Interactions of Adriamycin, Cytochrome c, and Serum Albumin with Lipid Monolayers Containing Poly(ethylene glycol)-Ceramide

Hongxia Zhao, Patricia M. Dubielecka, Tim Söderlund, and Paavo K. J. Kinnunen
Helsinki Biophysics and Biomembrane Group, Institute of Biomedicine, University of Helsinki, FIN-00014 Helsinki, Finland

ABSTRACT Poly(ethylene glycol)$_{2000}$C$_{20}$ceramide (PEG-Cer) containing monolayers at an air/water interface were characterized by measuring their surface pressure versus area/molecule (π-*A*) and surface potential versus area/molecule (ΔV-*A*) isotherms. The behavior of π-*A* as well as ΔV versus lipid density (ΔV-*n*) and ΔV-π isotherms for PEG-Cer are in keeping with two transitions of the lipopolymer, starting at $\pi \approx 9$ and 21 mN/m. We also investigated the effects of PEG-Cer on the binding of adriamycin, cytochrome c and bovine serum albumin to monolayers containing varying mole fractions X of PEG-Cer. PEG-Cer impedes the penetration of these ligands into lipid monolayers with similar effects at both X = 0.04 and 0.08. This effect of PEG-Cer depends on the conformation of the lipopolymer and the interactions between the lipid surface and the surface-interacting molecule as well as the size of the latter.

INTRODUCTION

Adsorbed or grafted hydrophilic polymers such as polyethylene glycol (PEG) immobilized at the interface between biofluids and biomaterials have gained considerable attention. This is because of their unique biological inertness, which is considered to result from hydrophilicity and chain mobility as well as lack of ionic charges (Desai and Hubbell, 1991). This inertness allows construction of biocompatible surfaces (for reviews see Torchilin et al., 1995; Woodle, 1995; Sadzuka, 2000). In addition to efforts aiming at practical applications, these polymers have been subjected to both theoretical (Alexander, 1977; de Gennes, 1980; Jeon et al., 1991; Szleifer, 1997a; Halperin, 1999) and experimental (Du et al., 1997; Wong et al., 1997; Majewski et al., 1998; Baekmark et al., 1995, 1999; Wiesenthal et al., 1999; Naumann et al., 1999) studies. Polymer-modified lipids serve as good models for grafted polymers of low molecular weight, where the grafting density of the polymer chains can be varied and quantitatively controlled by simply varying the ratio of unmodified to polymer-modified lipid within a mixed monolayer or a bilayer (Kuhl et al., 1994; Kenworthy et al., 1995; Majewski et al., 1997). Inclusion of phospholipids with grafted PEG chains into phospholipid liposomes (forming so-called stealth liposomes) prolongs their half-time in circulation and increases their efficiency in drug delivery (for reviews, see Torchilin et al., 1995; Woodle, 1995; Sadzuka, 2000). This effect has been attributed to the repulsive hydrophilic barrier around the liposome provided by the covalently attached PEG, which prevents liposomes from cell adhesion and from being opsonized by proteins (Senior et al., 1991; Du et al., 1997).

The above effect of PEG-conjugated lipids has been recognized to depend on the molecular weight of the PEG moiety as well as on the density of grafted PEG on the membranes (Kenworthy et al., 1995). Also the structure of the lipid anchor is important (Webb et al., 1998; Adlakha-Hutcheon et al., 1999). Leakage of the anticancer drug vincristine from liposomes containing PEG-ceramide (PEG-Cer) is less than from liposomes containing 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)] (PEG-DSPE) (Webb et al., 1998). The longer ceramide acyl chains seem to provide more efficient anchoring to the liposomes. PEG-conjugated ceramides have been demonstrated to promote bilayer formation in mixtures with non-bilayer-forming lipids (Holland et al., 1996a) and to regulate fusion of liposomes as well as liposomes and cells (Holland et al., 1996b).

A close simulation of PEG-liposome surfaces is a lipid monolayer or bilayer on a solid support with the grafted PEG moieties protruding from the surface and the hydrophobic tails of these molecules remaining inserted into the surface monolayer (Majewski et al., 1998; Kuhl et al., 1998; Baekmark et al., 1995, 1999). Lipid monolayers at the air/water interface have well-defined composition as well as lateral packing density and allow us to study various processes such as drug-ligand and protein-lipid interactions in the membrane/water interface under precisely controlled conditions (for review see Brockman, 1999). Recently, polymer-grafted lipids (lipopolymers) and their mixtures with different lipids were subjected to Langmuir-balance studies (Baekmark et al., 1995, 1999; Majewski et al., 1998) and were found to form stable films that exhibit a complex phase behavior (Baekmark et al., 1997; Wiesenthal et al., 1999; Naumann et al., 1999).

Adsorption of drugs and proteins into membrane surfaces and their behavior at interfaces as well as interactions with lipids are of interest in relation to cell membrane organization and functions (for reviews see Kinnunen, 1991; Kinnunen et al., 1994). In this study we compared the binding

Submitted September 4, 2001, and accepted for publication April 17, 2002.

Address reprint requests to Dr. Paavo K.J. Kinnunen, Helsinki Biophysics and Biomembrane Group, Institute of Biomedicine, P.O. Box 63 (Haartmaninkatu 8), FIN-00014 University of Helsinki, Finland. Tel.: 358-9-19125400; Fax: 358-9-19125444; E-mail: paavo.kinnunen@helsinki.fi.

0006-3495/02/08/954/14 $2.00

of three soluble molecules, adriamycin, cytochrome c, and bovine serum albumin (BSA) to PEG-Cer-containing monolayers. Adriamycin is a commonly used anticancer drug that bears a positive charge and interacts strongly with membranes containing acidic phospholipids (Goormaghtigh et al., 1980; De Wolf et al., 1991; Mustonen and Kinnunen, 1991). Adriamycin decreases acyl chain order in an acidic phospholipid membrane, thus implying disruption of the local membrane structure and altered physical state of membrane lipids (De Wolf et al., 1991). These membrane interactions could result in changes in lipid organization, and may also play a role in the antitumor activity of this drug (De Wolf et al., 1991). Membrane penetration of adriamycin is strongly dependent on lipid packing (Mustonen and Kinnunen, 1993). Drug-lipid interactions also contribute to efficiency of encapsulation of adriamycin into vesicles (Hernandez et al., 1991).

Cytochrome c (cyt c) is a well-characterized peripheral protein of the inner mitochondrial membrane that associates only weakly with zwitterionic phosphatidylcholine membranes (Mustonen et al., 1993). In keeping with its net positive charge and the presence of cationic clusters on its surface cyt c binds with a high affinity to acidic phospholipids (for review see Kinnunen et al., 1994). Adriamycin has been shown to reverse the binding of cyt c to cardiolipin at equimolar drug-lipid concentrations (Goormaghtigh et al., 1982). It has been suggested that the association of adriamycin and cyt c with acidic lipids involves similar mechanisms, with both hydrophobic as well as electrostatic interactions being involved (Mustonen et al., 1993). Yet, hydrophobicity appears to contribute less to the membrane association of adriamycin. Intriguingly, recent results show that cyt c is also centrally involved in apoptosis (Kluck et al., 1997; Yang et al., 1997), its release from mitochondria representing the rate-limiting step in the commitment of a cell to programmed cell death (Liu et al., 1996; Kluck et al., 1997; Yang et al., 1997). The other protein investigated in the present study is BSA. It is considerably larger than cyt c, with a molecular weight of ~66,000. BSA is the main component of plasma, constituting 50–60% of the total protein in blood. It promotes the aggregation and fusion of liposomes (Schenkman et al., 1981).

We report here on the characterization of compression isotherms for PEG-Cer-containing monolayers residing on an air/water interface. We also compare the effects of a PEG-Cer conjugate on the binding of adriamycin, cyt c, and BSA into phospholipid monolayers.

MATERIALS AND METHODS

Materials

Hepes, EDTA, horse heart cyt c (mainly oxidized form), BSA (essentially fatty acid free), and adriamycin were from Sigma Chemical Co. (St. Louis, MO). The purities of adriamycin, cyt c, and BSA were >97%, >95%, and >96%, respectively. Egg yolk phosphatidylcholine (eggPC, purity > 99%), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-(*N*-4-nitrobenz-2-oxa-1,3-diazol)aminocaproyl-*sn*-glycero-3-phosphocholine (NBD-PC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly (ethylene glycol) 5000] (POPE-PEG_{5000}) with PEG of molar mass of 5000 Da covalently attached via a carbamate linkage to POPE were from Avanti Polar Lipids (Alabaster, AL). 1-O-(2′-(ω-Methoxypolyethylene $glycol_{(2000)}$) succinoyl-2-*N*-arachidoylsphingosine (PEG-Cer) was from Northern Lipids (Vancouver, Canada). The purity of the above lipids was checked by thin layer chromatography on silicic-acid-coated plates (Merck, Darmstadt, Germany) developed with chloroform/methanol/water (65/25/4, v/v/v). Examination of the plates after iodine staining and, when appropriate, upon UV illumination revealed no impurities.

Measurement of compression isotherms

Compression isotherms for lipid monolayers were recorded using a commercial monolayer system (μTroughS, Kibron, Helsinki, Finland) with a rectangular trough (total area 120 cm^2) and symmetrical compression with two barriers. Lipids were spread onto the subphase in chloroform, and the solvent was allowed to evaporate for ~15 min. Surface pressure versus surface area (π-A) isotherms were recorded continuously, compressing the film at a rate of 4 $Å^2$/chain/min. The curves represent the means of at least three individual experiments. Standard deviations for π-A isotherms were less than $\pm$4 $Å^2$.

Measurement of surface potential

The surface potential versus surface area (ΔV-A) isotherms were recorded during the compression isotherm with the vibrating plate technique (μSpot, Kibron), as described previously (Brockman, 1994). The aqueous subphase was 5 mM Hepes, 0.1 mM EDTA, pH 7.4, and the volume was 20 ml. All measurements were performed at ambient temperature. The curves represent the mean of at least three individual experiments. The standard deviation for the values of ΔV was less than $\pm$15 mV.

Fluorescence microscopy of lipid monolayers

For fluorescence microscopy of the lipid monolayers the above Langmuir trough was placed on the stage of a Zeiss IM-35 inverted microscope. The quartz-glass window in the bottom of the trough was positioned over a Nikon extra long working distance 20× objective. A 450–490-nm bandpass filter was used for excitation and a 520-nm longpass filter for emission. Images were recorded with a Peltier-cooled 12-bit digital CCD camera (C4742–95, Hamamatsu, Japan) interfaced to a computer and operated by the software (HiPic 5.0.1) provided by the manufacturer.

The indicated phospholipids, as well as PEG-Cer and NBD-PC (X = 0.02) as a fluorescent marker were mixed in chloroform and subsequently applied on the air-water interface using a microsyringe. After equilibration for 10 min the monolayer was compressed symmetrically at a rate of 4 $Å^2$/chain/min. The compression was stopped after reaching the desired values for π, and the monolayer was allowed to settle for a another 10 min before recording the fluorescence images. During this equilibration period a decrease (~0.2–1.0 mN/m) in surface pressure was observed, the magnitude of the decrement in π depending on the film composition as well as the pressure range. This decrease in π represents the reorganization and relaxation of the monolayer toward the free energy minimum after the compression. Accordingly, it is essential to note that the probe distributions observed are unlikely to represent true equilibrium states. However, as identical compression rates and equilibration times were used in each experiment the results thus obtained should be amenable for comparison. All measurements were performed at ambient temperature (~+24°C). The average relative fluorescence emission intensities (RFIs) were obtained

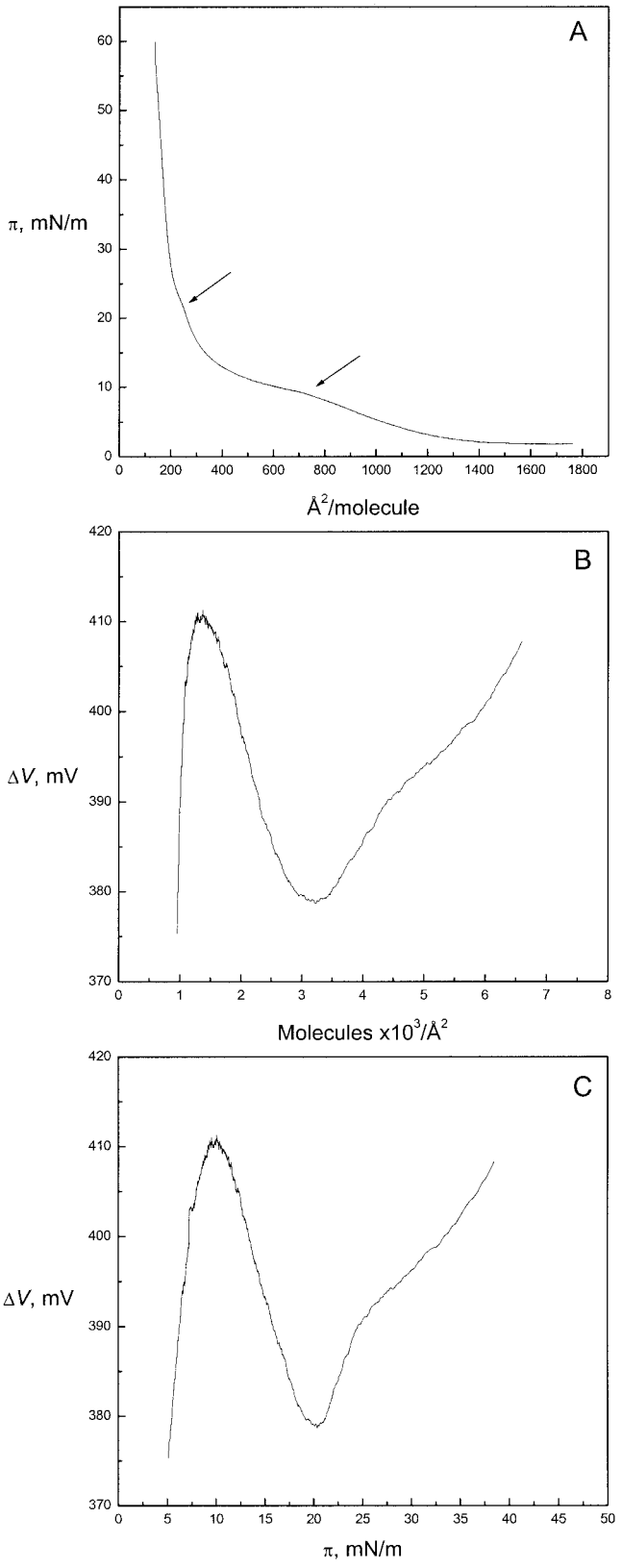


FIGURE 1 Compression (π-A) isotherm (A) and surface potential ΔV versus lipid density (ΔV-n) isotherm (B) and ΔV versus surface pressure (C) for a PEG-Cer monolayer. The lipid was spread as a monolayer on the surface of 5 mM Hepes, 0.1 mM EDTA, pH 7.4, and subsequently compressed at a rate of 4 Å^2/chain/min at ambient temperature (~+24°C). The curves represent the means of at least three individual experiments. The standard deviations for π-A and ΔV-A isotherms were less than ±4 Å^2 and ±15 mV, respectively.

from the images recorded under identical conditions and analyzed by the HiPic 5.0.1 software.

Penetration of ligands into phospholipid monolayers

Penetration of adriamycin and proteins into monomolecular phospholipid films was measured using magnetically stirred circular Teflon wells (multiwell plate, subphase volume 1.2 ml and total area 3.14 cm^2, Kibron). Surface pressure (π) was monitored with a Wilhelmy wire attached to a microbalance connected to a Pentium PC. Lipids were spread on the air-buffer (5 mM Hepes, 0.1 mM EDTA, pH 7.4) interface in chloroform (~1 mg/ml) and were allowed to settle for ~15 min so as to equilibrate at different initial surface pressure (π_0) before the injection of either adriamycin, cyt c, or BSA into the subphase. The final concentrations of the above compounds in the subphase were 10, 0.5, and 13 μM, respectively. The increments in π after the injection of adriamycin or proteins were complete in ~30 min, and the difference between the initial surface pressure (π_0) and the value observed after the penetration of the above ligands into the films was taken as $\Delta\pi$. The data are represented as $\Delta\pi$ versus π_0 (Brockman, 1999). These measurements yield also the value for π_c, critical surface pressure above which the ligand in question no longer can penetrate into the lipid monolayer. All measurements were performed at ambient temperature (~+24°C). Each data point represents the mean of triplicate measurements. The standard deviation varied between 0.1 mN/m and 0.8 mN/m and for the sake of clarity is not shown.

After the equilibration of the penetration of adriamycin, cyt c, and BSA into lipid monolayers, a 100-μl sample of the subphase was withdrawn through a small-diameter hole drilled in the side of the wells. For adriamycin the sample taken from the subphase was diluted into 700 μl with 5 mM Hepes, 0.1 mM EDTA, pH 7.4, and the concentration of the drug determined spectrophotometrically by absorbance at 480 nm using molar extinction coefficient of 11,500 M^{-1} cm^{-1}. For cyt c the sample from the subphase was diluted into 200 μl with 5 mM Hepes, 0.1 mM EDTA, 0.5% SDS, pH 7.4. The standards of cyt c were prepared in the same buffer and the concentrations of the protein quantitated by measuring absorbance at 410 nm. The concentration of BSA in the subphase was determined by NanoOrange protein quantitation kit (Molecular Probes, Leiden, The Netherlands). Fluorescence was measured using a microplate reader (Tecan, Hombrechtikon, Switzerland) with excitation and emission wavelengths of 485 and 595 nm, respectively. All measurements were repeated three times.

RESULTS

Characterization of lipid monolayers

Both PEG (Kim and Cao, 1993) as well as PEG-lipids readily spread to form monolayers on the air/water interface (Baekmark et al., 1995). We first recorded the pressure-area (π-A) isotherm for a pure PEG-Cer monolayer (Fig. 1 A). Two discontinuities are evident, marked by arrows. The onset of the first discontinuity is at $\pi \approx 9$ mN/m whereas the second occurs at $\pi \approx 21$ mN/m. In parallel to the π-A data, we recorded the corresponding surface potential versus area/molecule (ΔV-A) isotherms (Brockman, 1994). These data are shown also as ΔV versus lipid density n (Fig. 1 B)

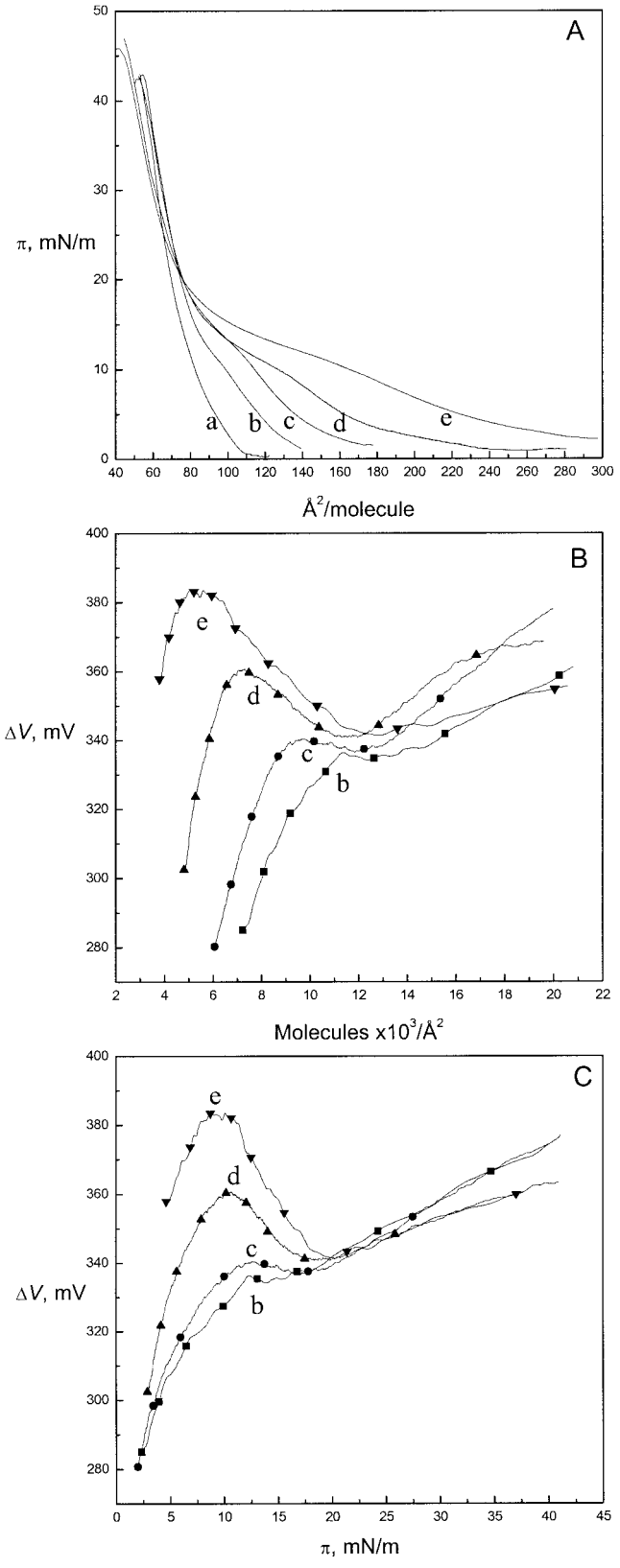


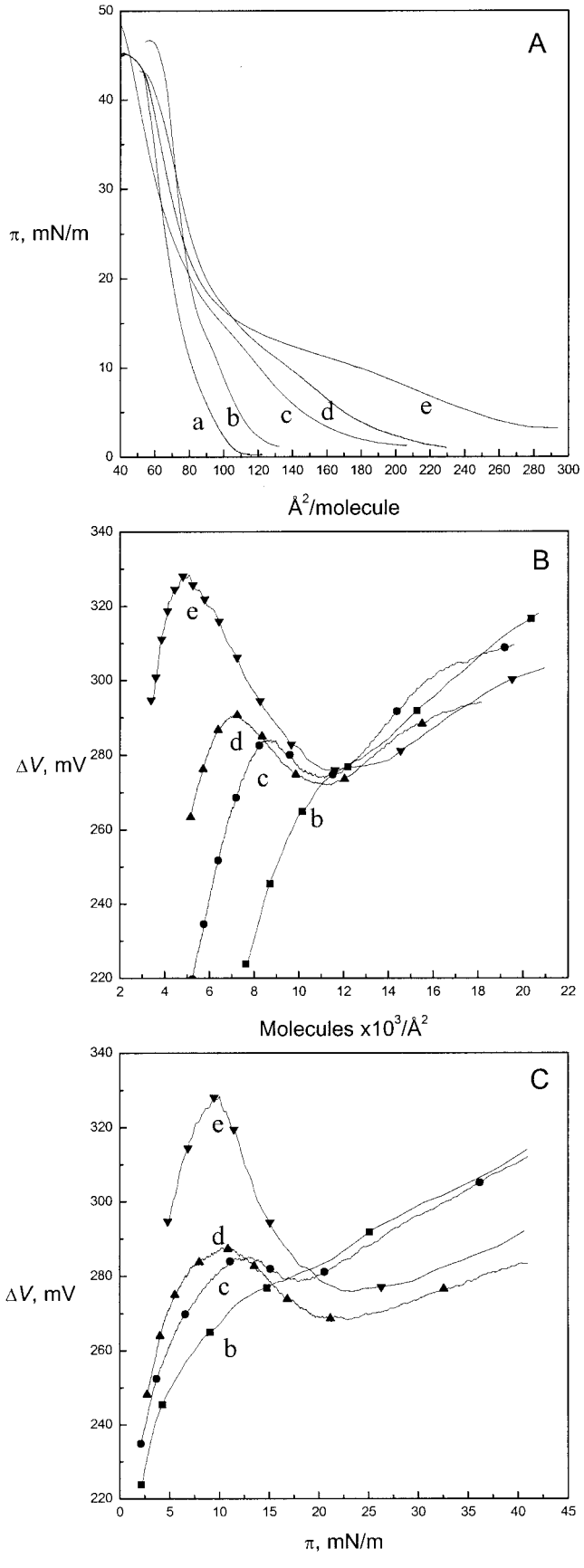
FIGURE 2 Effect of PEG-Cer on the π-A (A), ΔV-n (B), and ΔV-π (C) behavior of eggPC monolayers. Lipid compositions are as follows: (a) eggPC; (b) eggPC:PEG-Cer (■, 98:2, molar ratio); (c) eggPC: PEG-Cer (●, 96:4); (d) eggPC:PEG-Cer (▲, 92:8); (e) eggPC:PEG-Cer (▼, 88:12). Lipids were spread on the surface of 5 mM Hepes, 0.1 mM EDTA, pH 7.4. The lipid monolayers were compressed at a rate of 4 Å^2/chain/min at ambient temperature (~+24°C). The curves represent the means of at least three individual experiments.

as well as ΔV-π isotherms (Fig. 1 C). Similarly to the π-A isotherm two pronounced discontinuities are evident at lipid densities of ~1.4 and ~3.3 molecules × 10^3/Å^2 (Fig. 1 B), corresponding to $\pi \approx 9$ and 21 mN/m (Fig. 1 C), thus suggesting pure PEG-Cer monolayers to undergo two phase transitions (Fig. 1 C).

To study the effects of PEG-Cer on the behavior of eggPC (Fig. 2) and eggPC/POPG (96:4, molar ratio, Fig. 3) films, we measured compression isotherms of lipid films at $X_{PEG\text{-}Cer}$ = 0.02, 0.04, 0.08, and 0.12. Introduction of PEG-Cer (X = 0.02) causes a pronounced increase in the average area/molecule in the lipid monolayer. The first transition at $\pi \approx 9$ mN/m characteristic of the pure PEG-Cer monolayer is clearly evident also in the mixed films. Increasing $X_{PEG\text{-}Cer}$ augments the increase in A/molecule, and the initiation of the low surface pressure transition becomes more pronounced (Figs. 2 A and 3 A). However, the high surface pressure transition cannot be resolved in the π-A isotherms for the mixed monolayers containing PEG-Cer. Interestingly, both transitions are evident in the ΔV-n and ΔV-π isotherms, evident as maxima and minima at π ~9 and 21 mN/m for the PEG-Cer-containing films (Figs. 2, B and C, and 3, B and C). The increment in ΔV is augmented at $\pi \approx 9$ mN/m with increasing $X_{PEG\text{-}Cer}$ (Figs. 2 B and 3 B), similarly to the A/molecule at the low surface pressure transition (Fig. 4 A), yielding decreased critical lipid densities (Figs. 2 B and 3 B). Accordingly, at $\pi \approx 9$ mN/m the average area/PEG-Cer decreases with $X_{PEG\text{-}Cer}$ (Fig. 4 B). While increasing $X_{PEG\text{-}Cer}$ in the monolayers augments the changes in ΔV between the two transitions there are only minor differences between films containing POPG or eggPC (Fig. 4 C). In contrast to the low surface pressure transition, the high-pressure transition in ΔV-n isotherms at ~21 mN/m occurs at similar lipid densities for all PEG-Cer-containing monolayers (Figs. 2 B and 3 B).

Fluorescence microscopy of lipid monolayers

Morphology of the two-dimensional domains of phospholipid monolayers is sensitive to lipid phase transitions as well as to the chemical composition of the films (Weis, 1991). To study whether phase separation processes in a PEG-Cer monolayer at the air/water interface were evident and whether these could be visualized by observing the lateral distribution of the fluorescent lipid analog NBD-PC (X = 0.02), we examined the film as a function of π by fluorescence microscopy. However, fluorescence images were homogeneous at all surface pressures, thus indicating the absence of phase separation processes at least at this

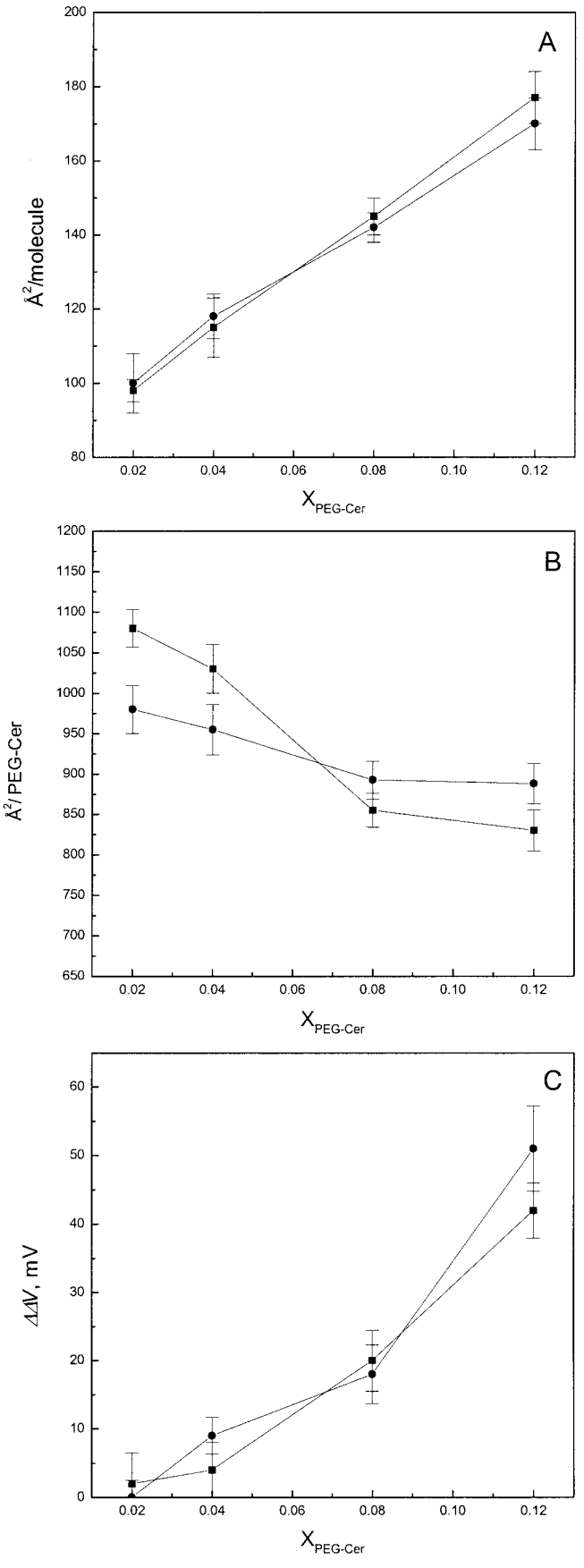


resolution (data not shown). Yet, relative emission intensity as a function of π revealed an interesting behavior (Fig. 5). When the surface pressure is close to 2 mN/m, the fluorescent intensities are very low. Upon compression the fluorescence intensities increase and at $\pi \approx 9$ mN/m there is a steep increment in NBD emission. Upon increasing π further also the fluorescence intensities increase, in keeping with the increment in the surface density of the fluorescent lipid. If the fluorescence of NBD would not be influenced by the polymer headgroup its emission intensity should increase linearly with reciprocal surface area. Another smaller discontinuity in RFI versus π is evident at $\pi \approx$ 18–19 mN/m. However, this is not the case, but clear discontinuities are evident at $\pi \approx 9$–12, and 18–19 mN/m (Fig. 5), corresponding to the phase transitions indicated by the compression isotherms. To obtain further insight into the effects of PEG-Cer on eggPC films, we studied the lateral distribution of NBD-PC also in the mixed monolayers. Similarly to neat PEG-Cer film, the images of these mixed monolayers both in the presence (X = 0.04 and 0.08) and absence of PEG-Cer were homogeneous with no visible phase separation (data not shown).

Penetration of adriamycin into phospholipid monolayers

To study the effects of PEG on the properties of lipid surfaces, we compared the penetration of adriamycin into eggPC monolayers and films containing PEG-Cer ($X_{PEG\text{-}Cer} = 0.04$ and 0.08). Intercalation of adriamycin into lipid monolayers spread at the air/water interface to different initial surface pressures (π_0) was observed by measuring the increment in surface pressure ($\Delta\pi$) subsequent to the addition of this drug into the subphase. The dependence of $\Delta\pi$ for the zwitterionic eggPC monolayer as a function of π_0 was linear, with critical packing preventing the increment in surface pressure extrapolating to $\pi_c \approx 35$ mN/m (Fig. 6 *A*). For lipid monolayers containing PEG-Cer (X = 0.04 and 0.08) $\Delta\pi$ was significantly reduced (Fig. 6 *A*), with insignificant difference between the two mole fractions of PEG-Cer and without effect on π_c. Adriamycin is cationic and has a high affinity to acidic lipids (Goormaghtigh et al., 1980). Accordingly, we studied the intercalation of adriamycin also into monolayers containing POPG (X = 0.04). Presence of this content of the latter phospholipid bearing

FIGURE 3 Effect of PEG-Cer on the π-*A* (*A*), ΔV-*n* (*B*), and ΔV-π (*C*) isotherms for eggPC/POPG monolayers. Lipid compositions are as follows: (*a*) eggPC:POPG (96:4, molar ratio); (*b*) eggPC:POPG:PEG-Cer (■, 94:4:2); (*c*) eggPC: POPG:PEG-Cer (●, 92:4:4); (*d*) eggPC:POPG:PEG-Cer (▲, 88:4:8); (*e*) eggPC:POPG:PEG-Cer (▼, 84:4:12). Lipids were spread on the surface of 5 mM Hepes, 0.1 mM EDTA, pH 7.4. Monolayers were compressed at a rate of 4 Å^2/chain/min at ambient temperature (~+24°C). The curves represent the means of at least three individual experiments.



an acidic headgroup had minor effect on the penetration of adriamycin into the monolayers (Figs. 6 *B* and 7 *B*). Interestingly, the $\Delta\pi$ versus π_0 dependence for the penetration of adriamycin into monolayers containing both PEG-Cer and POPG was very different from that in the absence of the acidic phospholipid (Fig. 6 *B*). At $\pi_0 < 20$ mN/m, $\Delta\pi$ due to adriamycin was less for PEG-Cer-containing lipid monolayers compared with eggPC/POPG monolayer. Accordingly, at $\pi_0 = 10$ mN/m the increment $\Delta\pi$ after the injection of adriamycin underneath the lipid monolayers with PEG-Cer ($X_{PEG\text{-}Cer} = 0.04$ and 0.08) was very low. However, when π_0 was increased, $\Delta\pi$ was augmented reaching a maximum at $\pi_0 \approx 19$–21 mN/m (Fig. 6 *C*). When π_0 exceeded this pressure range, the changes in $\Delta\pi$ for phospholipid monolayers containing PEG-Cer became similar to those observed for eggPC/POPG monolayers, with no difference between the two mole fractions of PEG-Cer (Fig. 6 *C*). We also quantified the amount of adriamycin adsorbed to the monolayers. The content of adriamycin in eggPC and eggPC/POPG films decreased with increasing π_0 (Fig. 7). Consistently with the effect of PEG-Cer on the changes in $\Delta\pi$, the amount of drug adsorbed to the lipid monolayers was decreased for PEG-Cer-containing monolayers (Fig. 7), with minor differences between data at $X_{PEG\text{-}Cer} = 0.04$ and 0.08 (for the sake of clarity, only data at $X_{PEG\text{-}Cer} = 0.04$ are shown). In the presence of PEG-Cer, only a weak dependence on π_0 was evident.

Penetration of proteins into phospholipid monolayers

We then compared the penetration of cyt c and BSA into the lipid monolayers at the air/water interface. Although cyt c is considered as a paradigm for the electrostatic binding of peripheral proteins to membranes (for review see Kinnunen et al., 1994), also hydrophobic interactions have been demonstrated to contribute (Mustonen et al., 1993). Increments in surface pressure ($\Delta\pi$) as a function of initial surface pressure (π_0) after the injection of cyt c into the subphase underneath eggPC monolayers are illustrated in Fig. 8 and reveal the dependence of the interaction of cyt c with the monolayers as a function of π_0 to be biphasic. Similarly to adriamycin, cyt c has a high affinity to acidic lipids (Kinnunen et al., 1994). Accordingly, POPG (X = 0.04) was incorporated into the lipid monolayers. This content of POPG had only a minor effect on the penetration of cyt c

FIGURE 4 The values for area/molecule (*A*) and area/PEG-Cer (*B*) at the low surface pressure transition $\pi \approx 9$ mN/m and magnitude of the changes in surface potential ($\Delta\Delta V$) between the maximum and minimum in ΔV at $\pi \approx 9$ and 21 mN/m, respectively (*C*), depicted as a function of the mole fraction of PEG-Cer in the lipid monolayers. The lipid compositions are eggPC/PEG-Cer (■) and eggPC/POPG/PEG-Cer (●). Each data point represents the mean of triplicate measurements, with the error bars indicating ±SD.

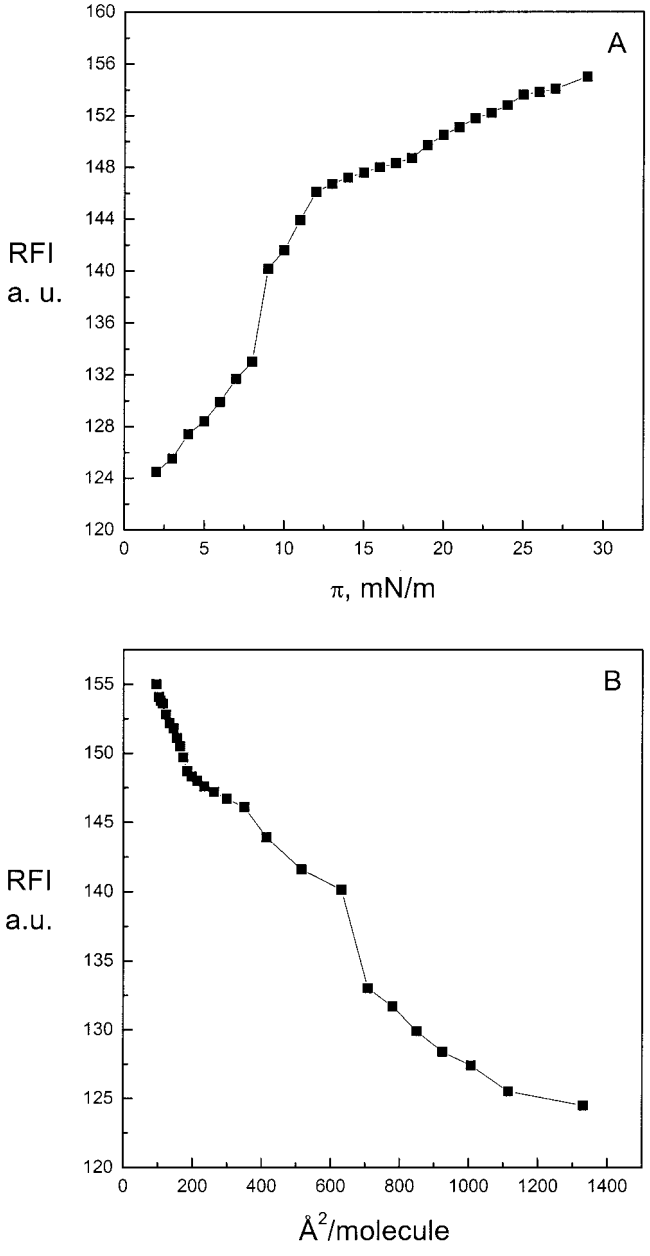


FIGURE 5 Increment of RFIs during the compression of a pure PEG-Cer monolayer, illustrated as a function of surface pressure (*A*) or mean molecular area (*B*). PEG-Cer and the fluorescent probe NBD-PC (X = 0.02) were spread on the surface of 5 mM Hepes, 0.1 mM EDTA, pH 7.4, and the lipid monolayer was compressed at a rate of 4 Å^2/chain/min at ambient temperature (~+24°C). Each data point represents the means of three individual experiments. The standard deviation is below 2 (RFI, arbitrary unit) and for the sake of clarity is not shown.

into the monolayers. The increments of surface pressure due to the intercalation of cyt c into the lipid monolayers was reduced in the presence of PEG-Cer (X = 0.04 and 0.08) at surface pressures $\pi_0 < 20$ mN/m with insignificant difference between the two mole fractions of PEG-Cer (Fig. 8 *B*). However, similarly to adriamycin at $\pi_0 \geq 20$ mN/m PEG-Cer seems to have no effect. The same behavior was observed for phospholipid monolayers containing POPE-PEG_{5000} (X = 0.04 and 0.08) instead of PEG-Cer, with little difference between these two lipopolymers (data not shown). The amount of cyt c bound to monolayers diminished with increasing π_0 (Fig. 8 *C*). Further decrement was evident in the presence of PEG-Cer at surface pressures $\pi_0 < 20$ mN/m, again with a minor difference between the two mole fractions of PEG-Cer (Fig. 8 *C*; for the sake of clarity, only the data at $X_{PEG\text{-}Cer} = 0.04$ were shown). Similarly to the changes in $\Delta\pi$, PEG-Cer had no effect on the quantity of cyt c adsorbed to the monolayers at $\pi_0 \geq 20$ mN/m.

The above behavior of cyt c was then compared with that of the significantly larger BSA. Changes in $\Delta\pi$ due to the penetration of BSA into an eggPC monolayer (data not shown) were nearly identical to those measured for an eggPC/POPG ($X_{PG} = 0.04$) monolayer, which revealed a linear $\Delta\pi$ versus π_0 behavior (Fig. 9 *A*). The increments of surface pressure due to the penetration of BSA into phospholipid films were attenuated in the presence of PEG-Cer (X = 0.04 and 0.08, Fig. 9 *A*), and the values for critical surface pressures π_c were ~20, 18, and 17 mN/m for eggPC/POPG monolayers (X_{POPG} = 0.04) and at $X_{PEG\text{-}Cer} = 0.00$, 0.04, and 0.08, respectively. There were no significant differences between the two mole fractions of PEG-Cer. For phospholipid monolayers containing POPE-PEG_{5000}, the penetration of BSA was dramatically decreased, and $\Delta\pi$ due to BSA is nearly absent when the mole fraction of POPE-PEG_{5000} is X = 0.08 (data not shown). Similarly to cyt c, the amount of BSA bound to lipid monolayers decreased with increasing π_0, with further decrement in the presence of PEG-Cer at surface pressures < 20 mN/m (Fig. 9 *B*). Above this latter surface pressure, minor differences in the quantity of this protein adsorbed to lipid monolayers were evident. There were insignificant differences at $X_{PEG\text{-}Cer} = 0.04$ and 0.08 for all the initial surface pressures measured (Fig. 9 *B*; for the sake of clarity, only data at $X_{PEG\text{-}Cer} = 0.04$ are shown).

DISCUSSION

A number of theoretical models have been forwarded to describe the behavior of surface-adsorbing and nonadsorbing polymers (Alexander, 1977; de Gennes, 1980; Milner, 1991; Halperin, 1992; Halperin et al., 1992). In brief, pancake → cigar transition for surface-adsorbing and mushroom → brush transition for non-surface-adsorbing polymers have been suggested. The pancake → cigar transition is predicted to take place as a first-order transition, associated with the coexistence of brushes and two-dimensional semidilute regions (Halperin, 1992). This should give rise to a plateau in the π versus A plot. The mushroom → brush transition for non-surface-adsorbing polymers has been suggested to be a continuous process, and thus no plateau should be observed in compression isotherms (Carignano and Szleifer, 1995). PEG chains adsorb to the air/water

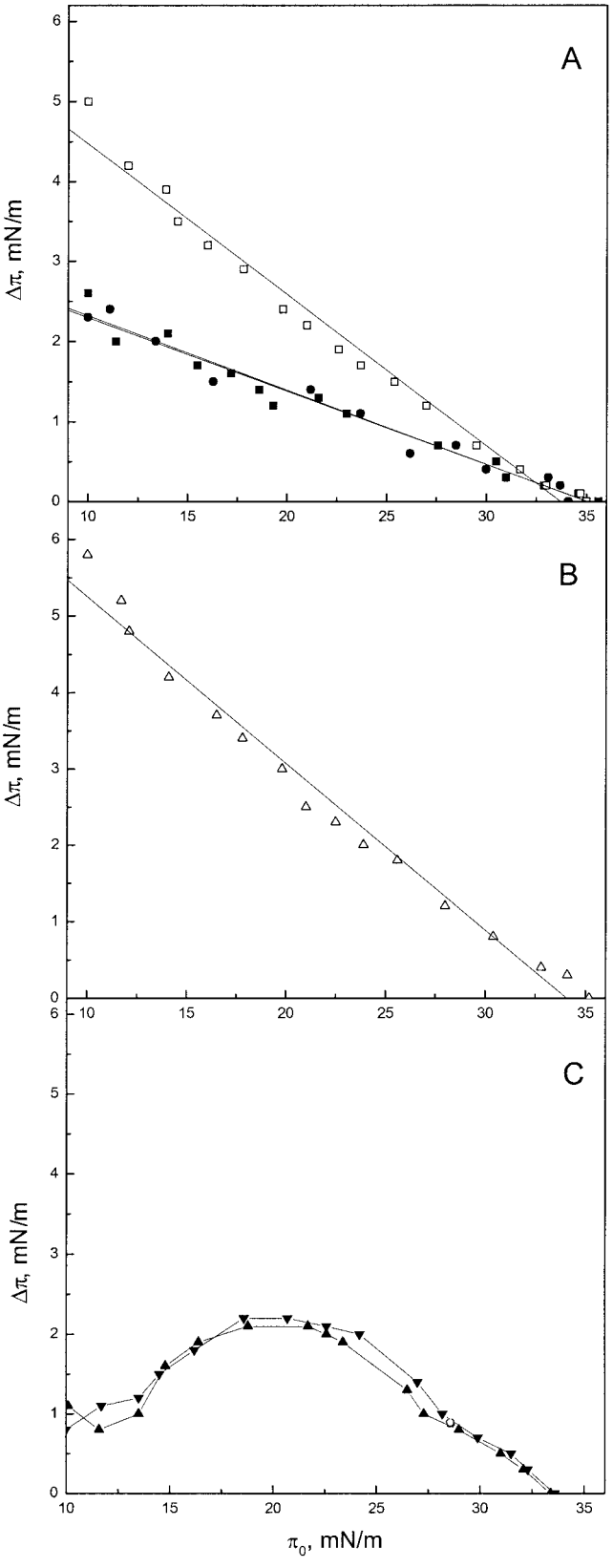


FIGURE 6 Effect of PEG-Cer on the penetration of adriamycin into lipid monolayers. Changes in the surface pressure ($\Delta\pi$) of lipid monolayer due to the addition of 10 μM adriamycin into the subphase (5 mM Hepes, 0.1 mM EDTA, pH 7.4) are illustrated as a function of the initial surface pressure (π_0). Lipid compositions are as follows: (*A*) eggPC (□), eggPC:PEG-Cer (■, 96:4, molar ratio), and eggPC:PEG-Cer (●, 92:8); (*B*) eggPC:POPG (Δ, 96:4); (*C*) eggPC:POPG:PEG-Cer (▲, 92:4:4) and eggPC:POPG:PEG-Cer (▼, 88:4:8).

interface at low packing density and a pancake → cigar transition was suggested, revealed by the plateau at $\pi \approx 10$ mN/m in the π versus A plot (Bijsterbosch et al., 1995; Faure et al., 1998). Baekmark et al. (1995) also observed the adsorption of PEG but interpreted the low surface pressure transition as a pancake → mushroom transition. As discussed previously (Carignano and Szleifer, 1995), special care is needed in trying to describe these polymer layers with scaling concepts, and there is no easy definition of the scaling regimes, such as mushroom and brush regimes for short-chain-length polymers. This could be the reason for the different interpretations of similar data. The grafted PEG polymer was found to be nonadsorbing in liposomes (Needham et al., 1992), which may due to the prevailing surface pressure in liposomes (Blume, 1979; Konttila et al., 1988; Brockman, 1999).

At low surface pressure, the behavior of the phospholipid/lipopolymer monolayers in the presence of PEG-Cer is mainly determined by the very large headgroup of the lipopolymer adsorbed onto the air/water interface. The low surface pressure transition would thus occur at a much lower lipid density for a pure PEG-Cer monolayer than for the mixed phospholipid/lipopolymer monolayers (Figs. 1–3). As the polymer density continues to increase, the chains start to interact and finally extend further away from the interface (Alexander, 1977; de Gennes, 1980). Further compression should limit the conformational entropy of the polymer chains, and they become more rigid. Expulsion of lipopolymer from the monolayer plane at very high surface pressures may occur, as suggested (Majewski et al., 1997). Wiesenthal et al. (1999) reported recently the high surface pressure transition to depend on the condensation of alkyl chains of the lipopolymer and considered this transition to be native to lipopolymers, requiring both a lipid and a polymer moiety in the same molecule. Furthermore, only lipopolymers with a hydrophobic anchor of saturated alkyl chains were observed to exhibit this high surface pressure transition, whereas for lipopolymers with unsaturated chains it was absent. Similarly, at high surface pressure the behavior of lipid monolayers in the presence of PEG-Cer should be determined by both the headgroup and the lipid alkyl chain. Similarly to the behavior reported for DSPE-EO_{45}-containing films (Baekmark et al., 1995), our results on pure PEG-Cer at the air/water interface revealed a high surface pressure transition at ~21 mN/m, which is abolished in the π-A isotherms for the mixed phospholipid/PEG-Cer monolayers. Interestingly, the changes in the ΔV-A and ΔV-π data recorded for neat PEG-Cer films were evident also for mixed monolayers of the polymer-grafted lipid and

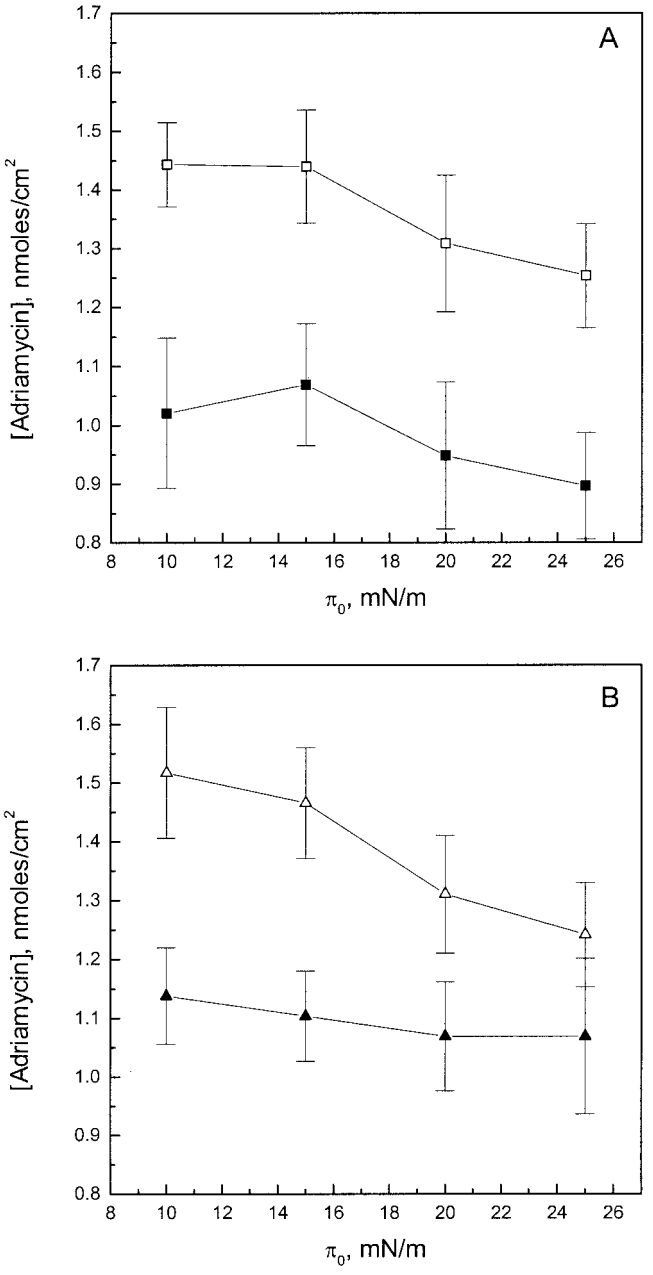


FIGURE 7 Surface concentrations of adriamycin adsorbed to the lipid monolayers at different initial surface pressures. Lipid compositions are eggPC (□), eggPC: PEG-Cer (■, 96:4, molar ratio), eggPC:POPG (Δ, 96:4), and eggPC: POPG: PEG-Cer (▲, 92:4:4). Each data point represents the mean of triplicate measurements, with the error bars indicating ±SD.

phospholipids (Figs. 2 and 3), and ΔV-A data recorded at $X_{PEG\text{-}Cer}$ from 0.02 to 0.12 do comply with the behavior of neat PEG-Cer monolayer (Figs. 1–3), exhibiting the high pressure transition. The ΔV-n and ΔV-π behavior in the presence of PEG-Cer thus lends support to two transitions for the PEG-grafted ceramide as a function of lipid lateral packing (Figs. 1–3). Accordingly, the increment in ΔV at low packing densities should correspond to the increasing surface density of the grafted chains. The subsequent and pronounced decrement in ΔV, from ~410 to ~380 mV (Fig. 1), between surface pressures of ~9 and ~21 mN/m, respectively, would reflect a significant change in the conformation of the polymer moiety of PEG-Cer in the monolayer, analogously to that reported for mixed monolayers of dimyristoylphosphocholine and ceramide with different *N*-acyl chains (Holopainen et al., 2001). Following the minimum at ~21 mN/m the augmented values for ΔV would thus correspond to a gradually increasing packing density of the PEG-chains in another conformational state. The decline in the increment of ΔV in the ΔV-π data at ~25 mN/m (Fig. 1 *C*) could reflect completion of the formation of the clusters of alkyl chains of PEG-Cer (Naumann et al., 2001).

Finally, the emission intensity RFI versus π for the fluorescent lipid analog NBD-PC reveals a pattern, which is in keeping with the above conformational transitions of the PEG chains (Fig. 5 *B*). More specifically, values for RFI do not increase linearly with the decrease in mean molecular area but exhibit discontinuities at $\pi \approx 9$–12 and 18–19 mN/m. As NBD emission is sensitive to the polarity of the environment (Fery-Forgues et al., 1993), the RFI versus *A*/molecule data are likely to signal changes in the interface imposed by the different states of the lipopolymer.

Although pronounced effects of the high surface pressure transition were evident in the $\Delta\pi$-π_0 data for eggPC/POPG/PEG-Cer films after the addition of adriamycin into the subphase, this transition does not influence the behavior of $\Delta\pi$-π_0 for eggPC/PEG-Cer monolayers. Adriamycin penetrates into eggPC films up to $\pi_c \approx 35$ mN/m. Although the values for $\Delta\pi$ are reduced in the presence of PEG-Cer the observed π_c remains unaffected (Fig. 6 *A*). Interestingly, POPG has a very dramatic effect on the $\Delta\pi$-π_0 data for adriamycin in the presence of PEG-Cer (Fig. 6 *C*). When π_0 is in the range of 10–16 mN/m the increment of surface pressure due to adriamycin is higher for eggPC/PEG-Cer monolayers (Fig. 6 *A*) than for eggPC/POPG/PEG-Cer monolayers (Fig. 6 *C*). Moreover, in the presence of PEG-Cer and POPG, $\Delta\pi$ caused by the penetration of adriamycin into lipid monolayer is very low at $\pi_0 = 10$ mN/m but becomes augmented when π_0 is gradually increased to 20 mN/m. However, quantitation of adriamycin adsorbed to lipid monolayers reveals significant amounts of this drug to be adsorbed to the lipid monolayers at these initial surface pressures, with little dependence on π_0 (Fig. 7 *B*). The apparent discrepancy between the $\Delta\pi$-π_0 data and the amount of adriamycin and the two proteins adsorbed to the films is of interest. Some molecules may interact only with the headgroups of lipid monolayers and should therefore induce relatively minor changes in $\Delta\pi$. In contrast, insertion into the hydrophobic region of lipid monolayers causes a larger increment in $\Delta\pi$. Comparison of the data obtained by these two techniques can thus be used to resolve whether ligand-membrane interactions include insertion into the films. At very high lipid packing densities the ligands would

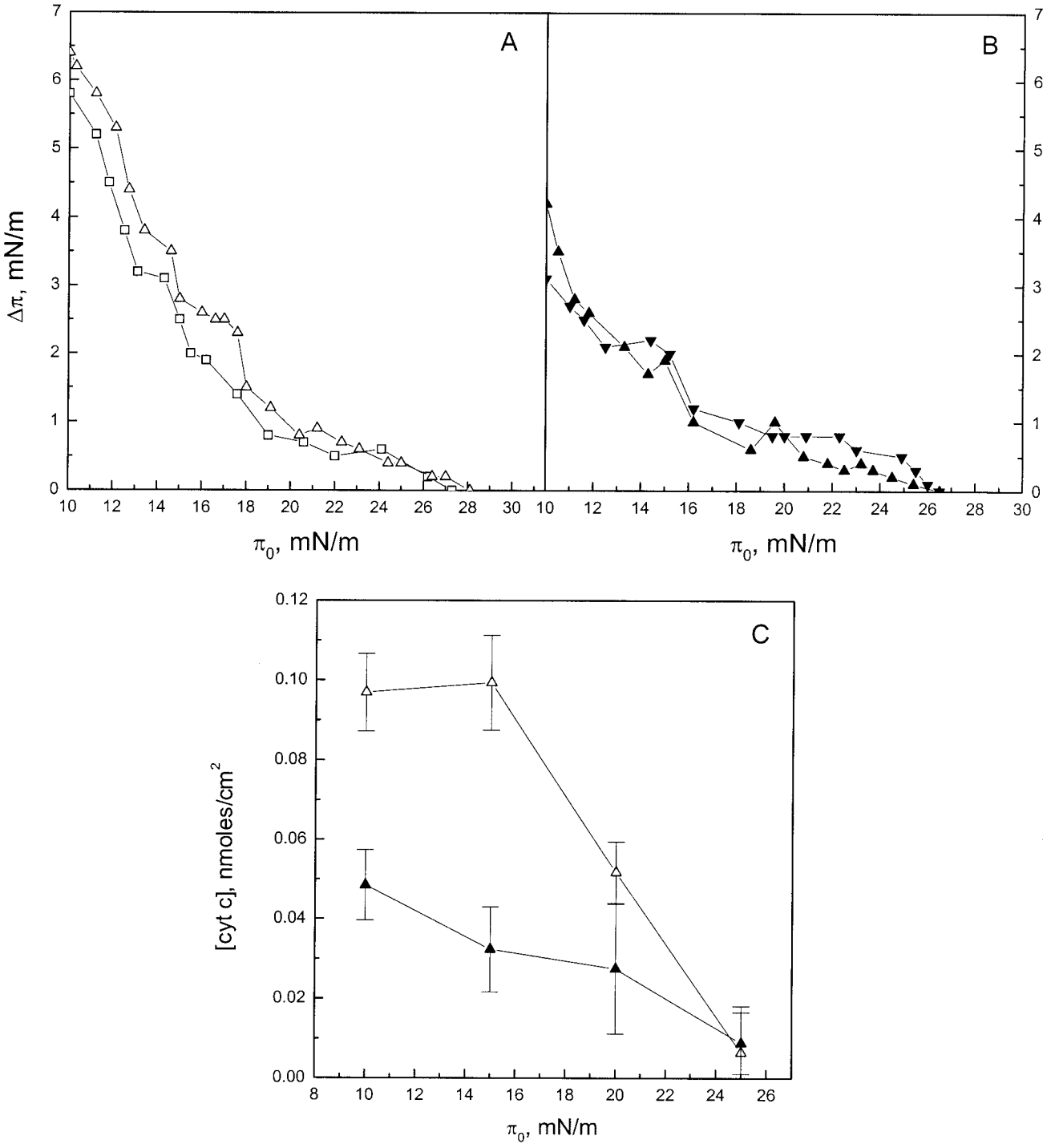


FIGURE 8 Effect of PEG-Cer on the penetration of cyt c into lipid monolayers. Changes in the surface pressure ($\Delta\pi$) of lipid monolayers due to the addition of 0.6 nmol of cyt c (corresponding to final concentration of 0.5 μM) into the subphase (5 mM Hepes, 0.1 mM EDTA, pH 7.4) are illustrated as a function of the initial surface pressure (π_0). Lipid compositions are as follows: (*A*) eggPC (□) and eggPC:POPG (Δ, 96:4); (*B*) eggPC:POPG:PEG-Cer (▲, 92:4:4) and eggPC:POPG:PEG-Cer (▼, 88:4:8). Also shown are the surface concentrations of cyt c adsorbed to the lipid monolayers at different initial surface pressures (*C*). Each data point represents the mean of triplicate measurements, with the error bars indicating ±SD.

interact only with the headgroups of lipid monolayers and thus at $\geq\pi_c$ the surface pressure is expected to remain unaffected although adriamycin and the two proteins do adsorb to the interface (Figs. 6–9). However, adriamycin induced small surface pressure increments at low surface pressures whereas quantitation of the ligand revealed sig-

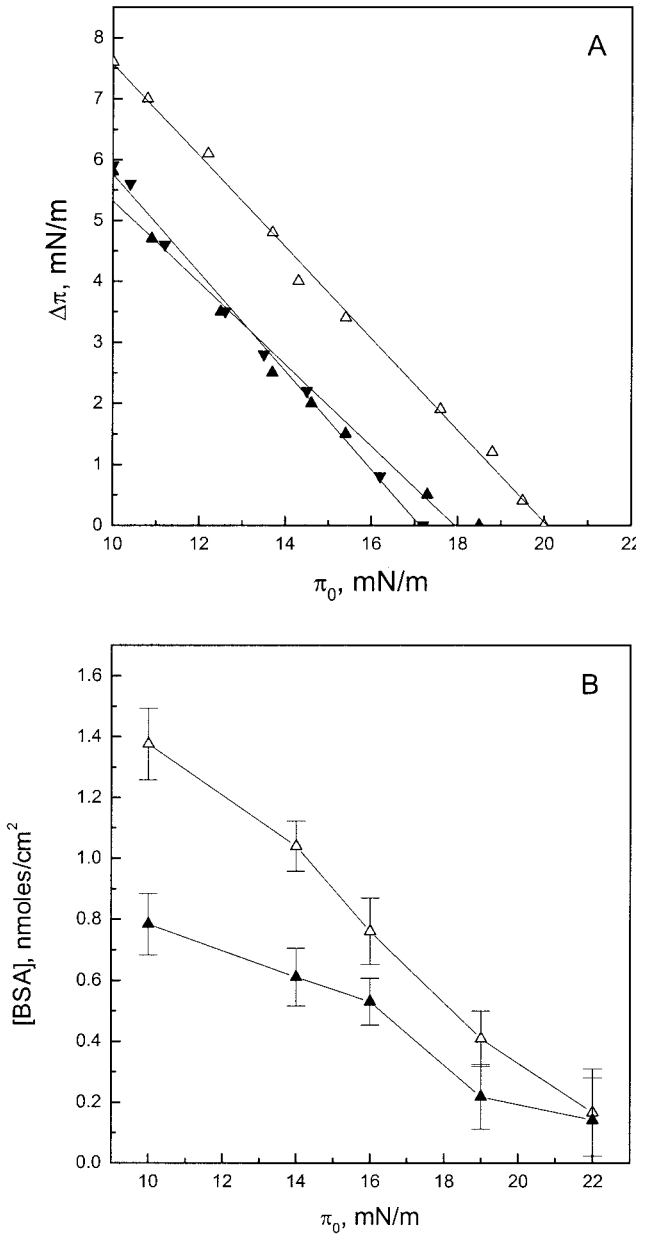


FIGURE 9 Effect of PEG-Cer on the penetration of BSA into lipid monolayers. (*A*) Changes in the surface pressure ($\Delta\pi$) of lipid monolayers due to the addition of 15.6 nmol of BSA (final concentration of 13 μM) into the subphase (5 mM Hepes, 0.1 mM EDTA, pH 7.4) are illustrated as a function of the initial surface pressure (π_0). Lipid compositions are eggPC:POPG (Δ, 96:4), eggPC:POPG:PEG-Cer (▲, 92:4:4), and eggPC:POPG:PEG-Cer (▼, 88:4:8). (*B*) Also shown are the surface concentrations of BSA adsorbed to the lipid monolayers at different initial surface pressures. Each data point represents the mean of triplicate measurements, with the error bars indicating ±SD.

nificant adsorption of this drug to the films containing PEG-Cer (Figs. 6 and 7). This discrepancy suggests that the $\Delta\pi$-π_0 data is in this case likely to reflect reorganization processes in the monolayer, and perhaps also conformational changes of the PEG chains. It is possible that at surface pressures of <20 mN/m, a significant fraction of POPG may reside beneath the polymer chain of PEG-Cer. This could involve hydrogen bonding between POPG and PEG polymer chains rather than the ceramide headgroup because similar $\Delta\pi$-π_0 behavior was observed for another lipopolymer, POPE-PEG_{5000} (data not shown). Electrostatic attraction would thus pull also adriamycin beneath the PEG, causing no increment in π (Fig. 6 *C*). The mutual plane-plane ring-stacking interactions of the adriamycin molecules (Menozzi et al., 1984) and the, to some extent, positively cooperative binding to membranes (De Wolf et al., 1991) may also contribute. When the area available underneath the PEG moiety is reduced with increasing π_0 the fraction of POPG accommodated underneath the polymer decreases, causing the values for $\Delta\pi$ to increase.

The penetration of adriamycin into eggPC/POPG/PEG-Cer monolayers is distinctly different from that of the two proteins cyt c and BSA. The present study suggests that the different molecular size of proteins and adriamycin is one factor determining their different penetration into lipid monolayers in the presence of the acidic phospholipid POPG. The significance of the size of a membrane-interacting protein has been emphasized previously (Halperin, 1999). Comparing $\Delta\pi$ versus π_0 data recorded with and without PEG-Cer, the values for BSA, the largest molecule used, change less than those for cyt c and adriamycin. This suggests that the larger the ligand, the smaller is the effect of PEG-Cer on surface pressure changes due to the penetration of the ligands into lipid monolayers. However, also other differences in the lipid-binding ligands must be considered. Several models have been forwarded to describe protein adsorption on tethered polymer layers (Jeon and Andrade, 1991; Jeon et al., 1991; Szleifer, 1997a,b,c; Halperin, 1999; Leckband et al., 1999). It is generally assumed that proteins would not change their conformation upon contacting the surface. It has been pointed out by Haydon and Taylor (1963) that the hydrophobic side chains of amino acids in the peptide chains would not be long enough to penetrate into the hydrophobic region of a phospholipid monolayer without some degree of unfolding. Accordingly, the increment in surface pressure following the addition of a protein underneath a lipid monolayer at the air/water interface is generally accepted to result from an intercalation of at least part of the protein molecule into the lipid film, with partial unfolding. At very low surface pressures, there is initially penetration of the whole protein molecule into the film, perhaps also involving a partial unfolding of the protein into the interface (Quinn and Dawson, 1970). At higher pressures the protein would adsorb in a different way into the monolayers with more limited penetration of either hydrophobic amino acid side chains or unfolded regions of the peptide chain. Interestingly, a linear $\Delta\pi$ versus π_0 dependence is not observed for the penetration of cyt c into eggPC and eggPC/POPG monolayers, which thus differs from the behaviors of adriamycin and BSA. This implies

that upon lipid binding cyt c undergoes conformational changes, which are dependent on lateral lipid packing. Another possibility is that the nature of cyt c-lipid interaction depends on π, these possibilities being mutually nonexclusive. Although $\Delta\pi$ is smaller in the presence of PEG-Cer, the lack of linear $\Delta\pi$ versus π_0 dependence still remains.

The possibility of a phase separation to be induced by adriamycin and the two proteins in monolayers in the absence and presence of PEG-Cer was studied using fluorescence microscopy (data not shown). EggPC/POPG monolayers in the presence and absence of PEG-Cer remained homogeneous at all surface pressures following the injection of adriamycin into the subphase. Similarly, there was no phase separation upon the addition of cyt c at $\pi_0 = 15$ mN/m. However, when π_0 was increased to 25 mN/m, phase separation due to cyt c was evident with no dependence on the presence of PEG-Cer. For BSA, the lipid monolayers were homogeneous in the absence of PEG-Cer whereas phase separation took place for PEG-Cer-containing films. The different organization of monolayers due to the binding of cyt c in the absence of PEG-Cer are in keeping with the nature of the interaction of this protein with lipids to depend on surface pressure. At low surface pressures cyt c intercalates into the monolayer and associates with both eggPC and POPG. When the surface pressure increased the protein mainly interacts with the lipid headgroups, preferentially with POPG. Because the negative charge density of the films is augmented with increasing π, we may expect the formation of a POPG-enriched domain to be induced upon the binding of cyt c to the mixed films. In keeping with this no phase separation was observed for an eggPC/NBD-PC monolayer after the injection of cyt c into the subphase. Finally, in the presence of PEG-Cer the behavior of lipid monolayers is more complex and is determined by factors such as the conformational transitions of PEG chains and lateral packing of lipid monolayers as well as ligand binding. The latter in turn depends on both surface pressure and the conformational states of PEG chains.

To conclude, our data demonstrate the packing-density-dependent phase transition for PEG-Cer in monolayers on the air/water interface. Our findings further show that the conformation of the PEG chains is an important determinant for the repulsive effects of PEG-Cer on the penetration of adriamycin and cyt c, driven by both electrostatic and hydrophobic forces. Our data also reveal this repulsive effect of PEG-Cer to disappear at $\pi_0 \approx 25$ mN/m, which could correspond to the pressure for the beginning of the formation of two-dimensional physical networks of this lipopolymer at the air/water interface (Naumann et al., 2001). This is in keeping with computer simulations that indicate that a more rigid polymer fails to form a dense protective cloud over the liposome surface that would prevent opsonizing particles from contacting liposome (Torchilin et al., 1994). Our findings provide information aiding the design of stealth liposomes containing PEG-Cer for improved drug delivery, as well as for the design of biocompatible surfaces with reduced protein adsorption. The protective effects of this amphipathic polymer depend on three key parameters: 1) the conformation of PEG polymer, 2) the interaction forces between lipid surface and ligand, and 3) the molecular size of the ligand. Accordingly, the incorporation of well-balanced contents of PEG-Cer into lipid monolayers or liposomes should effectively diminish the association of proteins and drugs with the lipid-grafted surfaces.

The technical assistance of Kaija Niva is appreciated.

This study was supported by the Finnish Academy and Technology Development Fund (TEKES).

REFERENCES

Adlakha-Hutcheon, G., M. B. Bally, C. R. Shew, and T. D. Madden. 1999. Controlled destabilization of a liposomal drug delivery system enhances mitoxantrone antitumor activity. *Nat. Biotechnol.* 17:775–779.

Alexander, S. 1977. Adsorption of chain molecules with a polar head, a scaling description. *J. Phys. (France).* 38:983–987.

Baekmark, T. R., G. Elender, D. D. Lasic, and E. Sackmann. 1995. Conformational transitions of mixed monolayers of phospholipids and poly(ethylene oxide) lipopolymers and interaction forces with solid surfaces. *Langmuir.* 11:3975–3987.

Baekmark, T. R., T. Wiesenthal, P. Kuhn, A. Albersörfer, O. Nuyken, and R. Merkel. 1999. A systematic infrared reflection-adsorption spectroscopy and film balance study of the phase behavior of lipopolymer monolayers at the air-water interface. *Langmuir.* 15:3616–3626.

Baekmark, T. R., T. Wiesenthal, P. Kuhn, T. M. Bayerl, O. Nuyken, and R. Merkel. 1997. New insights into the phase behavior of lipopolymer monolayers at the air/water interface: IRRAS study of a polyoxazoline lipopolymer. *Langmuir.* 13:5521–5523.

Bijsterbosch, H. D., V. O. de Haan, A. W. de Graaf, M. Mellema, F. A. M. Leermakers, M. A. Cohen Stuart, and A. A. van Well. 1995. Tethered adsorbing chains: neutron reflectivity and surface pressure of spread diblock copolymer monolayers. *Langmuir.* 11:4467–4473.

Blume, A. 1979. A comparative study of the phase transitions of phospholipid bilayers and monolayers. *Biochim. Biophys. Acta.* 557:32–44.

Brockman, H. 1994. Dipole potential of lipid membranes. *Chem. Phys. Lipids.* 73:57–79.

Brockman, H. 1999. Lipid monolayers: why use half of a membrane to characterize protein-membrane interactions? *Curr. Opin. Struct. Biol.* 9:438–443.

Carignano, M. A., and I. Szleifer. 1995. On the structure and pressure of tethered polymer layers in good solvent. *Macromolecules.* 28: 3197–3204.

de Gennes, P. G. 1980. Conformations of polymers attached to an interface. *Macromolecules.* 13:1069–1075.

Desai, N. P., and J. A. Hubbell. 1991. Solution technique to incorporate polyethylene oxide and other water-soluble polymers into surfaces of polymeric biomaterials. *Biomaterials.* 12:144–153.

De Wolf, F. A., M. Maliepaard, F. van Dorsten, I. Berghuis, K. Nicolay, and B. de Kruijff. 1991. Comparable interaction of doxorubicin with various acidic phospholipids results in changes of lipid order and dynamics. *Biochim. Biophys. Acta.* 1096:67–80.

Du, H., P. Chandaroy, and S. W. Hui. 1997. Grafted poly(ethylene glycol) on lipid surfaces inhibit protein adsorption and cell adhesion. *Biochim. Biophys. Acta.* 1326:236–248.

Faure, M. C., P. Bassereau, M. A. Carignano, I. Szleifer, Y. Gallot, and D. Andelman. 1998. Monolayers of diblock copolymer at the air/water interface: the attractive monomer-surface case. *Eur. Phys. J. B.* 3:365–375.

Fery-Forgues, S., J. P. Fayet, and A. Lopez. 1993. Drastic changes in the fluorescence properties of NBD probes with the polarity of the medium: involvement of TICT state? *Photochem. Photobiol. A Chem.* 70: 229–243.

Goormaghtigh, E., P. Chatelain, J. Caspers, and J. M. Ryusschaert. 1980. Evidence of a specific complex between adriamycin and negatively-charged phospholipids. *Biochim. Biophys. Acta.* 597:1–14.

Goormaghtigh, E., M. Vandenbranden, and J. M. Ruysschaert. 1982. Adriamycin inhibits the formation of non-bilayer lipid structures in cardiolipin-containing model membranes. *Biochim. Biophys. Acta.* 685:137–143.

Halperin, A. 1992. The phase behavior of tethered chains: an overview. *Macromolecular Rep*. A29:107–116

Halperin, A. 1999. Polymer brushes that resist adsorption of model proteins: design parameters. *Langmuir.* 15:2525–2533

Halperin, A., M. Tirrell, and T. P. Lodge. 1992. Tethered chains in polymer microstructures. *Adv. Polymer Sci.* 100:31–71.

Haydon, D. A., and J. L. Taylor. 1963. The stability and properties of bimolecular lipid leaflets in aqueous solution. *J. Theor. Biol.* 4:281–296.

Hernandez, J., A. Marti, and J. Estelrich. 1991. Interaction of doxorubicin with lipid systems. *Bioconjugate Chem.* 2:398–402.

Holland, J. W., P. R. Cullis, and T. D. Madden. 1996a. Poly(ethylene glycol)-lipid conjugates promote bilayer formation in mixtures of non-bilayer-forming lipids. *Biochemistry.* 35:2610–2617.

Holland, J. W., C. Hui, P. R. Cullis, and T. D. Madden. 1996b. Poly(ethylene glycol)-lipid conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine. *Biochemistry.* 35:2618–2624.

Holopainen, J. M., H. L. Brockman, R. E. Brown, and P. K. J. Kinnunen. 2001. Interfacial interactions of ceramide with dimyristoylphophatidylcholine: impact of the *N*-acyl chain. *Biophys. J.* 80:765–775.

Jeon, S. I., J. H. Lee, J. D. Andrade, and P. G. de Gennes. 1991. Protein-surface interactions in the presence of polyethylene oxide. I. Simplified theory. *J. Colloid Interface Sci.* 142:149–158.

Jeon, S. I., and J. D. Andrade. 1991. Proteins-surface interactions in the presence of polyethylene oxide. *J. Colloid Interface Sci.* 142:159–166.

Kenworthy, K., K. Hristova, D. Needham, and T. J. McIntosh. 1995. Range and magnitude of the steric pressure between bilayers containing phospholipids with covalently attached poly(ethylene glycol). *Biophys. J.* 68:1921–1936.

Kim, M. W., and B. H. Cao. 1993. Additional reduction of surface tension of aqueous polyethylene oxide (PEO) solution at high polymer concentration. *Europhys. Lett.* 24:229–234.

Kinnunen, P. K. J. 1991. On the principles of functional ordering in biological membranes. *Chem. Phys. Lipids.* 57:375–399.

Kinnunen, P. K. J., A. Köiv, J. Y. A. Lehtonen, M. Rytömaa, and P. Mustonen. 1994. Lipid dynamics and peripheral interactions of proteins with membrane surfaces. *Chem. Phys. Lipids.* 73:181–207.

Kluck, R. M., E. Bossy-Wetzel, D. R. Green, and D. D. Newmeyer. 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science.* 275:1132–1136.

Konttila, R., I. Salonen, J. A. Virtanen, and P. K. J. Kinnunen. 1988. Estimation of the equilibrium lateral pressure in liposomes of 1-palmitoyl-2-[10-(pyren-1-yl)-10-ketodecanoyl]-*sn*-glycero-3-phosphocholine and the effect of the phospholipid phase transition. *Biochemistry.* 27: 7443–7446.

Kuhl, T. L., D. E. Leckband, D. D. Lasic, and J. N. Israelachvili. 1994. Modulation of interaction forces between bilayers exposing short-chained ethylene oxide headgroup. *Biophys. J.* 66:1479–1488.

Kuhl, T. L., J. Majewski, J. Y. Wong, S. Steinberg, D. E. Leckband, J. N. Israelachvili, and G. S. Smith. 1998. A neutron reflectivity study of polymer-modified phospholipid monolayers at the solid-solution interface: polyethylene glycol-lipids on silane-modified substrates. *Biophys. J.* 75:2352–2362.

Leckband, D., S. Sheth, and A. Halperin. 1999. Grafted poly(ethylene oxide) brushes as nonfouling surface coatings. *J. Biomater. Sci.-Polym. Ed.* 10:1125–1147.

Liu, X., C. N. Kim, J. Yang, R. Jemmerson, and X. Wang. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell.* 86:147–157.

Majewski, J., T. L. Kuhl, M. C. Gerstenberg, J. N. Israelachvili, and G. S. Smith. 1997. Structure of phospholipid monolayers containing poly(ethylene glycol) lipids at the air-water interface. *J. Phys. Chem. B.* 101: 3122–3129.

Majewski, J., T. L. Kuhl, K. Kjaer, M. C. Gerstenberg, J. Als-Nielsen, J. N. Israelachvili, and G. S. Smith. 1998. X-ray synchrotron study of packing and protrusions of polymer-lipid monolayers at the air-water interface. *J. Am. Chem. Soc.* 120:1469–1473.

Menozzi, M., L. Valentini, E. Vannini, and F. Arcamone. 1984. Self-association of doxorubicin and related compounds in aqueous solution. *J. Pharmacol. Sci.* 73:766–770.

Milner, S. T. 1991. Polymer brushes. *Science.* 251:905–914.

Mustonen, P., and P. K. J. Kinnunen. 1991. Activation of phospholipase A2 by adriamycin in vitro. *J. Biol. Chem.* 266:6302–6307.

Mustonen, P., and P. K. J. Kinnunen. 1993. On the reversal by deoxyribonucleic acid of the binding of adriamycin to cardiolipin-containing liposomes. *J. Biol. Chem.* 268:1074–1080.

Mustonen, P., J. Lehtonen, A. Koiv, and P. K. J. Kinnunen. 1993. Effects of sphingosine on peripheral membrane interaction: comparison of adriamycin, cytochrome *c*, and phospholipase A_2. *Biochemistry.* 32: 5373–5380.

Naumann, C. A., C. F. Brooks, G. G. Fuller, W. Knoll, and C. W. Frank. 1999. Viscoelastic properties of lipopolymers at the air-water interface: a combined interfacial stress rheometer and film balance study. *Langmuir.* 15:7752–7761.

Naumann, C. A., C. F. Brooks, G. Fuller, T. Lehmann, J. Ruhe, W. Knoll, P. Kuhn, O. Nuyken, and C. W. Frank. 2001. Two dimensional physical networks of lipopolymers at the air/water interface: correlation of molecular structure and surface rheological behavior. *Langmuir.* 17: 2801–2806.

Needham, D., T. J. McIntosh, and D. D. Lasic. 1992. Repulsive interactions and mechanical stability of polymer-grafted lipid membranes. *Biochim. Biophys. Acta.* 1108:40–48.

Quinn, P. J., and R. M. C. Dawson. 1970. An analysis of the interaction of protein with lipid monolayers at the air/water interface. *Biochem. J.* 116:671–680.

Sadzuka, Y. 2000. Effective prodrug liposome and conversion to active metabolite. *Curr. Drug Metab.* 1:31–48.

Schenkman, S., P. S. De Araujo, A. Sesso, F. H. Quina, and H. Chaimovich. 1981. A kinetic structural study of two-step aggregation and fusion of neutral phospholipid vesicles promoted by serum albumin at low pH. *Chem. Phys. Lipids.* 28:165–180.

Senior, J., C. Delgado, D. Fisher, C. Tilcock, and G. Gregoriadis. 1991. Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly-(ethylene glycol)-coated vesicles. *Biochim. Biophys. Acta.* 1062:77–82.

Szleifer, I. 1997a. Protein adsorption on surfaces with grafted polymers: a theoretical approach. *Biophys. J.* 72:595–612.

Szleifer, I. 1997b. Protein adsorption on tethered polymer layers: effect of polymer chains architecture and composition. *Physica A.* 244:370–388.

Szleifer, I. 1997c. Polymers and proteins: interactions at interfaces. *Curr. Opin. Solid State Mater. Sci.* 2:337–344.

Torchilin, V. P., V. G. Omelyanenko, M. I. Papisov, A. A. Bogdanov Jr., V. S. Trubetskoy, J. N. Herron, and C. A. Gentry. 1994. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. *Biochim. Biophys. Acta.* 1195:11–20.

Torchilin, V. P., M. I. Papisov, A. A. Bogdanov, V. S. Trubetskoy, and V. G. Omelyanenko. 1995. Molecular mechanism of liposome and immunoliposome steric protection with poly(ethylene glycol): theoretical and experimental proofs of the role of polymer chain flexibility. *Stealth Liposomes.* 51–62.

Webb, M. S., D. S. Saxon, F. M. P. Wong, H. J. Lim, Z. Wang, M. B. Bally, S. L. Choi, P. R. Cullis, and L. D. Mayer. 1998. Comparison of different hydrophobic anchors conjugated to poly(ethylene glycol): ef-

fects on the pharmacokinetics of liposomal vincristine. *Biochim. Biophys. Acta.* 1372:272–282.

Weis, R. M. 1991. Fluorescence microscopy of phospholipid monolayer phase transitions. *Chem. Phys. Lipids.* 57:227–239.

Wiesenthal, T., T. R. Baekmark, and R. Merkel. 1999. Direct evidence for a lipid alkyl chain ordering transition in poly(ethylene oxide) lipopolymer monolayers at the air/water interface obtained from infrared reflection absorption spectroscopy. *Langmuir.* 15:6837–6844.

Wong, J. Y., T. L. Kuhl, J. N. Israelachvili, N. Mullah, and S. Zalipsky. 1997. Direct measurement of a tethered ligand-receptor interaction potential. *Science.* 275:820–822.

Woodle, M. C. 1995. Sterically stabilized liposome therapeutics. *Adv. Drug Delivery Rev.* 16:249–265

Yang, J., X. Liu, K. Bhalla, C. N. Kim, A. M. Ibrado, J. Cai, T. I. Peng, D. P. Jones, and X. Wang. 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science.* 275:1129–1132.