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Phospholipids as emulsifiers for micro/nano droplets suitable for biotechnological systems integration

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ABSTRACT. – We studied the emulsifying properties of palmitoyl oleoyl phosphatidylcholine (POPC) using the biocompatible compounds water and squalene as immiscible fluid phases. We tested the solubility limit of POPC in squalene and its equilibrium distribution between bulk phases. POPC is dissolvable in squalene up to 0.3% (w/v) with an ultrasonication procedure. Above this limit, aggregates of >1 µm are formed which are resistant to prolonged ultrasonication in size and quantity. Emulsifying properties of POPC were elaborated by measuring the droplet size ranges of emulsions. Nanofluidics was studied by pressure driven transport of nanometer sized emulsion droplets through defined nanochannels whereby droplet sizes 500 nm can be produced. The mechanical properties of the emulsifying phospholipid monolayer at the water/squalene interface were studied by profile analysis tensiometry (PAT). The dynamic interfacial tension was measured and the adsorption isotherms were established from long-time approximations of the diffusion-controlled adsorption. With PAT a critical aggregation concentration was determined in the same range as the solubility limit, which was measured by dynamic light scattering. The minimum interfacial tension for POPC as emulsifier was found to be below 1 mN/m. Thus, it can be concluded that phospholipids are suitable emulsifiers for microfluidics and produce adsorbed layers of remarkably small interfacial tension.

Key-words: Digital micro and nanofluidics, Adsorption kinetics, Drop profile analysis tensiometry, Oil/water interface, Nanoemulsions

Phospholipides comme émulsifiants pour les micro/nano-gouttelettes appropriés pour l'intégration des systèmes biotechnologiques

RÉSUMÉ. – Nous avons étudié les propriétés émulsifiantes de palmitoyl oléoyl phosphatidylcholine (POPC) en utilisant de l'eau et du squalène comme phases fluides non miscibles et biocompatibles. Nous avons testé la limite de solubilité du POPC en squalène et sa répartition d'équilibre entre les phases volumiques. POPC est soluble dans le squalène jusqu'à 0.3% (p/v) avec une procédure d'ultrasons. Au-dessus de cette limite, des agrégats de > 1 µm sont formés qui sont résistants aux ultrasons prolongée en diamètre et en quantité. Les propriétés émulsifiantes de POPC ont été élaborées par la mesure des diamètres des gouttelettes des émulsions. La nanofluidique a été étudiée par les transports entraîné par pression de gouttelettes d'émulsion de taille nanométrique au travers des nanocanaux définies par lesquels des gouttelettes de taille 500 nm peuvent être produites. Les propriétés mécaniques de la monocouche de phospholipides émulsifiant à l'interface eau/squalène ont été étudiées par la tensiométrie d'analyse du profil d'une goutte (PAT). La tension interfaciale dynamique a été mesurée et les isothermes d'adsorption ont été établies à cause des approximations de longue date de l'adsorption contrôlée par la diffusion. Avec PAT, une concentration critique d'agrégation a été déterminée dans la même gamme que la limite de solubilité, qui a été mesurée par diffusion dynamique de la lumière. La tension interfaciale minimale pour POPC comme émulsifiant a été jugée inférieure à 1 mN/m. Ainsi, on peut conclure que les phospholipides sont des émulsifiants appropriés pour la microfluidique et de produire des couches adsorbées de la tension interfaciale remarquablement faible.

Mots-clés : Micro- et nanofluidique digitale, la cinétique d'adsorption, tensiométrie d'analyse du profil d'une goutte, interface huile/eau, nanoémulsion

I. INTRODUCTION

Digital microfluidics and nanofluidics dealing with biological compounds like native proteins or cells have an urgent demand for compartmentalization techniques that are biocompatible or even biodegradable to be adapted to integration with biological systems. Standard water in oil emulsions in microfluidics using detergents, alcohols and mineral oil derived organic phases do not meet these requirements and additionally risk losses of biological functions by e.g. protein denaturation or destruction of biological membranes. Therefore, biocompatible and biointegratable compounds and dispersing techniques are needed for the next step of biotechnological systems integration.

Phospholipids, part of biological membranes, are effective natural surface-active agents (surfactants) and may improve

biocompatibility in emulsions. Most recent developments in digital microfluidics increasingly employ phospholipids as emulsifiers [Matosevic and Paegel, 2011; Punnamaraja and Steckl, 2011; Thiam *et al.*, 2012], but have not yet reached the level of full biocompatibility regarding the oil phase. Therefore, this study focuses on full biotechnological systems integration regarding the choice of all excipients. Emulsion particle size and emulsion stability are greatly dependent on the choice of the oil component in the emulsion as well as on the emulsifier. It is reported that the oil/water interfacial tension and particle size of the emulsion are inversely proportional as studied for squalene and other natural oils [Chung *et al*., 2001].

Knowledge of the interfacial behavior of phospholipid monolayers at oil/water interfaces is necessary to understand their role as emulsifiers in emulsions and droplet stability. Therefore it is useful to know the maximum film pressure achievable by adsorbing monolayers as well as the critical aggregation concentration of the selected phospholipid in an oil system. The adsorption of these emulsifiers to a fluid interface is commonly studied by determining the dynamic interfacial tension between the two phases by using e.g. profile analysis tensiometry (PAT) [Handa *et al*., 1989; He *et al*., 2008; Li *et al*., 1996a-c; Miller, 1981; Miller *et al*., 1994; Mitsche *et al*., 2010]. This tensiometer determines the volume, surface area and interfacial tension of a pendent or buoyant drop from the shape of its axisymmetric profile.

The aim of this work is to study the mechanical properties of the emulsifying phospholipid monolayer at the water/squalene interface for improved conditions of formation and stability of emulsions to be used in biocompatible digital microfluidics.

II. MATERIAL AND METHODS

II.1. Materials

Squalene with a purity ≥ 98% was purchased from Sigma-Aldrich (Taufkirchen, Germany). The phospholipid 1-palmitoyl-2-oleoyl-sn-glycero-3 phosphocholine (POPC) was obtained from Lipoid (Ludwigshafen, Germany). N-(Lissamine Rhodamine B sulfonyl)1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (LRh-PE), a head group labelled phospholipid, was purchased from Invitrogen (Darmstadt, Germany). The lipids were received as a powder and dissolved in ethanol, with an estimated purity of 99%, from AppliChem (Darmstadt, Germany). Bidistilled water with the quality for injectable drugs (WFI) was used for the preparation of buffers and cleaning procedures.

II.2. Preparation of emulsions

For the production of water/oil (w/o) emulsions, phospholipids had to be dissolved in squalene (oil phase). POPC was added to squalene by dissolving 0.3-1 g either directly into 1 ml oil using ultrasound [Fox *et al*., 2011] or via the film method. For the latter method an adequate quantity of stock solution of POPC in ethanol was added to a round-bottom flask. The organic solvent was removed by rotary evaporation until a dry and homogeneous film was generated on the wall of the round-bottom flask. Then, squalene and a few glass beads were added to the flask before placing it in a water bath for 30 min at 60°C and stirred to suspend the lipid film. The oil mixture was then sonicated in a Sonorex RK 514 Transistor ultrasonicating water bath (Bandelin electronic, Berlin, Germany) until the phospholipid was completely dissolved, monitoring by particle size and particle counting measurements. To monitor the quantity of dissolved phospholipid and its distribution in subsequent analytic steps, we used 0,1 mol% fluorescently labelled LRh-PE in the lipid-mixture.

Emulsions were prepared with an ultrasonic processor UP200S (Hielscher Ultrasonics, Teltow, Germany) using different volumes of water (0.2-2 vol.%) as dispersed phase. Optimal parameters setting were found to be an amplitude of 50% and a sonication cycle of 0.5 s per second for a sonication time of 20-30 min under argon atmosphere and cooling environment.

Subsequently, emulsions were optionally extruded once by pushing the suspension in a syringe (Liposofast basic, Avestin Europe, Mannheim, Germany) through a polycarbonate Nuclepore Track-Etch Membrane (Whatman International, Maidstone, UK) with a nominal pore diameter of 200 nm.

II.3. Size of emulsion droplets

Emulsions were investigated by photon correlation spectroscopy (PCS) using the Nano SZ90 Zetasizer (Malvern Instruments, Worcestershire, UK). A sample of the emulsion was diluted by 1:5 or 1:10 with squalene before thermal equilibration for 5 min at 20°C in the PCS device. The z-average of the size, the polydispersity index (PDI) and the count rate were determined.

II.4. Water/oil interfacial tension

To investigate the adsorbed layers at the water/oil interface, a drop tensiometer technique was used. Dynamic interfacial tension was measured by using the profile analysis tensiometer PAT-1D (Sinterface Technologies, Berlin, Germany). Because the accuracy of measured physical values depends on the calibration quality of the instrument, the tensiometer was calibrated as described by Loglio *et al*. [2003] and Vrânceanu *et al*. [2007]. The absence of surface active impurities in water or squalene was tested before each measurement by dynamic surface measurements over long times at 20°C. For the water/air interface a nearly constant value of γ = 72.5 mN/m and for the squalene/air interface a value of γ = 31.4 mN/m was found which are in good concordance with the literature [Shafrin and Zisman, 1967].

The main principle of this method is to determine the surface tension of a liquid from the shape of a pendent or sessile drop, which is described by the Young-Laplace equation. During the measurement, the local radii of curvature along the drop profile with their corresponding vertical height with respect to a reference plane were determined from the recorded images. The density difference of the two immiscible liquids and the acceleration due to gravity were defined parameters which determine the local hydrostatic pressure difference across the curved interface. With the PAT fitting software, the model profile is calculated by a fourth-order Runge-Kutta integration algorithm from the Laplace equation [Loglio *et al*., 2001].

III. RESULTS AND DISCUSSION

III.1. Emulsion particle size

The water/oil (w/o) emulsions were prepared by sonication as described above with 0.3 mg/ml POPC as emulsifier

in squalene. The average size, polydispersity index (PDI) and the count rate of the emulsion particles are summarized in Table 1. It is evident that the average sizes of the emulsion droplets were dependent on the volume of the dispersed phase. Emulsions generated with 0.2 vol.-% of the aqueous phase have smaller droplets than those with 2 vol.-% aqueous phase. Additionally, the number of counted droplets increased with increasing volume of the dispersed phase. The PDI, which is an indicator for the distribution of particle sizes, increased as well with higher volume fractions of water, demonstrating an increasingly heterogeneous emulsion. The droplet size of the emulsions with 2 vol.-% of dispersed phase (water) was reduced about half with a single extrusion through 200 nm pores of a polycarbonate membrane. Thereby also the number of counted particle reduced and the distribution of particle size became more homogeneous, which was reflected by smaller PDI values.

III.2. Characterization of POPC at the water/squalene interface by interfacial tension measurement

The adsorption of the biological emulsifier POPC to a water/squalene interface was studied by determining the dynamic interfacial tension between the two bulk phases. For that, a buoyant oil drop was generated at the tip of a capillary in an aqueous medium. Results of the measurements for different concentrations of POPC (c_0) as a function of time are presented in Fig. 1a and 1b, respectively. As can be seen in Fig. 1a, the initial decrease rate of γ increases with c_0 whereas the equilibrium interfacial tension decreases with c_0. However, in Fig. 1b, the decrease rate of γ seems to be lower for c_0 = 10 mg/ml than for 5 mg/ml. This artefact stems from the difficulty to start the surface tension measurement immediately after the formation of the o/w interface, since PAT can only be started after the disappearance of drop profile oscillations. Therefore, the start of the PAT measurement fluctuates especially at high c_0 so that only the equilibrium interfacial tension can be extracted from Fig. 1b, but not the decrease rate.

In Fig. 2a-d shapes of a droplet for 0.3 mg/ml POPC and a constant volume of 40 mm³ are shown for different time points. At the beginning of the measurement the droplet was least distorted by buoyancy and the surface tension was at its maximum (Fig. 2a). Over time the surface tension decreased resulting in an enlarged area of the droplet. After 5,000 s the droplet became thinner and maximally stretched and a few seconds after the last image separated from the capillary (Fig. 2d).

PAT can be also used to measure the surface area-pressure isotherm at the liquid/liquid interface. The interfacial pressure Π of the phospholipid monolayer is related to the surface tension via the relation

$$\Pi = \gamma_0 - \gamma, \qquad (1)$$

where γ_0 indicates the surface tension of the pure fluid.

III.3. Adsorption isotherms of POPC at the water/squalene interface

The determination of adsorption isotherms requires equilibrium adsorption data. In a diffusion controlled process, the equilibrium interfacial tension can be calculated by the extrapolation of the $\gamma\left(1/\sqrt{t}\right)$ plot to infinite time, based on the long-time approximation for diffusion-controlled

Table 1: Overview of PCS-measurements for w/o-emulsions with 0.3 mg/ml POPC dissolved in squalene before and after extrusion through a 200 nm polycarbonate membrane. Mean values of particle size (z-average), PDI and count rate were calculated from n independent emulsion samples being averaged with the respective standard deviation (s.d.), with each sample being measured 3 times with PCS.

No. of samples *n*	Extrusion	Vol.-% water	Size ± s.d. (nm)	PDI ± s.d.	Count rate ± s.d.
5	-	0.2	515.2 ± 40.1	0.12 ± 0.02	369.2 ± 73.5
7	-	2	846.8 ± 147.2	0.37 ± 0.07	611.9 ± 195.7
2	200 nm	2	437.2 ± 63.1	0.20 ± 0.03	470.2 ± 99.4

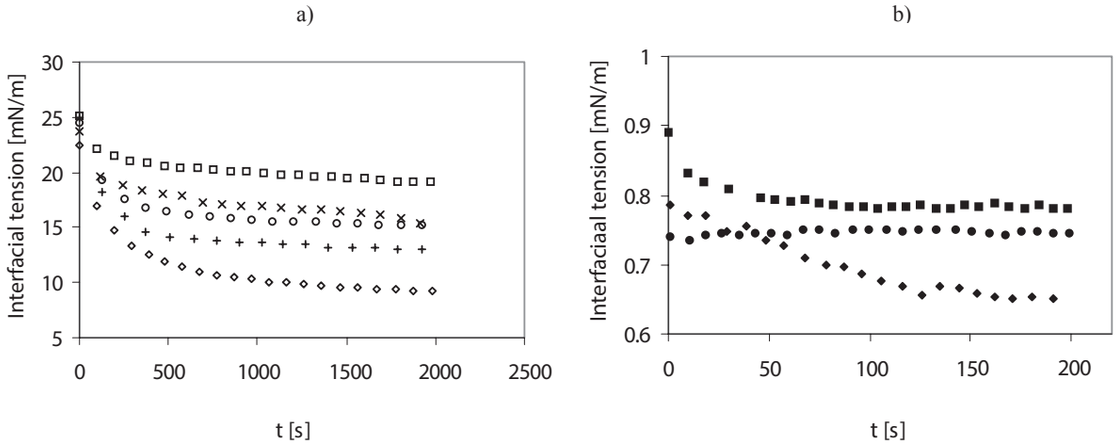

Figure 1: *Dynamic interfacial tension as a function of adsorption time for POPC in squalene at the interface with water for different concentrations c_0 (□) 0.0001mg/ml, (×) 0.001mg/ml, (❍) 0.01mg/ml, (+) 0.015mg/ml, (◇) 0.3mg/ml, (■) 3mg/ml, (●) 5mg/ml, (◆) 10mg/ml. a) 0.0001 mg/ml ≤ c_0 ≤ 0.3 mg/ml and b) 3 mg/ml ≤ c_0 ≤ 10 mg/ml.*

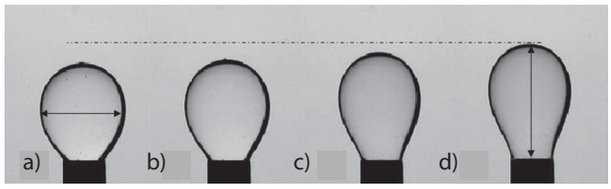

Figure 2: *Temporal development of the profile of a squalene droplet in water with a constant volume of 40 mm³ with a POPC bulk concentration c_0 = 0.3 mg/ml: a) 0 s, b) 200 s, c) 3000 s and d) 5000 s. The adsorption of the emulsifier POPC at the water/squalene interface is monitored by the dynamical interfacial tension which was measured for the different points as a) 22.69 mN/m, b) 18.29 mN/m, c) 14.23 mN/m and d) 12.36 mN/m. Image scale is given by the outside-diameter of the capillary with 2.0 mm.*

adsorption [Fainerman *et al*., 1994; Miller *et al*., 1994], which is given by the Hansen-Joos equation

$$\left[\frac{d\gamma}{d(1/\sqrt{t})}\right]_{t\to\infty} = \frac{RT\Gamma^2}{c_0}\sqrt{\frac{\pi}{4D}}, \qquad (2)$$

where $\gamma(t)$ denotes the dynamic interfacial tension, R the gas constant, T the absolute temperature, Γ the interfacial concentration, c_0 the emulsifier bulk concentration and D the diffusion coefficient. Fig. 3 shows the dynamic interfacial tension as a function of $1/\sqrt{t}$ for POPC in squalene at the interface to water. The linear relationship between $\gamma(t)$ and $1/\sqrt{t}$ is valid only for time periods of $t > 100$ s. The linear extrapolation and its intercept with the ordinate determines the equilibrium interfacial tension for infinite time. From the extrapolated interfacial tension values, an adsorption isotherm of POPC was obtained according to Gibbs

$$\Gamma = -\frac{c_0}{RT}\left(\frac{\partial\gamma}{\partial c_0}\right)_{p,T} = -\frac{1}{RT}\left(\frac{\partial\gamma}{\partial \ln c_0}\right)_{p,T}, \qquad (3)$$

as shown in Fig. 4 (solid line). The critical aggregation concentration (CAC) appears at about 3 mg/ml where any concentration increase does not result in further changes of the interfacial tension. According to eq. (3), a minimum area per molecule A at the CAC was obtained with a value of 253 Å² per molecule. The surface area per lipid molecule is given by

$$A = \frac{1}{N\Gamma}, \qquad (4)$$

where N is the Avogadro number.

To describe the experimental data in a second theoretical model, the Langmuir isotherm was used. The Langmuir adsorption isotherm is the basis for most of the adsorption kinetic models for surfactants and given by

$$\Gamma = \Gamma_\infty \frac{c_0}{a_L + c_0}, \qquad (5)$$

where Γ_∞ is the maximum interfacial concentration (saturation adsorption) and a_L the Langmuir adsorption constant, representing the concentration at which half of the interfacial coverage has been reached [Li *et al*., 1996a]. Using the equation of state of an ideal surface layer [Li *et al*., 1996c] the interfacial tension in the equilibrium can be calculated. In Fig. 4 the equilibrium interfacial tension (broken line) is presented as a function of initial bulk concentration c_0 of POPC at the water/squalene interface. For the Langmuir adsorption isotherm (eq. 5), an area of 256 Å² per molecule was obtained which coincides reasonably well with the result of the Gibbs adsorption isotherm (eq. 3).

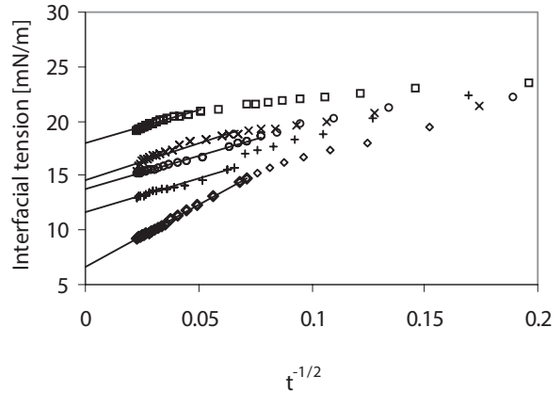

Figure 3: *Dynamic interfacial tension as a function of time ($1/\sqrt{t}$) for POPC in squalene at the interface with water for different concentrations c_0 (□) 0.0001mg/ml, (×) 0.001mg/ml, (○) 0.01mg/ml, (+) 0.015mg/ml, (◇) 0.3mg/ml.*

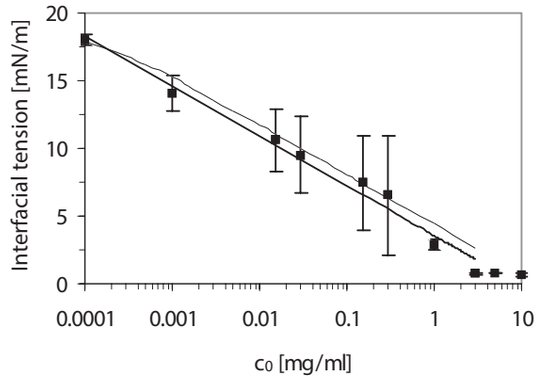

Figure 4: *γ-log c_0 curves for POPC in squalene at the interface with water. Black squares, experimental data with standard deviations; solid line, Gibbs adsorption isotherm according to eq. (3); thin solid line, Langmuir adsorption isotherm according to eq. (5).*

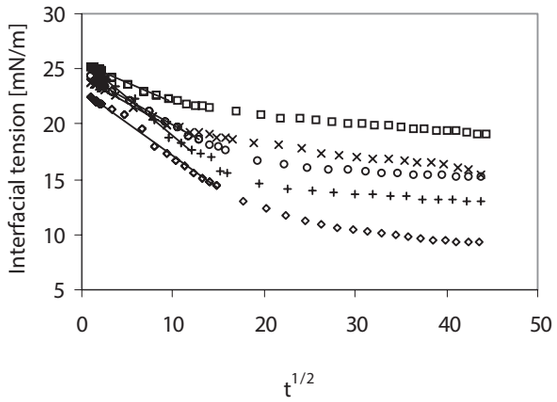

Figure 5: *Dynamic interfacial tension as a function of* $\sqrt{t}$ *for POPC in squalene at the interface with water for different concentrations* c_0 *(□) 0.0001mg/ml, (×) 0.001mg/ml, (○) 0.01mg/ml, (+) 0.015mg/ml, (◇) 0.3mg/ml.*

III.4. Diffusion-controlled adsorption model

For the diffusion-controlled adsorption mechanism a linear dependence of γ on $\sqrt{t}$ is expected [Thiam *et al.*, 2012] which is represented by the relation

$$\left[\frac{d\gamma}{d\sqrt{t}}\right] = -2RTc_0\sqrt{\frac{D}{\pi}}, \qquad (6)$$

In this case the diffusion coefficient can be obtained by using only the bulk concentration without any knowledge of the adsorption isotherm. Fig. 5 shows a set of experimental curves for different concentrations of POPC. With the obtained diffusion coefficient the surface concentration Γ can be calculated, according to eq. (2).

III.5. Solubility of POPC in squalene

The phospholipid POPC was solubilized completely in 1 ml squalene as described in section 2.2. After ultrasonication for 30 min the sample was measured by PCS. For further dissolution of aggregates, the sample was treated with prolonged sonication procedure in the water bath until a considerable decrease of aggregates could be detected. Table 2 presented the particle sizes, sonication time as well as the PDI and the count rate for every sample before and after the additional treatment. For concentrations $c_0 \leq 3$ mg/ml the count rate can be in the same range as for pure squalene, provided mixing is initiated by suitable conditions, i.e. formation of a thin lipid film produced by evaporation of a solvent (ethanol). For $c_0 \geq 3$ mg/ml extended ultrasonication did not lead to a considerable decrease in the count rate of particles. Therefore, the critical aggregation concentration (CAC) assessed by PAT is in the range of $c_0 = 3$ mg/ml is in accordance with the results of dissolution procedures and detection of aggregates by PCS.

IV. CONCLUSIONS

In digital microfluidics two-phase systems like water in oil emulsions are increasingly widespread to enable a multitude of functions based on discretisation and compartmentalisation in liquid processes. Up to now, conventional, detergent based emulsifiers or organic solvents are added to the system which often cause negative effects like denaturation of the analysed proteins or adsorption to the phase interface. Instead of these currently employed emulsifiers, phospholipids or mixtures of different phospholipids, who represent natural emulsifiers, can be applied for the two-phase systems to avoid these undesired concomitants and to enable full biotechnological system integration.

The presented results demonstrate that dynamic interfacial tension is a suitable method to assess the interfacial adsorption of bio-surfactants in natural oils [Dopierala *et al.*, 2011]. Phospholipids are an interesting and promising emulsifier for microfluidic or nanofluidic multiphase flow systems. They are especially interesting for biotechnological systems integration. The summarized results evidence the benefits of using phospholipids, but also point out open questions and challenges on the way for broader use in microflows in biological (nano)systems integration and bioengineering.

V. NOMENCLATURE

$\gamma(t)$ dynamic interfacial tension, Γ interfacial concentration of emulsifier, Γ_∞ maximum interfacial concentration, a_L Langmuir adsorption constant, A area per molecule, c_0 bulk concentration of emulsifier, CAC critical aggregation concentration, D diffusion coefficient, PAT profile analysis tensiometry, PDI polydispersity index, POPC palmitoyl

Table 2*: PCS measurements for the determination of the solubility limit of POPC in squalene. Mean values of particle size, PDI and count rate were calculated from 3 independent experiments of solubility and the respective samples.*

Experiment	C_0 (mg/ml)	Size (nm)	PDI	Count rate
Pure squalene	-	4.4	0.44	3.1
Thin POPC film (after rotary evaporation) dissolved in squalene	3.0	285.4	1.00	26.8
+ after 2 hours sonication	3.0	376.9	0.66	11.1
POPC as bulk lipid dissolved in squalene	3.0	1686.0	0.51	160.0
+ after 6 hours sonication	3.0	877.5	0.71	24.3
Thin POPC film (after rotary evaporation) dissolved in squalene	10.0	3196.0	0.86	867.4
+ after 12 hours sonication	10.0	1486.0	0.35	553.7

oleoyl phosphatidylcholine, w/o water in oil, *R* gas constant, *t* time, *T* absolute temperature, *p* pressure.

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