF release always showed a transient state of enhanced permeability of the liposomal bilayers, for which about 40–60 min was needed to achieve a CF release plateaux for a PC/GP weight ratio ranging from 9:1 to 7:3. This behavior was possibly due to the release of the flourescent dye encapsulated into the vesicles through holes or channels created in the membrane and was not due to bilayer fusion. The incorporation of surfactant monomers into coated membranes may directly induce the formation of hydrophilic pores or may merely stabilize transient holes, in agreement with the concept of transient channels suggested by Schubert et al. [29]. It is noteworthy that this effect took place in all cases regardless of the amount of GP present in the system. The only difference was the fact that the time needed to achieve the aforementioned CF release plateaux increased with the percentage of GP in the system. Hence, permeability alterations were studied 60 min after the addition of surfactant to the systems at 25 °C. The release of CF in these systems in the absence of $T_{X\text{-}100}$ 60 min after preparation was negligible.

To determine the partitioning of $T_{X\text{-}100}$ between lipid bilayers and the aqueous phase we first studied the validity of the equilibrium partition model proposed by Lichtenberg [26] and Almog et al. [27], based on Eq. (1) for the systems studied. This equation may be expressed by $L/S_B = (1/K)(1/S_W) - 1$. Hence, this validity requires a linear dependence between L/S_B and $1/S_W$; this line should have a slope of $1/K$, intersect with the L/S_B axis at -1 and intersect with the $1/S_W$ axis at K.

To test the validity of this model for the systems investigated, coated liposomes (at various PC/GP weight ratios) were mixed with varying sublytic $T_{X\text{-}100}$ concentrations (S_T). The resulting surfactant-containing vesicles were then spun at 140 000 g at 25 °C for 2 h to remove the vesicles. No PC was detected in the supernatants by TLC-FID [24]. The $T_{X\text{-}100}$ concentration in the supernatants (S_W) was determined by high-performance liquid chromatograph [30] and its concentration in the lipid bilayers was calculated ($S_B = S_T - S_W$). The S_B and S_W values obtained (over the same range of PC and $T_{X\text{-}100}$ concentrations used to determine K) were plotted in terms of the dependence of L/S_B on $1/S_W$. Straight lines were obtained for each system tested ($r^2 = 0.990$, 0.993 and 0.991 for the PC/GP weight ratios 9:1, 8:2 and 7:3, respectively). These straight lines were dependent on L and always intersected the L/S_B axis at -0.97 ± 0.11. Both the linearity of these dependances and the proximity of the intercept to -1 support the validity of this model to determine K for these surfactant/liposome systems.

To determine the Re, S_W and K values, a systematic study of permeability changes of CF-containing liposomes was performed for liposomes coated with increasing amounts of GP (PC/GP weight ratio ranging from 9:1 to 7:3). In each case the concentration of PC in the liposomes varied from 0.5 to 5.0 mM. The CF release curves for the PC/GP weight ratio 7:3 as a function of $T_{X\text{-}100}$ concentration are given in Fig. 1 (the curves for the other systems are not shown). The surfactant concentrations producing different percentages of CF release were obtained graphically and were plotted versus the PC concentration. An acceptable linear relationship was established in each case. The straight lines obtained corresponded to Eq. (4) from which Re and K were determined. The Re, K and S_W values as well as the regression coefficients (r^2) of the straight lines for the PC:GP weight ratios 9:1, 8:2 and 7:3 are given in Table 1.

The Re values always increased as the release of the trapped dye increased. Furthermore, rising GP proportions in the system led (for the same interaction step) to a progressive increase in Re. As for K, these values decreased as the release of the trapped dye increased in all cases. Increasing GP proportions resulted in a decrease in this parameter. In addition, the S_W values always increased as the percentage of CF released increased, in line with the data reported for the interaction of pure PC liposomes with $T_{X\text{-}100}$ [14, 16]. Increasing GP proportions in the system resulted in a progressive increase in S_W. The fact that higher free surfactant concentrations were needed to reach the same liposome alterations suggests that the GP coating structure acts as a physical barrier that progressively protects these bilayers, in agreement with our previous transmission electron microscopy observation [10–12]. Furthermore, the fact that the S_W values were always lower than the surfactant critical micelle concentration (0.15 mM) [14] indicates that the interaction was mainly ruled by the action of surfactant monomers in all cases.

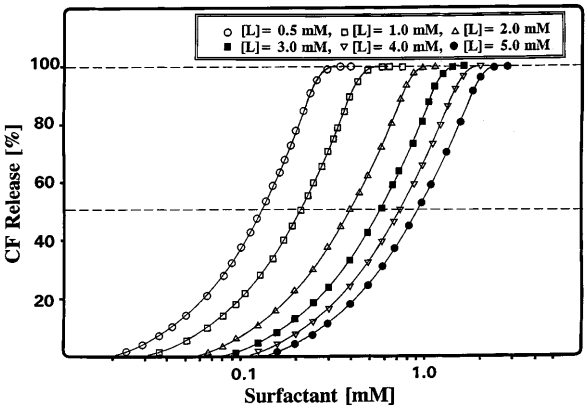

Fig. 1 Percentage changes in 5(6)-carboxyfluorescein (*CF*) release induced by Triton X-100 ($T_{X\text{-}100}$) in phosphatidylcholine (*PC*) liposomes (PC concentration ranging from 0.5 to 5.0 mM) coated with glycoprotein (*GP*) at the PC/GP weight ratio 7:3. Lipid concentration 0.5 mM (○), 1.0 mM (□), 2.0 mM (△), 3.0 mM (■), 4.0 mM (▽) and 5.0 mM (●)

Table 1 Effective surfactant-to-lipid molar ratio in the bilayers (*Re*), partition coefficient (*K*) and surfactant concentration in the aqueous medium (S_W) parameters as well as the regression coefficients (r^2) resulting in the interaction of Triton X-100 with phosphatidylcholine (*PC*) liposomes coated with increasing proportions of glyroprotein (*GP*). PC/GP weight ratios: 9:1, 8:2, and 7:3

5(6)-carboxy fluorescein release (%)	*Re*			*K*			S_W			r^2		
	PC:GP weight ratio			PC:GP weight ratio			PC:GP weight ratio			PC:GP weight ratio		
	9:1	8:2	7:3	9:1	8:2	7:3	9:1	8:2	7:3	9:1	8:1	7:3
10	0.03	0.03	0.04	1.62	1.50	1.43	0.018	0.020	0.027	0.990	0.992	0.999
20	0.05	0.06	0.07	2.38	2.26	2.18	0.020	0.025	0.031	0.993	0.995	0.991
30	0.08	0.09	0.09	2.75	2.60	2.50	0.027	0.032	0.033	0.993	0.992	0.995
40	0.11	0.12	0.13	2.83	2.79	2.61	0.035	0.039	0.044	0.992	0.995	0.992
50	0.15	0.16	0.17	3.00	2.87	2.77	0.046	0.048	0.052	0.999	0.995	0.996
60	0.19	0.20	0.21	2.95	2.82	2.75	0.054	0.059	0.063	0.994	0.998	0.997
70	0.22	0.23	0.24	2.91	2.79	2.73	0.062	0.067	0.071	0.995	0.999	0.992
80	0.26	0.28	0.29	2.86	2.73	2.68	0.072	0.080	0.084	0.996	0.993	0.997
90	0.28	0.30	0.31	2.80	2.71	2.63	0.078	0.086	0.090	0.992	0.990	0.991
100	0.37	0.38	0.40	2.70	2.52	2.40	0.100	0.109	0.119	0.989	0.993	0.991

The variation in the release of the trapped dye (percentage) versus *Re* when varying the PC/GP weight ratio in the system from 9:1 to 7:3 is shown in Fig. 2. The *Re* values reported for pure PC liposomes are also included [14]. A linear relationship between these two parameters was established up to 90% CF release, regardless of the proportion of GP added to the system; however, the presence of increasing amounts of GP led (for the same interaction step) to a rise in the *Re* values. Given that the surfactant capacity to alter the permeability of liposomes is inversely related to the *Re* values, the increasing presence of GP reduced this capacity; hence, the protection of liposomes against $T_{X\text{-}100}$ increased with the proportion of GP in the bilayers. The fact that the *Re* curves showed a similar trend to that exhibited by the curve for pure PC liposomes suggests that the presence of increasing amounts of GP almost did not affect the mechanism of interaction between surfactant and PC bilayers; however, this increasing presence progressively reduced the surfactant activity on these bilayer structures.

The variations in the *Re* and *K* values versus the percentage of GP assembled with liposomes are plotted in Figs. 3 and 4, respectively. A linear relationship was established between the *Re* and *K* values and the percentage of GP assembled; hence, both the surfactant ability to alter the permeability of liposomes and its affinity with these bilayer structures showed an inverse linear dependence on the amount of GP assembled over the range of PC/GP weight ratios investigated. These findings underline the progressive protective effect caused by this exopolymer on PC liposomes as well as the homogeneity of the coating structure formed, in agreement with our previous studies [11, 12].

The fact that at 100% CF release the surfactant always showed lower *K* values than those for 50% CF release (Fig. 4) could be attributed to the progressive saturation of the bilayers by the surfactants (the

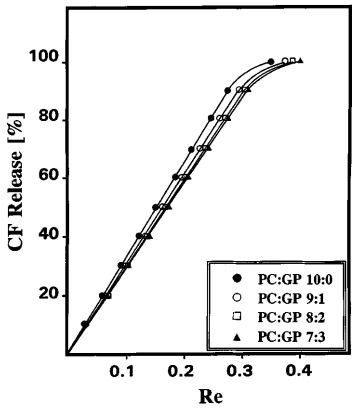

Fig. 2 Variation in the percentage of CF release of liposomes coated with increasing proportions of GP due to the action of $T_{X\text{-}100}$ versus the effective surfactant-to-PC molar ratio (*Re*). PC/GP weight ratios: 10:0 (●), 9:1 (○), 8:2 (□) and 7:3 (▲)

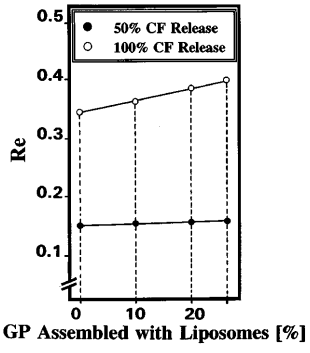

Fig. 3 Variation in the effective surfactant-to-PC molar ratio (*Re*) versus the percentage of GP assembled with liposomes. 50% CF release (●), 100% CF release (○)

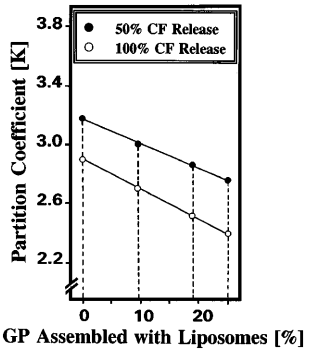

Fig. 4 Variation in the surfactant partition coefficients (K) between bilayers and the aqueous phase versus the percentage of GP assembled with liposomes. 50% CF release (●), 100% CF release (○)

amounts of surfactants in the aqueous phase increased more than in the bilayers). This behavior is in line with that reported by Paternostre et al. [6], when studying the interaction of the nonionic surfactant octyl glucoside (OG) with PC liposomes and with our previous studies on the effects of OG and $T_{X\text{-}100}$ with these bilayer structures [14, 31]. It is noteworthy that this effect occurred regardless of the GP assembled with liposomes and that the difference between the K values for 50 and 100% CF release increased with the proportion of GP assembled (from 0.3 for 9.4% GP assembled with liposomes to 0.37 for 24.9% GP assembled with these bilayer structures). As a consequence, the process of saturation of bilayers by the surfactant was directly affected by the presence of increasing amounts of GP assembled with liposomes.

From these findings we may conclude that the GP structure that coated PC liposomes when these vesicles were incubated with this exopolymer acted as a physical barrier. This barrier hampered the action of $T_{X\text{-}100}$ against the PC vesicles, reducing its sublytic activity as well as its affinity with these bilayer structures. However, the mechanisms of interaction of surfactant with liposomes were almost unaffected by the increasing presence of this exopolymer.

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