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Phase behavior of DSPC/$PEG_{40}St$ mixtures at higher emulsifier contents

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ABSTRACT

Phase behaviors of 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and polyoxyethylene(40)stearate ($PEG_{40}St$) were investigated with Langmuir monolayer isotherms and Brewster angle microscopy (BAM) imaging at DSPC/$PEG_{40}St$ molar ratios ranging from 9:1 to 5:5. Two plateaus were found in the Langmuir isotherms which were relatively shorter for the 9:1 mixture and extended significantly by increasing the $PEG_{40}St$ content, indicating that the $PEG_{40}St$ squeezed out whereas more emulsifier retained in the monolayer at higher $PEG_{40}St$ contents. A strong hysteresis was observed when the mixed monolayers were subjected to compression-expansion cycles. The degree of hysteresis for the first cycles also increased with increasing $PEG_{40}St$ content in the monolayer. Gray scale intensities in the Brewster angle microscopy images were determined for pure DSPC and pure $PEG_{40}St$ and a scale was established to better interpret the morphologies for the mixtures. Bud and vessels formed during the $PEG_{40}St$ squeezed out upon compression. Upon expansion, $PEG_{40}St$ and DSPC is reappeared on the monolayer. When considered BAM images together with the Langmuir isotherm, $PEG_{40}St$ molecules were found to be well distributed within the DSPC molecules at lower DSPC/$PEG_{40}St$ mole ratios and mostly phase separated at higher mole ratios. It was concluded that higher $PEG_{40}St$ content would be advantageous for the design of an efficient and cheaper ultrasound contrast agents.

1. Introduction

Ultrasound is a diagnostic imaging modality widely used in medicine because it is non-invasive, low risk, low cost, and portable technique providing real-time imaging [1]. However, it suffers from poor image quality because blood, healthy liver, spleen and kidney have similar acoustic properties [2]. The image quality can be improved using microbubbles as ultrasound contrast agents [3]. However, low stability of the microbubbles limits their use for extended period of time in clinical settings. There are mainly two components playing major role in microbubble stability, namely, gas core and the shell [4]. Use of gases with low blood dissolution relatively improved the microbubble stability; however, the shell needs to be redesigned for more stable and effective microbubbles [4].

The shell of a microbubble can be biocompatible protein, polymer, or lipid [1]. Previous studies showed that microbubbles coated with protein and polymer shells demonstrated gas loss due to formation of cracks on the shell under ultrasonic pulses. Additionally, protein coated microbubbles tend to adhere into vasculature [1,5]. On the other hand, lipid coated microbubbles were found highly echogenic due to their soft shells resulting from weak intra-lipid interactions [2,6–9]. It is well-understood that phospholipids form liposomes [10,11]. Addition of emulsifiers can transform the bilayers into a monolayer as in microbubbles [12–15]. Tween-40, lipopolymers such as DSPE-PEG_{5000}, DSPE-PEG_{2000}, and Polyoxyethylene-40-stearate ($PEG_{40}St$) were used as emulsifier in microbubble formulations [16–22]. The large size of the hydrophilic polar headgroups of the polyethyleneoxide (PEG) chain leads the emulsifier to acquire a more cone-shape conformation causing the bilayers to convert into monolayers [23]. Hence, phospholipids together with the emulsifiers can form a monolayer around the gas bubbles.

Not all lipids are miscible with emulsifiers [11]. For instance, it was shown that emulsifier is only miscible with expanded phase lipids with carbon number of the acyl chain less than 16 but not with condensed phase lipids [11,19]. Immiscibility of the emulsifier with the condensed phase lipid was attributed to the bulkiness of the PEG-chains of the emulsifier [4]. It appears that stable microbubbles can be produced if gas bubbles are stabilized by the condensed phase lipids with sufficient amount of the emulsifier to form a condensed/cohesive layer around the gas bubble.

A lipid-to-emulsifier mole ratio of 9:1 is generally used for the microbubble composition [4,18,20,24–28]. The phase formation, miscibility, and microbubble stability of this composition have still been under investigation. Emulsifier content less than 10% (9:1 mixture) is

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however small to reveal the effect of emulsifier on the phase behavior of the lipid-emulsifier mixtures because the majority of the shell component, more than 90%, is composed of lipids alone. Therefore, higher emulsifier contents are needed to gain more significant information about the effect of the emulsifier on the monolayer cohesiveness. To the best of our knowledge, the phase behavior of DSPC/$PEG_{40}St$ mixture with more than 15% of the emulsifier [11] have not been reported in the current literature.

In the present work, DSPC/$PEG_{40}St$ mixtures in a range from 9:1 to 5:5 mol ratios were prepared and their miscibility were investigated using surface pressure-area isotherms coupled with the Brewster angle microscopy (BAM) imaging. Two plateaus were found in the Langmuir isotherms indicating the squeeze out of the $PEG_{40}St$ molecules from the monolayer. Plateaus were relatively shorter for the 9:1 mixture and extended significantly by increasing the $PEG_{40}St$ content. In our recent paper, we developed a quantification method to estimate the squeeze out amount of $PEG_{40}St$ from Langmuir isotherms [29]. Almost 93%, 82%, and 53% of PEG40 St displaced for the 9:1, 7:3, and 5:5 mixtures, respectively, at the end of the first collapse plateau [29]. Remaining $PEG_{40}St$ squeezed out at the end of the second collapse plateau, where 20% of $PEG_{40}St$ still contained within the 5:5 composition [29]. Here, a scale was established on the Brewster angle microscopy (BAM) images of the monolayers to better interpret the morphologies of the mixtures. It was shown that $PEG_{40}St$ molecules were well distributed within the DSPC molecules at lower DSPC/$PEG_{40}St$ mole ratios and mostly phase separated at higher mole ratios. Compression-expansion cycles up to surface pressures below and above the collapse plateaus showed a solid monolayer at the higher surface pressures and produced cracks during expansions. These cracks were bare sub phase. It was concluded that increasing $PEG_{40}St$ content would be advantageous to design more stable lipid based microbubbles and reduce the cost for the ultrasound contrast agents.

2. Materials and methods

2.1. Materials

1,2 Distearoyl-sn-glycero-3-phoshhocholine (DSPC, 99%) and Polyoxyethylene-40- stearate ($PEG_{40}St$) were purchased from Sigma Aldrich (St. Louis, MO). Chloroform ($CHCl_3$, 99.4%) was purchased from Merck and used as a solvent to prepare spreading solutions. Ultrapure water used as sub phase was produced by Millipore purification system with specific resistivity of 18 MΩ cm. The required amounts of DSPC (MW, 790.16 g/mole) and $PEG_{40}St$ (MW, 2044 g/mole) were calculated for the 9:1; 8:2; 7:3; 6:4 and 5:5 M ratios of the mixtures and dissolved in chloroform so that the resulting final solid concentration was 0.7 mg/ml. After chloroform addition, the vials were immediately sealed with screw cap to avoid chloroform evaporation and kept in the freezer at -22 °C. The sealed vials were homogenized using a bath sonicator before spreading the solution at the air-water interface and warmed up to the room temperature under continuous stirring with the cap closed. About 30 to 50 µl of solution was spread over the water subphase to reach the desired initial mean molecular area.

2.2. Langmuir monolayer isotherms

The experimental procedure for the Langmuir isotherms of the monolayers was reported in our previous paper [29]. Briefly, Langmuir-Blodgett system (KSV minitrough, Finland) with two movable teflon barriers was employed to study the phase behavior of the binary systems and their pure components. The system was enclosed in a plexyglass box to minimize possible contamination of air-monolayer-water interface and the disturbance of the monolayer by the air currents. The Langmuir trough was filled with ultrapure water with specific resistivity of 18 MΩ cm produced by a Millipore purification system. Cleanness of the air-water interface was confirmed by closing and opening the barriers on the pure water and ensuring that surface pressure readings do not differ by more than ± 0.1 mN/m. The lipid solutions were spread on the water subphase via Hamilton micro syringe. The chloroform was allowed to dry for 20 min to obtain monolayers at the air-water interface. The surface pressure-area (π-A) isotherms were obtained via symmetric compression of the monolayers by the two barriers. A compression speed of 5 mm/min was used in all experiments. Each isotherm was performed 4–5 times to ensure reproducibility of the isotherms.

The cycle experiments of the isotherms were carried out by periodic compression followed by an expansion. For these experiments, a monolayer was compressed until the surface pressure reached the targeted pressure of either 30 mN/m or 50 mN/m, then the monolayer was allowed to equilibrate for 20 min and expanded to the initial pressure while the surface pressure was maintained constant. After allowing 20 min to equilibrate, the subsequent compression and expansion cycles were performed.

2.3. Brewster angle microscopy (BAM) images of the monolayers

Morphology of the monolayers were investigated by Brewster Angle Microscope (KSV Optrel BAM300) mounted on the Langmuir trough (KSV minitrough, Finland). Standard HeNe laser was used as the light source emitting linearly p-polarized light with an intensity of 10 mW at a wavelength of 632.8 nm. High quality Glan-Thompson polarizers were used with the polarization ratio of 10^{-6}. The images of the monolayers were captured by the CCD digital monochrome video camera (EHD®kamPro02) attached to the instrument with a resolution of 768 × 494 pixels. The images were acquired using an objective (Mitutoyo, Japan) with 10× magnification (WD: 33.5 mm, NA: 0.28, FOV: 400 × 300 µm).

For the BAM images, the microscope was firstly adjusted to the incident beam fixed at Brewster angle of water (53°) for no reflection from the surface, resulting in completely dark background. After spreading the monolayer, reflected lights from the monolayer surface passing through a second polarizer were recorded with the CCD camera for the same settings. Experiments were carried out at 22 ± 1 °C using circulating water bath. The BAM images of the samples were stored digitally at every 30 s. Representative images were selected for evaluations. The gray scale intensity of the images were determined using the ZEN® software (Carl Zeiss, Germany).

3. Results and discussion

The miscibility behaviors of DSPC and $PEG_{40}St$ mixtures were investigated at molar ratios of DSPC/$PEG_{40}St$ ranging from 9:1 to 5:5. Fig. 1a shows the surface pressure (π)-mean molecular area isotherms for pure DSPC, pure $PEG_{40}St$, and DSPC/$PEG_{40}St$ binary mixtures at different molar ratios, as also reported in our previous paper [29]. The discontinuities as the main turning points for the mixed monolayer isotherms and the locations of the isotherms in the low and high compressibility regions were shown separately in Fig. 1b for the 8:2 mixture as an example. Fig. 1c shows the compression modulus (C_s^{-1}) for the pure components and 8:2 mixture in particular as an example, which was used to detect phase transitions [30,31]. The compression modulus is defined by Eq. (1) [30,32,33]

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

where A is mean molecular area at that particular surface pressure. As seen in Fig. 1a and b, pure DSPC monolayer demonstrated liquid-condensed (LC) phase at the air-water interface at room temperature due to strong cohesive interactions between C_{18} aliphatic chains [32,34,35]. From the compression modulus, as shown in Fig. 1c, the collapse pressure for DSPC was found to be about 59 mN/m, at which C_s^{-1}