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Original article

Interfacial characterization of the molecular interactions in mixed monolayers of coumarin and phospholipids

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ABSTRACT

Coumarin (CMR) is a small molecule with diverse biological functions as anti-tumor, anti-fungal, anticoagulant and antimicrobial activities. CMR is poorly water soluble molecule with low bioavailability. Lipid-based colloidal carriers play an important role in the delivery of hydrophobic compounds. Langmuir technique is an effective method to evaluate interactions between drugs and lipids. In this study, we used 1,2-dipalmitoyl-sn-glycero-3-phospho-rac-(1-glycerol) (DPPG), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), and 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) lipids. Interfacial characterization was carried out based on surface pressure (π)-area (A) isotherms of pure and mixed films. Topographical analysis was performed using atomic force microscopy. The miscibility of the mixed films was dependent on their composition. The mixed films were stable due to the intermolecular attractive interactions. Our results contribute to the understanding of CMR-lipid interactions aiming to obtain stable colloidal carriers for drug delivery.

1. Introduction

Plant-derived compounds are known for their antimicrobial activity, treatment, and prevention of infections (Borges et al., 2005; Helander et al., 1998; de Souza et al., 2005). Bioactive compounds, such as benzopyrone class, can be obtained from natural sources as essentials oils extracted from plants (Gill and Holley, 2004; Friedman et al., 2003).

The development of new therapeutic drugs is driven to maximize therapeutic effects and minimize side effects. The production of new active pharmaceutical ingredients is expensive, slow and often uncertain. The natural products played critical roles in modern drug development (Pattni et al., 2015; Fylaktakidou et al., 2004). Coumarin (CMR) and its derivatives are widely distributed throughout nature and exhibit diverse therapeutic applications such as central nervous system stimulants, anti-HIV therapy, anti-coagulant, antitumor, antibacterial and antifungal activities (Musicki et al., 2000; Kostova et al., 2006; Fylaktakidou et al., 2004; Musa et al., 2008). CMR (1,2-benzopyrone) is considered unsuitable for therapeutic use due to its high toxicity, low solubility and limited stability (Hoult and Paya, 1996; Usui, 2006).

Lipid-based colloidal nanostructures are used as carriers for hydrophobic active substances with prospective benefits in biomedicine, cosmetics, and food industry (Rosler et al., 2012; Foldvari et al., 1990; Mozafari et al., 2008; Taylor et al., 2005). In addition, lipid-based colloidal carriers can be targeted to pathological tissues while reducing side effects and dose frequency.

Supported phospholipid monolayers have practical applications such as biomolecular separation (Harlan et al., 1995; Zaitsev et al., 1995), biosensors (Yasuzawa et al., 2000), drug delivery (Torchilin, 2012), and bio-functionalization (Findlay and Barton, 1978). Phospholipids are one of the essential components of the plasma membrane and lipid-based colloidal nanostructures.

The phospholipids used in this study were chosen due to their different size, charge and molecular shapes. Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are the most important neutral phospholipids found in living organisms (Dowhan, 1997). The abundance of PE is highly variable among organisms and cell types. PC lipids are predominantly found in animal cell membranes (Uran et al., 2001). Phosphatidylglycerol (PG) is a lipid mainly responsible for maintaining membrane lipid

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surface charge density and permeability to ions (Wada and Murata, 2007).

In this study, we have carried out Langmuir isotherm measurements to analyze the molecular interactions in mixed monolayers composed of phospholipids and CMR. The excess area (ΔAE), excess Gibbs free energy (ΔGe) and excess Gibbs free energy of mixing ($\Delta Gmix$) were calculated. In addition, topographical analysis of pure and mixed films was performed by atomic force microscopy (AFM).

2. Materials and methods

2.1. Materials

Coumarin (99% purity), 1,2-dipalmitoyl-sn-glycero-3-phospho-rac-(1-glycerol), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine and 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (all phospholipids with purity > 99%) were purchased from Sigma–Aldrich (Saint Louis, USA). Ultra-pure water (18.2 MΩ.cm^{-1}) was obtained from a Synergy Milli-Q system (Billerica, USA).

2.2. π-A and ΔV-A isotherms

CMR and lipids solutions were prepared in chloroform at a concentration of 1 mg.mL^{-1}. Subsequently, different solutions of CMR/lipids at different molar ratios (X_{CMR} = 0, 0.250, 0.375, 0.500, 0.675, 0.750, 0.875 and 1) were obtained. Then, 10 μL of these fractions were deposited uniformly on a citrate (citric acid)-phosphate (sodium phosphate) buffer (pH 7.4 and ionic strength 10^{-3} M) subphase (v = 20 mL) using a Hamilton micropipette. The experiments were performed under symmetrical compression at a constant speed of 15 mm.min^{-1} (temperature 23 °C ± 0.2 °C), after waiting 15 min to ensure evaporation of the solvent. Isotherms were conducted on a Kibron Langmuir trough (Microtrough XS, Helsinki, Finland). The surface pressure was measured with accuracy of ±0.01 mN.m^{-1}. Surface potential was measured by vibrational plate method (Microspot, Kibron, Helsinki, Finland) with an accuracy of ±0.1 mV. π-A and ΔV-A isotherms were repeated in triplicate with a standard deviation of ~5%, using at least three different solutions.

2.3. Morphological analysis of LB films

The pure and mixed monolayers were compressed and maintained at target pressure of π = 20 mN.m^{-1}. Subsequently, Langmuir films were transferred onto freshly cleaved mica, dipping the substrate at a constant rate of 2 mm.min^{-1} in order to control the formation of single monolayers. All measurements were performed with a SPM 9700 atomic force microscopy (Shimadzu instruments Co. ltd, Japan) in a non-contact mode. Cantilevers with a silicon AFM probe (Nanoworld, Japan, resonant frequency = 300 kHz, spring constant = 42 N.m^{-1}) were used. The images (512 points per line) were collected with a scan rate of 1.0 Hz in a scan area of 5.0 × 5.0 μm on at least two different samples on four different areas on each sample In addition, images were obtained and analyzed using AFM Gwyddion software (Necas, 2008).

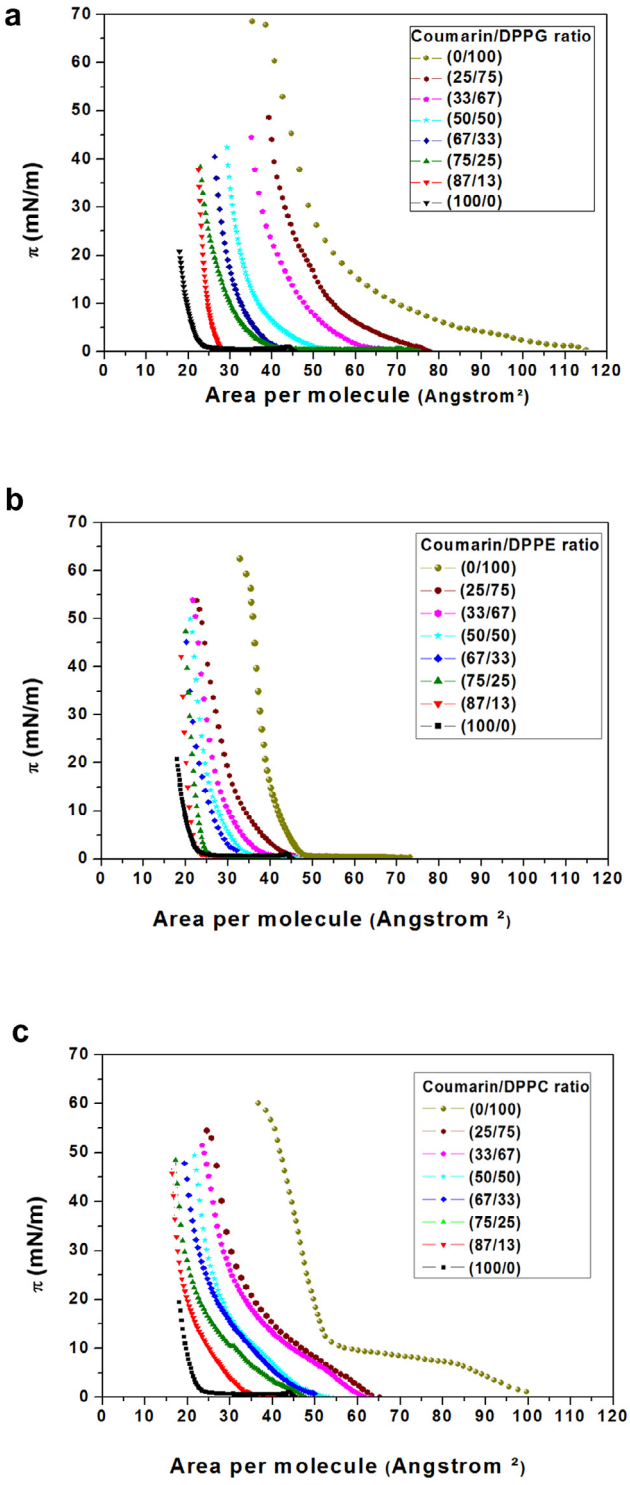

Fig. 1. π-A isotherms of mixed monolayers CMR/DPPG (a), CMR/DPPE (b) and (c) CMR/DPPC at different concentrations.

3. Results and discussion

3.1. Compression isotherms

π-A isotherms of the pure and mixed monolayers versus molecular area are shown in Fig. 1. CMR/DPPG isotherms showed a similar trend to that of pure DPPG lipid (Fig. 1a). Similar phase transitions were observed for all CMR/DPPG mixtures. DPPG and DPPC showed collapse pressures ($\Delta\Pi$coll) at 52 mN.m^{-1} and 60 mN.m^{-1}, respectively. A transition ($\pi \cong 3$ mN.m^{-1}) from liquid expanded to condensed phase was found for pure DPPE monolayer with a Δ. coll at ≅55 mN.m^{-1}. The values obtained for Δ. coll of pure lipids are in agreement with the literature (Vollhardt et al., 2000; Nowotarska et al., 2014).

$\Delta\Pi$coll is strongly dependent on the composition of the mixed monolayers and decreases proportionally to CMR molar ratio. From

the data presented in Fig. 1a and b it was noticed a steeper rise at x_{CMR} = 0.87 for DPPG and 0.75 for DPPE due to CMR incorporated on these monolayers resulting in a more organized state (Takao et al., 1995).

The interfacial behavior of the isotherms was evaluated considering the difference in the polar region since the studied lipids have identical hydrophobic moieties. DPPE and DPPC are zwitterionic lipids (Fig. 2). DPPG is composed of an anionic polar head (Fig. 2). The polar groups influence the miscibility between the studied molecules resulting in different molecular arrangements (Bouffioux et al., 2007) and lipid packing (Hazell et al., 2016). As expected, DPPG and DPPC mixed films resulted in expanded monolayers (Fig. 1a and c). Pan et al. (2012) demonstrated that DPPG headgroup areas are larger than phosphatidylcholine counterparts as a result of the repulsive electrostatic interactions between charged PG headgroups. DPPC hydrophobic sections are kept farther apart minimizing the lateral cohesive interactions (Myers, 1999). Conversely, DPPE mixed films result in solid-phase monolayers (Fig. 1b).

3.2. Surface potential measurements

The surface potential as function molecular area (ΔV-A) is shown in Fig. 3a. ΔV values obtained for all studied phospholipids were similar to previous reports (Andrade et al., 2006; Nowotarska et al., 2014). A gradual decrease of the ΔV (460 mV to 275 mV) as a function of CMR molar fraction is observed for DPPG. ΔV is susceptible to the orientation of both polar and nonpolar groups.

A linear dependence of the ΔV for DPPE and DPPC as a function of CMR concentration is observed in Fig. 3b and c, respectively. A decreased of ΔV was obtained for high CMR concentration. In addition, DPPE [DPPC] floating monolayers result in ΔV ~ 604 [605] mV and for X_{CMR} = 0.87 about of 334 [351] mV. A reduction of the surface potential can be explained through hydrogen bond occurring between CMR and zwitterionic lipid molecule, *i.e.*, between CMR (C=O) acting as hydrogen receptor group and protonated lipid headgroup (e.g. NH^{3+} found in the DPPE molecules) (Boggs, 1987). DPPG have the ability to form hydrogen bonds between the glycerol moiety and phosphate oxygen of neighboring phosphatidylglycerol lipids (Zaraiskaya and Jeffrey, 2005). DPPE can form inter- and intramolecular hydrogen bonds. Strong intermolecular interactions result in an increase in the liquid-crystalline phase transition temperature, affecting the stability and membrane permeability (Leekumjorn and Sum, 2006). The amine group (hydrogen-donor) of the DPPE can interact strongly with the phosphate/carbonyl groups or water (hydrogen-acceptor). In addition, choline shows a hydrophobic hydration around the CH_3 groups, and for amine occurs a competition of hydrogen bonds with water and oxygen atoms in the headgroups (Leekumjorn and Sum, 2006).

CMR interacts with the monolayers tail since it has a lipophilic structure with an octanol-water partition coefficient (log P_{ow}) of = 2.54 (Rabtti, 2012). The binding of molecules to phospholipid tails results in changes of the apparent dipole moment (Hidalgo et al., 2004). The apparent dipole moment is defined by the following formula:

$$\Delta V = \frac{\mu}{A.\varepsilon 0} + \Psi \quad (1)$$

where ΔV is the surface potential, μ is the apparent dipole moment, A is the area per molecule, ε0 is the vacuum permittivity, and Ψ is the double-layer contribution. The double-layer contribution was calculated according to Andrade et al. (2005).

From the data obtained from Eq. 1 we could observe that the relationship of the mixed apparent perpendicular dipole moment (μ) to perpendicular moment of pure phospholipid monolayers (μ_0) shifts according to the monolayer compression (Geraldo et al., 2013) (Fig. 4). The μ/μ_0 relationship was calculated

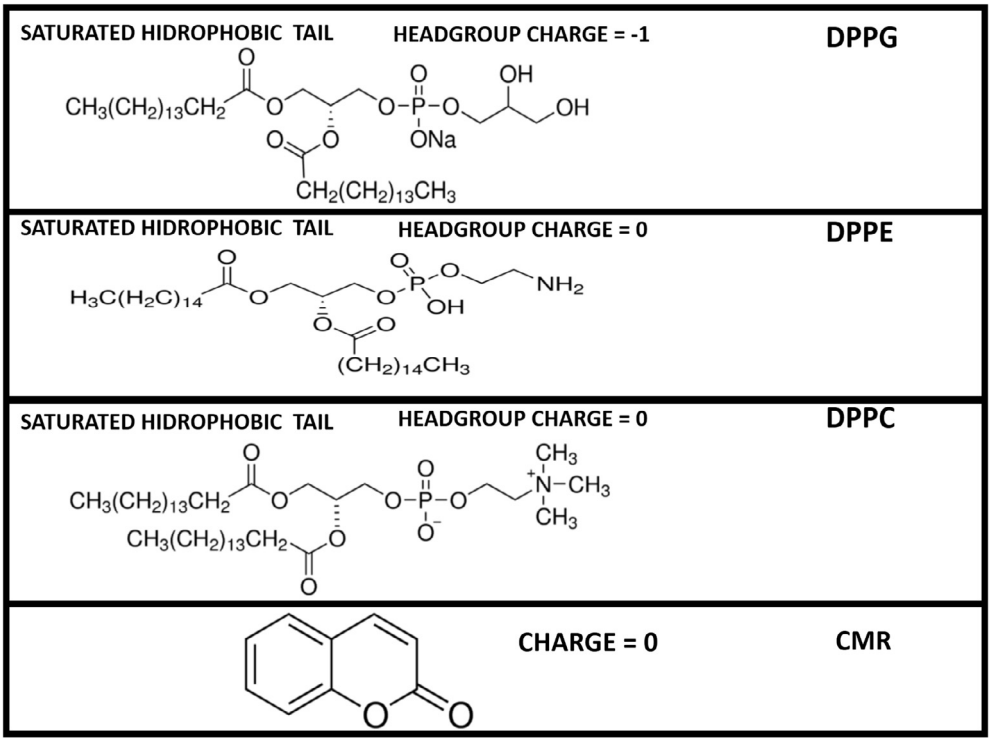

Fig. 2. Phospholipids and coumarin molecular structures. DPPC and DPPC have a cylindrical molecular shape and DPPE has a cone-shaped geometry.

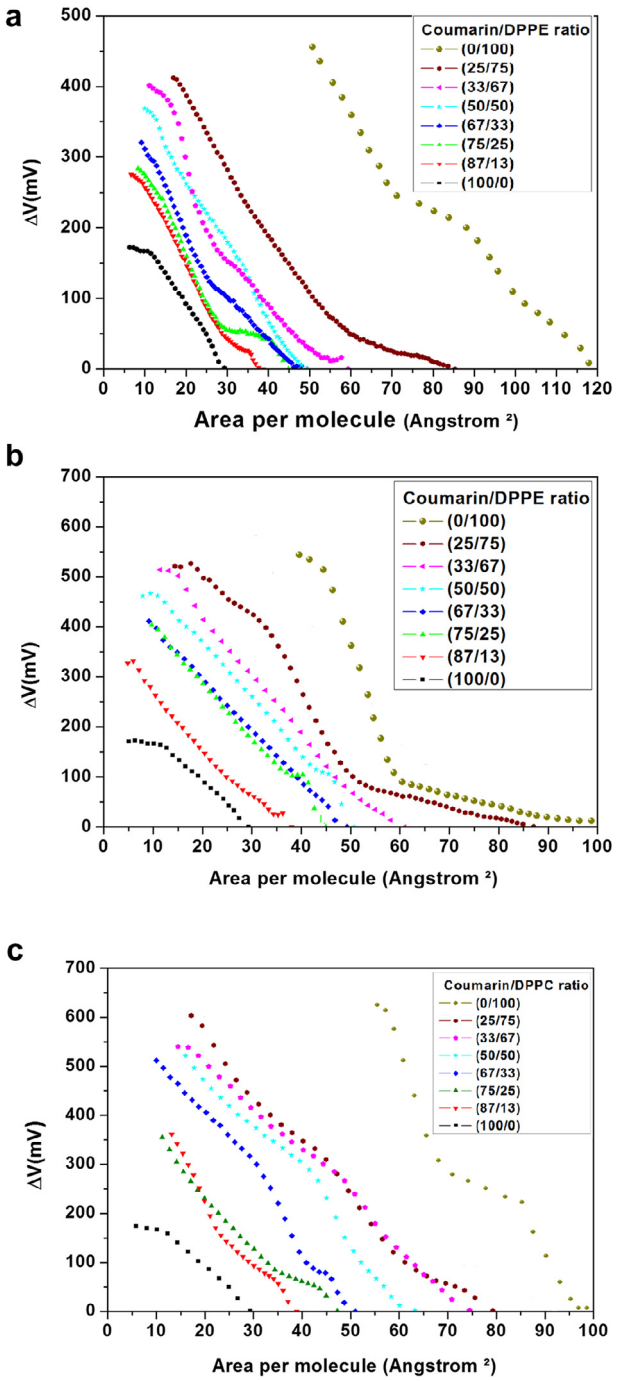

Fig. 3. ΔV-A of CMR/DPPG (a), CMR/DPPE (b) and CMR/DPPC (c) at different concentrations.

considering the molecular area at π = 20mN/m (highest $\Delta\Pi$coll for pure CMR film). The orientation of the intrinsic molecular dipole (μ) of the molecules forming the film and the organizational structure of water molecules in the subphase is known to play a fundamental role. Of note, μ is considered to be the vector sum of dipole moments arising from the hydrated polar group and hydrocarbon chain ($\mu = \mu_{polar} + \mu_{hydrocarbon\ chain}$). In addition, DPPG, DPPC and DPPE have similar hydrophobic tail. Thus, it was reasonable to assume that the apolar group contribution to the overall ΔV was the same. The differences in ΔV-A curve profiles were due to their different μ_{polar} contributions.

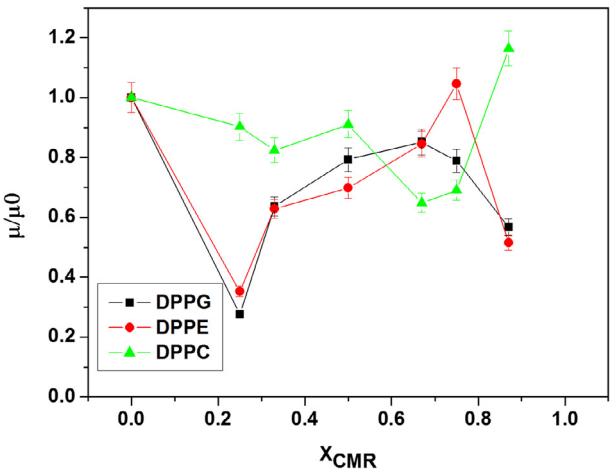

Fig. 4. Apparent dipole moment of pure phospholipid monolayers at π = 20mN/m.

3.3. Thermodynamic analysis

The thermodynamic analysis was performed based on mean molecular area (mma), excess area (*ΔAE*), excess Gibbs free energy (*ΔGe*) and excess Gibbs free energy of mixing (*ΔGmix*) at π = 5, 10, 15, and 20 mN/m. The deviations from linearity of the mma indicate the miscibility of the components according to additivity rule (Szczes et al., 2012) (Fig. 3). *ΔAE* can be calculated by

$$\Delta AE = A_{1,2} - Aid = A_{1,2} - (X_1A_1 + X_2A_2), \Delta AE = A_{1,2} - X_1A_1 - X_2A_2 \quad (2)$$

where $A_{1,2}$ is the mma, *Aid* is the ideal mixed monolayer, X_1 and X_2 are the mole fractions of the components 1 and 2, respectively. A_1 and A_2 are the corresponding area per molecule of the pure monolayers on the same surface pressure. ΔAE values becomes zero when the substances form an ideal mixture or are immiscible (Jones and Chapman, 1996).

The molecular area as a function of the molar ratios of CMR/DPPG is shown in Fig. 5a. It could be seen a negative deviation from mma (X_{CMR} = 0.33–0.67) due to an increase in the molecular attractive interactions. CMR/DPPG films were considered partially miscible and non-ideal floating monolayers since linearity was not observed. The results for CMR/DPPE mixed films exhibited different breaking points at X_{CMR}= 0.25 and 0.67 (Fig. 5b). *ΔAE* shows an improvement in the packing efficiency or even geometrical accommodations occurred at lower X_{CMR} (Fig. 6) (Andrade et al., 2006; Chou and Chang, 2000).

A positive deviation from *ΔAE* was observed for CMR/DPPC mixed films as a result of a more immiscible behavior (Figs. 5c and 6c). The extent of deviation was dependent on the surface pressure since at higher pressures the molecules become more compact and the effects of intermolecular interactions in the packaging become less evident (Chou and Chang, 2000). The repulsive behavior indicates a phase separation favoring to molecules clustering (Mishra et al., 2012). Similar results have been obtained with other coumarins (Sarpietro et al., 2011; Chakraborty et al., 2012).

In order to analyze the thermodynamic characteristics, we calculated *ΔGe* and *ΔGmix* (Baldyga and Dluhy, 1998; Dynarowicz-Latka et al., 2001; Maget-Dana, 1999) as follow:

$$\Delta Gmix = \Delta Gid + \Delta Ge, \quad (3)$$

where *ΔGid* is the ideal variation of the Gibbs free energy of mixing given by

$$\Delta Gid = kT(X_1 lnX_1 + X_2 lnX_2), \quad (4)$$

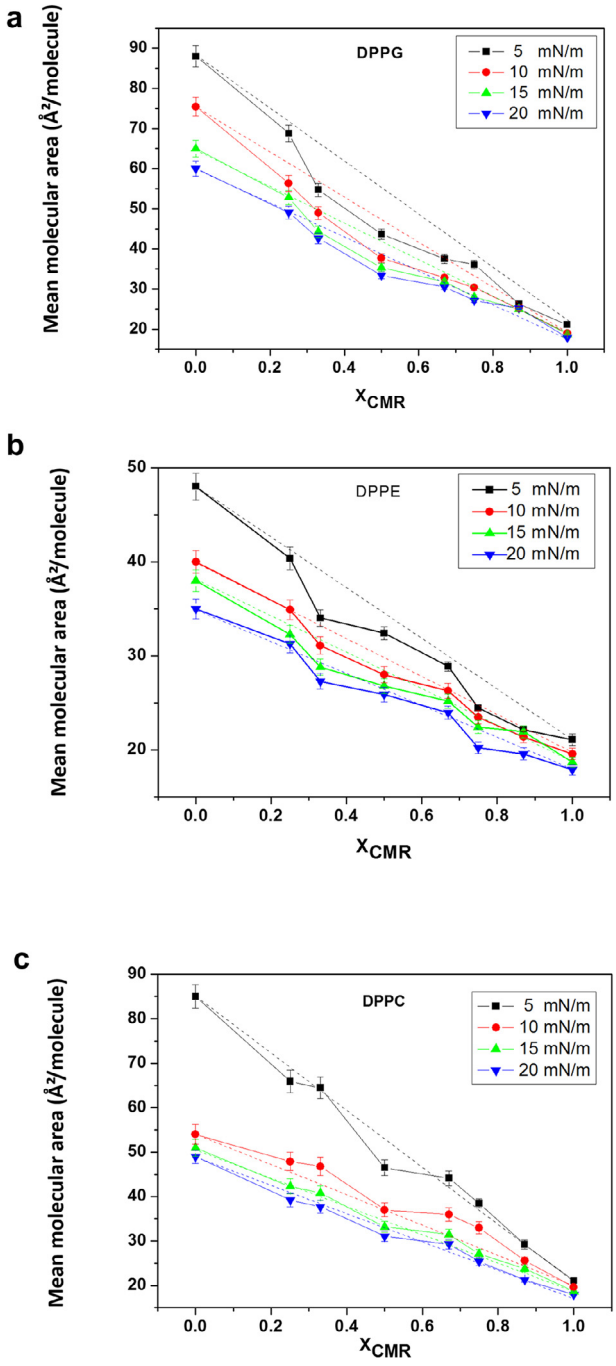

Fig. 5. Mean area per molecule as a function of mole fraction of coumarin in the mixed monolayers at a fixed surface pressure: CMR/DPPG (a), CMR/DPPE (b) and CMR/DPPC (c) at different concentrations (full line: real mean molecular area; dotted line: ideal mean molecular area).

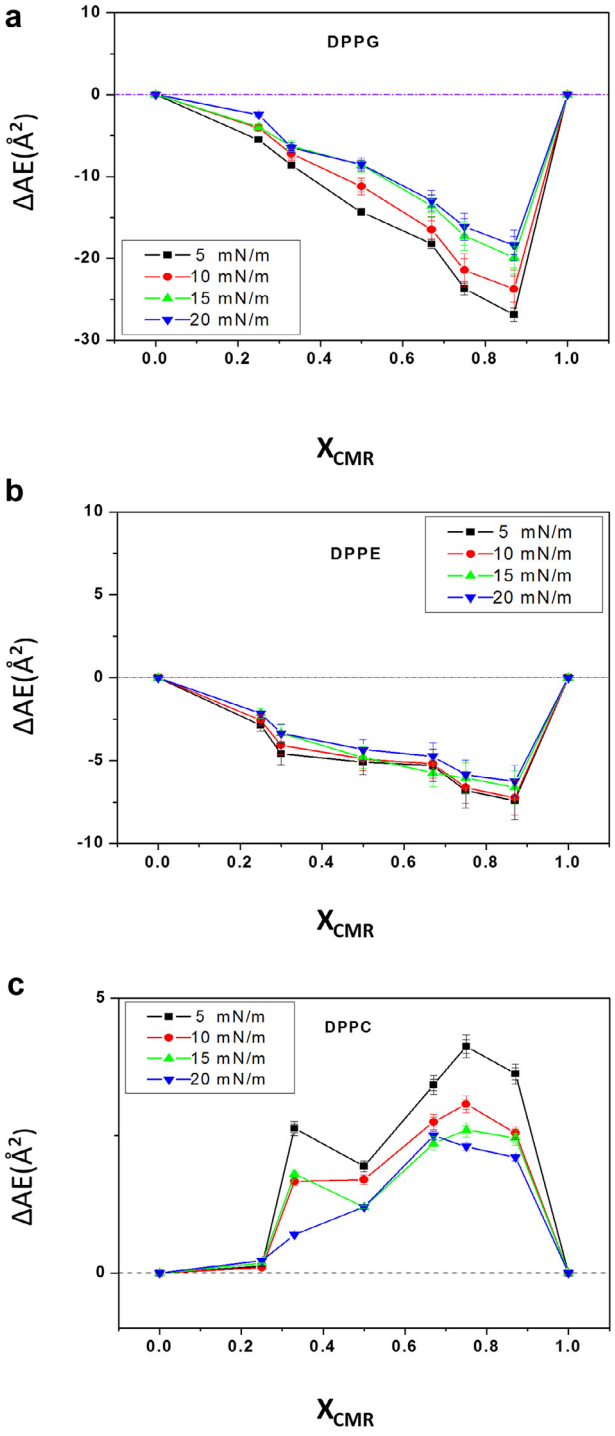

Fig. 6. Excess molecular area of the mixed monolayers of CMR/DPPG (a), CMR/DPPE (b) and CMR/DPPC (c) at different concentrations.

where K represents the Boltzmann constant and T is the absolute temperature. *ΔGe* represents the excess Gibbs free energy or contribution of the intermolecular interactions between molecules in the mixture. If *ΔGe* has a negative value, the existing molecular interactions between the components are attractive type while a positive variation implies a repulsive behavior. *ΔGe* is defined by the following formula:

$$\Delta Ge = \int_0^{\pi} [A_{1,2} - (X_1A_1 + X_2A_2)]d\pi, \quad (5)$$

ΔGe and *ΔGmix* for CMR/DPPG mixed films indicate a negative deviation from $X_{CMR} \geq 0.25$ to $X_{CMR} = 1.0$ (Figs. 7a and 8a). *ΔGe* and *ΔGmix* negative values indicate that CMR/DPPE monolayers are thermodynamically stable (Fig. 7b). *ΔGe* and *ΔGmix* values were positive for DPPC mixed monolayers indicating immiscibility (Figs. 7c and 8c). The distance between the molecules is dependent on the phospholipid head group that contributes to the repulsive interactions (Myers, 1999; Boggs, 1987).

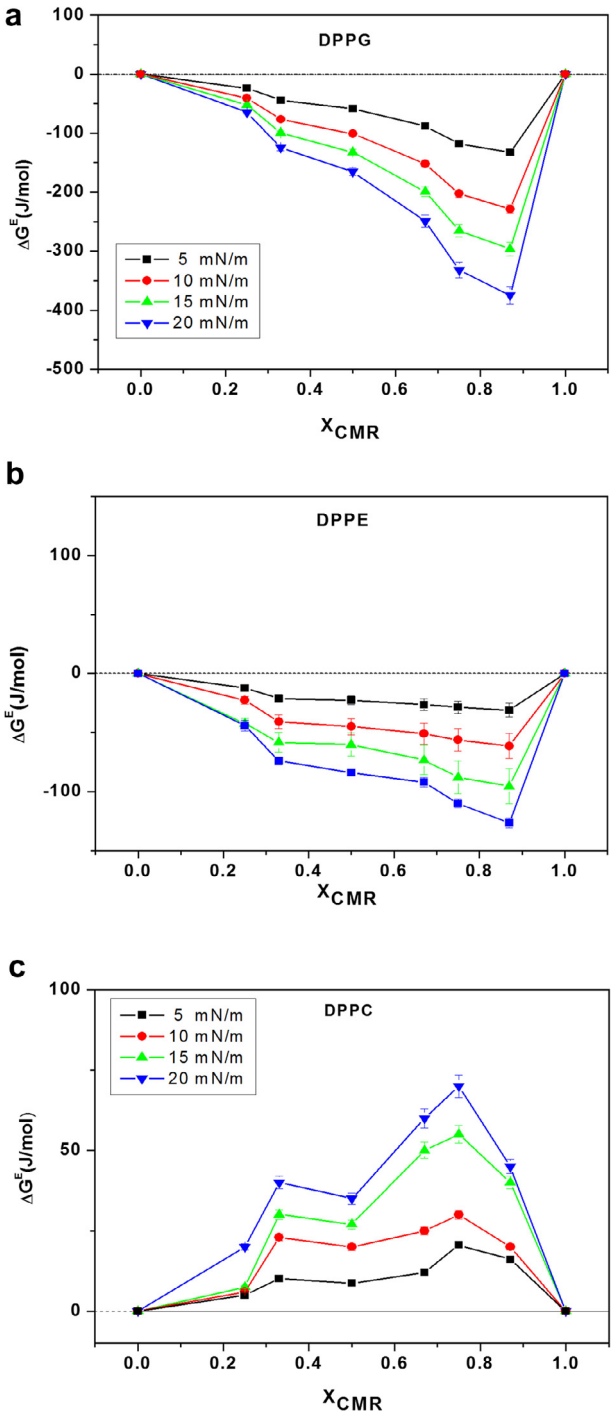

Fig. 7. ΔGe of CMR/DPPG (a), CMR/DPPE (b) and CMR/DPPC (c) at different concentrations.

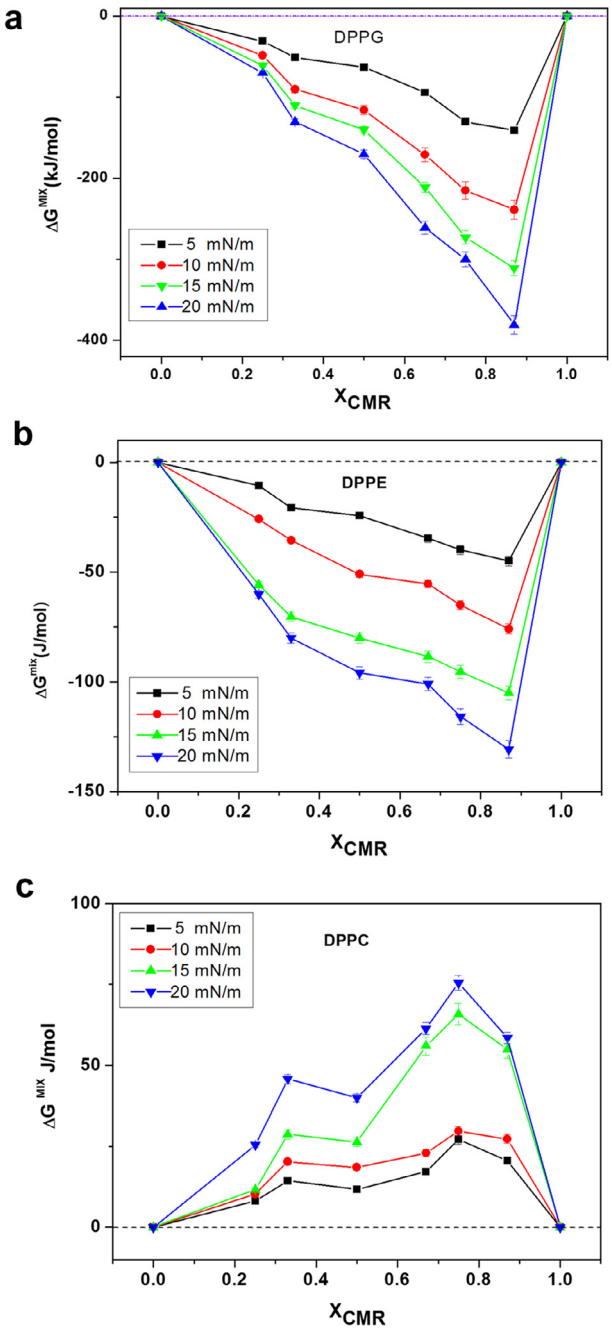

Fig. 8. ΔGmix of CMR/DPPG (a), CMR/DPPE (b) and CMR/DPPC (c) at different concentrations.

3.4. AFM analysis

Fig. 9 shows AFM images of the pure phospholipids monolayers transferred at $\pi = 20$ mN/m. Pure phospholipids show a uniform pattern with the root mean square (rms) equivalent to 0.219 nm, 0.060 nm and 0.054 nm for DPPG, DPPE and DPPC, respectively (Fig. 9). We obtained rms ~ 0.045 nm ($X_{CMR} = 0.87$) equivalent to a smooth monolayer topography (Fig. 10a). On the other hand, CMR/DPPE and CMR/DPPC films correspond to rough surfaces with rms = 0.1754 nm and 0.072 nm, respectively (Fig. 10b and c). The measurements were repeated in triplicate with a standard deviation of ~5% for rms.

4. Conclusions

Stability, miscibility and topographical characteristics of pure and mixed monolayers were evaluated by associating Langmuir-Blodgett and AFM techniques. The more effective thermodynamic association was obtained for DPPG/CMR mixed monolayers due to a favorable attractive intermolecular interaction. The differences in the molecular structure of the phospholipids explain the distinct behavior of the interaction between molecules. The coumarin was

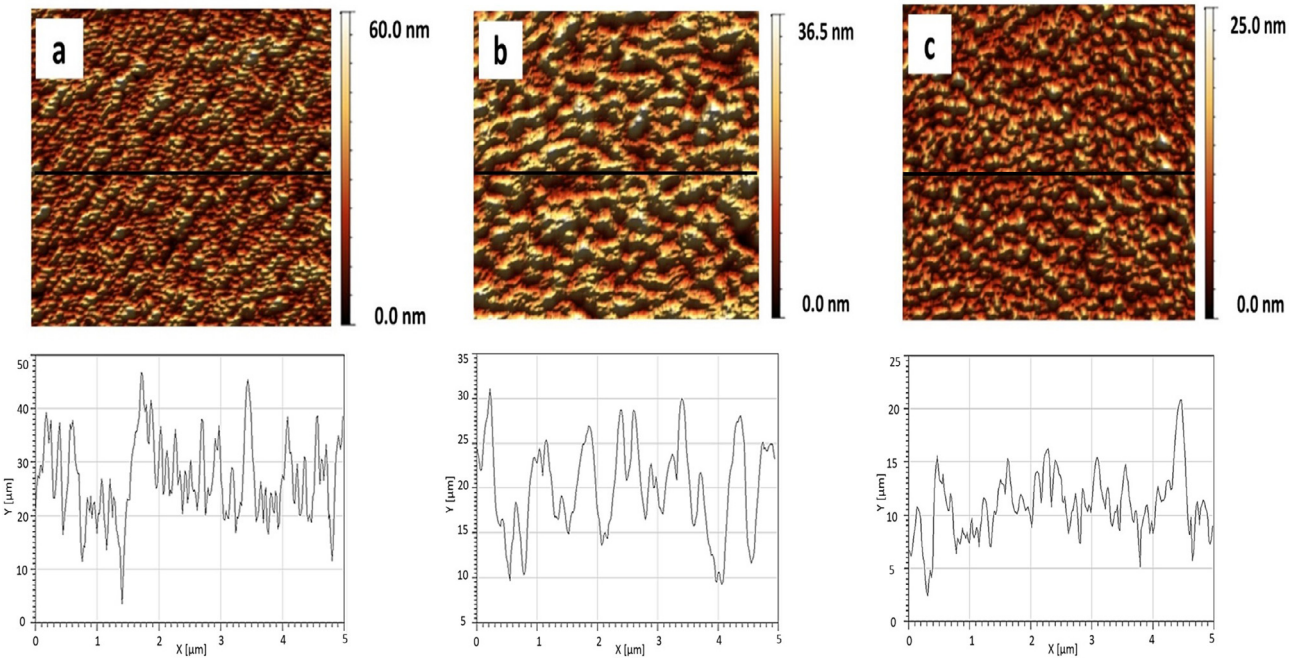

Fig. 9. Topographical images of pure DPPG (a), DPPE (b), and DPPC.

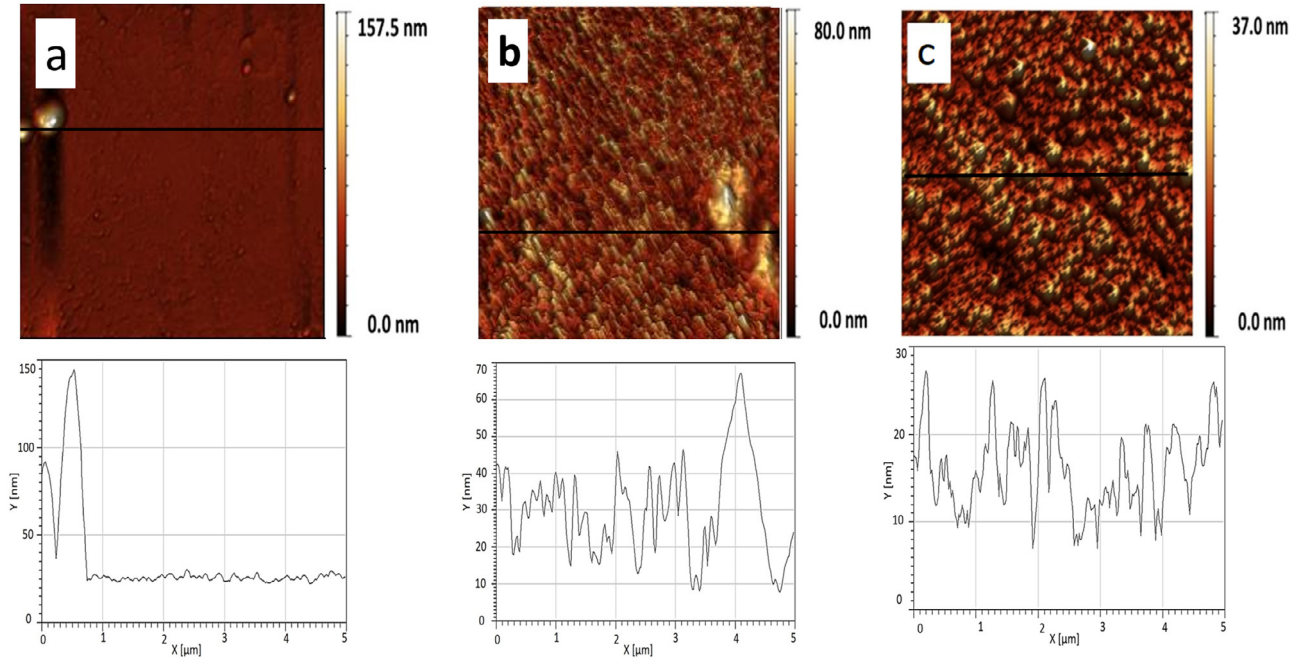

Fig. 10. Topographical images of the mixed monolayers: CMR/DPPG (a), CMR/DPPE (b) and CMR/DPPC (c). The mole fraction of coumarin is equivalent to 0.87.

able to alter the dipole moment, packing of the molecules and aggregate formation.

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