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Oil/water "hand-bag like structures": how interfacial rheology can help to understand their formation?

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Bicompartmental structures, named "hand-bag like structures" (HBS), where phospholipid membranes stabilise both an oily and a water phase, were firstly observed in cationic emulsions thanks to cryomicroscopy. The emulsion formulation was found to play a crucial role: no HBS were observed neither in the absence of cationic lipid nor in the presence of DOTAP, while oleylamine and DSPE-PEG were found to increase the proportion of HBS compared to stearylamine. The HLB value and the unsaturation of the lipids acyl chains then appeared as some key parameters for HBS formation. Interfacial rheology measurements have given additional information on the interfacial film such as its cohesion (E_e), interactions with the adjacent phases (E_{ne}) and relaxation time τ, which was found to be the most discriminating parameter. τ was indeed significantly longer for the samples containing no HBS. This suggests that the ability of the interface to rearrange could play a role in HBS formation.

Key words: Emulsion – Alkylamines – o/w bicompartmental structures – Drop tensiometer.

The design of emulsions is a convenient way to get water insoluble molecules dispersed at the molecular level without using organic solvents. The multiple applications of emulsions in many fields account for this interesting ability. In therapeutics, however, and especially in the case of intravenous (IV) administration, drastic constraints limit the formulation and the challenge is often to ensure a long-term stability to the system. Benita *et al*. proposed an interesting solution to this problematic some years ago, with submicronic cationic emulsions [1]. In these systems, the presence of cationic lipids stabilizes the o/w interface and favors the formation of droplets of low size (200 nm). The colloidal size as well as the use of phospholipids as emulsifiers allows these emulsions to be suitable for IV injection [2]. In fact, the formulation of such systems was characterized by the presence in excess of three surface-active agents stabilizing the triglycerides (TG) droplets: egg phospholipids (Lipoid E80), a cationic lipid and poloxamer 188 [3]. Cryomicroscopy experiments (cryoTEM) of these cationic emulsions have revealed the existence of unexpected bicompartmental structures (called "hand-bag like structures", HBS) where phospholipid membranes stabilise both an oily and a water compartment [4]. These new systems, which were never described before the work of Teixeira *et al*., may be of interest in drug delivery when both hydrophilic and lipophilic molecules are needed to be released together at the same therapeutic target (i.e. intracellularly).

In the present study, the emulsion has been prepared with other lipids and observed in cryoTEM to try to determine the parameters required for the formation of the HBS (i.e. charge, HLB, etc.). Besides the individual physicochemical characteristics of the interfacial lipids, additional information has been searched with the aid of interfacial rheology experiments. The technique of the rising drop tensiometer enabled three parameters to be quantified: τ, the relaxation time of the interface; E_e, the interaction forces existing between the lipids, thus corresponding to the cohesion of the monolayer; and E_{ne} the intensity of the interactions of the lipids with the adjacent phases, oil and water. Thus this model brought new insight in the complex question of the HBS formation and must be considered as one of several ways to help understand the mechanism of HBS formation.

I. MATERIALS AND METHODS

1. Chemicals

Several types of lipids were used for the oily phase of the emulsion: Lipoid E80 (EPC) (MW = 707 ± 3) was purchased from Lipoid GmbH (Ludwigshafen, Germany), stearylamine (SA) (MW = 269.5) from Sigma (L'Isle-d'Abeau, France), oleylamine (OA) (MW = 267.5) from Fluka (L'Isle-d'Abeau, France), DOTAP from Sigma (United States), DSPE-PEG from Avanti polar lipids (United States) and triacylglycerols (TG) (MW = 505 ± 3) from Société industrielle des oléagineux (Saint-Laurent-Blangy, France). EPC is a complex mixture of egg phospholipids with various acyl chains (mainly C_{16} and C_{18}) in which PC is the main constituent (minimum: 80%). OA is a mixture of primary amines (98% purity, 70% oleylamine content). Oil includes medium chain TG synthesized from two saturated fatty acids: capric acid (C10:0) and caprylic acid (C8:0).

The water phase was composed of distilled water, poloxamer 188 from BASF (Ludwigshafen, Germany), and glycerol from Fluka (Sigma, L'Isle-d'Abeau, France).

2. Emulsion preparation

The submicron emulsions were prepared according to a previously described procedure [5]. The oily and aqueous phases were prepared separately and heated to 70°C, then mixed and stirred with a magnetic stirrer. The final emulsions were obtained after mixing with ultraturrax (Janke & Kunkel,

Table I - Composition of the emulsions (%, w/w). DSPE-PEG represented 2.5 mol % of total phospholipid content. The proportions of SA, OA and DOTAP were calculated in order to have the same number of positive charges in each cationic emulsion at pH 7.4 (40 mol % of total phospholipid content).

Composition		Samples					
		EPC	EPC/DOTAP	EPC/SA	EPC/PEG	EPC/OA	EPC/OA/PEG
Oily phase	EPC	2.00	2.00	2.00	1.82	2.00	1.82
	DSPE-PEG	-	-	-	0.18	-	0.18
	DOTAP	-	1.32	-	-	-	-
	SA	-	-	0.50	-	-	-
	OA	-	-	-	-	0.50	0.50
	TG to	10.0	10.0	10.0	10.0	10.0	10.0
Water phase	Poloxamer 188	1.68	1.68	1.68	-	1.68	-
	Glycerol	2.25	2.25	2.25	2.25	2.25	2.25
	Water to	100	100	100	100	100	100
Emulsifier proportion in oily phase		20.0	33.2	25.0	20.0	25.0	25.0

Table II - Composition of the oily phases used in the rheology experiments (%, w/w).

Composition		Samples					
		EPC	EPC/DOTAP	EPC/SA	EPC/PEG	EPC/OA	EPC/OA/PEG
Oily phase	EPC	4.00E-04	4.00E-04	4.00E-04	4.00E-04	4.00E-04	4.00E-04
	DSPE-PEG	-	-	-	4.00E-05	-	4.00E-05
	DOTAP	-	1.00E-04	-	-	-	-
	SA	-	-	1.00E-04	-	-	-
	OA	-	-	-	-	1.00E-04	1.00E-04
	TG to	10.0	10.0	10.0	10.0	10.0	10.0
Emulsifier proportion in oily phase		4.00E-03	5.00E-03	5.00E-03	4.40E-03	5.00E-03	5.40E-03

IKA-Labortechnik T 45N, Vanves, France) and homogenisation in a microfluidiser (M100s, Microfluidics Corp., Moizon, France) under four bars pressure and four microfluidisation cycles. *Table I* gives the composition of the different formulations studied (%, w/w).

Droplets size was measured by Quasi Elastic Light Scattering (QELS) using a Nanosizer (N4Plus, Coultronics). The mean droplet size was found to be 165 nm (± 50 nm).

3. Cryomicroscopy experiments

An emulsion drop was deposited on air glow-discharged grid coated with a perforated carbon film [6]. The grid was mounted on a guillotine-like frame and the emulsion excess blotted with a filter paper. Then the frame was released and the grid plunged into liquid nitrogen cooled liquid propane. The grid was transferred from liquid propane to the Gatan transfer chamber and loaded in a Gatan 626 stage. The samples were observed in a Philips CM12 electron microscope operated at 100 kV. Micrographs were recorded at a magnification of 35,000 on Kodak image plate S0 163 developed 12 min in D19 full strength.

4. Value calculations of HLB and acyl chains unsaturation ratio

The HLB value of each interfacial lipid was calculated according to Davis method [7], the different chemical groups being endowed with additive numbers:

$$HLB = \sum_i H_i - \sum_i L_i + 7 \qquad \textbf{Eq. 1}$$

where H_i is the hydrophilic and L_i the lipophilic individual group numbers. The resulting HLB of the interfacial film of the emulsion (HLB_{mix}) is then calculated as follows:

$$HLB_{mix} = \sum_i W_i HLB_i \qquad \textbf{Eq. 2}$$

where W_i and HLB_i are the weight fraction and the HLB of the i^{th} component, respectively.

The ratio of the molar proportion of saturated /unsaturated acyl chains in the interfacial film, $R_{s/u}$, is calculated as follows:

$$R_{s/u} = \sum_i S_i N_i / \sum_i US_i N_i \qquad \textbf{Eq. 3}$$

where S_i and US_i are the molar proportion of saturated and unsaturated chains respectively, in individual lipids and N_i the number of acyl chains of the given lipid (actually, one or two depending on the lipid used).

5. Interfacial rheology: rising drop tensiometer

The drop tensiometer (Traker, IT concept, France) allowed the determination of the interfacial tension by analyzing the axial symmetric shape (Laplacian profile) of the rising drop of phospholipid/TG solution (oily phase, density = 0,94) in water phase. It is note worthy that for technical reasons the interfacial lipid concentration in the oily phase had to be strongly decreased comparatively to the initial emulsion (*Table II*). Unlike the formulation conditions, neither poloxamer, nor glycerol were added to the water phase. The experiments were carried out at