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ARTICLE

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Thin liquid films and monolayers of DMPC mixed with PEG and phospholipid linked PEG

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Abstract In this work thin liquid films (TLFs) and monolayers at the air/water interface formed by dimyristoylphosphatidylcholine (DMPC) and by DMPC mixed with poly ethylene glycols (PEGs) and dimyristoylphosphatidylethanolamine (DMPE) linked PEGs were studied. Film forming dispersions were composed of two types of particles: liposomes and micelles. TLFs stability, threshold concentration C_t (i.e., the minimum one for stable film formation), and hydrodynamic behavior were measured. At equivalent conditions, DMPC films were Newton black films (real bilayers), while DMPE-PEGs films were much thicker with free water between the monolayers. DMPE-PEG addition to DMPC films caused both C_t decrease (depending on PEG moiety length and Mw) and change of TLF formation mechanism. TLFs' hydrodynamic behavior also strongly depended on DMPE-PEG content and Mw. It was observed that thinning of the DMPC and DMPE-PEGs films continued to different film types and thickness, being much thicker for the latter films. Addition of free PEGs (PEG-200/6000) did not alter TLF type or stability, but changed TLF thinning time, confirming that free PEGs with Mw < 8000 could not penetrate in the membrane and alter "near-membrane" water layer viscosity. Monolayer studies showed improved formation kinetics of both adsorbed and spread films, decrease of surface tension (equilibrium and dynamic), and of film compression/decompression histeresis area in DMPE-PEGs monolayers compared with DMPC pure films. Our study shows that combining the models of phospholipid TLFs and monolayers provide the opportunity to investigate the properties of membrane surface and to clarify some mechanisms of its interactions with membrane-active agents.

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Introduction

Poly ethylene glycols (PEGs) with different molecular weight are one of the most commonly used membrane active agents in the modern pharmacy, medicine, molecular biology, and biophysics (Arnold et al. 1983; Arnold et al. 1985; Marsh 2001). The effects of PEGs on the membrane structure and properties strongly depend of the formulation form of the PEG moiety, free or phospholipid (PL) linked PEG.

PEG moieties of phospholipid linked PEGs, extended in "mushroom" (at low PL-PEG surface concentration) or "brush" (at high PL-PEG concentration) chain conformation from the membrane plane toward the water solution, form at the membrane surface thick hydrophilic layer (Chapman and Jones 1995; Kuhl et al. 1994; Johnson and Edwards 2001). It was responsible for the increased steric disjoining pressure among PL linked PEG coated surfaces measured with surface force apparatus technique (Kuhl et al. 1994). Thus, the hydrophilic layer creates a steric barrier preventing the adsorption of lipoproteins and opsonins to the liposome surface, making the liposomes invisible for the reticuloendothelial system responsible for the uptake of foreign particles out of the blood flow. Thus, the so-called "stealth" liposomes are obtained, with increased stability and prolonged lifetime in the blood flow circulation (from hours to days), making them promising drug vehicles (Chapman and Jones 1995; Johnson and Edwards 2001; Chung et al. 2004). Stealth liposomes were used as vehicles of previously known anticancer and antifungal drugs (doxorubicin, alphameticin, etc.) for which increased pharmacological effect and decreased toxicity were obtained (Chapman and Jones 1995; Cattel et al. 2004; Wang et al. 2005). It was also found that apart from "stealth" bilayer liposomes, PL linked PEGs, pure or mixed (at high mol content) with phospholipids,

form "stealth" normal monolayer micelles, which could also be used as drug vehicles (Szeifer et al. 1998; Marsh 2001; Johnson and Edwards 2001; Montesano et al. 2001; Belsito et al. 2000). Recently, PL linked PEGs were used for obtaining "stealth" erythrocytes as universal blood substituent (Szeifer et al. 1998).

Free PEGs are well known fusion agents (Arnold et al. 1983; Arnold et al. 1985; Hui et al. 1999; Malinin et al. 2002) in model membrane systems and cell cultures. However, there are also numerical reports (Semenchenco 1988) concerning the cryoprotective action of low molecular PEGs (up to PEG-1000). Recently, Kuhl et al. (1996) on the base of previous results of other researchers (Arnold et al. 1983, 1985) and surface force apparatus experiments with phosphatidyl choline solid supported membranes conclude that only free PEGs with molecular weight (Mw) 8,000–10,000 induce membrane fusion by depletion attraction osmotic pressure mechanism. Free PEGs with Mw < 8,000 alter the structure and the viscosity of the "near membrane" free and bound water without penetrating the membrane, while free PEGs with Mw > 10,000 are sufficiently hydrophobic and directly penetrate in the membrane surface.

Two model membrane systems widely used for studying the effects of membrane active compounds are phospholipid thin liquid films (TLFs) and monolayers at the air/water interface. TLFs, being of several types, are composed of two mutually adsorbed, plane-parallel oriented "head-to-head" PL monolayers (Fig. 1), thus being structurally analogous to the *cis*-monolayers apposition occurring at the onset of membrane fusion and at close intermembrane adhesion (Naydenova et al. 1990; Exerowa and Krugliakov 1998). Despite the successful use of phospholipid TLFs as model system (Lalchev 1997; Exerowa and Krugliakov 1998) in biology and medicine (regarding membrane interactions, fusion and adhesion processes, bio-surfactant action at interfaces, etc.) there are to date only two studies (Nikolova and Jones 1996; Nikolova and Jones 1998) of TLFs formed by dimyristoyl phosphatidylethanolamine (DMPE) linked PEGs relating to film thickness and gas permeability. In these works, the hydrodynamic behavior and stability of the films were not discussed. PL monolayers (Fig. 1d) can be regarded as half of TLFs or black lipid membranes (Lalchev 1997; Lalchev 2004) and appear to be a preferred model for studying the interactions among phospholipids in the membrane plane.

The aim of the current work was to study TLFs and monolayers at the air/water interface formed by dimyristoylphosphatidylcholine (DMPC) mixed with DMPE linked PEGs or with free PEGs. The stability and hydrodynamic behavior of pure DMPC films were compared with those of PEG-containing films (free or DMPE linked PEGs). The surface tension (equilibrium and during compression/decompression) of pure DMPC monolayers and PEG-containing monolayers was also measured. TLFs and monolayers formed by pure DMPE linked PEGs were also obtained and their properties were compared with those of pure DMPC and DMPC/DMPE-PEG mixed films.

DMPC was chosen because it is a typical liposome forming phospholipid commonly used in biophysics and pharmacy (Chapman and Jones 1995; Johnson and Edwards 2001) which at physiological temperature is in liquid–crystalline phase state (Cevc et al. 1990), in contrast to other lipids, such as DMPE, which has main phase transition temperature as high as 49°C (Cevc 1987; Wilkinson and Nagle 1981). Liposomes composed of phosphatidylcholines and PE-PEGs have been used for a long time as in vivo drug carriers (Kenworthy et al. 1995; Bergstrandt 2003; Warriner et al. 1998). The film forming dispersions were composed, depending on DMPE-PEG mol content, of two types of particles: liposomes and micelles (Montesano et al. 2001; Belsito et al. 2000). Both PL linked PEGs (DMPE-PEG_{550}, DMPE-PEG_{2000}, and DMPE-PEG_{5000}) and free PEGs (PEG-400, PEG-1500, PEG-2000, PEG-5000) were used with PEG moiety Mw < 8000.

Materials and methods

Materials

DMPC, DMPE linked PEGs, DMPE-PEG_{550}, DMPE-PEG_{2000}, and DMPE-PEG_{5000}, were purchased from "Avanti Polar Lipids" and PEG-400, PEG-1500, PEG-2000, PEG-6000 from "Sigma". NaCl was purchased from "Merck". Solutions were made with bidestilled water with conductivity less than 1.10^{-6} S/cm.

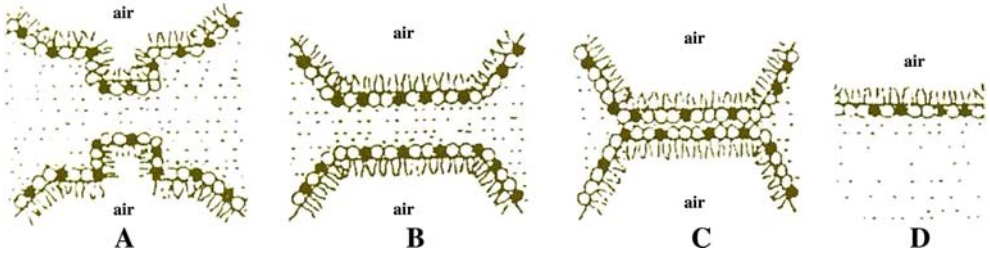

Fig. 1 Schematic representation of TLFs and monolayers at the air/water interface used in our study. Two types of TLFs are shown: Common Black Film (**b**) and Newton Black Film (**c**). Thick TLF with black spot (**a**) is also presented. **d** shows phospholipid monolayer, which can be regarded as a half of bilayer film

Determination of the type of the particles composing film forming dispersions

Several film forming dispersions were used, mixture of DMPC with less than 10 mol% DMPE-PEG, with 20 and 80 mol% DMPE-PEG. Experiments were performed according to the protocol of Montesano et al. (2001) and Belsito et al. (2000) by measuring with SPEKOL –11, the optical density at 400 nm (OD_{400}) of the dispersions of DMPC mixed with DMPE-PEGs. According to the above authors, by measuring OD_{400} (PE-linked PEG mol%) dependence one can qualitatively study the change of the ratio liposomes/micelles when altering PE-PEG mol content. They showed that a straightforward interpretation of OD_{400} changes with increasing content of PEG-lipid in the dispersions shows that the initial rapid drop corresponds to a decrease in overall size of the lipid aggregates attributed to disaggregation of the liposomes and that the closed bilayer vesicles of reduced size convert to micelles when OD_{400} becomes very low.

Thin liquid films

TLFs were formed by the method of Scheludko and Exerowa (Exerowa and Krugliakov 1998) using the modified measuring cell of Lalchev et al. (1997)as previously described. A biconcave drop (50 µl volume) of the phospholipid dispersion (pH 6.8–7.0; $C_{el} = 0.5$ M NaCl) was incubated into the cylinder of the measuring cell at $T = 37$°C for 30 min. After sucking the solution from the drop, thick TLF is formed (Fig. 1). Further, the film spontaneously gets thinner and after some characteristic film thinning time, t_{0-1} (s), critical film thickness is reached (ca. 300 Å). Then a black spot (BS), local thinning in the film, appears (as schematically shown in Fig. 1a), expands with characteristics rate to fill up the whole area of the film. The kinetic of this process was measured by BS expansion time t_{1-2} (s) detecting the time from the formation of the first BS to the moment of its expansion to the whole film area, i.e., to black film formation. At different experimental conditions two types of stable black films can be formed: common black films, CBFs, (Fig. 1b) and Newton black films, NBFs, (Fig. 1c).

The probability (W) for formation of stable black films depends strongly on the phospholipid concentration, C, (Lalchev 1984; Exerowa and Krugliakov1998) and can be calculated by the equation $W = (\Delta N/N$, where N is the total number of trials (at least 50 for each concentration) and ΔN is the number of trials in which stable black films are formed. Thus, W varies between 0 and 1 indicating that the films always rupture ($W = 0$) and that the films are always formed stable ($W = 1$). The dependence $W(C)$ is extremely steep which allowed to define a threshold concentration (C_t) as the minimum phospholipid concentration at which $W = 1$ and stable films are always formed (Lalchev 1984). It is proven that $W(C)$ dependence is sensitive to the composition of the film forming dispersion, molecular shape, and phase state of the film forming PLs, pH, electrolyte concentration, applied pressure, etc. (Lalchev 1997; Exerowa and Krugliakov 1998).

Monolayers

Monolayers at the air/water interface (spread and adsorbed) of pure DMPC and DMPC mixed with free PEGs or DMPE linked PEGs were formed in the Langmuir and the surface tension γ (mN/m) was measured by the method of Wilhelmy with accuracy ±0.5 mN/m, as previously described (Christova et al. 1998). The surface tension–time and γ(C) dependences were recorded. The dynamic behavior of the monolayers, i.e., maximum (γ_{max}) and minimum (γ_{min}) surface tension, after compression/decompression of the film from 100 to 20% of the initial film surface area with compression rate 3 min/cycle, and the histeresis area (A_H) during compression/decompression cycle of the monolayer were measured. Experiments were performed at $T = 37$°C, pH 6.8–7.0, and electrolyte concentration $C_{el} = 0.5$ M NaCl.

Results and discussion

Thin liquid films

Evaluation of the ratio of liposomes and micelles in the film forming dispersions

Experiments were performed with film forming dispersions of DMPC mixed with DMPE-PEGs, composed of two types of particles: liposomes or/and micelles. The type of the dispersion particles was regulated by varying the DMPE-PEG mol content and was determined experimentally by measuring the optical densities (OD_{400}) of the dispersions at 400 nm. The technique was applied by Montesano et al. 2001 and Belsito et al. 2000 for determining the type and the ratio among the particles composing dispersions of DPPE linked PEGs mixed with DPPC. OD_{400} (DMPE-PEG mol%) dependence is shown in Fig. 2.

The upper region of the plot curved part corresponds to dispersions consisting predominantly of liposomes (Montesano et al. 2001; Belsito et al. 2000). With increasing DMPE-PEG content, OD_{400} decreases due to the decreased size of the dispersion forming particles transforming from bilayer liposomes to micelles. When the whole dispersion is composed by micelles the plateau of minimum OD_{400} is reached. It can be seen that the plateau was reached at lower concentration for DMPE-PEG_{2000} (35 mol%) in comparison with DMPE-PEG_{550} and DMPE-PEG_{5000} (73 mol%). Similar non-linear dependence of the particle transformation from liposomes to micelles on PEG moiety molecular weight was

also observed for dispersions of DPPE-PEGs with DPPC (Montesano et al. 2001; Belsito et al. 2000). In the current work, for most of the experiments with DMPE-PEG containing dispersions, three concentrations of DMPE linked PEGs were used, less than 10 mol% DMPE-PEG (where dispersions are formed predominantly by liposomes), 20 mol% (where probably mixture of liposomes and micelles exist in the dispersions), and 80 mol% DMPE-PEG (where DMPC/DMPE-PEG dispersions consist of micelles only).

TLFs of DMPC mixed with DMPE-PEGs

$W(C)$ curves of TLFs of pure DMPC and pure DMPE-PEG dispersions are shown in Fig. 3.

The threshold concentrations, C_t, of films from DMPC/DMPE-PEGs mixed dispersions are listed in Table 1. It can be seen in Fig. 3 that all mixed DMPC/DMPE-PEGs used formed stable black films at threshold concentration values lower than the threshold concentration for DMPC films (2.9×10^{-4} M DMPC). For DMPE-PEGs films the value of C_t decreased with increasing PEG moiety length and molecular weight and the lowest value of C_t was obtained for DMPE-PEG_{5000} (4×10^{-6} M). It is important to note that at constant electrolyte concentration ($C_{el}=0.5$ M NaCl) the type of the black films formed by DMPE-PEGs and DMPC were different (Table 1). DMPC films were Newton black films, with no free water core between the phospholipids monolayers (Fig. 1c), while the films of the shortest DMPE linked PEG-550 were common black films. Both NBFs and CBFs were formed by the mechanism of fluctuation BS formation (Exerowa and Krugliakov 1998). However, the films of longer chain DMPE-PEG_{2000} and DMPE-PEG_{5000} were formed not by the BS formation mechanism, but by continuous thinning without black spot formation until equilibrium black CBF-like films were obtained. This behavior probably could be explained in terms of strong steric repulsion disjoining pressure arising between the phospholipid monolayers due to the overlapping of hydrophilic polymer "brushes". Such effects were observed for PL linked PEG_{2000} (Kuhl et al. 1994), for DPPE-PEG_{2000} and DMPC/ DPPE-PEG_{2000} mixture (Nikolova and Jones 1998), three-block co-polymer surfactants (Exerowa and Krugliakov 1998), etc.

The threshold concentration values and the formation mechanisms (Table 1) of mixed DMPC/DMPE-PEG films depend on both DMPE-PEG content and on the PEG moiety length and molecular weight.

The C_t of mixed films was lower than that of DMPC films and higher in comparison with pure DMPE-PEG films. Analogous to DMPE-PEG black films, the lowest C_t value (0.5×10^{-5} M) was obtained for the longest chain PL linked PEG, for 80 mol% DMPE-PEG_{5000}. When the DMPE-PEG concentration was increased to some DMPE-PEG concentration ($C^{min}_{DMPE\text{-}PEG}$), TLFs thinned not to bilayer NBFs (characteristic for DMPC), but to the much thicker CBFs or CBF-like films (characteristic for DMPE linked PEGs, Table 1). CBFs were formed at $C_{DMPE\text{-}PEG550}{}^{min}\geq9$ mol%, and CBF-like films formed by continuous thinning were obtained at $C\geq7$ mol% DMPE-PEG_{2000} and at $C\geq3$ mol% DMPE-PEG_{5000}. We also observed that both DMPE-PEG_{2000} and DMPE-PEG_{5000} were able to induce film thinning to CBFs by BS formation but at extremely low concentrations (less than 0.1 mol%, data not shown). For the three DMPE-PEGs the minimal $C^{min}_{DMPE\text{-}PEG}$ was lower than 10 mol%, i.e., the film forming dispersions were mainly composed of liposomes (Montesano et al. 2001; Belsito et al. 2000). It can be seen that $C^{min}_{DMPE\text{-}PEG}$ decreased with increasing PEG moiety molecular weight (Table 1).

The hydrodynamic behavior of mixed DMPC/DMPE-PEG films also strongly depend on the mol% of

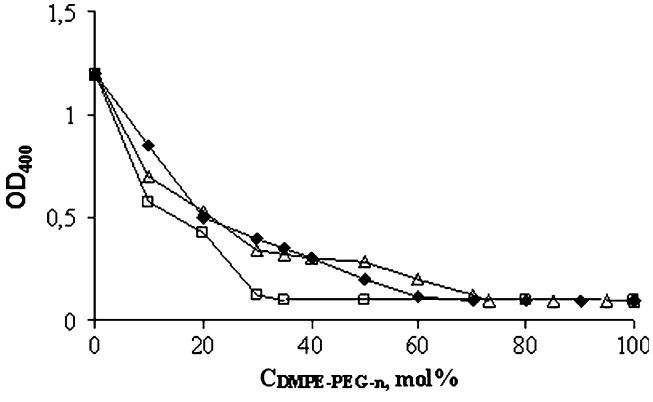

Fig. 2 OD_{400} ($C_{DMPE\text{-}PEG\text{-}n}$) dependence of mixed dispersions DMPC/DMPE-PEG. Data labels- DMPE-PEG_{550} (*filled diamond*), DMPE-PEG_{2000} (*open square*), DMPE-PEG_{5000} (*open triangle*). Experiments were performed at total lipid (DMPC + DMPE-PEG) concentration $C=1000$ μg/ml, $T=37$°C, $C_{el}=0.5$ M NaCl, pH 6.8–7.0

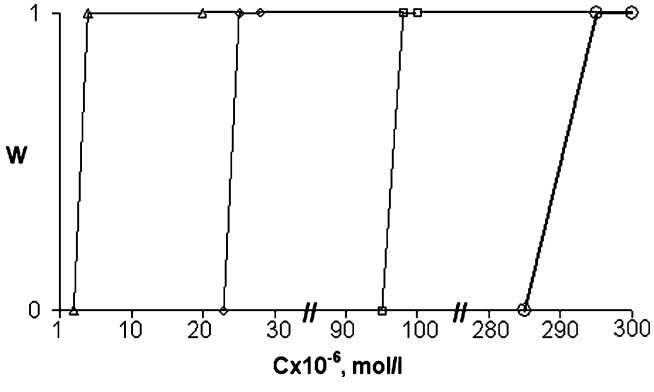

Fig. 3 Comparison between $W(C)$ dependences of DMPC and DMPE linked PEGs films at $C_{el}=0.5$ M NaCl . Data labels: (*O* and *thick line*) DMPC (the film is NBF, Fig. 1c); (*open square*) DMPE-PEG_{550} (the film is CBF, Fig. 1b); (*open diamond*) DMPE-PEG_{2000} (the film is CBF-like); and (*open triangle*) DMPE-PEG_{5000} (the film is CBF-like). Experiments were done at $T=37$°C, $C_{el}=0.5$ M NaCl, pH 6.8–7.0 with film diameter $d_f=200$ μm

Table 1 Type C_t, t_{0-1}, t_{1-2} of TLFs stabilized by DMPC and DMPC/DMPE-PEG mixtures with different molecular weight

Composition	TLF type	C_t (mol/l)	t_{0-1} (s)	t_{1-2} (s)	Formation mechanism
DMPC	NBF	2.9×10^{-4}	30	1	Fluctuation BS(one spot) formation
DMPE-PEG_{550}	CBF	9.6×10^{-5}	40	6	Fluctuation BS(2–5 spots) formation
DMPC + DMPE-PEG_{550} (9 mol%)[a]	CBF	2×10^{-4}	31	4	Fluctuation BS (2–5 spots) formation
DMPC+ DMPE-PEG_{550} (20 mol%)	CBF	1.2×10^{-4}	38	5	Fluctuation BS (2–5 spots) formation
DMPC + DMPE-PEG_{550} (80 mol%)	CBF	9.8×10^{-5}	40	6	Fluctuation BS (2–5 spots) formation
DMPE-PEG_{2000}	CBF-like	2.5×10^{-5}	*75*[b]		Continuous thinning without BS formation
DMPC + DMPE-PEG_{2000} (7 mol%)[a]	CBF-like	7×10^{-5}	*45*		Continuous thinning without BS formation
DMPC + DMPE-PEG_{2000} (20 mol%)	CBF-like	3.2×10^{-5}	*60*		Continuous thinning without BS formation
DMPC + DMPE-PEG_{2000} (80 mol%)	CBF-like	2.7×10^{-5}	*70*		Continuous thinning without BS formation
DMPE-PEG_{5000}	CBF-like	4×10^{-6}	*85*		Continuous thinning without BS formation
DMPC + DMPE-PEG_{5000} (3 mol%)[a]	CBF-like	1×10^{-5}	*45*		Continuous thinning without BS formation
DMPC + DMPE-PEG_{5000} (20 mol%)	CBF-like	7×10^{-6}	*70*		Continuous thinning without BS formation
DMPC + DMPE-PEG_{5000} (80 mol%)	CBF-like	5×10^{-6}	*70*		Continuous thinning without BS formation
Free PEG-6000	CBF	5×10^{-3}	720	14	Fluctuation BS(2–5 spots) formation

Experiments were performed at $T=37°C$, $C_{el}=0.5$ M NaCl, pH 6.8–7.0, $d_f=100$ μm
[a]It denotes the minimum concentration $C^{min}_{DMPE\text{-}PEG}$ above which TLFs thinned to CBFs or CBF-like films (but not to NBFs)
[b]Numbers in italics shows the time (t_{0-2}) from thick film formation to equilibrium CBF-like film formation

DMPE linked PEG. The results concerning film thinning time (t_{0-1}) and BS expansion time (t_{1-2}) of DMPC/DMPE-PEG_{550} films are shown in Fig. 4.

It can be seen that t_{0-1} increased linearly from 30 s (for pure DMPC) to 40 s (above 42 mol% DMPE-PEG_{550} plateau value of t_{0-1} was observed). At 9 mol% DMPE-PEG_{550}, the minimum at which the films thinned to CBFs (not to NBFs as for DMPC), $t_{0-1}=32$ s. The value of BS expansion time, t_{1-2}, steeply increased from 1 s (for NBFs of pure DMPC) to plateau value of 6 s (at ≥30 mol% DMPE-PEG_{550}) for the mixed films. At 9 mol% DMPE-PEG_{550} where the films (and the spots respectively) became common black t_{1-2} value was 4 s.

Figure 5a represent the dependences of DMPC film thinning time and BS expansion time on DMPE-$PEG_{2000/5000}$ mol%.

For concentrations up to 7 mol% DMPE-PEG_{2000} and up to 3 mol% DMPE-PEG_{5000}, typical CBFs were observed with linearly increasing t_{0-1} values to ca. 37 s and t_{1-2} values to ca. 9 s. Above 7 mol% DMPE-PEG_{2000} and 3 mol % DMPE-PEG_{5000}, we observed that the films thinned continuously to CBF-like films without formation of black spots. In this case, we recorded the CBF-like formation time on the mol% of DMPE-PEGs, increasing from 45 s to a plateau value of 75 and 85 s at 80 mol% DMPE-PEG_{2000} and 75 mol% DMPE-PEG_{5000}, respectively (Fig. 5b), which was equal to the film formation times from individual DMPE-PEGs.

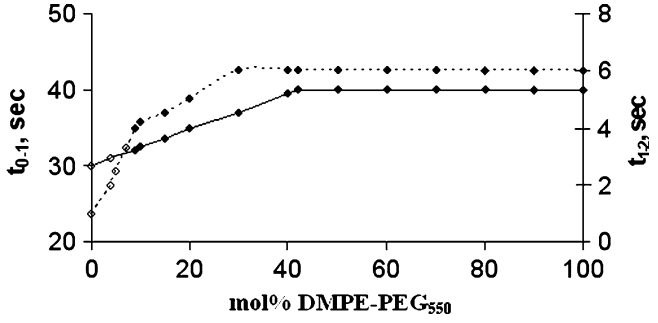

Fig. 4 Film thinning time (t_{0-1}, *full line*) and BS expansion time (t_{1-2}, *dashed line*) dependences on mol% DMPE-PEG_{550} in DMPC/DMPE-PEG_{550} mixed TLFs with film diameter $d_f=200$ μm. Data labels designate film thinning to NBFs (*open diamond*) or to CBFs (*filled diamond*). Experiments were done at $T=37°C$, $C_{el}=0.5$ M NaCl, pH 6.8–7.0

TLFs of DMPC in presence of free PEGs

None of the free PEGs used in our study, PEG-200, PEG-400, PEG-1500 and PEG-6000, did change neither DMPC film type and stability nor BS expansion time

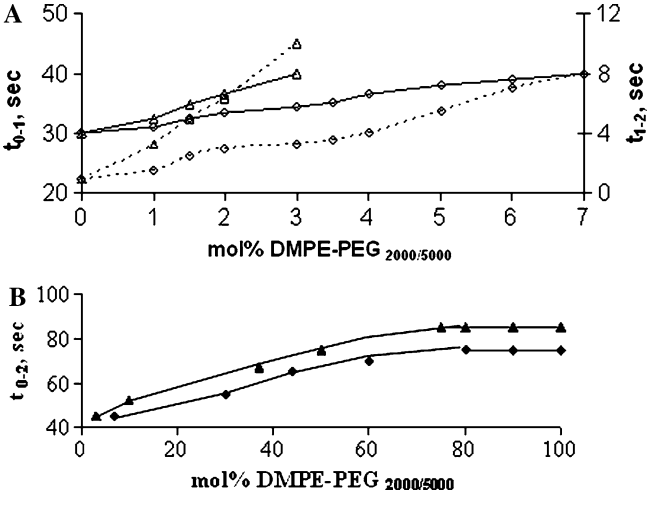

Fig. 5 Film thinning time (t_{0-1}, *full line*, **a**), BS expansion time (t_{1-2}, dashed line, **a**), film formation time (t_{0-2}, **b**) dependences on mol% of DMPE-$PEG_{2000/5000}$ (*open diamond* and *filled diamond* represent DMPE-PEG_{2000} and *open triangle* and *filled triangle* represent DMPE-PEG_{5000}). *Open symbols* are used for CBFs, while *closed symbols* are used for CBF-like films formed by continuous thinning. Experiments were done at $T=37°C$, $C_{el}=0.5$ M NaCl, pH 6.8–7.0, film diameter $d_f=200$ μm

values. The only effect of the PEG molecules present in the film liquid subphase was the increase of film thinning time, t_{0-1}, (Fig. 6).

The t_{0-1} increase was proportional to the PEG molecular weight, which increased dramatically from 30 s for pure DMPC films to 180 s in the presence of 10^{-2} M PEG-6000. These results are in agreement with the well known data for the viscosity increase of solutions in the presence of free PEGs and other solvated linear polymers (Eliassi and Modaress 1988).

The lack of effect of PEGs molecules on the film type and BS expansion time is in agreement with the fact that PEG-200, PEG-400, and PEG-1500 do not form black films, only high molecular PEG-6000, at concentrations as high as 5×10^{-3} M, could form common black films (Table 1).

Monolayers

Monolayers of DMPC mixed with DMPE-PEGs

The monolayers used, adsorbed and spread, were from DMPC, DMPE-PEG, and mixed DMPC/DMPE-PEG monolayers. Mixed monolayers content of DMPE-PEGs was less than 10 mol% (in concentrations at which TLFs thinned to CBF/CBF-like films but not to NBFs, Table 1), 20 mol%, and 80 mol% DMPE-PEG (at which mixed liposome/micelles dispersions and dispersions of micelles only were formed in the samples, respectively, Fig. 2). Because the effects at 20 and 80 mol% DMPE-PEG on the monolayer surface tension (both at equilibrium and during compression/decompression) were practically identical. Thus the results for 20 mol% DMPE-PEG are only shown below.

The $\gamma(C)$ dependences of adsorbed and $\gamma(t)$ dependences of spread (200 Å^2 area per molecule) monolayers of DMPC, DMPE-PEG, and mixed DMPC/DMPE-PEG are represented at Fig. 7a and b, respectively.

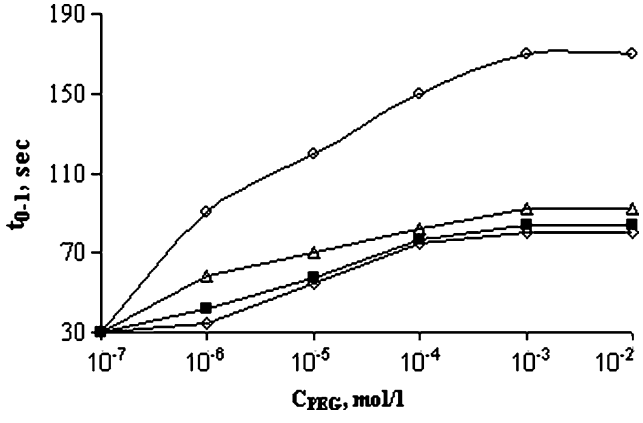

Fig. 6 Effect of free PEGs with different molecular weight on the DMPC film thinning time. Films were with diameter $d_f = 200$ μm. Data labels: *open diamond* (PEG-200), *filled square* (PEG-400), *open triangle* (PEG-1500), o (PEG-6000). Experiments were done at $T = 37$°C, $C_t = 200$ μg DMPC/ml, $C_{el} = 0.5$ M NaCl, pH 6.8–7.0

The comparison of $\gamma(C)$ dependences (Fig. 7a) showed that the saturation ($\gamma = 68.5$ mN/m) occurred at the lowest concentration (10^{-8} M DMPC) for pure DMPC monolayers. The result is in agreement with the data for the low solubility and low critical micelle concentration of DMPC (Chapman and Jones 1995), determining lower degree of adsorption in comparison with DMPE-PEGs. We also observed increased surface activity of DMPE-linked PEGs compared to individual DMPC and DMPE monolayers (data not shown). For DMPE-PEG monolayers the γ values decreased with increasing PEG moiety Mw, the lowest γ (64 mN/m) was obtained for DMPE-PEG$_{5000}$. As can be seen, the inclusion of DMPE-PEGs in the mixed DMPC/DMPE-PEG monolayers decreased γ of the saturated DMPC monolayer proportionally to the DMPE-PEG content and PEG moiety Mw. At 20 mol% DMPE-PEG, the $\gamma(C)$ dependences of pure DMPE-PEG and of the mixed DMPC/DMPE-PEG monolayers were almost identical.

Comparing $\gamma(t)$ dependences of spread monolayers (Fig. 7b) it can be seen that DMPC monolayers reached equilibrium surface tension $\gamma_{eq} = 58$ mN/m after equilibration time (t_{eq}) of 8 s. The presence of DMPE-PEGs in the monolayers resulted both in improving the kinetics of film spreading and decreasing of γ_{eq} value (being slight for DMPE-PEG$_{550}$, and very strong for DMPE-PEG$_{2000/5000}$). Both effects were also proportional to the DMPE-PEG content in the film. At 20 mol% DMPE-PEG, the film spreading kinetics and γ_{eq} were practically the same as for pure DMPE-PEG monolayers. It can be seen that at ≥20 mol% DMPE-PEG the kinetics of film spreading was the same for all DMPE-linked PEGs used ($t_{eq} = 5$ s), while γ_{eq} value depends on PEG moiety Mw and lowest γ_{eq} of 42 mN/m for the highest molecular weight DMPE-PEG$_{5000}$ was reached. These results are in agreement with the data for the increase of the effective molecular area and lateral steric repulsion force between PL linked PEGs with the increase of PEG moiety length and molecular weigh (Kuhl et al. 1994; Marsh 2001). The latter could result in accelerated kinetics of film spreading and uniform molecular packing at the air/water interface, necessary for reaching low equilibrium surface tension (Majewski et al. 1997).

Five consecutive compression/decompression (from100 to 20% of the initial surface area) cycles were conducted with DMPC, DMPE-PEG, and mixed DMPC/DMPE-PEG spread monolayers, and for each cycle the surface tension of the films at minimal (γ_{min}) and maximal (γ_{max}) surface area were measured. Since the results from third to fifth cycle were identical, only the results for the first three cycles are represented at Fig. 8a and b.

DMPC pure monolayers reached for the first compression/decompression cycle $\gamma_{min} = 31$ mN/m and $\gamma_{max} = 60$ mN/m. It is seen that only DMPC/DMPE-PEG$_{550}$ mixed films showed for the first cycle higher values of both minimal and maximal surface tension in comparison with the DMPC monolayers. In contrast,

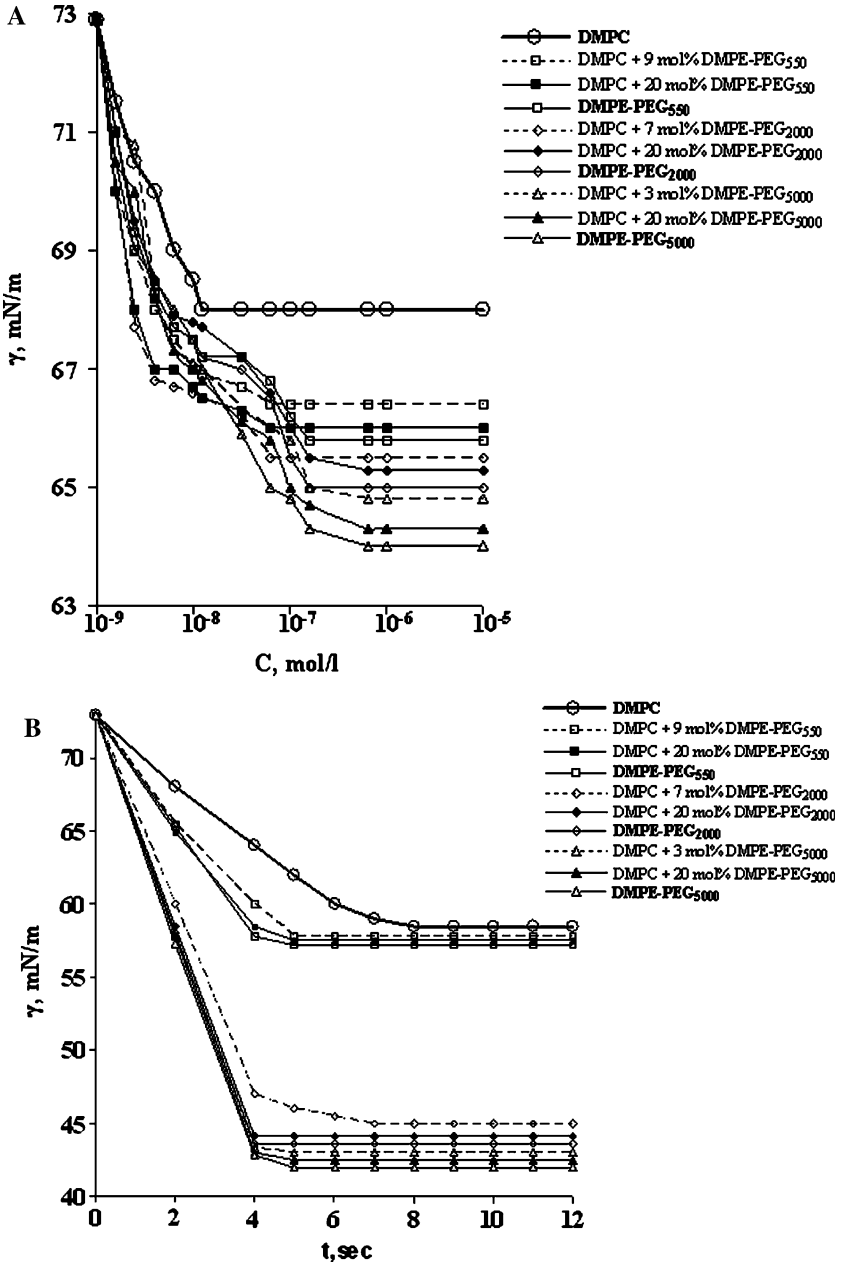

Fig. 7 Comparison of $\gamma(C)$ dependences of adsorbed (**a**) and of γ(t) dependences of spread (**b**) DMPC pure (*O* and *thick line*) monolayers and mixed DMPC/DMPE-PEG monolayers: DMPE-PEG_{550} pure (*open square*), DMPC + 20 mol% DMPE-PEG_{550} (*filled square*), DMPC + 9 mol% DMPE-PEG_{550} (*open square* and *dashed line*); DMPE-PEG_{2000} pure (*open diamond*), DMPC + 20 mol% DMPE-PEG_{2000} (*filled diamond*), DMPC + 7 mol% DMPE-PEG_{2000} (*open diamond* and *dashed line*); DMPE-PEG_{5000} (*open triangle*), DMPC + 20 mol% DMPE-PEG_{5000} (*filled triangle*), DMPC + 3 mol% DMPE-PEG_{5000} (*open triangle* and *dashed line*). Spread monolayers surface density corresponded to 200 $Å^2$ per molecule. Experiments were done at $T = 37°C$, $C_{el} = 0.5$ M NaCl, pH 6.8–7.0

DMPE-PEG_{2000} and DMPE-PEG_{5000} monolayers, pure and mixed with DMPC, showed lower values of γ_{min} and γ_{max} in comparison with DMPC monolayers for all compression/decompression cycles. The latter effects could be explained in terms of the significantly better spreading of the films containing high molecular weight DMPE-linked $PEG_{2000/5000}$. For the DMPC/DMPE-$PEG_{2000/5000}$ mixed films, decreasing of γ_{min} and γ_{max} values in comparison with DMPC films was proportional to the PEG moiety Mw, the lowest $\gamma_{min} = 20$ mN/m and $\gamma_{max} = 35$ mN/m were reached for DMPC/DMPE-PEG_{5000} films. The recorded differences in compression/decompression behavior between DMPC/DMPE-PEG_{550} and DMPC/ DMPE-$PEG_{2000/5000}$ films are in agreement with our data for the different effects of low molecular weight DMPE-PEG_{550} and DMPE-$PEG_{2000/5000}$ molecules on the hydrodynamic behavior and formation mechanism of the TLFs.

Compression/decompression histeresis area (A_h) values of pure DMPC, pure DMPE-PEG, and mixed DMPC/DMPE-PEG monolayers are shown in Table 2.

It can be seen that A_h of DMPC films (0.978 mN) was higher in comparison with DMPE-PEG pure monolayers. With increase of PEG moiety Mw, A_h of DMPE-PEG monolayers increased, probably due to increased cohesive interaction among PEG chains, and maximal $A_h = 0.393$ mN occurred for DMPE-PEG_{5000} films. It must be noted that the qualitative difference that mixed DMPC/DMPE-PEG films showed higher histeresis areas than pure DMPE-PEG monolayers (which could

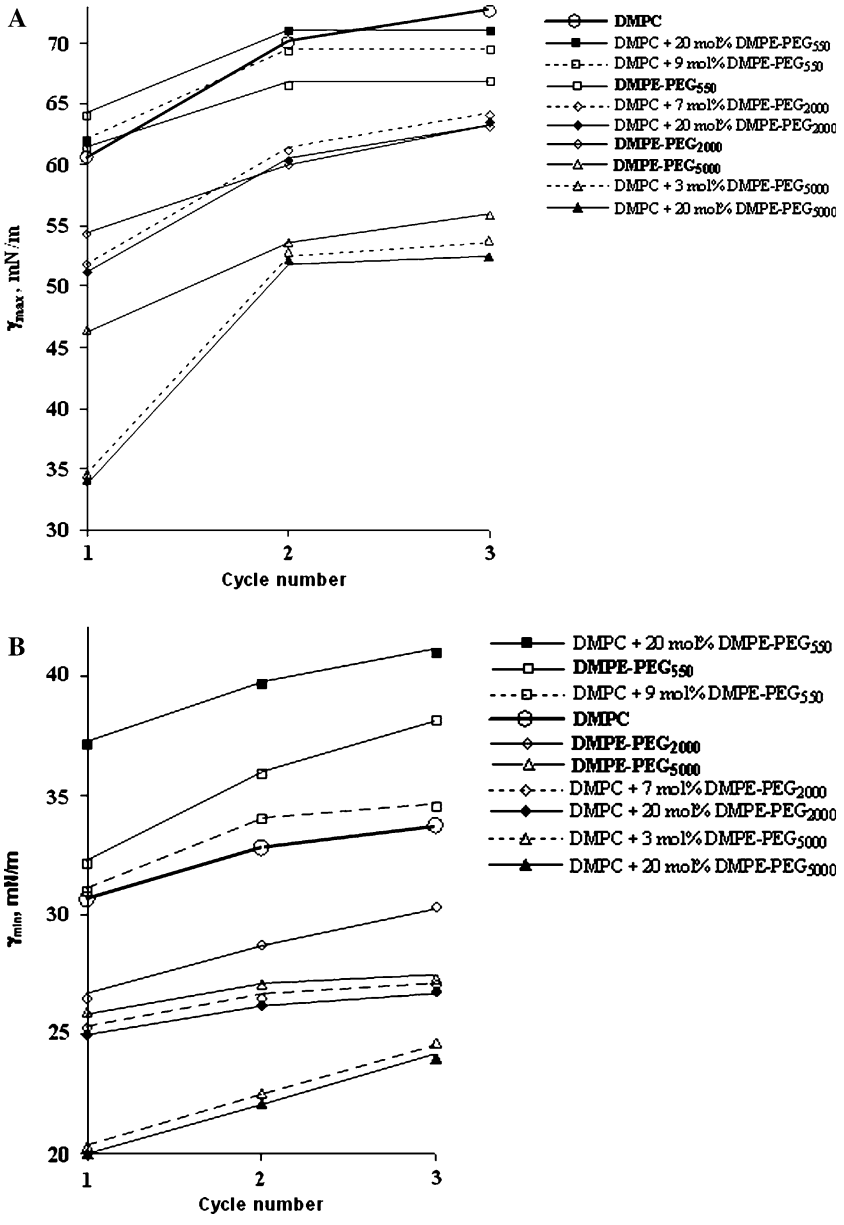

Fig. 8 Comparison of γ_{min} (cycle number) dependences (**a**) and γ_{max} (cycle number) dependences (**b**) of spread monolayers of DMPC pure (*O* and *thick line*) monolayers and DMPE-PEG containing monolayers: DMPE-PEG_{550} pure (*open square*), DMPC + 20 mol% DMPE-PEG_{550} (*filled square*), DMPC + 9 mol% DMPE-PEG_{550} (*open square* and *dashed line*); DMPE-PEG_{2000} pure (*open diamond*), DMPC + 20 mol% DMPE-PEG_{2000} (*filled diamond*), DMPC + 7 mol% DMPE-PEG_{2000} (*open diamond* and *dashed line*); DMPE-PEG_{5000} pure (*open triangle*); DMPC + 20 mol% DMPE-PEG_{5000} (*filled triangle*); DMPC + 3 mol% DMPE-PEG_{5000} (*open triangle* and *dashed line*). Spread monolayers surface density corresponded to 200 $Å^2$ per molecule. Experiments were done at $T = 37°C$, $C_{el} = 0.5$ M NaCl, pH 6.8–7.0

Table 2 A_h of spread monolayers (200 $Å^2$ per molecule) of DMPC and lipid-linked PEGs with different molecular weight after compression/decompression cycle from100 to 20% of the initial monolayer area

Composition	A_h (mN)
DMPC	0.978
DMPE-PEG_{550}	0.157
DMPC + DMPE-PEG_{550} (9 mol%)	0.612
DMPC + DMPE-PEG_{550} (20 mol%)	0.385
DMPE-PEG_{2000}	0.368
DMPC + DMPE-PEG_{2000} (7 mol%)	0.608
DMPC + DMPE-PEG_{2000} (20 mol%)	0.476
DMPE-PEG_{5000}	0.393
DMPC + DMPE-PEG_{5000} (3 mol%)	0.687
DMPC + DMPE-PEG_{5000} (20 mol%)	0.691

Data are averaged over five consecutive cycles for each of the monolayers. Experiments were performed at $T = 37°C$, $C_{el} = 0.5$ M NaCl, pH 6.8–7.0

be related with different structuring of the lipids on the interface due to different liposome/micelle content in the dispersions), and that a quantitative calculations on monolayer compressibility, visco-elastic modulus, etc. were not performed since, as was mentioned in Materials and methods, the values of π_{max} were monitored during monolayer compression from 100 to 20% of the initial area with compression rate 3 min cycle, i.e., under non-equilibrium conditions (fivefold compression of the monolayer area for 90 s).

Monolayers of DMPC in presence of free PEGs

We found that none of the PEGs used, PEG-200, PEG-400, PEG-1500, and PEG-6000, showed penetration to DMPC films even at PEG concentration in the subphase

as high as 10^{-2} M. The investigation of the adsorption of free PEGs to pure air/water interface showed that only high molecular weight PEG-6000 was capable of decreasing surface tension by about 6 mN/m at concentrations as high as 8.10^{-4} M, while the rest PEGs showed no significant effect on γ up to 10^{-2} M (Fig. 9).

This result correlates with the fact that of the free PEGs used, only PEG-6000 was capable of forming stable common black films. These data and our results demonstrated that free PEGs did not alter the type and stability of DMPC TLF but only film thinning time t_{0-1} values agreed with the data (Kuhl et al. 1996; Hui et al. 1999) that free PEGs with Mw less than 8,000 could not penetrate in the membrane surface and just alter the viscosity of the "near membrane" water layer.

Conclusions

In this work, TLFs and monolayers at the air/water interface formed by DMPC and its mixtures with DMPE linked PEGs or with free PEGs were studied. For all experiments the PEG moiety Mw was less than 8000.

Experiments were conducted at three concentrations of DMPE-PEGs in the film forming dispersions, less than 10 mol% (where dispersions are formed predominantly by liposomes), 20 mol% (where mixture of liposomes and micelles exists in the dispersions) and 80 mol% (where only micelles exist in sample dispersions).

The choice of the DMPC/DMPE-PEG mixture is motivated by the fact that liposome and micelle forming mixtures of phosphatidylcholines and PE-PEGs with equal acyl chain length are developed for the purposes of in vivo drug delivery (Bergstrandt 2003; Kenworthy et al. 1995) and are already used for preparation of lamellar (Lα, g) hydrogels, applied in healing, drug delivery, cosmetics, etc. (Keller et al. 1997; Warriner et al. 1998). Of course, in view of mixing DMPC and DMPE phospholipids in the studied DMPC/DMPE-PEG mixture, the behavior of the films obtained should be influenced not only by the presence of PEG moiety, but also by the different head groups and phase transition temperatures between the two lipids. Being covalently linked to the amino group of DMPE, the effect of PEG moiety dominates which causes the vanishing of the specific features of PE group, and the lipid-polymer conjugate properties (e.g., phase transition temperature, lateral diffusion coefficients, critical micelle concentration, etc.) are determined mainly by the balance between the acyl chains and PEG moiety (Ashok et al. 2004; Kenworthy et al. 1995; Pantusa et al. 2003; Soong and Macdonald 2005). Thus, the dimyristoyl chains of the DMPE-PEG conjugate provide its anchoring to the DMPC monolayers in our model systems and in the pharmaceutically used liposomes. In view of the data published (e.g., that DMPE-PEG conjugate induces formation of inverted cubic Im3m phase by dielaidoylphosphatidylethanolamine, DEPE, Koynova et al. 1997) at low concentrations and stable bilayer phase by dioleoylphosphatidylethanolamine and DEPE (eliminating the non-bilayer ones) at higher concentrations (Holland et al. 1996; Koynova et al. 1997), the DMPE/DMPE-PEG mixture can also be an interesting topic for further studies by monolayers and TLFs.

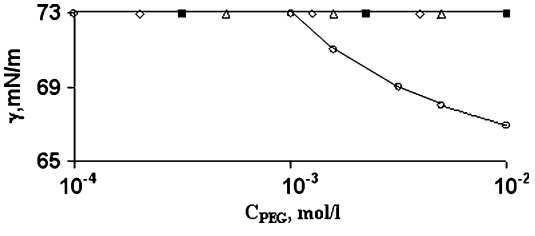

Fig. 9 γ(C) adsorption isotherms of Free PEG-200 (*open diamond*), PEG-4000 (*filled square*), PEG-1500 (*open triangle*), and PEG-6000 (*o*) at pure air/liquid interface. Experiments were done at $T = 37°C$, $C_{el} = 0.5$ M NaCl, pH 6.8–7.0

All the TLFs studies used DMPE linked PEGs formed by stable black TLFs. The threshold concentration C_t, was smaller for all DMPE-PEGs films than that for DMPC films and decreased with increase of PEG moiety Mw (Fig. 3). Under the same experimental conditions (e.g., capillary pressure), the thinning process of the DMPC films continues the formation of NBFs (real bilayers, Fig. 1c) while DMPE-PEGs films form much thicker CBFs or equilibrium CBF-like films (both with free water core between the monolayers, Fig. 1b). In addition, while DMPE-PEG_{550} forms CBFs by the mechanism of fluctuating BS formation (characterized by film thinning time t_{0-1} and BS expansion time t_{1-2}), higher molecular weight samples of DMPE-$PEG_{2000/5000}$ form CBF-like films by continuous thinning (characterized by total film formation time t_{0-2}) without BS formation. Thus, without direct measurement of the film thickness, we observed that the thinning at the same capillary pressure of the DMPC and DMPE-PEGs films continued to different film types and thickness, being much thicker for the latter films, which is probably due to the appearance of steric repulsion between long PEG chains of the monolayers of these films. When included in DMPC films, DMPE-PEG_{550} increased t_{0-1} and t_{1-2} of the mixed films and changed the film type formed. The thinning process in the mixed films reached, not to the bilayer NBFs, but to thicker CBFs. Moreover, the higher molecular DMPE-$PEG_{2000/5000}$ molecules changed the black film formation mechanism, which is not by BS formation but reaches CBF-like films by continuous thinning (Table 1). We recognized a minimal DMPE-PEG concentration ($C^{min}_{DMPE\text{-}PEG}$) for each case, at which the onset of the above effects was observed. The $C^{min}_{DMPE\text{-}PEG}$ depends on the mol content and Mw of the PEG moiety in the mixture (Table 1), being less than 10 mol% for the three samples studied where film forming dispersions consist mainly of liposomes (Fig. 2).

Monolayer studies found improved formation kinetics of both adsorbed and spread films and decrease of surface tension values (equilibrium, minimal and maximal) depending on the PEG moiety Mw and content of DMPE linked PEG in the monolayers (Figs. 7, 8, Table 2). It was also observed that the film compression/decompression histeresis area in DMPE-PEG containing monolayers decreased in comparison with DMPC pure monolayers. These differences are in correspondence with the data for the hydrodynamic behavior and formation mechanism of the TLFs obtained from the three DMPE linked PEGs studied. However, it was revealed that the effects of 20 and 80 mol% DMPE-PEGs presence in the monolayers (both equilibrium and during compression/decompression) were practically identical and hence no differences were observed in the monolayer properties on the type of DMPC/DMPE-PEG dispersions despite of their composition (liposomes/micelles mixture or micelles only).

Free PEGs (PEG-200, PEG-400, PEG-1500, and PEG-6000) added to DMPC films did not alter DMPC film type and stability, they only increased the film thinning time, t_{0-1}, proportionally to the PEG molecular weight. None of the free PEGs used penetrated in DMPC spread monolayers and only PEG-6000 was capable of forming stable CBFs and reducing the surface tension of the air/water interface at concentrations higher than 10^{-4} M. These results agreed with literature data (Kuhl et al. 1996; Hui et al. 1999) that free PEGs with Mw less than 8,000 could not penetrate the membrane surface and just alter the viscosity of the ''near membrane'' water layer.

In general, as our results in this paper demonstrate, the coupling of amphiphile lipid molecule (DMPE) to PEG molecule significantly alters the surface properties of DMPE-PEG complex molecule, which added to phospholipid monolayers or TLFs causes drastic changes in their properties and behavior. Our study also shows that combining the models of phospholipid TLFs and monolayers provide the opportunity to investigate the properties of membrane surface and to clarify some mechanisms of its interactions with membrane-active agents.

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