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The presence of PEG-lipids in liposomes does not reduce melittin binding but decreases melittin-induced leakage

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Abstract

Poly(ethyleneglycol) (PEG), anchored at the surface of liposomes via the conjugation to a lipid, is commonly used for increasing the liposome stability in the blood stream. In order to gain a better understanding of the protective properties of interfacial polymers, we have studied the binding of melittin to PEG-lipid-containing membranes as well as the melittin-induced efflux of a fluorescent marker from liposomes containing PEG-lipids. We examined the effect of the polymer size by using PEG with molecular weights of 2000 and 5000. In addition, we studied the role of the anchoring lipid by comparing PEG conjugated to phosphatidylethanolamine (PE) which results in a negatively charged PEG-PE, with PEG conjugated to ceramide (Cer) which provides the neutral PEG-Cer. Our results show that interfacial PEG does not prevent melittin adsorption onto the interface. In fact, PEG-PE promotes melittin binding, most likely because of attractive electrostatic interactions with the negative interfacial charge density of the PEG-PE-containing liposomes. However, PEG-lipids limit the lytic potential of melittin. The phenomenon is proposed to be associated with the change in the polymorphic tendencies of the liposome bilayers. The present findings reveal that the protective effect associated with interfacial hydrophilic polymers is not universal. Molecules like melittin can sense surface charges borne by PEG-lipids, and the influence of PEG-lipids on liposomal properties such as the polymorphic propensities may be involved in the so-called protective effect.

Keywords: Poly(ethylene glycol); Liposome; Melittin; Efflux; Binding; Spectroscopy

1. Introduction

Liposomes used as drug vectors are often coated with hydrophilic polymers. This strategy leads to an increased stability of the drug carrier and to an extended period of circulation in the blood stream

Abbreviations: PEG, poly(ethyleneglycol); PE, phosphatidylethanolamine; Cer, ceramide; PLA2, phospholipase A2; PEG2000, PEG with a molecular weight of 2000; PEG5000, PEG with a molecular weight of 5000; PEG2000-Cer, *N*-myristoyl-sphingosine-1-[succinyl-(methoxy poly(ethyleneglycol))2000]; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; PEG2000-PE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethyleneglycol)2000]; PEG5000-PE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethyleneglycol)5000]; SRB, sulforhodamine B; EDTA, ethylenediaminetetraacetic acid; LUV, large unilamellar vesicle; PC, phosphatidylcholine

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[1–3]. Initially inspired by the long circulation time of red blood cells, gangliosides, primarily the glycolipid GM1, were introduced into vesicles to provide the interfacial protective coating [4,5]. Subsequently, it has been found that simpler and less expensive macromolecules such as poly(ethyleneglycol) (PEG) can cause a similar protective effect and also lead to 'stealth' liposomes [6,7]. Despite the fact that stealth liposomes are generated by covering the membrane surface with grafted hydrophilic macromolecules, our current understanding of the origin of the protective effect is limited. The nature of the grafted macromolecules has an influence on the protective properties. In the case of PEG, differences in the molecular weight, grafting density, and the anchoring group of the PEG-lipids have been shown to lead to differences in their protective effect [3,8–13]. A commonly used anchor for PEG is the phosphatidylethanolamine (PE) molecule since the amino moiety of the polar head group of PE is easily exploited for the coupling [1]. The performances of PEG-PEs have been found to be sensitive to the linkage group as chemical stability is affected [8]. The coupling reaction usually transforms the positively charged ammonium group into a neutral carbamate linkage and as a consequence, the PEG-PE molecule bears a negative charge due to its phosphate group [7]. Recently, a neutral analogue has been synthesized by coupling the PEG moiety to the sphingolipid ceramide (Cer), PEG-Cer [10]. The comparison between PEG-PE and PEG-Cer revealed some differences in the pharmacokinetics of liposomal vincristine formulations [10]. Experimental and theoretical studies [2,3,9,11,12, 14–16] have shown that the grafting density of interfacial PEG influences protein binding. Interfacial polymers in the brush regime severely inhibit protein adsorption onto the surface. However, at grafting densities which are usually used to generate stealth liposomes, the interfacial polymers were shown to be in fact in the mushroom regime [16,17]. The inhibition of protein adsorption observed in this case has been explained in terms of excluded surface [16] as well as spacing and overlapping of the grafted PEG chains [9].

In parallel, the presence of interfacial PEG does not appear to provide a universal protection against adsorption of molecules onto coated vesicles. PEG-lipids were shown to limit the adsorption of hydrophilic polymers such as monoacylated PEG [16], of proteins such as albumin, cytochrome *c*, and fibronectin [3,9,12], and even of cells such as erythrocytes and macrophages [3]. On the other hand, PEG-lipids showed practically no effect on the release of propidium trapped inside vesicles when these vesicles were exposed to human serum [18], or on the release of vesicle-entrapped calcein when the vesicles were exposed to poly(2-ethylacrylic acid) [19]. Similarly, the enzymatic activity of phospholipase A2 (PLA2) is not inhibited but rather promoted by interfacial PEG-PEs incorporated into vesicles [20,21]. It has been proposed that the size and the shape of the protein as well as the protein–surface interactions can be critical parameters influencing the protective properties of interfacial PEG [9,15,16]. The understanding of the origin of these observed differences is necessary to have a rational approach in the design, development and optimization of interfacial macromolecules leading to more efficient stealth liposomes.

Recently, we have examined the origin of the protective effect of interfacial PEG [16]. Experimental studies of binding of monoacylated PEG to liposomes containing PEG-PE, combined with Monte Carlo simulations of the PEG-coated surface, led to the conclusion that the decreased binding of monoacylated PEG to PEG-lipid-containing liposomes results from the occlusion of the liposome accessible surface area. The 'surface crowding' caused by PEG limits the membrane association of monoacylated PEG that represented a crude model for acylated proteins in this context. The observed effect was sensitive to the size and the surface concentration of interfacial PEG. At concentrations typically used to generate PEG-stabilized vesicles (≤ 10 mol% of PEG-PE with a PEG molecular weight of 2000 (PEG2000) and ≤ 6 mol% of PEG-PE with a PEG molecular weight of 5000 (PEG5000)), it was found that the hydrophilic polymer remains in the so-called 'mushroom' regime. This conclusion implies that the PEG monomer density perpendicular to the membrane reaches a maximum at about 1–2 nm above the liposome surface, leaving a monomer-depleted layer at the proximity of the water–liposome interface. This depletion layer may have an impact on the protective efficiency of the interfacial polymers. A further increase in the PEG-lipid concentration

does not lead to a transition from the 'mushroom' to the 'brush' regime as the vesicles become then unstable and the formation of micelles can be observed [16,22].

In order to gain a better insight into the role played by interfacial PEG, we have investigated the effect of PEG-lipids on the binding and the lytic activity of melittin. This 26 amino acid peptide that is the major component of bee venom forms in membranes an α-helix with a secondary amphipathic character [23]. This structural feature is shared with segments of complement system proteins [24,25]. We have examined how the presence of interfacial PEG modulates, first, the affinity of melittin for liposomes and, second, the extent of melittin-induced leakage of a fluorescent marker entrapped in liposomes. The effects of the PEG molecular weight and the type of PEG anchor on these processes were investigated as well. The present results bring some new insights into the understanding of the protective effect of interfacial PEG.

2. Materials and methods

N-Myristoyl-sphingosine-1-[succinyl(methoxy poly(ethyleneglycol))2000] (PEG2000-Cer) as well as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Northern Lipids Inc. (Vancouver, BC, Canada). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethyleneglycol)2000] (PEG2000-PE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethyleneglycol)5000] (PEG5000-PE) were obtained from Avanti Polar Lipids (Birmingham, AL, USA). Melittin was purified from bee venom (Sigma, St. Louis, MO, USA) by ion exchange chromatography and then desalted according to the high-performance liquid chromatography procedure previously described [26]. Sulforhodamine B (SRB) was purchased from Molecular Probes, Inc. (Eugene, OR, USA). Ethylenediaminetetraacetic acid (EDTA) was bought from Aldrich (Milwaukee, WI, USA).

2.1. Binding experiments

PEG-lipid/POPC mixtures were prepared by combining the appropriate volumes of benzene/methanol (95/5, v/v) solutions of individual lipids and then lyophilizing the organic solvents. The indicated proportions of PEG-lipids refer to the molar percentages relative to the total amount of lipids. The lipid mixture was hydrated in a HEPES buffer (50 mM HEPES, 136 mM NaCl, and 5 mM EDTA, pH 7.4). Large unilamellar vesicles (LUVs) of about 100 nm diameter were obtained by extrusion. The intrinsic fluorescence of the tryptophan group of melittin was used to quantify the binding of melittin to lipid vesicles as described previously [27,28]. Briefly, melittin was suspended in the HEPES buffer and its concentration was determined by UV absorption at 280 nm ($\varepsilon = 5570\ M^{-1}\ cm^{-1}$). For the binding experiments, the solution was diluted to a working concentration of 4.7 μM in a 10×10-mm quartz cuvette. Lipid aliquots were added stepwise to the melittin solution and the tryptophan emission spectrum was recorded after each addition of lipid ($\lambda_{excitation} = 280$ nm). An emission spectrum obtained from a blank (i.e. without melittin) was recorded and then subtracted from the tryptophan emission spectrum to eliminate the Raman band of water and correct light-scattering effects. The emission wavelength was determined according to the center of gravity of the top 25% of the emission band.

2.2. Leakage experiments

The extent of melittin-induced leakage was examined by recording the release of SRB from liposomes [29,30]. LUVs were prepared as described above except that a SRB-containing buffer (80 mM SRB, 50 mM HEPES, 20 mM NaCl, and 5 mM EDTA, pH 7.4) was used to hydrate the lipids. The SRB-containing vesicles were separated from free dye molecules using a column filled with Sephadex G-50 fine gel swollen in a HEPES buffer (50 mM HEPES, 136 mM NaCl, and 5 mM EDTA, pH 7.4) which was isosmolar to the SRB-containing buffer. Both buffers had an osmolarity of about 350 mOsm/kg.

The fraction of dye-loaded LUVs that eluted from the column was diluted in the HEPES external buffer to obtain a final lipid concentration of about 3–10 μM in the cuvette. The exact lipid concentrations were determined by the Fiske–Subbarow assay [31]. The high concentration of encapsulated SRB led to

self-quenching of its fluorescence, which resulted in a low background fluorescence intensity for the vesicle dispersion (I_B). The addition of an aliquot of melittin solution to the vesicle dispersion caused the release of SRB into the outside medium. This leakage was monitored by measuring the increased SRB fluorescence intensity (I_F) resulting from its dilution outside the vesicles. After a time course of 10 min, Triton X-100 (0.1 vol%) was added to release all SRB molecules trapped inside the vesicles and then the corresponding total fluorescence intensity (I_T) was measured. The percentage of SRB release was calculated according to:

$$\% \text{ release} = 100(I_F - I_B)/(I_T - I_B)$$

In using this equation, it is assumed that melittin-induced leakage proceeds via an all-or-none mechanism, as previously observed for melittin-induced leakage with several lipid systems, including some negatively charged vesicles [29,32]. If the efflux mechanism is affected by interfacial PEG, the reported efflux should be considered as an approximation. The excitation and emission wavelengths of SRB were 565 and 586 nm, respectively. All the fluorescence measurements were performed on a SPEX Fluorolog-2 spectrometer, equipped with a mini-sample stirrer. Both excitation and emission band path widths were set to 0.5 nm.

2.3. Infrared spectroscopy

POPC and PEG-lipid mixtures were hydrated with a HEPES buffer (50 mM, 136 mM NaCl, and 5 mM EDTA) pH 7.4 prepared in D_2O, to obtain a final lipid proportion in the sample of 50% (w/w). The samples were subjected at least twice to a freeze–thaw cycle (liquid nitrogen/room temperature) during which they were repeatedly vortexed to ensure their homogeneous hydration. An aliquot of a sample was transferred between two CaF_2 windows separated by a Teflon ring of 5 μm thickness. The IR spectra were recorded on a Bio-Rad FTS-25 spectrometer equipped with a water-cooled global source and a medium-band mercury–cadmium–telluride detector. For each spectrum, 100 scans with a resolution of 2 cm^{-1} were coadded and Fourier-transformed using a triangular apodization function. Fourier deconvolution was applied to the C=O stretching band re-

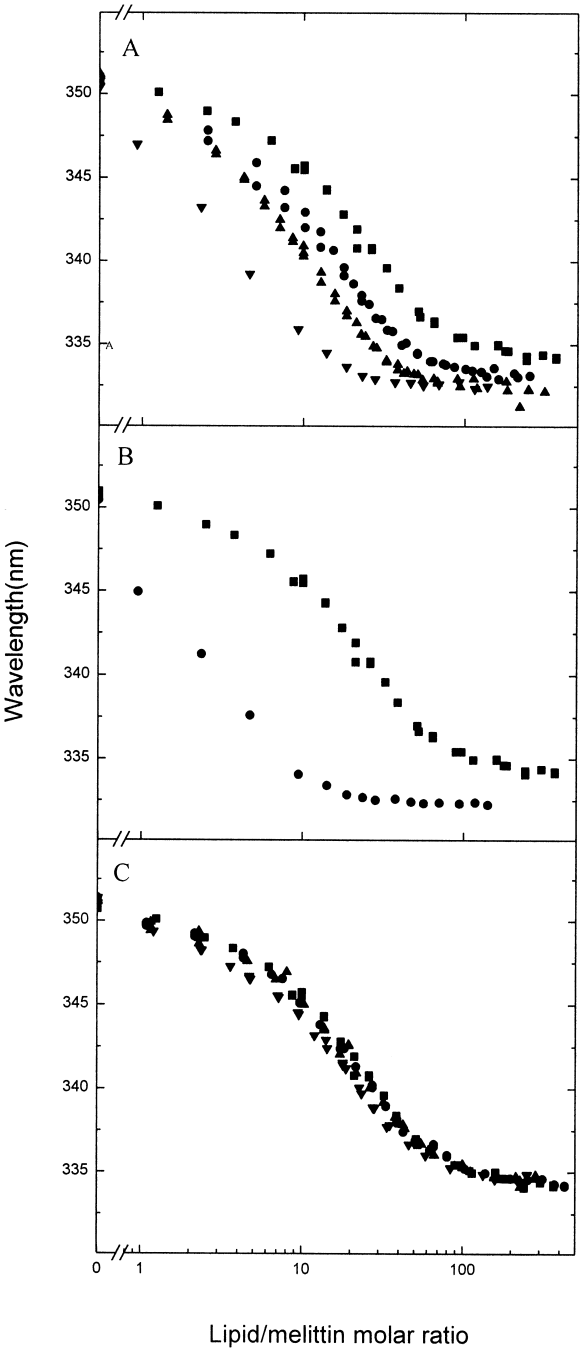

Fig. 1. Influence of interfacial PEG on melittin association to POPC vesicles. The shift of the fluorescence maximum of melittin is displayed as a function of increasing lipid concentration. (A) POPC vesicles (■), POPC vesicles containing 2% (●), 6% (▲) and 10% (▼) PEG2000-PE, respectively. (B) POPC vesicles (■), POPC vesicles containing 6% PEG5000-PE (●). (C) POPC vesicles (■), POPC vesicles containing 2% (●), 6% (▲) and 10% (▼) PEG2000-Cer, respectively.

gion to highlight the presence of the two components.

3. Results and discussion

3.1. Binding of melittin to POPC liposomes containing PEG-PE and PEG-Cer lipids

The effect of the presence of interfacial PEG-lipids on melittin binding to POPC vesicles was examined (Fig. 1). The association of melittin with the vesicles was probed by the shift of the fluorescence maximum of its ^{19}Trp. For free melittin in aqueous solution the maximum of the Trp fluorescence was 351 nm, a value typical for monomeric melittin for which ^{19}Trp is exposed to the aqueous environment [27,28]. Upon addition of vesicles, the fluorescence maximum shifted progressively to reach a plateau at about 344 nm when virtually all the melittin molecules were associated with the lipid membrane. For pure POPC vesicles, the lipid/melittin molar ratio at which half of the wavelength shift was observed ($R_{0.5}$) was found to correspond to 18, in agreement with previous findings obtained by Trp fluorescence [27] and circular dichroism [33]. When PEG2000-PE was incorporated into POPC vesicles (Fig. 1A), we observed an increased affinity of melittin for the lipid matrix, despite the increase in surface crowding. This phenomenon is clearly observed from the significant shift of the Trp fluorescence binding curve towards smaller lipid/melittin ratios when PEG2000-PE is introduced in the POPC matrix. It has been shown that the shift of the maximum of the fluorescence band cannot be translated in a straightforward manner into the absolute proportion of membrane-bound melittin [34]. However the fluorescence shift reports faithfully the relative extent of melittin binding and has been used to determine the trends of the influence of interfacial PEG on melittin binding. $R_{0.5}$ is reduced by a factor of six when 10% PEG2000-PE is incorporated into POPC vesicles ($R_{0.5} = 3$) in comparison to pure POPC vesicles ($R_{0.5} = 18$). This increased binding of melittin in the presence of PEG2000-PE is a progressive effect; at 6% PEG2000-PE, the value of $R_{0.5}$ is about 9 (Fig. 1A). This phenomenon is sensitive to the size of PEG grafted to the vesicular surface. Incorporation of 6% PEG5000-PE into POPC vesicles led to an $R_{0.5}$ of about 2 compared to an $R_{0.5}$ of about 9 for 6% PEG2000-PE (Fig. 1B). It should be noted that POPC vesicles containing 6% PEG5000-PE or 10% PEG2000-PE did not display the full sigmoidal shape as observed for the other binding isotherms but the fluorescence maximum shift was abrupt upon the addition of the first lipid aliquots.

When PEG is anchored to a Cer moiety, the increase in melittin binding with increasing PEG-Cer content is much more limited (Fig. 1C). In the presence of 6% PEG2000-Cer, no significant difference compared to pure POPC vesicles is observed whereas a proportion of 10% PEG2000-Cer leads to a small decrease of $R_{0.5}$ from 18 (pure POPC) to about 15.

These results clearly indicate that surface crowding induced by the presence of interfacial PEG does not prevent melittin adsorption. Actually, in the case of PEG-PE, the opposite effect was observed and the affinity of melittin towards membranes containing this PEG-lipid is promoted. It is well established that the introduction of negatively charged lipids in membranes increases melittin affinity [35,36]. This increase is in the same order of magnitude as that observed with PEG-PE, and it is likely that electrostatic interactions between the negatively charged phosphate group of PEG-PE and the positively charged residues of melittin are responsible for the enhanced affinity. This conclusion is supported by the absence of an effect on melittin binding in the case of the neutral PEG-Cer. It has been found that PEG-PE-containing vesicles made of phosphatidylcholine (PC) displayed a negative electrophoretic mobility, conversely to neutral PC vesicles [7,10]. The value of the mobility for PEG-PE-containing vesicles was considerably smaller than those obtained for bilayers containing negatively charged phospholipids such as phosphatidylglycerol. This effect has been associated with the hydrodynamic drag caused by the interfacial PEG [7]. However, measurements of the interfacial surface charge using the fluorescent probe TNS [7] have actually shown that PEG-PE and phosphatidylglycerol provide the same surface potential. This finding is reasonable because the charge carried by PEG-PE should lie in a plane located at the head group level. Therefore, for molecules like melittin that insert into a membrane at the

interfacial level, the local surface potential is therefore expected to be significantly negative.

Our measurements also show that surface crowding does not prevent melittin binding, even for the neutral PEG-Cer lipids. The polymer depletion layer predicted by previous Monte Carlo simulations may be the origin of the lack of the protective effect of PEG-lipids against melittin binding. It has been established that membrane-bound melittin adopts a α-helical structure and that the N-terminal helical segment is inserted into the membrane whereas the C-terminal segment lies roughly parallel to the interface [23,37,38]. Molecular dynamics simulations and X-ray diffraction experiments have proposed that melittin is located at a position corresponding roughly to the position of the lipid carbonyl groups [37,39]. Therefore, the location of the adsorbed melittin is below the level where interfacial crowding is at its maximum; the maximum monomer density was estimated to be about 1 and 2 nm above the interface for PEG2000 and PEG5000, respectively [16]. The relative position of the peptide may be associated with the absence of a protective impact of interfacial PEG on melittin association. The effect of the size of the binding molecule will be discussed below in more general terms.

It should be noted that the presence of 6% PEG5000-PE in POPC vesicles promotes melittin binding slightly more than 6% PEG2000-PE. It was shown that this PEG5000-PE proportion is close to proportions leading to bilayer destabilization and micelle formation [16,22,40]. In this case, it is possible that local bilayer instabilities caused the enhanced binding that is observed.

3.2. *Infrared spectroscopy with PEG-PE and PEG-Cer/POPC mixtures*

In order to examine the effect of PE-lipids on the vesicle interface, we have analyzed the carbonyl stretching band in the IR spectra of the lipid mixtures (Fig. 2). This band shows two components, which are highlighted using Fourier self-deconvolution. The components at 1730 and 1745 cm^{-1} have been attributed to hydrogen-bonded and free carbonyl groups, respectively [41,42], and therefore the rel-

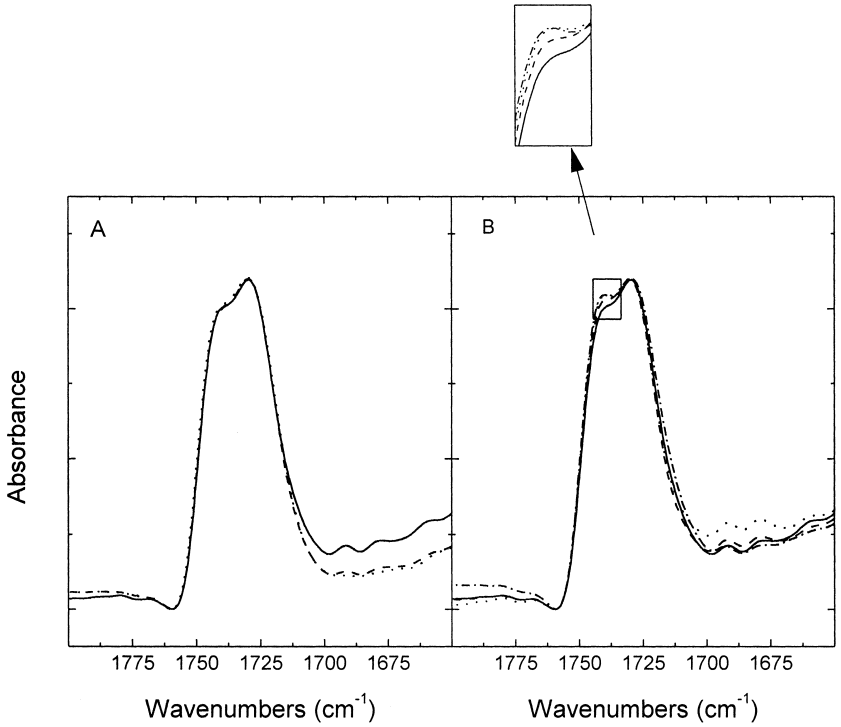

Fig. 2. Fourier self-deconvoluted carbonyl stretching region for (A) POPC vesicles (straight line), POPC vesicles containing 6% (dashed line) and 10% (dotted line) PEG2000-Cer, respectively. (B) POPC vesicles (straight line), POPC vesicles containing 6% (dashed line) and 10% (dotted line) PEG2000-PE and 10% POPE (dashed/dotted line), respectively.

ative intensities of these components reflect the hydration of the vesicle interface. The presence of 10% PEG2000-Cer in the POPC matrix does not affect at all the profile of the carbonyl stretching band (Fig. 2A). Results available from the literature suggest that Cer itself has a very limited effect on fluid PC bilayers when its molar fraction is less than 0.1 [43–45], in agreement with our present findings. In contrast to PEG-Cer, the presence of PEG2000-PE in POPC leads to an increase of the relative intensity of the component at 1745 cm^{-1} relative to that at 1730 cm^{-1} indicating an increased proportion of free carbonyl groups (Fig. 2B). This effect is progressive with the PEG2000-PE content. We have also recorded the spectrum of a POPE/POPC mixture, and for a molar content of 10%, the effects of PEG-PE and POPE are very similar. This increase in the proportion of non-hydrogen-bonded carbonyl groups may be related to a tighter lipid packing and the resulting dehydration of PE [46,47]. This molecular feature seems to be preserved for PEG2000-PE. The interfacial dehydration reported for PEG-PE is not observed for PEG-Cer. Therefore, it is likely that this effect is not caused by the hydrophilic polymer part but is rather related to the PE anchoring group. These results obtained by IR spectroscopy are consistent with recent fluorescence studies [48,49] indicating that probes at the interface of vesicles that contain PEG-PE experience restricted motions relative to those formed exclusively with PC. This phenomenon has been associated with the more densely packed surface and the less hydrated interface. This ordering effect of PEG-PE lipids at the membrane interface does not appear to be detected at the lipid acyl chain level [48]. It has also been reported that the presence of PEG-PE in liposomes limits their passive permeability to calcein [49], an effect that is consistent with an increased interfacial packing of the lipids.

3.3. *Melittin-induced leakage from POPC liposomes containing PEG-PE and PEG-Cer lipids*

Because of the considerable difference in melittin binding between vesicles incorporating PEG-PE and PEG-Cer, we have examined the protective impact of these interfacial PEGs on the melittin-induced leakage from vesicles by measuring the release of the fluorescent marker SRB. In Fig. 3 we show the efflux of SRB as a function of melittin concentration. In case of pure POPC vesicles, a melittin/lipid molar ratio of 1×10^{-3} is sufficient to induce the release of 50% of the encapsulated SRB and at a ratio of about 6×10^{-3} the release is practically complete. Such a dependence is in agreement with previous results obtained with calcein and carboxyfluorescein [30,50]. Despite the differences in the affinity of melittin towards PEG-PE- and PEG-Cer-containing liposomes, our data show that the melittin-induced efflux of SRB is reduced to a very similar extent for POPC vesicles containing 10% PEG2000-PE or 10% PEG2000-Cer relative to the bare liposomes (Fig.

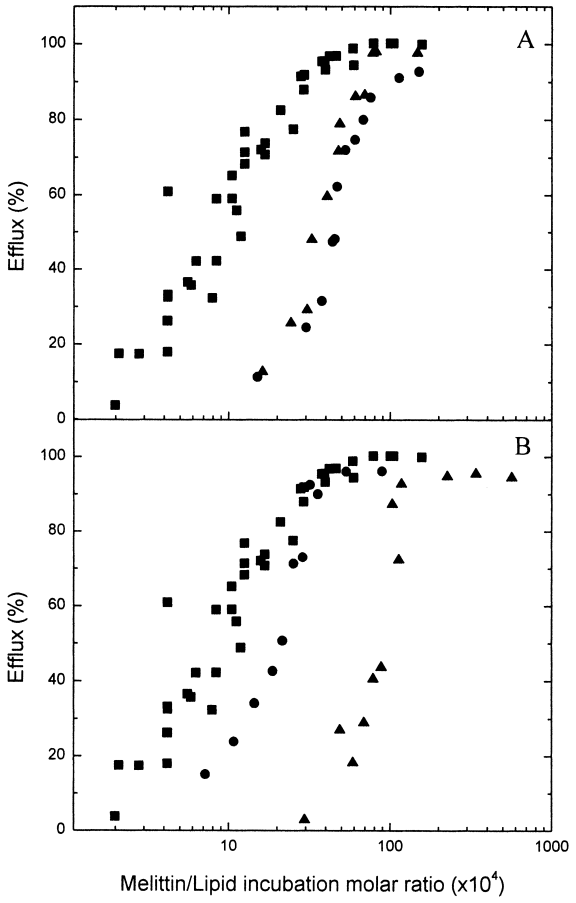

Fig. 3. Effect of interfacial PEG on melittin-induced leakage from POPC vesicles. The percentage of SRB efflux after 420 s is reported as a function of increasing melittin concentration. (A) POPC vesicles (■), POPC vesicles containing 10% PEG2000-PE (●) and 10% PEG2000-Cer (▲), respectively. (B) POPC vesicles (■), POPC vesicles containing 6% PEG2000-PE (●) and 6% PEG5000-PE (▲), respectively.

3A). In this case, the melittin/lipid ratio required to induce the release of 50% of the encapsulated material is 4×10^{-3}, an increase by a factor of four relative to POPC liposomes. The presence of interfacial PEG is therefore a way to control melittin-induced leakage. This permeability regulation is also dependent on PEG size. As shown in Fig. 3B, the protective effect of interfacial PEG against melittin-induced leakage is much more pronounced for PEG5000 than for PEG2000. In fact, the presence of 6% PEG5000-PE in the POPC liposomes leads to an increase of the melittin/lipid ratio required for 50% release of contents by about an order of magnitude (from 1×10^{-3} to 9×10^{-3}). At a ratio of 3×10^{-3}, the bare POPC liposomes are almost completely emptied by melittin whereas POPC liposomes containing 10% PEG5000-PE remain intact.

Interfacial PEG thus protects liposomes against melittin-induced lysis. This phenomenon is clearly not associated with a reduced binding of melittin towards liposomes as a consequence of the interfacial crowding because the opposite was shown in our binding measurements. At least three potential, not necessarily mutually exclusive explanations for this behavior can be proposed. First, it was shown recently that the presence of interfacial PEG inhibits the formation of non-lamellar phases [51,52]. This phenomenon is consistent with the polymorphism of PEG-lipids themselves as they usually have a tendency to form micelles [16,22], a macrostructure associated with amphiphilic molecules possessing a large hydrophilic moiety. The exact mechanism of melittin-induced release is not yet established, but it is possible that it entails the formation of structures similar to the inverted hexagonal phase. In fact, it has been shown that melittin can promote the formation of inverted hexagonal phases in some cases [53]. Therefore, the presence of interfacial PEG could inhibit melittin action through counterbalancing the tendency to form inverted phases and would, in this case, stabilize the bilayers. This hypothesis is supported by the observation that the protective effect is associated with the PEG group itself. We have found that the anchoring part has no significant influence whereas PEG5000-PE exhibits a more effective protection against melittin-induced lysis than PEG2000-PE. In parallel, it was also found that PEG5000-PE is a more effective inhibitor of inverted hexagonal phase formation than PEG2000-PE [52], supporting the hypothesis based on polymorphic propensities. Second, it is possible that interfacial PEG interferes with the diffusion of melittin at the bilayer interface. It has been proposed that melittin-induced lysis can be separated into two steps: (i) melittin adsorption onto the liposomes and (ii) a structural reorganization that may include the diffusion and aggregation of melittin molecules to form defects that lead to leakage [29,54]. Despite the fact that Monte Carlo simulations suggest maximum steric crowding above the level of adsorbed melittin [16], it is possible that the 'stalks' of the interfacial PEG shown to be in the mushroom regime act as obstacles and limit melittin diffusion. In this manner, the formation of melittin aggregates and therefore the formation of defects could be limited. This second explanation is however not supported by the time course of the release because, despite the well defined effect on the extent of efflux, the efflux kinetics of SRB are not affected significantly by the presence of interfacial PEG (data not shown). Third, considering the significant differences in affinity of melittin for vesicles containing PEG2000-Cer and PEG2000-PE, it may be surprising to obtain a similar protective effect with the two kinds of PEG-lipids. It has been shown that bilayers containing negatively charged lipids appear to be more resistant to membrane perturbations induced by melittin despite displaying an increased melittin affinity [29]. This phenomenon has been rationalized by proposing that the electrostatic interactions responsible for the greater affinity would also anchor the peptide at the interface and would prevent it from adopting the position required for the defect formation. In the case of PEG-PE, the electrostatic anchoring of melittin at the interface and the decreased propensities to form inverted phases due to the interfacial PEG could be both responsible for the reduction of melittin-induced leakage. PEG size has a large effect on melittin-induced permeability. However, the type of anchor segment does not play a significant role in melittin-mediated leakage. These observations suggest that the susceptibility of bilayers to the lytic action of melittin is modulated by the interfacial PEG groups themselves.

4. Conclusions

The results presented here indicate that the presence of interfacial PEG displays an ambivalent influence on vesicle–melittin interactions. On one hand, interfacial PEG either promotes the binding of the peptide when anchored to the negatively charged PE, a behavior associated with the anchoring moiety, or has no significant effect in case of the neutral PEG-Cer. On the other hand, PEG-lipids have a protective effect with regard to melittin-induced leakage, a property that is associated with the polymer moiety. The common explanation for the stability of PEG-coated vesicles in the blood stream is the steric crowding of the surface, which prevents the binding of factors that would be responsible for clearance or destabilization. It was shown that PEG-lipids limit the binding of bovine serum albumin, laminin, cytochrome *c* and fibronectin as well as the adhesion of erythrocytes, lymphocytes, and macrophages [3,9,12]. However, this protective effect is not present for smaller molecules like peptides or small proteins. The presence of PEG-PE does not affect the release rate of propidium from vesicles in human serum [18]. It was proposed that small plasma molecules such as peptides and lysolipids would be responsible for the leakage and that these molecules would not sense the steric barrier provided by PEG. Similarly, the release of carboxyfluorescein entrapped in vesicles can be induced by the protonation of poly(2-ethylacylic acid), and, in this case also, the perturbation of membrane integrity is not affected by the presence of interfacial PEG [19]. Along the same line, the binding of a palmitoylated peptide–ethanolamine conjugate was found to be relatively independent of the presence of interfacial PEG [16]. Furthermore, the results obtained with melittin indicate that the affinity of small peptides for vesicles is not necessarily diminished by interfacial PEG. Therefore, it is obvious that steric crowding is not sensed in a universal manner by different molecules, the size of the binding species being a factor influencing the binding behavior. Interfacial PEG appears to be less efficient for small molecules. It has been proposed that the inhibition of protein binding is a result of the occlusion of the accessible surface area [12,16]. The accessible area of a PEG-grafted surface was determined by two-dimensional projections of PEG-coated surfaces using Monte Carlo simulations, and it corresponds to 50 and 40% of the total area for surfaces covered with 10% PEG2000 and 6% PEG5000, respectively [16]. Despite this surface crowding, we did not observe a reduction in melittin binding. As melittin is a relatively small molecule and is bound at the interface level, this phenomenon may be associated with the presence of a polymer depletion layer near the vesicle interface as reported recently [16]. Alternatively, it has been proposed that the inhibition of protein binding occurs when grafted PEG chains overlap, a condition that is associated with the radius of gyration of the interfacial macromolecules [9]. The in-plane radius of gyration of interfacial PEG has also been proposed to vary with the distance from the interface [16]. It is smaller in close proximity of the surface and reaches a maximum at a distance of 1 and 2 nm from the surface for grafted PEG2000 and PEG5000, respectively. This dependence may play a role in the absence of protection provided by interfacial PEG in the mushroom regime, for relatively small molecules like melittin.

The results presented here also highlight that the presence of negative charges carried by the anchoring part of PEG-PE promotes the binding of melittin. This electrostatic contribution should be taken into account in the investigation of the interfacial properties of PEG-covered liposomes. For example, it has been shown recently that PEG2000-PE promotes the activity of PLA2, a 15 kDa enzyme [20,21]. Because it is well established that negatively charged lipids are PLA2 activators [55,56], it is necessary to examine whether electrostatic contributions, which play a role in melittin binding, could also be associated with the activation of PLA2. Despite the fact that steric crowding does not appear to be effective in reducing melittin binding, the presence of the hydrophilic macromolecules at the interface preserves the membrane integrity from a permeability point of view, likely because of the bilayer stabilization or diffusion limitation provided by interfacial PEG. This behavior is clearly related to the presence of PEG at the liposome interface as this effect is dependent on PEG size and very little on the anchoring group. The findings reported here illustrate the necessity to develop a three-dimensional picture of the liposome interface bearing grafted hydrophilic mac-

romolecules that should also include the polymorphic propensities of the lipid bilayer.

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