Interaction between C18 fatty acids and DOPE PEG2000 in Langmuir monolayers: effect of degree of unsaturation

Lai Ti Gew^{1,2} · Misni Misran²

Received: 5 December 2016 / Accepted: 1 June 2017 / Published online: 27 July 2017
© Springer Science+Business Media B.V. 2017

Abstract In this study, we address the effect of the *cis*-double bond in 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamide-*N*-[methoxy(polyethylene glycol)-2000, DOPE PEG2000 (DP), on the Langmuir monolayer of C18 fatty acids—namely, stearic acid (SA), oleic acid (L1), linoleic acid (L2), and linolenic acid (L3)—with the same head group but different degrees of saturation on their hydrocarbon chains. Negative values of Gibbs free energy of mixing (ΔG_{mix}) were obtained throughout the investigated ranges of the unsaturated C18 fatty-acid (L1, L2 and L3) mixed systems, indicating that very strong attractions occurred between molecules in the monolayers. The bend and kink effects from the *cis*-double bond(s) in the hydrocarbon chain affected the membrane fluidity and molecular packing in the monolayers, which resulted in a greater interaction between unsaturated C18 fatty acids and DP. The most thermodynamically stable mole composition of unsaturated C18 fatty acids to DP was observed at 50:1; this ratio is suggested to be the best mole ratio and will be subsequently used to prepare DP–C18 fatty-acid nanoliposomes. The presence of *cis*-double bonds in both hydrocarbon chains of DOPE in DP also created an imperfection in the membrane structure of lipid-drug delivery systems, which is expected to enhance lipid-based systems for antibody conjugation and drug encapsulation.

Keywords Intermolecular interactions · Langmuir–Blodgett film · Stealth liposomes · Atomic force microscopy · Membrane fluidity · Surface roughness

✉ Misni Misran
misni@um.edu.my

Lai Ti Gew
janeg@sunway.edu.my

¹ Department of Biological Sciences, School of Science and Technology, Sunway University, No. 5, Jalan Universiti, Bandar Sunway, 47500 Petaling Jaya, Selangor, Malaysia

² Department of Chemistry, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

1 Introduction

Pegylated-liposomes (or stealth liposomes) are well known as promising drug-delivery systems. Pegylated-liposomes prolong the lifetime circulation period of encapsulated drugs in the bloodstream, leading to cancer cell death [1–9]. Cancer therapeutic studies have shown that long-circulating drug-loaded stealth liposomes offer a better approach to cancer therapy than non-stealth liposomes [10–13]. For example, Doxil®, the first nano-pegylated liposomal drug, has shown increased anti-cancer efficacy, decreased drug toxicity, and a significantly improved therapeutic index in clinical studies [6, 14, 15]. The presence of polyethylene glycol (PEG) on the surfaces of liposomes also inhibits the non-specific and specific interactions of serum proteins on the liposome surface, preventing liposome–liposome aggregation during the drug-delivery process [2–4, 8, 15].

In preparing lipid-based formulations, the lipid molecular structures and properties, such as their hydrocarbon chain lengths and degrees of saturation, their head group sizes, and their charges should be considered. The molecular structures of lipids greatly affect the intermolecular interactions that occur between lipids and lipids, lipids and proteins, as well as lipids and the encapsulated drug in the lipid-protein-mixed system [16–19]. Molecular packing and membrane fluidity are significantly influenced by the degree of saturation on the hydrocarbon chains of the lipids, the sizes of their head groups, and their charges [20–23]. To obtain a stable lipid-based system, such as a liposomal drug carrier, lipid selection is rather important to ensure that the lipid-drug pairing is compatible in mixed systems.

DOPE PEG2000 (DP) has a PEG head group that consists of an average of 45 units of ethoxyl monomers, which is attached to a phospholipid with two C18 hydrocarbon chains with one *cis*-double bond on each chain (Fig. 1a). The molecular structures of the C18 fatty acids are simpler, consisting of a small –COOH head group with one 18-carbon hydrocarbon chain. Repulsions between DP and DP in the monolayer are expected to be greater than the –COOH head groups of the C18 fatty acids because of their chemical structure. There are at least three types of interactions that occur in the pure-DP monolayer, as illustrated in Fig. 1b: (1) head-to-head interactions, (2) tail-to-tail interactions, and (3) head-to-tail interactions.

Fig. 1 **a** Molecular structure of DP; **b** Three types of intermolecular interactions that occur between DP and DP: (1) Head-to-head; (2) tail-to-tail; (3) head-to-tail

PEG with saturated lipids, such as 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-amino(polyethylene glycol)-2000 (DSPE PEG2000), is commonly used in numerous preparations of stealth liposome (or immunoliposome) formulations for in vivo and in vitro cancer therapeutic studies [24–31]. PEGs with saturated lipids have no bends or kinks in their saturated lipid molecular structures that promote molecular packing, so they are easily inserted into the liposome membrane. Many applications of stealth/non-stealth liposomes (or immunoliposomes) in cancer/tumor research have been performed [10, 11, 32–36] but studies that have reported on the intermolecular interactions between PEG on lipid Langmuir monolayers are very limited.

In this study, we address the effect of the *cis*-double bond in DP on Langmuir monolayers of C18 fatty acids, namely stearic acid (SA), oleic acid (L1), linoleic acid (L2), and linolenic acid (L3) with the same head group but with different degrees of saturation on their hydrocarbon chains. Saturated SA has no double bonds in its C18 hydrocarbon chain, while L1 is an unsaturated fatty acid that has one double bond, and polyunsaturated fatty acids such as L2 and L3 contain two and three double bonds, respectively. The Langmuir technique is a powerful tool to determine the intermolecular interactions of mixed systems, which allow us to better understand their compatibility by quantitatively analyzing their thermodynamic stability using the obtained isotherms [37–41]. The monolayer films were transferred onto a solid substrate by using computer-controlled LB and then observed by tapping-mode atomic force microscopy (TM-AFM). We determined how DP molecules interact with each C18 fatty acid in the mixed system with the support of quantitative energetic data obtained with a fixed head group while varying the degree of unsaturation in the hydrocarbon chains of the C18 fatty acids. Using the insight from these studies, we suggest a precise ratio of C18 fatty acids to DP for preparation of stealth fatty-acid nanoliposomes in future studies. We hope that this suggested composition can serve as a reference for designing and developing effective polymer-grafted liposomal drug-carrier systems or for improving existing formulations.

2 Experimental

2.1 Materials

C18 fatty acids, such as SA, L1, L2, and L3 were purchased from Sigma-Aldrich. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamide-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt), DOPE PEG2000 (DP), (molecular weight of 2801.47 amu) was purchased from Avanti Polar Lipids, USA. DP is a white powder and was stored at -20°C when not in use. Double-distilled water processed using the NANOpure Diamond Ultrapure Water System was used as the subphase throughout.

2.2 LB monolayer/mixed monolayer measurements

Separate stock solutions of fatty acids and DP were prepared in analytical-grade chloroform that was purchased from Merck. Fatty-acid solutions of 25 μl with increasing concentrations (as stated in Table 1) were deposited randomly into the water subphase ($26 \pm 0.1^{\circ}\text{C}$) with a Hamilton microsyringe precise to 0.5 μl . After spreading, 25 μl of DP was added to each monolayer of fatty acid. The monolayers were left to equilibrate for approximately 5 min and then compression began. We estimated that the small amount of PEG was sufficient for the

PEG-lipid systems to obtain a long circulation effect in the bloodstream. Large amounts of PEG conjugated on liposomes are known to (1) disrupt the bilayer structure of mixed lipid liposomes, (2) restrict the adhesion of liposomes to the targeted site due to steric hindrance, and (3) increase the cost of PEG, which is rather expensive, making it economically nonfeasible to produce stealth liposomes on a larger scale [2, 42–45]. Consequently, a much lower concentration of PEG compared with the concentration of lipids was utilized in this study. In this experiment, the mole ratio of fatty acids to DP was kept constant with respect to increasing the moles of fatty acids; 10:1, 20:1, 30:1, to 100:1. The concentrations of L1 and DP solutions to prepare each mixed system are formulated in Table 1. Selected isotherms (mole ratio of fatty acids to DP) that showed significant trends were analyzed and plotted in graphs.

Surface pressure (Π) – molecular area (A) isotherms were obtained using a computer-controlled KSV 5000 (Finland) Langmuir balance furnished with symmetric barriers and a Teflon trough with a total area of 71,640 mm² (597 × 120 mm) equipped with a platinum Wilhelmy plate (19.62 × 10.00 mm) of 39.80 mm total length. Monolayers were compressed at a rate of 10 mm min⁻¹. Each experiment was repeated at least three times to ensure the isotherms were reproducible. The isotherms of the same mixtures will give the same limiting molecular area by extrapolating the linear slope of individual Π – A isotherms to zero surface pressure.

2.3 Deposition of C18 fatty acid/DP bilayer on oxidized silicon wafer for AFM imaging

Silicon (100) wafers (Sigma-Aldrich) were cut into approximately 5 × 1 cm pieces and placed in a furnace (Carbolite, Watertown, WI, USA) for 8 h at 900 °C to allow oxidation. The oxidized silicon wafer was clamped vertical to the subphase and immersed into the dipping well before spreading the monolayer material. After complete evaporation of the solvent, the floating layer was compressed at a rate of 10 mm min⁻¹ to reach a target surface pressure of monolayers and kept for 15 min to attain stability for deposition. The target surface pressures were different among the mixtures. By referring to isotherms and C_s^{-1} versus Π curves of each pure and mixed system (Figs. 2, 3, 4), the deposition was carried out at the liquid-condensed phase for the pure SA at a surface pressure of 45 mN m⁻¹ and at 35 mN m⁻¹ for mixed monolayers SA/DP. The liquid-expanded phase for DP was at 12 mN m⁻¹ and the liquid-condensed phase for unsaturated C18 fatty acids and their mixed systems was at 25 mN m⁻¹; for each, the selected surface pressures were for SA, SA/DP and for unsaturated C18 fatty

Table 1 Concentration of stock solutions of L1 and DP for each mixed system

Mole ratio of C18 fatty acids to DP	[L1], mg ml ⁻¹	[DOPE PEG2000], mg ml ⁻¹
20:1	0.4	0.2
30:1	0.6	0.2
40:1	0.8	0.2
50:1	1.0	0.2
60:1	1.2	0.2
90:1	1.8	0.2
100:1	2.0	0.2

acids and their mixed systems with DP at a mole ratio of 50:1. This mole ratio was chosen by referring to our ΔG_{mix} findings, where 50:1 is the most thermodynamically stable mole composition. The Y-type deposition of the bilayer LB film was performed at the targeted pressure as above with a dipping speed of 10 mm min^{-1} . All the transferred films were kept for at least 3 days in a dry, clean, and closed container before atomic force microscopy (AFM) imaging.

2.4 Tapping mode-AFM imaging

High-resolution imaging of bilayers was obtained by using the NanoScope®V scanning probe microscope controller (Bruker, USA) in tapping mode under ambient conditions after transferring them from the air/water interface to an oxidized silicon substrate. Silicon probes with aluminum coating (VISTA T190R, Canada) were used. The resonance frequency of the probe was 190 kHz and the force constant was 48 N m^{-1} . Images in height mode were collected simultaneously with 512×512 points at a scanning rate of 1.0 Hz per line. A series of AFM images were taken from different perspectives.

Fig. 2 The surface pressure - area (Π -A) isotherms of mixed monolayers (plotted in mole ratio of C18 fatty acids to DOPE PEG2000): **a** stearic acid/DOPE PEG2000, **b** oleic acid/DOPE PEG2000, **c** linoleic acid/DOPE PEG2000, and **d** linolenic acid/DOPE PEG2000, spread on a nanopure water subphase at 26°C

Fig. 3 **a** The surface pressure - area (Π - A) isotherm of the DOPE PEG2000 monolayer and **b** its compressibility modulus (C_s^{-1}), spread on a nanopure water subphase at 26 °C

Fig. 4 The compressibility modulus (C_s^{-1}) vs. surface pressure (Π) of mixed monolayers (plotted in mole ratio of C18 fatty acids to DOPE PEG2000): **a** stearic acid/DOPE PEG2000, **b** oleic acid/DOPE PEG2000, **c** linoleic acid/DOPE PEG2000, and **d** linolenic acid/DOPE PEG2000, spread on a nanopure water subphase at 26 °C

3 Results and discussion

3.1 Π -A isotherms

The Π -A isotherms of fatty-acid monolayers and mixed monolayers of various mole ratios of C18 fatty acids (SA, L1, L2, and L3) to DP on nanopure water subphases are presented in Fig. 2. The limiting molecular areas of pure SA, L1, L2, and L3 monolayers were stated in our most recent published work, which were 21, 43, 42, and 42 Å², respectively [46]. Limiting molecular areas were determined by extrapolating the linear slope of individual Π -A isotherms to zero surface pressure. For all the mixed monolayers, the molecular areas were also obtained by extrapolating the linear slope of individual Π -A isotherms to zero surface pressure (Table 2).

When DP was incorporated into the SA monolayer, the mean molecular area of the mixtures increased as the content of SA increased (Fig. 2a). The Π -A isotherm of pure SA increased rapidly from zero surface pressure to 50 mN m⁻¹ moving at a span of a mean molecular area of 27 to 20 Å². As compared to the isotherms in the presence of DP, for instance 90:1 and 100:1, the isotherms shifted away from pure SA, slightly to the right (increasing the mean molecular area) and a lower collapse pressure was observed. Shifting to the right showed that repulsion between the molecules SA and DP occurred in the mixtures. The effect of DP was clearly shown from 0 to 15 mN m⁻¹ in the mixed system isotherms, where the similar shape of the Π -A isotherm of DP can be observed in the mixed systems (Figs. 2 and 3). DP-DP interactions occurred first, followed by DP-C18 fatty-acid interactions. The mixed monolayers of unsaturated fatty-acid (L1, L2, and L3) isotherms (Fig. 2b-d) with the addition of DP shifted away from their pure fatty-acid isotherms due to the presence of PEG. While there was no noticeable difference between some of the mole ratios in the isotherms, at 5 mN m⁻¹ we observed a mixed monolayer containing <50:1 (mole ratio of unsaturated C18 fatty acids to DP) that had a larger mean molecular area compared with its respective pure monolayer. Profiles of C18 fatty acids to DP >50:1, which had a smaller mean molecular area, were also observed at a surface pressure of 5 mN m⁻¹. This showed that interactions occurred between the fatty acids and DP. The effect of DP was clearly shown from 0 to 15 mN m⁻¹ in all of the mixed system isotherms. DP-DP interactions occurred first, followed by DP-C18 fatty-acid interactions. This observation was also supported by the compressibility modulus (Fig. 4). Similar curves as observed in DP (Fig. 3) were also seen in most of the binary systems in Fig. 4a-d at surface pressures from 0 to 15 mN m⁻¹, which will be discussed later.

Table 2 Limiting molecular areas of mixed monolayers were obtained by extrapolating the linear slope of individual Π -A isotherms to zero surface pressure

Mole ratio	Limiting molecular areas, A ₁₂ , Å ²			
	SA/DP	L1/DP	L2/DP	L3/DP
20:1	42.0	45.0	53.6	21.3
30:1	32.0	44.0	30.0	25.0
40:1	40.0	39.0	37.5	25.0
50:1	32.0	24.7	24.0	23.8
60:1	30.0	26.4	32.0	25.0
90:1	38.0	24.0	25.5	20.0
100:1	39.0	25.5	20.0	23.6

No collapse pressure was observed in the DP isotherm (Fig. 3). The limiting molecular area of DP was found to be $594 \text{ \AA}^2$ (Fig. 3). The diameter of DP equals $27.5 \text{ \AA}$ (using the formula $A = \pi r^2$). The typical collapse pressure of a lipid monolayer (for example SA) will cause a rapid decrease in the surface pressure. However, there are some exceptions where the DP monolayer is in a liquid state; a horizontal break can be observed in the isotherm. This horizontal break of DP can be observed at approximately 12 mN m^{-1} with the support of a compressibility modulus profile (Fig. 3). The derivative of the pure-DP C_s^{-1} versus Π profile (Fig. 3) did not show a change at approximately 12 mN m^{-1} , which presumably corresponds to its collapse pressure, showing that the molecular size of DP was much larger than that of the C18 fatty acids. There were several breaks in the slopes that may indicate phase transitions, which can be observed at 6, 8.5, and 10.5 mN m^{-1} . This change was more obvious in the plot of C_s^{-1} versus Π (Fig. 3), indicating that the monolayer of DP was less compressible and disordered and the low C_s^{-1} values suggest that the DP molecular organization favored their arrangement in the liquid-expanded phase in subphase water at 26°C . We predicted that several interactions occurred due to the molecular structure of DP (Fig. 1) e.g., interactions between the large PEG head group of DP and another head group of DP. Additionally, the *cis*-double bonds of the DOPE hydrocarbon chains in one or two DP molecules provided kink effects that significantly influenced the molecular packing and miscibility. The isotherm of DP will be used as a reference to compare C18 fatty acid–PEG interactions that occur in our studies.

3.2 Compressibility analysis

The packing density of monolayers can be evaluated and analyzed in terms of the compression modulus C_s^{-1} , which is defined as [47, 48]:

$$C_s^{-1} = -A \left(\frac{d\Pi}{dA} \right) \quad (1)$$

The C_s^{-1} versus Π curves provide detailed information on the phase transitions of C18 fatty-acid/DP monolayers (Fig. 4a–d). C_s^{-1} can be classified into various phases, namely (a) liquid-expanded (LE) phase at surface pressures in the range 10 to 50 mN m^{-1} , (b) liquid (L) phase from 50 to 100 mN m^{-1} , (c) liquid-condensed (LC) phase from 100 to 250 mN m^{-1} and (d) solid (S) phase above 250 mN m^{-1} . In this work, the compression moduli were obtained by numerical calculations of the first derivative from the isotherm data points using the OriginPro-8 program.

At low surface pressures (0 to 10 mN m^{-1}), results similar to those of the DP compression moduli were observed in all of the mixed systems, as plotted in Fig. 4. These results showed that the effect of DP was very strong in all of the mixtures; intermolecular interactions began with DP–DP interactions and then DP–FAs occurred from 10 mN m^{-1} onward.

SA can easily form a solid phase because it contains a saturated hydrocarbon chain. With the presence of DP in SA monolayers, its mixtures were less compressible than pure SA, consecutively forming LE, L, and LC phases. The *cis*-double bonds of DOPE in DP increased the membrane fluidity, which prevented the molecules from packing

tightly and uniformly. Interestingly, the maximal values of the compression modulus for mixed systems of SA/DP of 90:1 and 100:1 were higher than those of other mixed monolayers of SA/DP (Fig. 4a). The molecular packing of SA/DP was favorable when the mole ratio of SA was higher; less bend and kink effects were contributed from the *cis*-double bonds of DOPE in the DP molecular structures to the monolayers.

The *cis*-double bonds in the unsaturated C18 fatty acids in the mixed systems increased the membrane fluidity and greatly affected the compressibility of the mixed monolayer. Pure-L1, -L2, and -L3 were in L phase. Mixed systems of L1/DP, L2/DP, and L3/DP had lower compression moduli compared with their pure monolayers, which were below 50 mN m^{-1} (Fig. 4b–d). The phases of the monolayers changed from L to LE phase. The L3/DP mixed systems had a similar compression-modulus value as the pure system. However, an exception was observed for a mole ratio of L3/DP at 100:1; its maximal value was higher than that of the pure system (Fig. 4d). The surface morphology of each mixture at a mole ratio of 50:1 can be observed in Fig. 9.

3.3 Miscibility of mixed monolayers

The miscibility of the mixed monolayer components can be determined by calculating the mean molecular area A_{12} .

For ideal mixing, A_{12}^{id} is defined as [47–49]:

$$A_{12}^{id} = A_1X_1 + A_2X_2 \quad (2)$$

where A_1 and A_2 are the mean molecular areas of single components at the same surface pressure and X_1 and X_2 are the mole fractions of components 1 and 2 in the mixed film, respectively.

These deviations can be described quantitatively by the excess mean molecular area (A_{ex}) values as:

$$A_{ex} = A_{12} - A_{12}^{id}. \quad (3)$$

Non-linear plots of A_{ex} (Fig. 5) showed the existence of interactions between the monolayer components of C18 fatty acids/DP. The strengths of these interactions were verified based on ΔG_{mix} (Fig. 6).

Negative deviations from ideal behavior were obtained for SA/DP (Fig. 5) over most of the ranges investigated. Exceptions were observed when the mole ratio of SA/DP was 90:1 and 100:1, where position deviation occurred. These two mixtures were immiscible and DP poorly interacted with SA molecules. This observation was consistent with the obtained positive values of ΔG_{mix} (Fig. 6).

Negative deviations were observed in unsaturated fatty acids (L1, L2, and L3) for all of their mixed systems. These deviations revealed non-ideal behavior and showed that the monolayer components were miscible. This observation was also supported by the negative values obtained for ΔG_{mix} (Fig. 6), which showed that strong intermolecular attractions occurred between unsaturated fatty acids and DP. These results will be further discussed in the following section regarding the thermodynamic stability of the mixed monolayer.

Fig. 5 Excess mean molecular area (A_{ex}) vs mole ratio of C18 fatty acids to DOPE PEG2000 mixed monolayers: ∇ = stearic acid/DOPE PEG2000, $\bullet$ = oleic acid/DOPE PEG2000, $\blacktriangle$ = linoleic acid/DOPE PEG2000, and $\square$ = linolenic acid/DOPE PEG2000, spread on a nanopure water subphase at 26 °C. Negative deviations from ideal behavior were observed for SA/DP over most of the range investigated, except when the mole ratios of SA/DP were 90:1 and 100:1, which showed position deviation. Negative deviations were observed in unsaturated fatty acids for all of the mixed systems. These deviations revealed non-ideal behavior and showed that the monolayer components were miscible

Fig. 6 Free energy of mixing (ΔG_{mix}) vs. mole ratio of C18 fatty acids to DOPE PEG2000 mixed systems: ∇ = stearic acid/DOPE PEG2000, $\bullet$ = oleic acid/DOPE PEG2000, $\blacktriangle$ = linoleic acid/DOPE PEG2000, and $\square$ = linolenic acid/DOPE PEG2000, spread on a nanopure water subphase at 26 °C. The values of ΔG_{mix} of SA/DP mixtures were almost negative throughout the range of binary systems; exceptions were observed at 90:1 and 100:1. Lower negative free energy of mixing values were obtained throughout the range of unsaturated C18 fatty acid mixed systems compared with the saturated SA mixed systems, indicating that very strong attractions occurred between the molecules in the monolayers because the bend and kink effects from the *cis*-double bond(s) in the hydrocarbon chains affected the molecular packing of the monolayers

3.4 Thermodynamic stability

Molecular interactions can be expressed quantitatively using thermodynamic analysis. The total Gibbs free energy of mixing ΔG_{mix} is defined by [47, 48]:

$$\Delta G_{\text{mix}} = \Delta G_{\text{id}} + \Delta G_{\text{ex}} \quad (4)$$

where

$$\Delta G_{\text{id}} = RT(X_1 \ln X_1 + X_2 \ln X_2) \quad (5)$$

and the excess Gibbs free energy of mixing, ΔG_{ex} , can be calculated from Π - A isotherms as:

$$\Delta G_{\text{ex}} = \int_0^{\Pi} [A_{12} - (X_1 A_1 - X_2 A_2)] d\Pi \quad (6)$$

where A_{12} , A_1 and A_2 represent the area of the mixed system and the areas of components 1 and 2, respectively and Π is the surface pressure of the monolayer. If the monolayer is ideally mixed, ΔG_{ex} should be zero.

The interactions in the investigated mixed systems were compared by analyzing the free energy of the mixing values, which were calculated and plotted in Fig. 6.

The values of ΔG_{mix} of SA/DP mixtures were close to negative throughout the tested ranges of the binary systems. Exceptions were observed at the two highest mole ratios of SA/DP, which were 90:1 and 100:1. A similar observation was apparent in their compression-modulus plot (Fig. 4a). Strong repulsions are expected when SA molecules are packed tightly and uniformly. This also suggests that DP molecules were attracted to the hydrophobic head group of SA in these two mixtures.

Lower negative free energy of mixing values were obtained throughout the range of the unsaturated C18 fatty acid (L1, L2, and L3) mixed systems compared with the saturated SA mixed systems (Fig. 6), indicating that very strong attractions occurred between the molecules in the monolayers. This observation was expected because the bend and kink effects from the *cis*-double bond(s) in the hydrocarbon chains affect the molecular packing of the monolayers. Greater interactions occurred between the unsaturated hydrocarbon chains of the fatty acids and the unsaturated DOPE in DP.

L1, L2, and L3 showed similar trends, becoming less negative as the moles of fatty acid were increased. Intermolecular interactions were greater when unsaturated C18 fatty acids interacted with DP. By extrapolating the curves of L1/DP, L2/DP, and L3/DP, we obtained 50:1 as the intersection point. We suggest that this ratio is favorable to prepare pegylated L1, L2, and L3 nanoliposomes; at this monolayer composition, the mixed systems are thermodynamically the most stable. Increasing the number of double bonds in the hydrocarbon chains of the fatty acids did not significantly contribute to interactions in the binary systems. As the moles of unsaturated C18 fatty acids increased (from 60:1 and higher), ΔG_{mix} had predominantly similar negative values.

3.5 AFM observation

AFM topography provides a morphological insight into the behavior of the pure and mixed systems (Figs. 7, 8, 9). One key feature of AFM imaging is the surface roughness measurements [50–52]. Surface roughness (R_a and R_q) is a commonly used parameter to characterize the features about the bilayer membrane. R_a and R_q provide valuable information about the surface morphology and surface interactions; however, they are calculated using different formulas.

Fig. 7 AFM images of pure C18 fatty-acids bilayer deposited on oxidized silicon wafer at scan size of $1 \mu\text{m} \times 1 \mu\text{m}$ with a data scale of 25 nm for: **a** stearic acid, **b** oleic acid, **c** linoleic acid, and **d** linolenic acid (this figure was published in [42] recently, it will be used as a comparison between surface topography of pure C18 fatty acids and their mixtures with DP)

R_a is the mean value of the surface relative to the center plane, calculated as [53]:

$$R_a = \frac{1}{L} \int_0^L |Z(x)| dx \quad (7)$$

R_q is the root mean square (rms) of surface measurements of peaks and valleys, defined as [53]:

$$R_q = \sqrt{\frac{1}{L} \int_0^L Z^2(x) dx} \quad (8)$$

where $Z(x)$ is the function that describes the surface profile analyzed in terms of height (Z) and position (x) of the sample over the evaluation length of L . R_q of a surface is similar to the roughness mean value, R_a , with the only difference being the mean squared absolute values of

Fig. 8 AFM images of pure DOPE PEG2000 bilayer deposited on oxidized silicon wafer obtained in scan size of **a** 5 $\mu\text{m} \times 5 \mu\text{m}$ with a data scale of 25 nm. A cross section was drawn on a selected DP molecule incorporated on the membrane depicted in **a** to obtain more information of the height and width of AS25 molecule. The height and width of this PEG were found to be approximately 500 $\times$ 286 nm, respectively as shown in **b** and **c**

the surface roughness profile. A comparison of R_a and R_q values of pure systems of C18 fatty acids (taken from our recent publication [53] for comparison purposes), DP and mixed systems of C18 fatty acids/DP (at a mole ratio of 50:1) in Table 3 were obtained by using NanoScope Analysis 1.5.

We observe that similar surface images were obtained for C18 fatty acids as they have a similar carboxyl head group (Fig. 7); however R_a values of C18 fatty acids increase when increasing the degree of saturation of C18 fatty acids [46]. Deposition of SA onto a solid substrate was performed at solid phase (45 mN m^{-1}) and unsaturated C18 fatty acids was carried out at liquid phase due to the presence of *cis*-double bond(s) in their respective chemical structures. The surface is relatively flat as the difference between R_a to the respective R_q of pure C18 fatty acids is far lower (about 0.1) than the mixed systems of C18 fatty acid/DP (about 0.15 to 0.2). However, the R_a and R_q values of DP are much higher due to its large head group and the difference between its R_a and R_q is 0.35 as more valleys and peaks are distributed on the surface morphology of pure DP. This could be the big PEG head groups of the DP span on the solid substrate as observed in Fig. 8a. The deposition was performed at the liquid-expanded phase (due to the nature of the DP chemical structure); hence the monolayer of DP was less compressible and disordered as illustrated in Fig. 10b. A large amount of identical spots are randomly distributed on the surface morphology of mixed systems of C18 fatty acids/DP (Fig. 9), presumably, these spots are the PEG head groups of DP. The molecules were arranged in a better manner as we transferred the monolayer from the water subphase onto the solid substrate at the liquid-condensed phase. For SA (a saturated C18 fatty acid), the R_a value changes dramatically from 0.215 (pure SA) to 0.602 nm (mixed system of SA/DP). The unsaturated L1 with one *cis*-double bond in its hydrocarbon chain also showed a dramatic increase in R_a value, from 0.249 (pure L1) to 0.594 (L1/DP). On the other hand, slight changes of R_a value of polyunsaturated C18 fatty acids (L2 and L3) were observed; 0.428 (pure L2) to 0.583 (L2/DP) and 0.460 (pure L3) to 0.556 nm (L3/DP). This observation is due to the PEG head group of DP creating a globular-like structure on the saturated SA membrane surface (Fig. 10c) after being mixed, causing an increase in the R_a value as the pure SA surface, as mentioned earlier, is relatively flat. The membrane fluidity

Fig. 9 AFM images of binary mixture of C18 fatty acids/DP (mole ratio of 50:1) bilayer deposited on oxidized silicon wafer obtained in a $5.0\ \mu\text{m} \times 5.0\ \mu\text{m}$ scan area with a data scale of 25 nm for: **a** stearic acid/DP, **b** oleic acid/DP, **c** linoleic acid/DP, and **d** linolenic acid/DP

increased with the incorporation of DP into the L1 membrane, which caused a noticeable change in the R_a value. As for the L2 and L3 membrane, the unsaturation induced a greater penetration of the PEG head group into the bilayer membrane. The polar PEG head groups surround the L2 and L3 polar carboxyl head groups in their membrane, respectively, therefore only a slight change in the R_a value is observed (Fig. 10d). R_a also indicated that the surface of the L1 membrane is slightly flatter than L2 and L3 by showing that the molecules of L1 are more ordered than L2 and L3. The R_a and R_q values of SA/DP are slightly bigger than the unsaturated fatty acids. The differences between the R_a and R_q values are about 0.15 to 0.2. These values support our energetic studies, as negative ΔG_{mix} values were obtained at a mole ratio of 50:1 for all C18 fatty acid/DP mixtures. We conclude that the intermolecular interaction of DP with respective C18 fatty acid in the membrane is not greatly affected by the degree of saturation.

AFM observations from this study support our LB quantitative analysis that indicated that DP was strongly incorporated into the C18 fatty acid membrane at a mole ratio of 50:1.

Fig. 10 A cartoon illustration of surface topography to describe the surface roughness values, R_a and R_q in Table 1. **a** A flat surface of pure C18 fatty acids, owing to the identical of carboxyl head-groups of fatty acid. **b** pure DP, that PEG head-groups tend to fold on itself; **c** saturated C18 fatty acid - SA/DP, the present of PEG head-group on SA bilayer membrane creating the globular-like structure on the membrane surface; and **d** unsaturated C18 fatty acid - L3/DP, the polar PEG head-groups cloak around L3 polar carboxyl head-group in the membrane

4 Conclusions

The *cis*-double bonds in the hydrocarbon chains of DOPE in DP introduced imperfections into the membrane structure causing the molecular packing to become less compressible and increasing membrane fluidity, which enhanced the lipid-system for antibody conjugation and drug encapsulation. Langmuir monolayers of lipid-DP mixed systems were successfully used to illustrate one aspect of pegylated fatty acid liposomes. Measuring surface roughness by AFM enabled us to elucidate the behavior of DP molecules in saturated and unsaturated C18 fatty acids bilayer membranes. The large PEG head group of DP formed a globular-like structure on the saturated SA bilayer but it surrounded the polar carboxyl head group of the unsaturated L3 bilayer. The investigation of DP-C18 fatty-acid mixtures enabled us to understand the stability of DP in saturated and unsaturated fatty-acid membranes in the pegylated lipid-based drug-delivery system. In this study, we conclude that the most thermodynamically stable composition of unsaturated C18 fatty acids and DP was 50:1. At this molecular ratio,

Table 3 Surface roughness R_a and R_q values of pure C18 fatty acids (taken from our recent publication [46], for comparison purposes), DP and mixed systems of C18 fatty acids/DP (obtained using NanoScope Analysis 1.5)

Pure systems	Surface roughness, nm		Mixed systems	Surface roughness, nm	
	R_a	R_q		R_a	R_q
SA	0.215	0.286	SA/DP	0.602	0.806
L1	0.249	0.303	L1/DP	0.594	0.746
L2	0.428	0.578	L2/DP	0.583	0.783
L3	0.460	0.575	L3/DP	0.556	0.708
DP	0.818	1.170			

they are more energetically stable and cost-efficient, as only a small amount of PEG will be required. Therefore, 50:1 is the best mole ratio to use for preparing DP-C18 fatty acid nanoliposomes.

Acknowledgements This study was financially supported by the Fundamental Research Grant Scheme (FP013-2015A) and UMRG Flagship (RP022C-16SUS), Malaysia. Gew would like to express her thanks to Phra Phrom for his blessing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

1. Chonn, A., Cullis, P.R.: Recent advances in liposome technologies and their applications for systemic gene delivery. *Adv. Drug Deliv. Rev.* **30**, 73–83 (1998)
2. Allen, C., Santos, N.D., Gallagher, R., Chiu, G., Shu, Y., Li, W., Johnstone, S., Janoff, A., Mayer, L., Webb, M., Bally, M.: Controlling the physical behavior and biological performance of liposome formulations through use of surface grafted poly(ethylene) glycol. *Biosci. Rep.* **22**, 225–250 (2002)
3. Moghimia, S.M., Szebenib, J.: Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. *Prog. Lipid Res.* **42**, 463–478 (2003)
4. Torchilin, V.P.: Recent advances with liposomes as pharmaceutical carriers. *Nat. Rev. Drug Discov.* **4**, 145–160 (2005)
5. Immordino, M.L., Dosio, F., Cattel, L.: Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. *Int. J. Nanomedicine.* **1**, 297–315 (2006)
6. Zhang, Y.: Stealth Liposomes: the silent nanobombers. *Trends in Bio/Pharm. Industry.* 19–24 (2008)
7. Chang, H.I., Yeh, M.K.: Clinical development of liposome-based drugs: formulation, characterization, and therapeutic efficacy. *Int. J. Nanomedicine* **7**, 49–60 (2012)
8. Nag, O.K., Awasthi, V.: Surface engineering of liposomes for stealth behavior. *Pharmaceutics* **5**, 542–569 (2013)
9. Anselmo, A.C., Mitragotri, S.: An overview of clinical and commercial impact of drug delivery systems. *J. Control. Release.* **190**, 15–28 (2014)
10. Ng, K.Y., Zhao, L., Liu, Y., Mahapatro, M.: The effects of polyethyleneglycol (PEG)-derived lipid on the activity of target-sensitive immunoliposome. *Int. J. Pharm.* **193**, 157–166 (2000)
11. Yanga, T., Choi, M.K., Cuia, F.D., Kim, J.S., Chung, S.J., Shimb, C.K., Kim, D.D.: Preparation and evaluation of paclitaxel-loaded PEGylated immunoliposome. *J. Control. Release* **120**, 169–177 (2007)
12. Manjappa, A.S., Chaudhari, K.R., Venkatarajua, M.P., Dantuluri, P., Nanda, B., Sidda, C., Sawant, K.K., Murthy, R.S.R.: Antibody derivatization and conjugation strategies: application in preparation of stealth immunoliposome to target chemotherapeutics to tumor. *J. Control. Release* **150**, 2–22 (2011)
13. Gunaseelan, S., Gunaseelan, K., Zhang, M.D.X., Sinko, P.J.: Surface modifications of nanocarriers for effective intracellular delivery of anti-HIV drugs. *Adv. Drug Deliv. Rev.* **62**, 518–531 (2010)
14. Working, P.K., Newman, M.S., Huang, S.K., Mayhew, E., Vaage, J., Lasic, D.D.: Pharmacokinetics, biodistribution and therapeutic efficacy of doxorubicin encapsulated in stealth® liposomes (Doxil®). *J. Liposome Res.* **4**, 667–687 (1994)
15. Allen, T.M., Cullis, P.R.: Liposomal drug delivery systems: from concept to clinical applications. *Adv. Drug Deliv. Rev.* **65**, 36–48 (2013)
16. Blume, G., Ceve, G.: Molecular mechanism of the lipid vesicle longevity *in vivo*. *Biochim. Biophys. Acta.* **1146**, 157–168 (1993)
17. Lee, A.G.: Lipid–protein interactions in biological membranes: a structural perspective. *Biochim. Biophys. Acta* **1612**, 1–40 (2003)
18. Seu, K.J., Cambrea, L.R., Everly, R.M., Hovis, J.S.: Influence of lipid chemistry on membrane fluidity: tail and headgroup interactions. *Biophys. J.* **91**, 3727–3735 (2006)
19. Lingwood, L., Simons, K.: Lipid rafts as a membrane-organizing principle. *Science.* **327**, 46–50 (2010)
20. Ma, G., Allen, H.C.: DPPC Langmuir monolayer at the air–water interface: probing the tail and head groups by vibrational sum frequency generation spectroscopy. *Langmuir.* **22**, 5341–5349 (2006)
21. Lingwood, D., Simons, K.: Lipid rafts as a membrane-organizing principle. *Science.* **327**, 46–50 (2010)

22. Barelli, H., Antonny, B.: Lipid unsaturation and organelle dynamics. *Curr. Opin. Cell Biol.* **41**, 25–32 (2016)
23. Sezgin, E., Levental, I., Mayor, S., Eggeling, C.: The mystery of membrane organization: composition, regulation and roles of lipid rafts. *Nat. Rev. Mol. Cell Biol.* **18**(6), 361–374 (2017). doi:10.1038/nrm.2017.16
24. Johnston, M.J., Semple, S.C., Klimuk, S.K., Ansell, S., Maurer, N., Cullis, P.R.: Characterization of the drug retention and pharmacokinetic properties of liposomal nanoparticles containing dihydrosphingomyelin. *Biochim. Biophys. Acta* **1768**, 1121–1127 (2007)
25. Bestman-Smith, J., Gourde, P., Desormeaux, A., Tremblay, M.J., Bergeron, M.G.: Sterically stabilized liposomes bearing anti-HLA-DR antibodies for targeting the primary cellular reservoirs of HIV-1. *Biochim. Biophys. Acta* **1468**, 161–174 (2000)
26. Loomis, K., Smith, B., Feng, Y., Garg, H., Yavlovich, A., Campbell-Massa, R., Dimitrov, D.S., Blumenthal, R., Xiao, X., Puri, A.: Specific targeting to B cells by lipid-based nanoparticles conjugated with a novel CD22-ScFv. *Exp. Mol. Pathol.* **88**, 238–249 (2010)
27. Lehtinen, J., Raki, M., Bergström, K.A., Uutelä, P., Lehtinen, K., Hiltunen, A., Pikkarainen, J., Liang, H., Pitkänen, S., Määttä, A.M., Ketola, R.A., Yliperttula, M., Wirth, T., Urtti, A.: Pre-targeting and direct immunotargeting of liposomal drug carriers to ovarian carcinoma. *PLoS ONE* **7**, 1–10 (2012)
28. Yan, F., Li, L., Deng, Z., Jin, Q., Chen, J., Yang, W., Yeh, C.K., Wu, J., Shandas, R., Liu, X., Zheng, H.: Paclitaxel-liposome-microbubble complexes as ultrasound-triggered therapeutic drug delivery carriers. *J. Control. Release* **166**, 246–255 (2013)
29. Kroon, J., Metselaar, J.M., Storm, G., van der Pluijm, G.: Liposomal nanomedicines in the treatment of prostate cancer. *Cancer Treat. Rev.* **40**, 578–584 (2014)
30. Foreman, M.B., Coffman, J.P., Murcia, M.J., Cesana, S., Jordan, R., Smith, G.S., Naumann, C.A.: Gelation of amphiphilic lipopolymers at the air–water interface: 2D analogue to 3D gelation of colloidal systems with grafted polymer chains. *Langmuir* **19**, 326–332 (2003)
31. Cavalcanti, L.P., Tho, I., Konovalov, O., Fossheim, S., Brandl, M.: Compressibility study of quaternary phospholipid blend monolayers. *Colloids Surf. B* **85**, 153–160 (2011)
32. Yoshizawa, Y., Kono, Y., Ogawara, K.I., Kimura, T., Higaki, K.: PEG liposomalization of paclitaxel improved its *in vivo* disposition and anti-tumor efficacy. *Int. J. Pharm.* **412**, 132–141 (2011)
33. Gaillard, P.J., Appeldoorn, C.C.M., Dorland, R., van Kregten, J., Manca, F., Vugts, D.J., Windhorst, B., van Dongen, G.A., de Vries, H.E., Maussang, D., van Tellingen, O.: Pharmacokinetics, brain delivery, and efficacy in brain tumor-bearing mice of glutathione pegylated liposomal doxorubicin. *PLoS ONE* **9**, 1–10 (2014)
34. Lundberg, B.B., Griffiths, G., Hansen, H.J.: Cellular association and cytotoxicity of anti-74-targeted lipid drug carriers in B lymphoma cells. *J. Control. Release* **94**, 155–161 (2004)
35. Lu, R.M., Chen, M.S., Chang, D.K., Chiu, C.Y., Lin, W.C., Yan, S.L., Wang, Y.P., Kuo, Y.S., Yeh, C.Y., Lo, A., Wu, H.C.: Targeted drug delivery systems mediated by a novel peptide in breast cancer therapy and imaging. *PLoS ONE* **8**, 1–10 (2013)
36. Girard-Egrot, A.P., Godoy, S., Blum, L.J.: Enzyme association with lipidic Langmuir–Blodgett films: interests and applications in nanobioscience. *Adv. Colloid Interf. Sci.* **116**, 205–225 (2005)
37. Wydro, P., Krajewska, B., Hąc-Wydro, K.: Chitosan as a lipid binder: a Langmuir monolayer study of chitosan–lipid interactions. *Biomacromolecules* **8**, 2611–2617 (2007)
38. Hąc-Wydro, K., Jędrzejek, K., Dynarowicz-Łątka, P.: Effect of saturation degree on the interactions between fatty acids and phosphatidylcholines in binary and ternary Langmuir monolayers. *Colloids Surf. B* **72**, 101–111 (2009)
39. Hąc-Wydro, K., Wydro, P.: The influence of fatty acids on model cholesterol/phospholipid membranes. *Chem. Phys. Lipids* **150**, 66–81 (2007)
40. Gew, L.T., Misran, M.: Albumin–fatty acid interactions at monolayer interface. *Nanoscale Res. Lett.* **9**, 218 (2014). doi:10.1186/1556-276X-9-218
41. Crawford, N.F., Leblanc, R.M.: Serum albumin in 2D: a Langmuir monolayer approach. *Adv. Colloid Interf. Sci.* **207**, 131–138 (2014)
42. Needham, D., Kim, D.H.: PEG-covered lipid surfaces: bilayers and monolayers. *Colloids Surf. B* **18**, 183–195 (2000)
43. Sriwongsitanont, S., Ueno, M.: Physicochemical properties of PEG-grafted liposomes. *Chem. Pharm. Bull.* **50**(9), 1238–1244 (2002)
44. Rovira-Bru, M., Thompson, D.H., Szleifer, I.: Size and structure of spontaneously forming liposomes in lipid/PEG-lipid mixtures. *Biophys. J.* **83**(5), 2419–2439 (2002)
45. Garbuzenko, O., Barenholz, Y., Priev, A.: Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer. *Chem. Phys. Lipids* **150**, 66–81 (2005)
46. Gew, L.T., Misran, M.: Energetic mixing of anti-SNAP25 on lipid monolayers: degree of saturation of C18 fatty acids. *Surf. Interface Anal.* **49**(5), 388–397 (2016). doi:10.1002/sia.6144

47. Davies, J.T., Rideal, E.K.: *Interfacial Phenomena*. Academic Press, New York (1963)
48. Gaines, G.L.: *Insoluble Monolayers at Liquid-Gas Interfaces*. Interscience, New York (1966)
49. Gupta, R.K., Manjuladevi, V.: Molecular interactions at interfaces. In: Aurelia, M. (ed.) *Molecular Interactions*, pp 81–104. InTech Open Access Publisher, Croatia (2012)
50. Buys, A.V., Rooy, M.J.V., Soma, P., Papendorp, D.V., Lipinski, B., Pretorius, E.: Change in red blood cell membrane structure in type 2 diabetes: a scanning electron and atomic force microscopy study. *Cardiovasc. Diabetol.* **12**, 25 (2013)
51. Abednejab, A.S., Amoabediny, G., Ghace, A.: Surface modification of polypropylene membrane by polyethylene glycol graft polymerization. *Mater. Sci. Eng. C* **42**, 443–450 (2014)
52. Johnson, D., Hilal, N.: Characterisation and quantification of membrane surface properties using atomic force microscopy: a comprehensive review. *Desalination* **356**, 149–164 (2015)
53. De Oliveira, R.R.L., Albuquerque, D.A.C., Cruz, T.G.S., Yamaji, F.M., Leite, F.L.: Measurement of the nanoscale roughness by atomic force microscopy: basic principles and applications. In: Victor, B. (ed.) *Atomic Force Microscopy - Imaging, Measuring and Manipulating Surfaces at the Atomic Scale*, pp. 147–175. INTECH Open Access Publisher, Croatia (2012)