



# Langmuir films of dipalmitoyl phosphatidylethanolamine grafted poly(ethylene glycol). In-situ evidence of surface aggregation at the air-water interface



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## ABSTRACT

The molecular packing-dependent interfacial organization of polyethylene glycol grafted dipalmitoylphosphatidylethanolamine (PE-PEGs) Langmuir films was studied. The PEG chains covered a wide molecular mass range (350, 1000 and 5000 Da).

In surface pressure-area ( $\pi$ -A), isotherms PE-PEG<sup>1000</sup> and PE-PEG<sup>5000</sup> showed transitions (midpoints at  $\pi_{m,t1} \sim 11$  mN/m, “**t1**”), which appeared as a long non-horizontal line region. Thus, **t1** cannot be considered a first-order phase transition but may reflect a transition within the polymer, comprising its desorption from the air-water interface and compaction upon compression. This is supported by the increase in the  $\nu_s$ (C-O-C) PM-IRRAS signal intensity and the increasing surface potentials at maximal compression, which reflect thicker polymeric layers. Furthermore, changes in hydrocarbon chain (HC) packing and tilt with respect to the surface led to reorientation in the  $PO_2^-$  group upon compression, indicated by the inversion of the  $\nu_{asym}(PO_2^-)$  PM-IRRAS signal around **t1**. The absence of a **t1** in PE-PEG<sup>350</sup> supports the requisite of a critical polymer chain length for this transition to occur. In-situ epifluorescence microscopy revealed 2D-domain-like structures in PE-PEG<sup>1000</sup> and PE-PEG<sup>5000</sup> around **t1**, possibly associated with gelation/dehydration of the polymeric layer and appearing at decreasing  $\pi$  as the polymeric tail became longer.

Another transition, **t2**, appearing in PE-PEG<sup>350</sup> and PE-PEG<sup>1000</sup> at  $\pi_{m,t2} = 29.4$  and 34.8 mN/m, respectively, was associated with HC condensation and was impaired in PE-PEG<sup>5000</sup> due to steric hindrance imposed by the large size of its polymer moiety.

Two critical lengths of polymer chains were found, one of which allowed the onset of polymeric-tail gelation and the other limited HC compaction.

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## 1. Introduction

Poly(ethylene glycol) (PEG), with different degrees of polymerization covalently bound to a phospholipid moiety, comprises a kind of lipopolymer (LP) widely used in liposome formulations for drug encapsulation and transport [5]. In these liposomes, PEG moieties are the polar head group and form a hydrophilic coating that stabilizes vesicles and increases their half-life in the circulatory system, preventing their self-aggregation and/or fusion as well as the nonspecific adsorption of serum proteins [6].

PEGylated phospholipids may also be important for building up model membranes containing a pseudo-glycocalix. Other lipids

covalently modified with hydrophilic polymers, including glycolipids with both linear and branched head, were considered good candidates to build up a model glycocalix [7]; however, the complex interactions between these hydroxylated polymers and possible adsorbed molecules complicate the study of their properties in such complex chemical environments. The use of PEG derivatives would avoid this disadvantage, although it would require their being stabilized in a lamellar, near planar, phase.

It was proposed that linear polymers attached to interfaces may be found in two different organizational schemes depending on the packing density [8,9], i.e. a random coil (mushroom) conformation at a low molecular packing, and a stretched (brush) conformation at higher molecular packings. Moreover, on the basis of polymer physics [10], it was predicted that, according to the composition and the proportion of PEGylated phospholipids in mixtures with other phospholipids, it would be possible to achieve these two

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conformations (“brush” or “mushroom”) not only in monolayers but also in liposomes [11]. A third conformation, the pancake, has also been predicted to exist in monolayers at the lowest surface pressures [12,13]. In addition, theoretical models make it possible to predict the thickness of the polymer layer arranged near the lipid-water interface, as well as the conformational and rheological changes undergone by the polymer, which depend on the molecular packing of the monolayer where it is anchored both with phospholipid [14] and with O-linked alkyl glycerol derivatives [13].

There is considerable information in the literature about different PEG lipopolymer derivatives, studied with different perspectives, methods [8,14–20] and physical conditions [2,3,8]. However, a complete analysis of the same set of molecules in similar conditions through a combination of techniques is lacking. This would help to better understand the molecular features underlying the control of surface organization of these biopolymers.

Therefore, in the present paper, we used dipalmitoyl phosphatidylethanolamine (PE), covalently modified with three PEGs differing only in the number of monomer subunits (7, 23 or 113 subunits for PEG<sup>350</sup>, PEG<sup>1000</sup> and PEG<sup>5000</sup>, respectively). The interfacial stability and the conformational equilibrium of LP were studied by lateral pressure vs. area ( $\pi$ -A) compression isotherms. Brewster angle microscopy (BAM), epifluorescence microscopy (EFM) and polarization modulation infrared reflection-absorption spectroscopy (PM-IRRAS) were combined, in conjunction with Langmuir balance experiments with different molecular packing conditions and at constant temperature, to evaluate the transitions occurring within both the lipid hydrocarbon chains (HC) and the polymer.

Note that, although Langmuir trough experiments have been widely applied to study the behavior of similar LPs at the air-water interface, the LP series studied in the present work have not been included in previous reports. This information not only completes already existing information, but is also useful to understand microscopic and spectroscopic data, which are the novel contributions of the present work.

## 2. Materials and methods

### 2.1. Materials

1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)] with average PEG molecular weights of 350, 1000 and 5000 (PE-PEG<sup>350</sup>, PE-PEG<sup>1000</sup>, PE-PEG<sup>5000</sup>) were purchased from Avanti Polar Lipids (Alabaster, Alabama). DiI-C<sub>18</sub>, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate was from Molecular Probes Inc. (Eugene, OR, USA). The general structure of the three PE-PEGs analyzed is summarized in Fig. S1 (Supplementary data).

### 2.2. Surface pressure vs mean molecular area ( $\pi$ -A) compression isotherms

#### 2.2.1. $\pi$ -A isotherm recording

Monomolecular layers were prepared and monitored with a Minitrough II (KSV Instruments Ltd., Finland) (see Supplementary data and ref [21], for a detailed description). Briefly, CHCl<sub>3</sub>:MeOH (2:1) solutions of pure compounds were spread on an unbuffered aqueous surface at 25 ± 1 °C. The interface was compressed at a 10 nm<sup>2</sup>/s constant compression rate. Lateral surface pressure ( $\pi$ ) was measured by the Wilhelmy plate method and the surface potential ( $\Delta V$ ) registered simultaneously by the voltage-measuring system (vibrating plate method; KSV Instruments, Helsinki, Finland). Reproducibility was within ±0.01 nm<sup>2</sup>, ±1 mN/m and ±1 mV for molecular area (A),  $\pi$  and  $\Delta V$ , respectively.

The surface parameters  $A_{\min}$  (minimum A occupied by an amphiphatic molecule in a monolayer at the closest molecular packing) and  $\pi_c$  (collapse pressure, the maximal  $\pi$  corresponding to  $A_{\min}$ ), were obtained from  $\pi$ -A isotherms.

#### 2.2.2. Compression modulus

The compression modulus K was calculated for each isotherm according to Eq. (1):

$$K = -(A_{\pi}) \cdot \left( \frac{\partial \pi}{\partial A} \right)_T \quad [1]$$

where  $A_{\pi}$  is the molecular area at  $\pi$ . K enables the elasticity of the monolayer to be inferred, and the bidimensional phase transitions from the  $\pi$ -A isotherm profile to be defined more precisely.

#### 2.2.3. Molecular area for the onset of a hypothetical mushroom-brush phase transition

For a surface-grafted polymer in a “mushroom” regime, the global approximation theory of Flory-Huggins was used [10] (for calculation details see Supplementary data).

### 2.3. Brewster angle microscopy (BAM) and epifluorescence microscopy (EFM)

A Brewster angle microscope (EP<sup>4</sup>, Nanofilm, Germany) was mounted over a Langmuir trough for direct interfacial observation. During isotherm compression,  $\pi$  was stopped at the desired value (5, 15 or 35 mN/m) and 5 min were allowed for film stabilization. BAM images were obtained through a 10× objective with 2  $\mu$ m lateral resolution and were recorded with a CCD camera.

For EFM experiments, monolayers were prepared from solutions doped with 1 mol% DiI-C<sub>18</sub>. A KSV Minisystems surface barostat was mounted on the stage of a Nikon Eclipse TE2000-U (Tokyo, Japan) microscope, which was supplied with 20 × long-working distance optics. The Teflon trough used had a 35-mm diameter quartz window at its base, which allowed the monolayer to be observed through the trough. The monolayer morphology was documented with a Nikon DS-5 M color video camera supporting a resolution up to 2560–1920 pixels, which represents 1231 × 923  $\mu$ m (Capture area).

### 2.4. PM-IRRAS spectroscopy

Polarization Modulation Infrared Reflection-Absorption Spectroscopy (PM-IRRAS) was performed essentially as described previously [21] (see also Supplementary data), with a KSV PMI 550 PM-IRRAS (KSV Instruments Ltd., Finland) mounted on a KSV Minitrough.

Spectra were collected with a resolution of 8 cm<sup>-1</sup>, at 100 kHz modulating frequency and a variable delay in the wavelength ( $\lambda$ ). The incidence angle was 80° and the maximum retardation wavelength was 1500 cm<sup>-1</sup> with a gap of 0.55 $\lambda$ . These parameters enabled a high resolution IR spectrum to be recorded within the 950 and 1900 cm<sup>-1</sup> wavenumber range in which most signals from phospholipids and PEG polar heads appeared. For an incidence angle of 80°, a positive-oriented band indicates a transition moment occurring preferentially in the plane of the surface, while a negative downward-oriented band reveals an orientation preferentially perpendicular to the surface [22]. The integrated absorption (I) of a peak reflects both the orientation of the molecules and the total number of molecules in the IR beam [23]. Hence, the product  $I \cdot A$  would be expected to be constant during the compression, provided there is no molecular reorientation during the compression. This was applied to the analysis of PO<sub>2</sub><sup>-</sup> peak reorientation.

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