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Short Communication

Interfacial pressure and phospholipid density at emulsion droplet interface using fluorescence microscopy

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ABSTRACT

Phospholipids are widely used to stabilize oil in water micron size emulsion droplets; the interfacial phospholipid density and tension of such droplets are difficult to estimate. In the present paper, we describe a simple approach by which the measurement of a micron size oil droplet interface fluorescence intensity provides directly both the interfacial phospholipid density and the interfacial tension. This method relies on two prior calibration steps: (i) the quantitative variation of the interfacial tension with fluorescence intensity at droplets interface through micro-manipulation techniques; (ii) the variation of interfacial tension with phospholipid density through monolayer isotherm. Here, we show the validity of this approach with the example of micron size oil droplets stabilized with a phosphatidylcholine phospholipid, in aqueous buffer.

1. Introduction

Characterization of liquid/liquid interfaces is required in emulsion science [1] and in many related industrial processes (e.g. food engineering, cosmetics and pharmaceutics). Moreover, the properties of liquid/liquid interfaces become also predominant when small droplets are manipulated in miniaturized devices [2]. Interfacial tension (IT) of the oil/water interface is a key parameter which can be tuned or modified thanks to surfactant monolayers located at the interface. IT variation is quantitatively related to the surfactant density through a "monolayer isotherm". Several experimental approaches have been used for decades to establish such monolayer isotherms at oil/water interface. The Langmuir trough is usually employed to study oil/water interface and obtain IT and interfacial surfactant densities at the macroscopic scale [3]. This device can also be coupled with fluorescence microscopy [4], in order to investigate phase transitions. Pendant drop techniques based on shape analysis have also been used to perform isotherm measurements on macroscopic (millimeter scale) drops [5,6] at the air/water interface and at the oil/water interface [7]. Small scale systems like emulsion micro-droplets require tools adapted to their size to investigate interfacial properties. Currently, micro-manipulation techniques allow carrying out IT measurements [8] on individual micron size droplets but give no information on the surfactant density. In addition, this measurement relies on deformation of the droplet that is aspirated into a pipette. It can result in a modification or complete destruction of the interface (e.g. in case proteins are present at the interface). To overcome these limitations, we describe here a simple and non-destructive approach by which a simple fluorescence intensity measurement of a micron size oil droplet interface provides simultaneously the interfacial phospholipid density and tension. This method relies on two prior calibration steps: (i) the variation, of IT with fluorescence intensity at droplets interface, F, IT(F), through micro-manipulation techniques; (ii) the variation of IT with phospholipid density, d, IT(d), through monolayer isotherm. The latter is obtained using a setup in which the monolayer at the oil/water interface is formed in a step by step process, increasing the amount of surfactants by successive depositions. Here, we demonstrate on micron size oil droplets in aqueous buffer stabilized by a phospholipid monolayer, that after these two calibration steps, both IT and d can be directly obtained by the simple measurement of F. We chose soybean oil to form droplets and phospholipids as stabilizing surfactants because they are both biological compounds. Soybean oil droplets stabilized by phospholipids are used in food industry and can be used to mimic lipid droplets found in cells of living organisms. In the following, we use "interfacial density" and

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"molecular area" as equivalent information since they are inverse of each other.

2. Methods

2.1. Materials

Phospholipids (1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (DOPE-RHO)) were purchased from Avanti Polar Lipids in chloroform and used as received. DOPE-RHO was selected as a low bleaching dye carrying the same aliphatic tails as DOPC. The ionic strength and pH of aqueous phase of the emulsion was imposed: the pH of the saline buffer (100 mM potassium chloride (Fluka)) was adjusted at 7.4 using 25 mM Hepes (Sigma Aldrich) and 40% potassium hydroxide solution (Fluka). All solutions were prepared with ultra pure water (Elga). Soybean oil (SO) (CAS 8001-22-7) was purchased from Sigma. It was purified using an activated magnesium silicate (Florisil 100–200 mesh from Sigma) column and stored under inert argon atmosphere at 4 °C. Purification of oil phase (SO and also hydrocarbons) is required before use in studies of oil–water interface (see e.g. [9,10]). SO is mainly composed of mixed triacylglycerides of different chain length (~90% of C_{18} unsaturated and 10% of C_{16} saturated acyl chains) [11]. Contaminant polar components that may compete with phospholipids at the droplet interface were removed from SO [12]. These contaminants may come from the degradation of triglyceride molecules that produce more polar molecules like monoglycerides or fatty acids in addition of contaminants present in the oil. The dynamic IT (IT versus time) was determined before and after purification (see Fig. S1 in ESI). It showed that the IT of unpurified oil decreased on a time scale of 10 s whereas it remained stable (28 ± 0.2 mN/m) in the case of purified oil. Temperature was kept constant during all experiments (21 ± 1 °C).

2.2. Droplets formation

Droplets were formed by shaking oil and buffer for 1 h in 3 mL glass vials. We used VORTEX-GENIE® 2 (Scientific Industries, Inc.) mixer with speed set at 6. Phospholipids (DOPC and PE-RHO) dissolved in chloroform were mixed and added to oil/chloroform (32%, v/v). Volume of chloroform coming with phospholipids can be neglected. The final concentration of phospholipids in emulsion droplets ranged from 75 μM to 375 μM. Buffer was then added to obtain 5% volume fraction of oil–chloroform in water. The droplets size was between 5 μm and 35 μm. With this protocol, the amount of phospholipid embedded in droplets and available for further adsorption at the interface is controlled. Also the presence of chloroform increases droplet density, favoring their sedimention instead of their creaming. This prevents their contamination at buffer/air interface.

2.3. Interfacial tension of droplets

Droplets' interfacial tension was measured using micromanipulation technique. The device was made of a micromanipulator and a pipette holder (Narishige, Japan) under a video microscope. As illustrated in Fig. 1, aspirating a single droplet in a micropipette provided a direct measurement of IT. Each droplet was gently captured with a micropipette through weak aspiration and lifted up in the buffer. Then, the aspiration was slowly increased using a syringe connected to the pipette, causing the oil droplet to enter inside the pipette. Once the oil meniscus into the pipette reached a hemispherical shape, further aspiration increase resulted in a sudden and complete suction of the droplet inside the pipette. The knowledge of this complete aspiration pressure threshold (P_{asp}), the micropipette diameter and the droplet diameter directly provides [8]: $IT = P_{asp}/[4 \times (1/D_p - 1/D_d)]$, where D_p and D_d denote the diameters of the pipette and droplet, respectively.

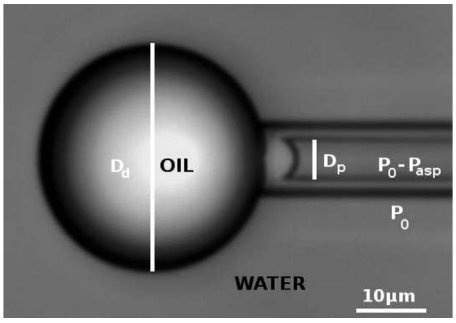

Fig. 1. Micrograph of a micromanipulated oil droplet during IT measurement: the aspiration threshold of the droplet is detected. P_0 is the pressure in the surrounding buffer and IT is expressed as $IT = P_{asp}/[4 \times (1/D_p - 1/D_d)]$.

The sizes of pipette and droplet were obtained by image analysis (ImageJ software provided by NIH). The pressure was measured with a pressure transducer (DP103, Validyne Eng. Corp., USA), the output voltage of which was monitored with a digital voltmeter.

2.4. Fluorescence intensity of droplet interface

The interfaces of droplets were labeled with 2% (w/w) fluorescent phospholipid fraction. Fluorescence images were recorded with a CCD camera (MicroMAX:782YHS), with exposure time of 400 ms. Droplets were shone individually away from other droplets to prevent their photobleaching. Interface fluorescence intensity was measured using ImageJ software. As shown in Fig. 2, a region of interest (ROI) was selected on each droplet. The ROI consisted of a partial circular ring, along the interface, with 260° angular extension and 3 pixels width.

The ROI was centered on the apex of the droplet in order to rule out any artefact due to proximity of the glass pipette. The intensity was averaged in the ROI. We checked that the mean intensity was unsensitive to either small angular or width changes of the ROI.

2.5. Interfacial tension of flat interface

A flat oil/water interface was formed in a glass petri dish (18.8 cm in diameter). The oil upper phase and water lower phase

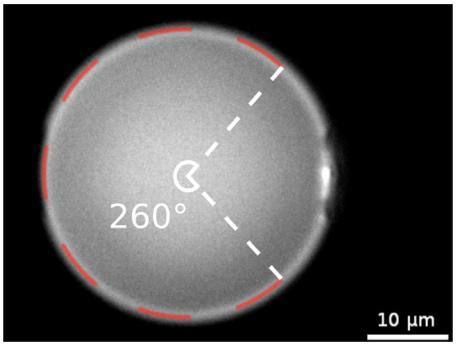

Fig. 2. Micrograph of a micromanipulated droplet, as recorded for interface's fluorescence intensity measurement. The circular red dashed line features the ROI location used to determine *F*, the mean value of interfacial fluorescence intensity. <!–<query>Fig. 2 will appear in black and white in print and in color on the web. Based on this, the respective figure caption has been updated. Please check, and correct if necessary.</query>–>(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

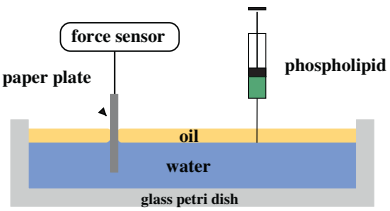

Fig. 3. Interfacial tension is measured in a Wilhelmy configuration during step by step deposition of phospholipids DOPC at the water–oil interface.

had 3 mm and 1 cm in height, respectively. IT was measured in a Wilhelmy configuration made of a paper plate coupled with a NIMA sensor (Fig. 3). The data were monitored with NIMA-plate software. The interface was thoroughly cleaned by aspiration. We checked surface purity by measuring surface tension of water consistent with tabulated value at 21 °C namely 72.8 mN/m. Subsequently, the paper plate was immersed in water and the oil was gently added on the top of water.

Phospholipids were deposited step by step at the oil/water interface with a microsyringe to increase the monolayer density. The deposition procedure was validated prior to perform these experiments (see Fig. S0 in ESI), using a Langmuir trough described elsewhere [13] and the Wilhelmy configuration described above. After each deposition, the monolayer was allowed to relax until surface pressure was stable. Fresh new solution of phospholipids was used to ensure no variation of the native concentration (10 mg/mL DOPC in chloroform). The successive depositions (0.5, 1.0 or 2.0 μL) of this stock solution were carried out, using microsyringes (Hamilton). The high diameter of the petri dish was selected in order to allow deposition of achievable volumes of the stock solution. Also, the meniscus effect on interface area can be neglected.

3. Results and discussion

Droplets observed by fluorescence microscopy had a radial intensity profile exhibiting a maximum at the interface (Fig. 2). Confocal microscopy observation of droplets confirmed that fluorescence was completely located at the interface of droplets (see Fig. S2 in ESI), with no staining of the bulk. As expected, the fluorescence intensity of the droplets interface was found to increase linearly with the fluorescent phopholipid fraction (see Fig. S3 in ESI). The investigated range of fluorescent lipid fraction spanned from 0.1% to 2.5%.

3.1. First calibration step, IT(F)

The variation of interfacial pressure with fluorescence intensity of droplets interface was first established. A set of measurements was carried out with various concentrations of phospholipids at the interface of droplets. Both fluorescence intensity and interfacial tension were measured successively as described in the previous section.

Results are presented in Fig. 4 showing the interfacial pressure (i.e. IT decrease due to surfactant monolayer) versus the inverse of the interface fluorescence intensity normalized by the droplet diameter (D_d), which is proportional to the molecular area of phospholipids. The whole shape of the curve is similar to isotherms of monolayers obtained at liquid interfaces [3] with usual Langmuir troughs. Note that for sake of simplicity, we used a phospholipid exhibiting no phase transition or coexistence at the investigated temperature [14].

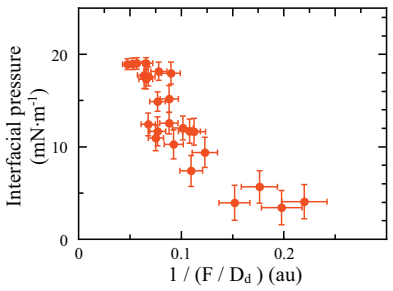

Fig. 4. Interfacial pressure as a function of the inverse of the interface fluorescence intensity normalized by the droplet diameter. This set of measurements involves 25 droplets with 26 ± 4 μm in diameter. IT's error bars are calculated from experimental uncertainties involved in the measurement. Error bars associated to abscissa represent 10% of reported value.

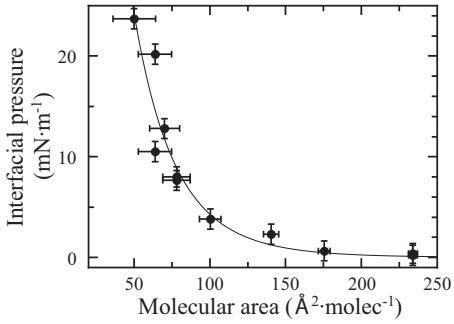

Fig. 5. Reference isotherm: interfacial pressure of a DOPC monolayer at the SO/water interface as a function of molecular area (A_m). IT's error bars are ±1 mN/m. Molecular area's error bars are associated with a percentage of deposited volumes. The solid line is the fitting phenomenological curve $a \times \exp(b/(x+c))$, with $a = 1.47 \times 10^{-10}$ mN/m, $b = 1.80 \times 10^4$ Å^2/molec and $c = 6.45 \times 10^4$ Å^2/molec.

3.2. Second calibration step, IT(d)

We also performed the isotherm measurement of the phospholipid monolayer at the oil–water interface as described above. For convenience, water was used instead of buffer A, since salt was shown to have no effect on SO/water interfacial tension [15]. We checked that SO/water and SO/buffer ITs were equal for a single deposition of phospholipids at the flat interface. We also checked that these ITs of SO droplets remained similar in water and in buffer, within experimental error. Results are shown in Fig. 5. The experiment was performed twice. These two isotherms were carried out through 5 and 6 successive depositions, respectively. Successive depositions of phospholipid at the interface resulted in the increase of error bars at low molecular area.

The minimum molecular area, corresponding to interface saturation with phospholipids is about 60 ± 15 Å^2/molec. This value fits well with the value of molecular area at collapse at air–water interface (50 Å^2/molec) reported by Nag and Keough [14]. Mixing of oil with aliphatic chains of phospholipids could account for higher values of molecular area at collapse [7]. Similarly, the onset of interfacial pressure in the monolayer at the oil–water interface occurred around 250 Å^2/molec, which is approximately twice the one reported by Nag and Keough [14] for DOPC monolayer at air–water interface. This difference in molecular area at pressure onset, between oil/water and air/water interfaces, has already been reported [3,4]. Note that the onset of the increase in interfacial pressure at high DOPC molecular areas is very similar to the behavior of DPPE[1] [3] and DPPC[2] [4] at the n-hydrocarbon/water interfaces.

[1] 1,2-Dihexadecanoyl-sn-glycero-3-phosphoethanolamine.
[2] 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine.

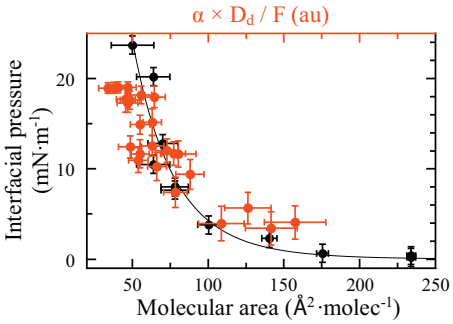

Fig. 6. Superimposition of rescaled fluorescence isotherm with the step by step deposition isotherm. The optimal overlap is presented with $\alpha = 716$. Error bars are described in above figures' caption.

Finally, the inverse fluorescence intensity scale (*X*-axis in Fig. 4) was matched with the inverse density scale (abscissa in Fig. 5) of the reference isotherm. For this purpose, a scaling factor $\alpha = 716$ au was applied to inverse fluorescence scale. The optimal value of α was determined through minimization of the mean difference in molecular area with respect to the fitting reference isotherm (see Fig. S4 in ESI). Note that α represents the fluorescence intensity per unit of surfactant density and appears as a feature of the experimental set-up. This rescaled fluorescence isotherm is presented in Fig. 6 (upper axis) merged with the reference isotherm shown in Fig. 5.

As can be seen in Fig. 6, isotherms obtained with the two different methods superimpose with each other within experimental errors. The validation of our method was carried out by comparing interfacial pressures obtained by fluorescence microscopy and measured by micropipette tensiometry. This validation is presented in Fig. S5 of ESI: fluorescence microscopy allows the determination of interfacial pressure within an accuracy of 20%. So, these results show that interfacial fluorescence intensity of droplets can be quantitatively related to interfacial density of phospholipids adsorbed at the interface of individual droplets. The accuracy of this method is mostly limited by the sensitivity and the dynamic range of the CCD. In the present case, using a standard cooled CCD camera, the determination of molecular area is already achieved with an accuracy of ± 15 Å^2/molec. This accuracy can be enhanced using higher quality CCD camera. It would directly lead to a better accuracy on interfacial pressure as well, and would reduce the fluorescence sensitivity limited range (high molecular area or low interfacial pressure) of the isotherm.

4. Conclusions

We have presented a simple method that provides the density of surfactants and interfacial pressure at oil droplets interface from fluorescence microscopy measurements. The pre-required calibration steps which relate, first, IT to fluorescence intensity and second, IT to interfacial density, are carried out in a simple way. The latter is reached without requirement of a Langmuir trough that can be replaced by common bench equipment. In a similar manner, fluorescence intensity is quantified without confocal microscope but instead with classical epifluorescence microscopy. To our knowledge, this approach is the first one allowing such measurements on micrometer scale droplets. Moreover, this method is not invasive since it does not imply any contact or deformation of droplets. Thus it could be directly applied in miniaturized integrated microfluidic devices for characterization and engineering purposes. In addition, we note that this method could also be applied at bubbles interfaces.

Acknowledgements

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2013.11.033.

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