New Bicompartmental Structures Are Observed When Stearylamine is Mixed with Triglyceride Emulsions

Helder Teixeira,[1] Catherine Dubernet,[1] Véronique Rosilio,[1] Simon Benita,[3] Jean Lepault,[2] Inge Erk,[2] and Patrick Couvreur[1,4]

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INTRODUCTION

Over the past 30 years, efforts have been made in conceiving colloidal systems for drug administration and targeting. A wide range of dispersed formulations have been designed including liposomes, nano- and microparticles, as well as single emulsions, and some of these systems are now even associated with marketed new drugs. Recently, lipids and especially cationic lipid-based systems have gained an increasing interest due to their ability to condense DNA and to allow efficient transfection at least *in vitro* and *exvivo* (1–3). Numerous investigations have been performed in order to characterize the supramolecular assemblies formed between nucleic acids and lipids and to find some structure–activity relationship (4).

In this context, we have recently developed a positively-charged emulsion based on lecithin, medium-chain triglycerides, poloxamer 188, and stearylamine as a delivery system for oligonucleotides. This emulsion, easy to prepare and well-tolerated, was found to dramatically improve stability of oligonucleotides in the presence of serum (5,6). With the aim to characterize the morphology of this emulsion, we have performed some cryomicroscopic examinations leading to the identification of unexpected structures. This rapid communication describes these new colloidal systems consisting of two compartments with an oily core and an aqueous phase associated with the same structure. Such items, which were never described before in the literature, could be useful for drug administration when both hydrophilic and lipophilic molecules need to be administered together.

MATERIALS AND METHODS

Materials

Medium-chain triglycerides (MCT) (Societé des Oleagineux, France), Lipoid E-80® (egg-phosphatidylcholine, PC, or DSPC) (Lipoid, Germany), poloxamer 188® (BASF, Germany), α-tocopherol (Fluka, France), Stearylamine (SA) (Sigma, USA) and 1,2-distearoyl-*sn*-glycero-3-phosphatidyl ethanolamine-N [poly(ethyleneglycol)2000] (DSPE-PEG) (Avanti polar lipids, USA). Dr. Daniel Scherman (Rhône Poulenc Rorer, Vitry-sur-Seine, France) kindly provided the polycation RPR 120535 (RPR C_{18}) (7). The chemical structures of the lipids used in the preparation of the medium chain triglyceride-based emulsions are presented in Figure 1.

Methods

Emulsion Preparation and Characterization

The submicron emulsions were prepared according to a previously described procedure (5). Briefly, the oil 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 ultra-turrax (Ikawerk T 45N, Vanves, France) and homogenization in a microfluidizer M-110S which exploits a technology based on a submerged jet principle (Microfluidics Corp., Moizon, France) under 4 microfluidization cycles at 4-bar pressure. The formulations (%,w/w) are presented in Table I. Size measurements were performed after dilution of the samples in glycerol 2.25% by photon correlation spectroscopy using a nanosizer (Nanosizer ND4, Coultronics, France). The ζ-potential was determined from their electrophoretic mobility in a Malvern Zetasizer® (Malvern Instruments, United Kingdom) after dilution in NaCl 10^{-3}M.

Cryomicroscopy Experiments

A drop of the emulsions was deposited on a glow-discharged grid coated with a perforated carbon film (8). 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 minutes in D19 full strength.

Surface Tension Measurements

The lipids were spread at constant area (16.6 cm^2) from chloroform–methanol (9:1 v/v) solutions on the glycerol 2.25% subphase. The addition of the spreading solvent alone to a clean surface produced no measurable change in the surface tension of the subphase. The surface tension of monolayers obtained by successive addition of lipid aliquots was measured by the Wilhemy plate method using a Krüss K 10 digital tensiometer equipped with a force transducer having a sensibility of 0.1 mN/m. The measurement cell was thermostated at 23°C through all measurements. The surface pressure (π) was deduced from the $\gamma_{\text{glycerol 2.25\%}} - \gamma_{\text{monolayer}}$ relationship.

[1] UMR CNRS 8612, Faculté de Pharmacie, Université Paris-Sud, Chatenay-Malabry.
[2] Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette.
[3] School of Pharmacy, University of Jerusalem, Israel.
[4] To whom correspondence should be adressed at UMR CNRS 8612, Université de Paris XI, Faculté de Pharmacie, 5, Rue J.B. Clément, 92296 Châtenay-Malabry, France. (e-mail: patrick.couvreur@cep.u-psud.fr)

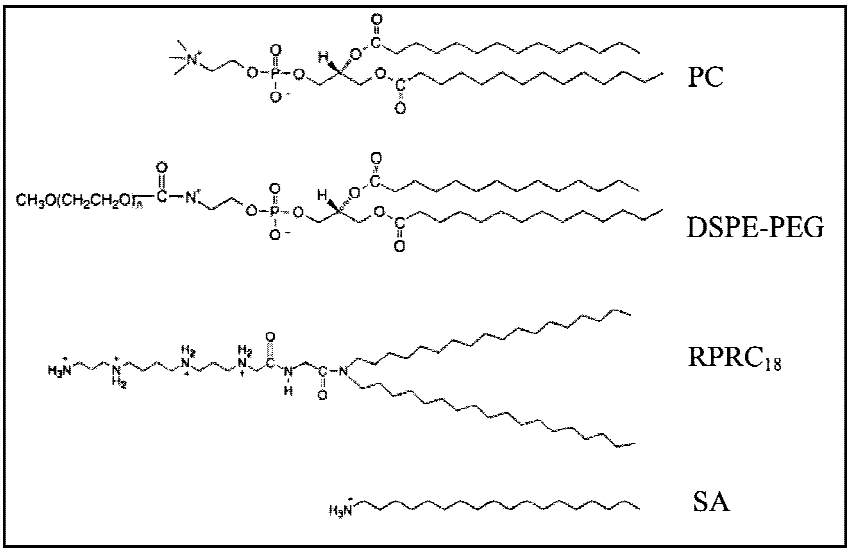

Fig. 1. Structure of the lipids used in the preparation of the emulsions.

RESULTS AND DISCUSSION

Cryomicroscopic observations of the PC negatively charged emulsion samples show the typical appearance of an O/W emulsion with droplets displaying a higher density to the electrons (Fig. 2a) and a size close to 150 nm (Table II).

On the contrary, positively-charged emulsions containing stearylamine (SA) revealed a completely different morphology. Some items were composed of an oily phase associated with a water compartment surrounded by a phospholipidic bilayer (Fig. 2b) which is sometimes round (Fig. 2b) or which sometimes appears with some breaks of curvature, thus forming a triangle (Fig. 2c) or a rectangle (Fig. 2d). The general appearance of these structures with a size of ca. 150 nm (Table II) is like a "handbag," the oily phase being the bag itself and the water compartment surrounded by the "bridle." The addition of DSPE-PEG to the formulation led to an emulsion sample with almost the same size (Table II), but with a clear increase of the number of handbag-type structures (Fig. 3). While the oily compartment is defined by a single layer, the water compartment is determined by either one or two layers. At the boundary with the oily compartment there is one layer; however, the water compartment is formed by two membranes at the boundary with the external aqueous medium. In order to investigate if these bicompartmental structures were always associated with a positive value of the zeta potential or if the nature of the cationic lipid is a key factor for formation of handbag-like structures, we performed an additional observation using a diacylated lipid (RPR C_{18}) bearing four positive charges (Fig. 1) instead of the monoacylated SA displaying only one positive charge (Fig. 1). Replacing SA by RPR C_{18} in the emulsion at the same positive charge ratio led to a return to a cryomicroscopic morphological appearance similar to that of the single PC anionic emulsion (spherical oily droplets with a size of 178 nm are observed). Thus, the formation of these unexpected handbag-like structures needs the presence of SA in the composition of the emulsion. Nevertheless, substitution of egg-PC by DSPC led to the disappearance of any handbag structure (Table II), suggesting that the nature of the acyl chains of the phospholipid used has an important role too.

Table 1. Composition of Submicron Emulsions Obtained by Microfluidization (%, w/w).

	PC	PC/SA	PC/SA/DSPE-PEG	DSPC/SA	PC/RPR C_{18}
PC[a]	2.00	2.00	1.82	—	2.00
DSPC	—	—	—	2.00	—
DSPE-PEG	—	—	0.18[c]	—	—
Stearylamine[b]	—	0.5	0.5	0.5	—
RPR C_{18}[b]	—	—	—	—	0.543
MCT to	10.00	10.00	10.00	10.00	10.00
Pluronic F-68	1.68	1.68	—	1.68	1.68
Glycerol	2.25	2.25	2.25	2.25	2.25
Water to	100.00	100.00	100.00	100.00	100.00

[a] Lipoid E-80.
[b] Represent 1.12×12^{21} charges per 100 ml of emulsion.
[c] Represent 2.5 mol% of total phospholipid content.

Finally, surface tension measurements were performed in order to understand the interfacial interactions involved in this phenomenon at the molecular level. As shown in Figure 4, the lipidic PC–SA and PC–SA–DSPE-PEG mixtures spread at the air–water interface behaved similarly and exhibited a higher compressibility than PC and PC–RPRC_{18} monolayers, as inferred from their much lower mean molecular area (0.8 nm^2) compared to those of PC and PC–RPRC_{18} mixtures (1.15 and 1.2 nm^2, respectively). In other words, at the same surface pressure, the presence of SA in the mixtures allowed the molecular surface density to increase. It has been previously shown that the PC polar headgroups strongly interact with the positively charged SA headgroups in the electric double bilayer (9). This interaction between two immiscible components favors molecular rearrangements at the interface, which might be responsible for the observed "handbag"-like structures. At low surface density, addition of DSPE-PEG molecules to the PC–SA mixture had no effect on the π-A isotherm profile. However, when the mixed monolayer was compressed towards a more condensed state, a pre-collapse (at about 36 mN/m) occurred (Fig. 4), demonstrating lipid separation and ejection of DSPE-PEG molecules from the interface. This separation in addition to the molecular rearrangements induced by the electrostatic inter-

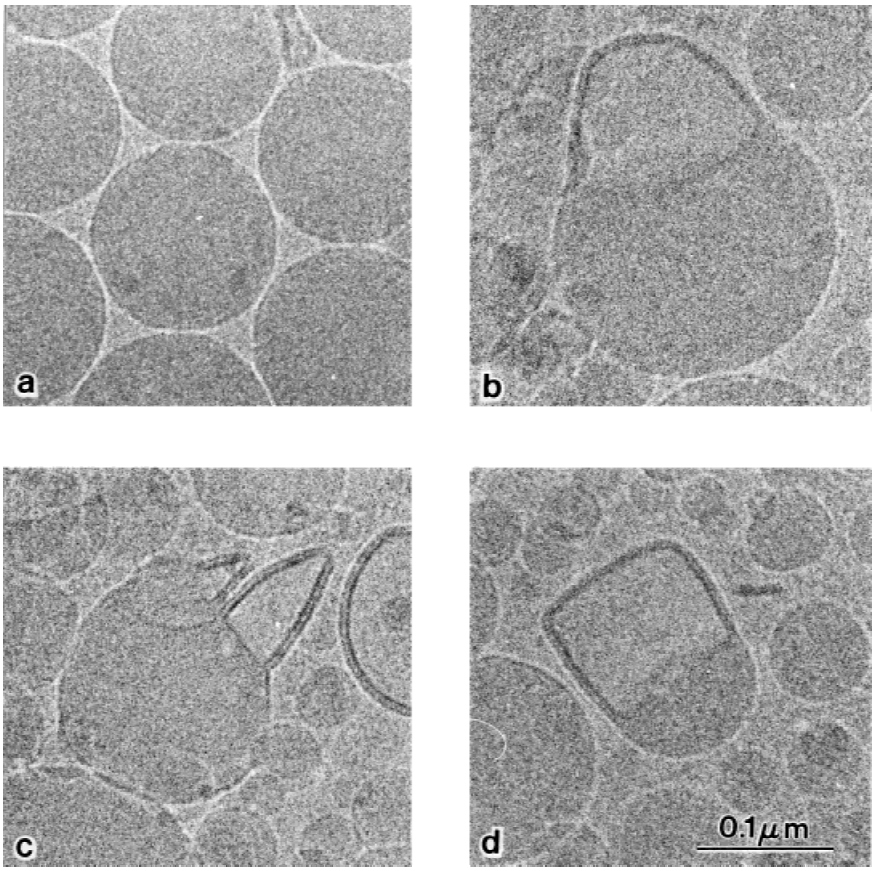

Fig. 2. Appearance of the structure of the droplets in PC emulsion (a) and the handbag-like structure observed in PC–SA or PC–SA–DSPE-PEG emulsions (b-d). Note that the "bridle" of the aqueous compartment may be roundshaped (b), or with some breaks of curvature, thus forming a triangle (c), or a rectangle (d).

actions seems to be related to the presence of hydrophilic PEG chains in the subphase. Unlike SA, $RPRC_{18}$ did not induce any condensation of PC monolayers. Obviously, electrostatic interactions at the interface between PC headgroups and positively charged $RPRC_{18}$ could not occur due to the localization of the $RPRC_{18}$ charges into the subphase.

How the formation of these bicompartmental structures occurs is actually under investigation, but it is hypothesized that SA could induce some separation of the lipidic mixture at the interface allowing the phospholipids to form the lamellar phase corresponding to the "bridle" of the structure. Such a hypothesis is supported by the fact that the formulation DSPC–SA did not show any separation. These two lipids should indeed be perfectly miscible since they both present a saturated acyl chain in C_{18}. The increase of handbag structures observed with DSPE-PEG-containing formulations could be interpreted as an increase in lipid separation as suggested by surface tension experiments. However, we cannot exclude that PEG itself also contributed to the stabilization of the structure. In the case of $RPRC_{18}$ containing emulsions the diacyl chains and the strong repulsion forces make that these molecules fill a more important area as observed in Figure 4. However, with this type of lipid, separation does not occur because the four positive charges of that lipid are rather localized in the water phase and not at the interface, as is the case with the monocationic SA.

Table 2. Size, ζ-potential, and Frequency of Bicompartmental Structures (f) Observed in Submicron Medium Chain Triglyceride-Based Emulsions: PC, PC/SA, DSPC/SA, PC/SA/DSPE-PEG, and $PC/RPRC_{18}$

	Size (nm)	ζ-potential (mV)	f[a]
PC	157 ± 49	−28 ± 1.2	0
PC/SA	141 ± 57	49 ± 1.6	+
DSPC/SA	173 ± 62	48 ± 1.2	0
PC/SA/DSPE-PEG	187 ± 68	50 ± 0.9	++
$PC/RPRC_{18}$	178 ± 65	59 ± 1.5	0

[a] *f* is the number of bicompartmental structures observed in cryomicroscopy expressed as a tendency (+ frequent, ++ very frequent, 0 absence of these structures).

CONCLUSION

Triglyceride lipid emulsions have been studied since a long time for parenteral feeding, and they have some advantages over other colloidal systems concerning their ease of preparation, excellent stability, and biopharmaceutics which are all well understood (10). The most significant drawback of these emulsions is, however, that they are not adapted for the delivery of water-soluble compounds. This is why hydropho-

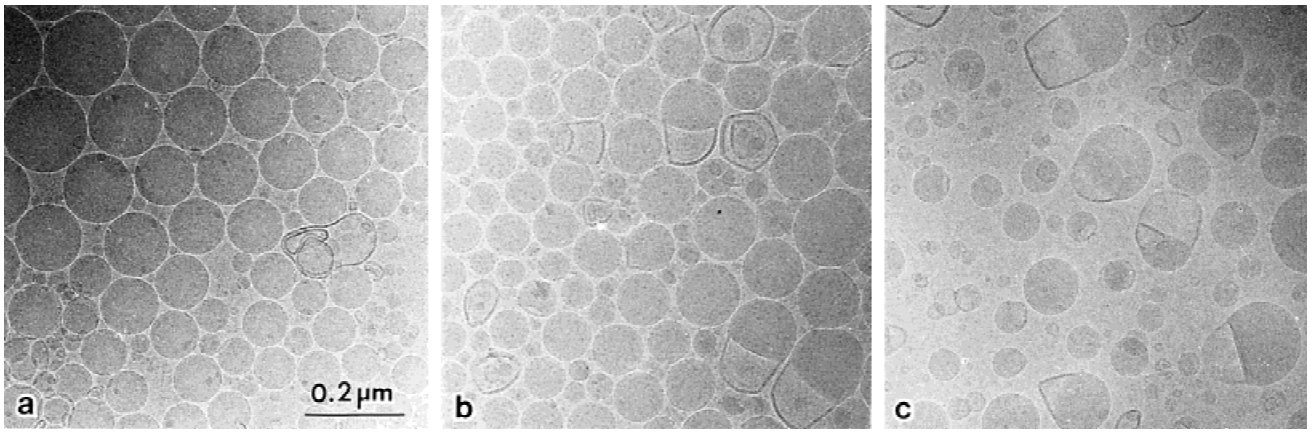

Fig. 3. Representative frames obtained in cryomicroscopy, giving an idea of the frequency of occurrence of handbag-like structures in (a) PC, (b) PC–SA, and (c) PC–SA–DSPE-PEG emulsions.

bic prodrugs or analogs of such compounds have been synthesized to allow their formulation as an emulsion. A typical example of this approach is the development of lipidic formulations of prodrugs of mitomycine C (11). Here we have identified that when containing SA, triglyceride-based emulsions are able to form unexpected bicompartmental structures with an oily phase and a water phase both associated with the same structure. These systems which were never described before may be of interest in drug delivery when both hydrophilic and lipophilic molecules are needed to be released together intracellularly.

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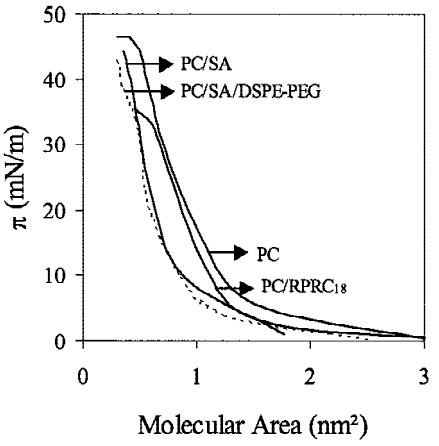

Fig. 4. π-A isotherms for PC and its mixtures with SA, DSPE-PEG, and $RPRC_{18}$ used for the preparation of cationic emulsions. Note the pre-collapse at 36 mN/m with PC–SA–DSPE-PEG mixed monolayer.

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