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Characterization of oligonucleotide/lipid interactions in submicron cationic emulsions: influence of the cationic lipid structure and the presence of PEG-lipids

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Abstract

We have recently described how oligonucleotide (ON) stability and release from O/W cationic emulsions are governed by the lipid composition. The aim of the present paper was to investigate the properties of the ON/lipid complexes through fluorescence resonance energy transfer (FRET), size, surface tension measurements and cryomicroscopy. Starting from a typical emulsion containing stearylamine as a cationic lipid, the influence of the lipid structure (monocationic molecules bearing mono or diacyl chains, or polycations) as well as of the presence of PEGylated lipids, were studied. The presence of a positive charge on the droplet surface clearly contributed to enhance the ON interaction with lipid monolayers and to bring the ON molecules closer to the interface. Hydrophobic interactions through the acyl chains were shown to further enhance the anchorage of the ON/lipid complexes. In contrast, the incorporation of PEGylated lipids acted as a barrier against the establishment of electrostatic bindings, the polyethyleneglycol chains acting themselves as interaction sites for the ON leading to

Abbreviations: DOPE, dioleylphosphatidyl ethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; SA, stearylamine; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphatidyl ethanolamine; DSPE-PEG, 1,2-distearoyl-*sn*-glycero-3-phosphatidyl ethanolamine-*N* [poly (ethylene glycol)2000]; MCT, medium chain triglycerides; pdT16-rho, hexadecathymidylate-rhodamine; PC, phosphatidyl choline (Lipoïd E80®); NBD-PE, *N*-(7-nitrobenz-2oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; RPR122535, RPRC18.

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hydrophilic complexes. Similar features were observed for the polycationic lipid, and cryomicroscopy revealed the existence of bridges of various intensities between the droplets of the emulsion containing either PEG or the polycation, probably because of the configuration of the ON at the interface.

Keywords: Cationic emulsion; Oligonucleotide delivery; Stearylamine; DSPE-PEG; FRET; Surface tension

1. Introduction

Anti-sense oligonucleotides (ON) have been used to selectively modulate the expression of genes and to inhibit the synthesis of cellular proteins. However, when using natural phosphodiester molecules, ON-based therapy is compromised by the rapid degradation of ON in biological fluids and by their inability to efficiently cross cell membranes due to both their hydrophilic character and large molecular structure [1,2]. The introduction of phosphorothioate linkages has been shown to confer nuclease resistance. However, non-specific interactions, mainly with plasma proteins, limit the application of phosphorothioates in vivo [3].

In the last decade, lipids and especially cationic lipid-based systems have gained increasing interest due to their ability to allow efficient ON delivery. The most successful method for ON delivery is the development of cationic liposomes composed of cationic lipids and a phospholipid helper such as DOPE (dioleylphosphatidyl ethanolamine) [4–7]. For instance, ON associated with cationic liposomes were shown to be delivered into the cytoplasm at an early stage of the endocytotic pathway with a marked increase of anti-sense activity [6] and efficient protection against nuclease attack [7].

In this way, we have recently developed cationic emulsions based on a triglyceride oil core stabilized by a phospholipid/cationic lipid mixture as a new delivery system for ON [8]. These emulsions would have the advantage over most of the cationic lipidic formulations of being well tolerated, either locally (ocular administration) or intravenously [9]. ON molecules can be efficiently associated with the oil droplets through an ion-pair formation over a wide range of length (up to 50-mer). In addition, the cationic lipid composition of the emulsion (nature of the hydrophilic head group and the acyl chains) has been varied in attempts to optimize the ON-emulsion decomplexation [10] and the incorporation of poly (ethylene glycol) (PEG)-based lipids has been proposed as a strategy to mask the net positive charge and to reduce the opsonization of the oil droplets [10,11]. This triglyceride-based emulsion has been shown to be an efficient system for the delivery of ON to tumoral cells (submitted).

Despite the great potential of cationic lipids as carriers for the delivery of ON, the characterization of the complexes formed between ON and lipids remains, in general, poorly studied and understood. Previous investigations have clearly shown that ON molecules induced aggregation and lipid mixing with cationic liposomes (DOPE/DOTAP [1,2-dioleoyl-3-trimethylammonium-propane]) and that, depending on the charge ratio and the lipid composition, the complex size varies significantly [12,13]. Concerning the morphological aspect, the formation of a complex at the surface of positively charged vesicles (DOPE/DOTAP/PE-PEG [phosphatidyl ethanolamine-*N* [poly (ethylene glycol)]) has been evidenced by freeze-fracture electron microscopy [13,16].

While only few reports are available concerning the physico-chemical characterization of cationic liposomes/ON complexes, none have dealt with the association of ON with cationic emulsions. In the present paper, the properties of the complexes formed between our new cationic-emulsions and a model oligohexadecathymidilate (pdT_{16}) were investigated through size, electron cryomicroscopy, fluorescence resonance energy transfer and surface tension experiments. Starting from a typical emulsion containing stearylamine as a cationic lipid, the influence of the lipid

structure (monocationic molecule bearing mono or diacyl chains, or polycation), as well as of the presence of PEGylated lipids, were studied. The final aim was to have a better insight on the nature of the interactions between ON and the interfacial lipids of the emulsions, as well as on the supramolecular assemblies induced by these interactions, in order to be able to better understand the mechanism involved in the ON release from these different emulsions [10].

2. Materials and methods

2.1. Chemicals

Samples of pdT_{16} and pdT_{16}-rhodamine (pdT_{16}-rho) were purchased from Eurogentec (France). 5′-End labeled ^{33}P-pT_{16} was synthesized using T4 polynucleotide kinase (Biolabs, UK) and ^{33}P ATP (Isotopchim, France). Submicron emulsions were prepared using medium-chain triglycerides (MCT), mainly composed of acylglycerides of C_8–C_{12} (Societé des Oleagineux, St. Laurent, Blangy, France), Lipoid E-80®, composed of egg phosphatidylcholine (PC) (Lipoid Ag, Ludwigshafen, Germany), poly (oxyethylene)-*b*-poly(oxypropylene)-*b*-poly (oxyethylene) poloxamer 188® (BASF, Ludwigshafen, Germany), DSPE-PEG 1,2-distearoyl-*sn*-glycero-3-phosphatidyl ethanolamine-*N* [poly (ethylene glycol)2000] (Avanti polar lipids, USA), DSPE (1,2-distearoyl-*sn*-glycero-3-phosphatidyl ethanolamine), Stearylamine (SA) and DOTAP [1,2-dioleoyl-3-trimethylammonium-propane] (Sigma, MO, USA). The chemical structures of the different cationic lipids used are presented in Fig. 1. The fluorescent dye NBD-PE [*N*-(7-nitrobenz-2oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine] was purchased from Molecular Probes (USA). The polycation RPR 122535 (RPR C_{18}) [15] was kindly provided by UMR CNRS — Aventis (Vitry-sur-Seine, France).

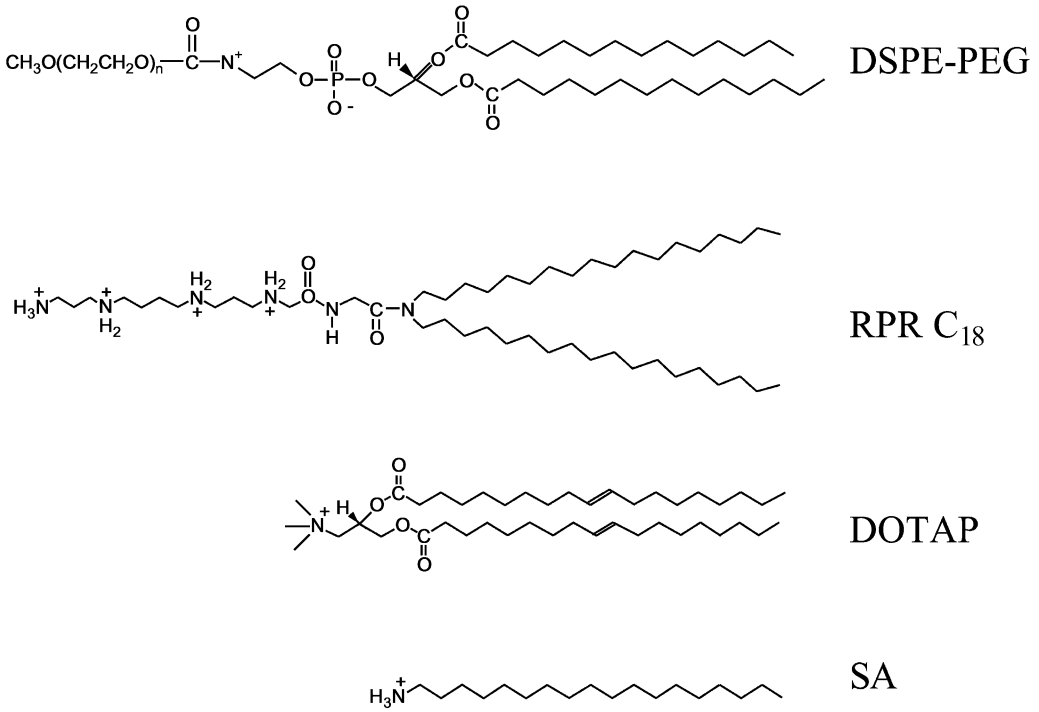

Fig. 1. Structure of the lipids used in the preparation of submicron emulsions.

2.2. Methods

2.2.1. Emulsions preparation and characterization

The submicron emulsions were prepared according to previously described procedure [8]. 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 (M100s, Microfluidics Corp., Moizon, France) under four microfluidization cycles at 4-bar pressure. The exact composition of the different formulations (%, w/w) are presented in Table 1. The droplet size distribution of the emulsions were estimated by photon correlation spectroscopy using a nanosizer (Nanosizer ND4, Coultronics, France). The samples were diluted in glycerol (2.25%) and analyzed at 20 ± 2°C.

2.2.2. Oligonucleotides association to submicron emulsions

The test for pdT_{16} association was performed at the end of the manufacturing process. Various volumes of an aqueous solution of pdT_{16} and a trace of ^{33}P-pdT_{16} (radiolabeled pdT_{16} was synthesized as described by Lappalainen et al. [7]) were added to 1 ml of the emulsions and incubated for 12 h at room temperature. Free pdT_{16} (in the external aqueous phase of the emulsion) was determined after separation by ultrafiltration/centrifugation (5 min at $7950 \times g$ through a porous membrane 30 000 Da cut-off, Ultrafree Millipore, USA) and counting of the radioactivity in the clear ultrafiltrate by liquid scintillation (Beckman LS 6000TA). Under these conditions, membrane binding and rejection was calculated for pdT_{16} aqueous solutions (1–100 μM) and the percentage recovery was 90%. The amount of ^{33}P-pdT_{16} in the whole emulsion was also determined by liquid scintillation so that the amount of ON associated with the emulsion could be obtained as follows:

$$ON_{ass}\% = \frac{ON_{total} - ON_{free}}{ON_{total}} \times 100$$

2.2.3. Cryomicroscopy experiments

A drop of the emulsion was deposited on an air glow-discharged grid coated with a perforated carbon film [16]. 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 micro-

Table 1
Composition of submicron emulsions obtained by microfluidization (%, w/w)

	PC	PC/SA	PC/DOTAP	PC/RPR C_{18}	PC/SA/PEG
PC[a]	2.00	2.00	2.00	2.00	1.82
DSPE-PEG[b]	–	–	–	–	0.18
Stearylamine[c]	–	0.5	–	–	0.5
DOTAP[c]	–	–	1.32	–	–
RPR C_{18}[c]	–	–	–	0.543	–
MCT to	10.00	10.00	10.00	10.00	10.00
Poloxamer 188	1.68	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 (egg-PC).
[b] Represent 2.5 mol.% of total phospholipid content.
[c] Represent 1.12×12^{21} charges per 100 ml of emulsion.

scope operated at 100 kV. Micrographs were recorded at a magnification of 35 000 on a Kodak image plate S0 163 and developed in 12 min in D19 full strength.

2.2.4. Fluorescence resonance energy transfer (FRET)

To investigate the localization of the ON associated with the emulsions, FRET studies have been performed using NBD-PE probe and pdT_{16} labeled at the 3′ end with rhodamine (pdT_{16}-rho). Prior to emulsion preparation, NBD-PE was incorporated into the oil phase (1 mol.% of PC). pdT_{16}-Rho was added to the final emulsions containing NBD-PE under a slight stirring into 96-well plates at the same fluorophore molar ratio. Thus, fluorescence spectra were recorded with an ultraviolet/visible microspectrofluorometer, as described elsewhere [17,18]. Briefly, excitation was achieved by an Ar^+ laser at 488.0 nm. A 100× objective and a luminous field diaphragm were used on the excitation path to focus the laser beam on a spot less than 2 μm in diameter. Fluorescence spectra were recorded in the region of 500–750 nm on a 1024-diode-intensified optical multichannel analyzer (Princeton Instruments). The beam power was reduced to approximately 0.1 μW by the use of neutral density filters.

Fluorescence energy transfer was evaluated by the fluorescence intensity ratio at 520 vs. 575 nm for each emulsion containing the labeled ON. Control experiments were performed by separately incubating the emulsions containing the NBD-PE probe on one hand, and the pdT_{16}-rho with the non-fluorescent emulsion on the other hand. Fluorescence intensities (FI) were recorded and the values were added so that a theoretical spectrum was obtained, allowing us to calculate a theoretical fluorescence intensity ratio (FIR) in conditions of absence of transfer. Experiments were repeated three times.

2.2.5. Surface tension measurements

The Wilhelmy plate method, as previously described [19] was used for all surface tension measurements at constant area. The surface pressure π was deduced from the $\pi = \gamma_0 - \gamma_M$ relationship, where γ_0 is the surface tension of the aqueous solution and γ_M is the surface tension in the presence of a spread or an adsorbed monolayer. The thin platinum plate was attached to a force transducer (Hewlett Packard). The measurements were taken without detaching the plate from the interface and the data were continuously plotted on a calibrated chart recorder. All reported surface tension values are the means of at least three measurements. The accuracy of the experiments was estimated to ± 0.1 mN/m. In adsorption experiments, pdT_{16} molecules were injected into the aqueous subphase (2.25% glycerol) through a side arm at 45° to the vertical and the interaction of the ON with the spread lipids was studied at two initial surface pressures, $\pi_i = 12 \pm 1$ mN/m and $\pi_i = 25 \pm 3$ mN/m, corresponding to expanded and condensed lipid monolayers, respectively, as previously established in preliminary compression isotherms (data not shown). The change in surface pressure ($\Delta\pi$) was calculated as an increment in surface pressure due to pdT_{16} adsorption into the monolayers. For each experiment, $\Delta\pi_{max}$ was defined as the maximum surface pressure change observed at equilibrium after the injection of 12.5 μM pdT_{16} in the subphase. The aqueous solution was prepared from ultrapure water obtained by osmosis from a Milli-RO6 Plus Millipore apparatus and then doubly distilled from permanganate solution.

3. Results

3.1. Physico-chemical characterization and pdT_{16} association efficiency

First, the optimization of the microfluidization process was performed [8]. After four microfluidization cycles at 4-bar pressure, this technique yielded monodisperse emulsions with a typical mean droplet size of 150–180 (±50 nm), as seen in Fig. 2a. pdT_{16} Association has been performed at the end of the manufacturing process. For all the concentrations (up to 125 μM), no significant changes in the size or the zeta potential (Fig. 2b) could be detected, except for the emulsion containing DSPE-PEG (PC/SA/PEG). Indeed, in this case, size progressively increased with ON

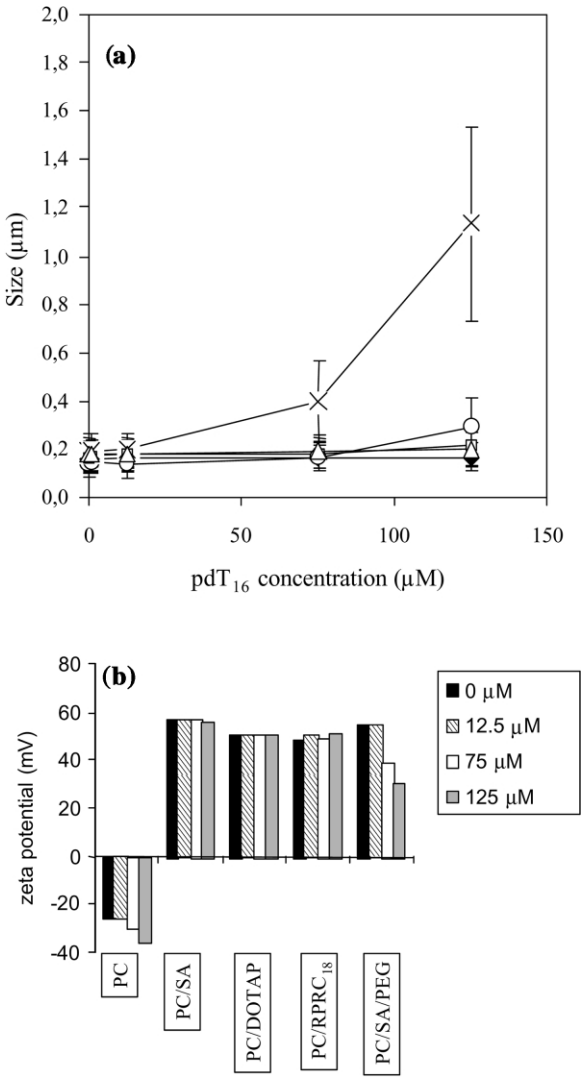

Fig. 2. Size variation (a) and zeta potential (b) along with pdT_{16} concentration. Key: (♦) PC; (○) PC/SA; (X) PC/SA/PEG; (□) PC/DOTAP; and (△)PC/RPR C_{18}.

concentration reaching more than 1 μm at 125 μM. The amount of ON associated with cationic submicron emulsions was close to 100% up to 125 μM ON: no ^{33}P-pdT_{16} radioactivity was found in the external aqueous phase after separation by ultrafiltration/centrifugation procedure. In contrast, for PC emulsions, negatively charged (Fig. 2b), the association efficiency was only 80% at 12.5 μM and decreased to approximately 70% at 125 μM ON [8].

3.2. Cryomicroscopy experiments

Fig. 3 shows electron micrographs of the cationic emulsions before and after ON association. In contrast to the PC/$RPRC_{18}$-emulsion, the formulations containing SA (PC/SA and PC/SA/PEG) as a cationic lipid display numerous bicompartmental structures, referred to as hand-bag-like structures [20]. The addition of ON (12.5 μM) did not significantly change the appearance of PC/SA emulsions (Fig. 3a,b). Conversely, the addition of ON to the PC/SA/PEG-emulsion induced the aggregation of constituting objects (Fig. 3c,d). During aggregation, the interaction between the droplets was strong enough to change the curvature of the droplets (arrow). Finally, in the case of PC/$RPRC_{18}$, an intermediate situation was found, intact oil droplets being ranged in strings (arrow) of 10–15 pieces when ON was added (Fig. 3f). PC and PC/DOTAP emulsions showed individualized spherical droplets of a typical emulsion, which were not modified by the presence of ON (data not shown).

3.3. Fluorescence energy transfer experiments (FRET)

Fluorescence energy transfer was evaluated by the fluorescence intensity ratio at 520 vs. 575 nm. In the case of fluorescence energy transfer, this ratio tends to decrease since the peak at 575 nm will proportionally increase. As pointed out in Fig. 4, the theoretical values (FIR_{the}) varied from one formulation to the other, and this must be related to the sensitivity of the NBD-PE probe to its environment, each cationic lipid being susceptible to modify the micro-pH close the interface. Concerning the experimental fluorescence intensity ratio (FIR_{exp}), as shown in Fig. 4, the values were found to be lower than the corresponding theoretical ones in PC/SA, PC/DOTAP and PC/$RPRC_{18}$ cationic emulsions, but were found to be unchanged in the case of PC-emulsion and PC/SA/DSPE-PEG, suggesting that fluores-

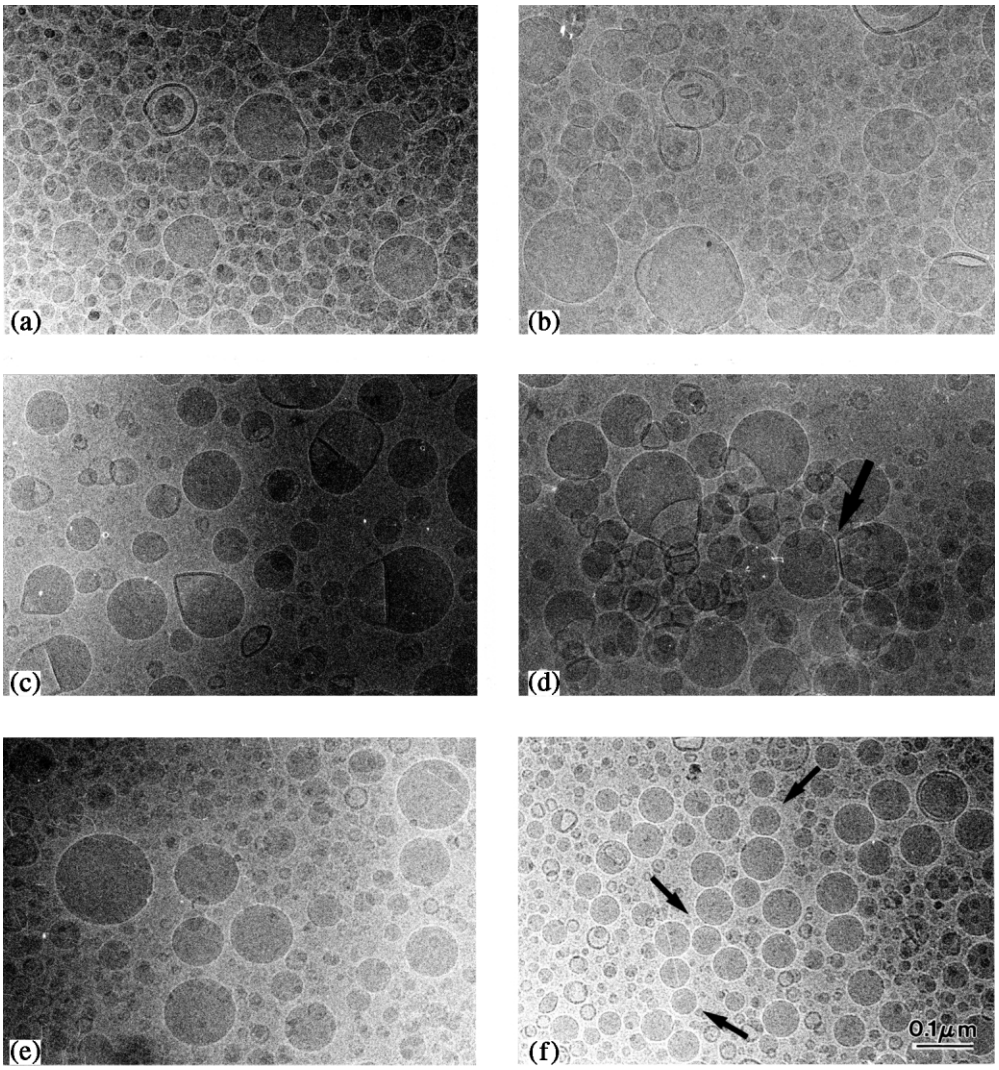

Fig. 3. Cryo-TEM micrographies of cationic emulsions before (left) and after (right) pdT_{16} association at 12.5 μM. Key: PC/SA (a,b); PC/SA/PEG (c,d); and PC/$RPRC_{18}$ (e,f).

cence energy transfer only occurred with PC/SA, PC/DOTAP and PC/$RPRC_{18}$ emulsions.

3.4. Surface tension measurements

Fig. 5 shows that in the absence (free interface) or in the presence of a PC monolayer, pdT_{16} injected into the subphase at the concentrations up to 125 μM had a very limited effect on the surface tension. Conversely, when the surface was covered by a PC/SA monolayer ($\pi_i = 12$ mN/m), pdT_{16} injection induced a significant increase in the surface pressure ($\Delta\pi_{max} = 6$ mN/m for 37.5 μM pdT_{16}).

The $\Delta\pi_{max}$ (maximum surface pressure change) following pdT_{16} adsorption into the different lipid monolayers was then studied at an ON concentration of 12.5 μM in the subphase, because it corresponded to the ON concentration used in the release experiment studies [10]. In Fig. 6, the

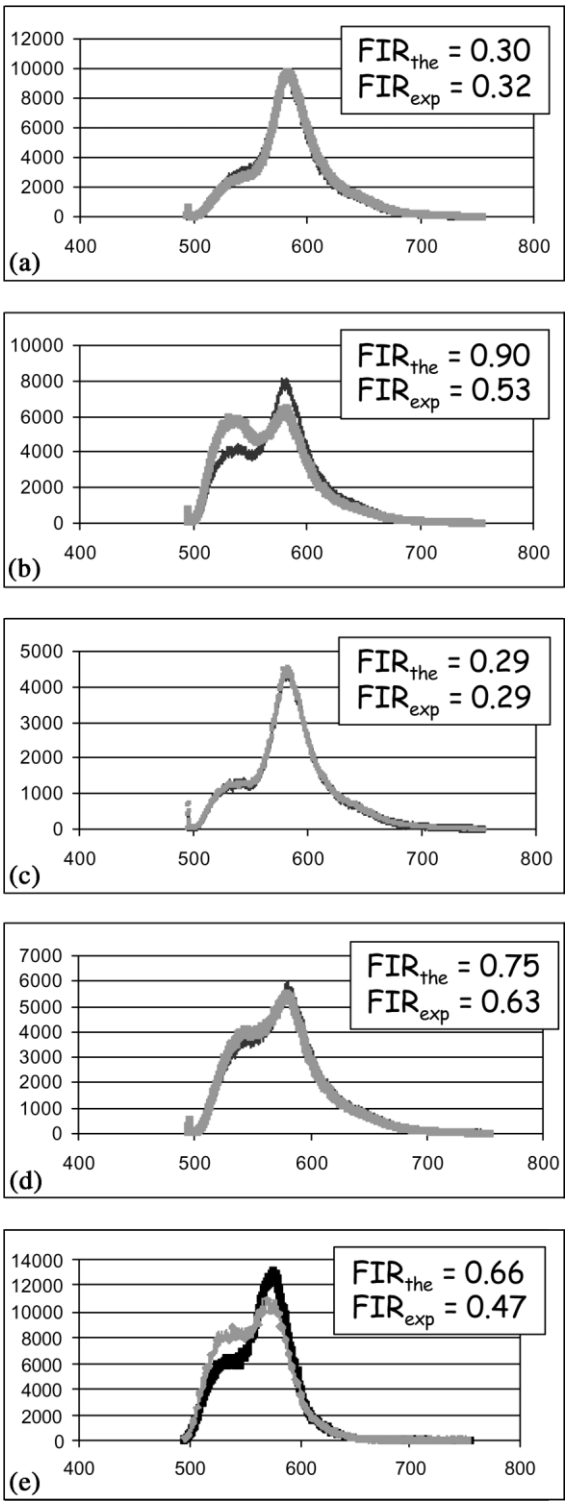

Fig. 4.

changes in surface pressure induced by pdT_{16} injected beneath pure PC, SA, DOTAP and $RPRC_{18}$ monolayers ($\pi_i = 12$ mN/m) are plotted against time. Whereas ON injection in the subphase had almost no effect on the surface pressure of a PC monolayer, a very fast increase in $\Delta\pi$ was observed when the ON was injected beneath a monolayer formed of cationic lipids such as SA or DOTAP. With those lipids, almost 70% of the maximum surface pressure change ($\Delta\pi_{max}$) was reached in less than 30 min following pdT_{16} injection. Inflexion points could be observed at approximately 300 min in the case of both PC and DOTAP monolayers. These might correspond to the final rearrangement of the ON molecules driven towards the interface, with the establishment of hydrophobic interactions. It must be noted that in both these cases, the nature of the acyl chain was quite similar (unsaturated, thus fluid at ambiant temperature). Conversely, the adsorption of pdT_{16} into the $RPRC_{18}$ monolayer was weak and was rapidly followed by molecule desorption from the monolayer as inferred from the initial increase in $\Delta\pi$ and then its decrease towards zero, suggesting that ON/polycationic amphiphile complexes were driven towards the water phase.

Similar features were observed when pdT_{16} was injected beneath mixed PC/SA, PC/DOTAP and PC/$RPRC_{18}$ monolayers at $\pi_i = 12$ mN/m (Fig. 7a). However, when the surface density was increased ($\pi_i = 25$ mN/m) the $\Delta\pi$ values appeared to be lower and even negative in the case of PC/$RPRC_{18}$ monolayers.

The presence of 2.5 mol.% DSPE-PEG in PC monolayer, led to an increase of $\Delta\pi_{max}$ reaching ~ 2 mN/m; when SA was added to this mixed

Fig. 4 Fluorescence intensity spectra obtained at $t = 3$ h from different emulsions containing NBD-PE as a fluorescent probe and incubated with pdT_{16}-rho at 5.6 μM. FIR is the fluorescence intensity ratio I_{520}/I_{575}. Theoretical values were obtained after the addition of the fluorescence intensity of an emulsion sample on the one hand, and the ON sample on the other hand separately, prepared under the same conditions as the mixed emulsion/ON samples. Grey: theoretical curve; black: experimental curve. (a) PC; (b) PC/SA; (c) PC/SA/PEG; (d) PC/DOTAP; and (e) PC/RPR C_{18}.

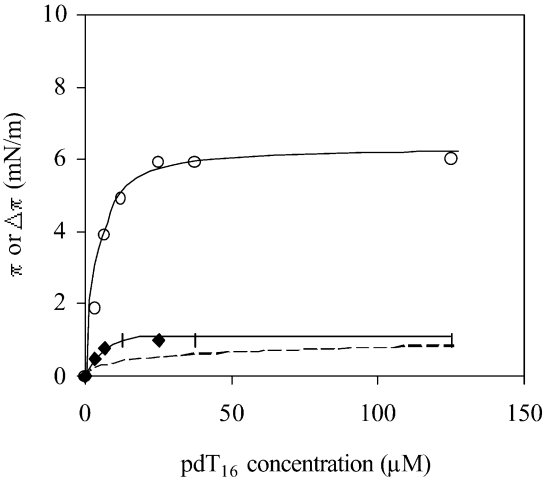

Fig. 5. Surface pressure changes ($\Delta\pi$) as a function of ON concentration in the subphase, in the absence (dashed line) and in the presence of a PC or PC/SA monolayer. Key: (♦) PC and (○) PC/SA.

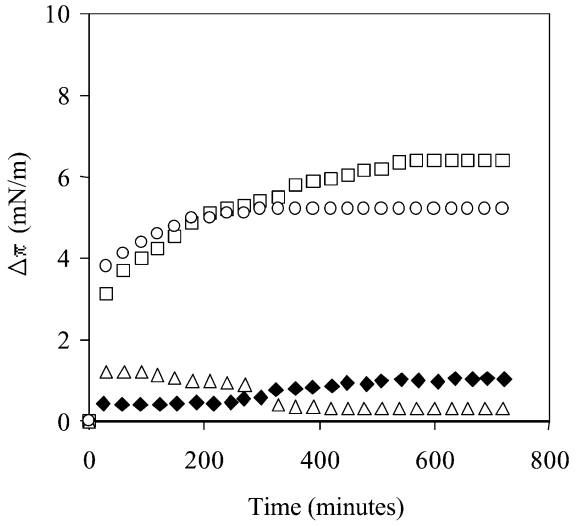

Fig. 6. Surface pressure changes ($\Delta\pi$)-time relationships after ON injection beneath pure lipid monolayers at the initial surface pressure (π_i) of 12 ± 1 mN/m. Key: (♦) PC; (○) SA; (□) DOTAP; and (△) RPR C_{18}.

monolayer, $\Delta\pi_{max}$ reached ~ 4 mN/m (Fig. 7b). For PC/PEG and PC/SA/PEG mixed monolayers, in a condensed state, $\Delta\pi_{max}$ decreased reaching negative values of approximately -1 and -3 mN/m, respectively. In comparison, the results obtained with PC/DSPE mixed monolayers indicated some contribution of the DSPE part of the PEGylated lipid in the overall interaction of PC/PEG with ON at low initial pressure, but not at a higher one ($\Delta\pi_{max} = 0.2$ mN/m).

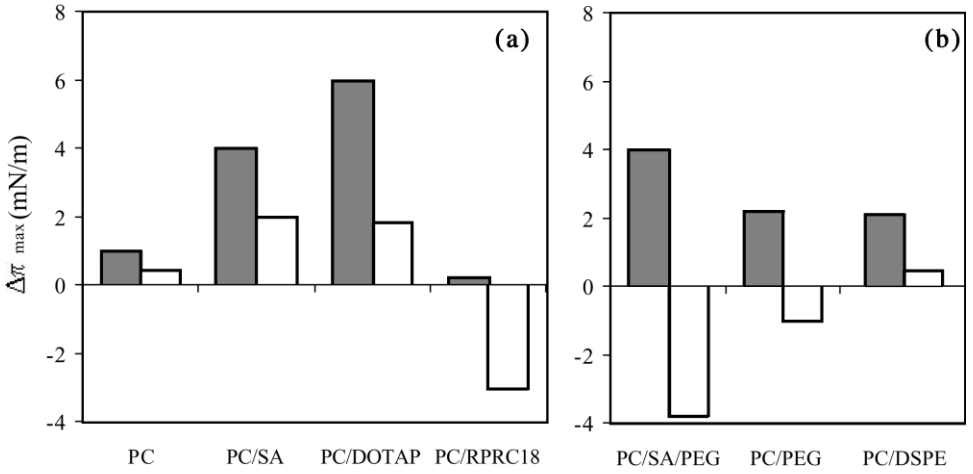

Fig. 7. Maximum surface pressure change ($\Delta\pi_{max}$) induced by pdT_{16} (12.5 μM) injected beneath pure and mixed monolayers. Key: (a) PC, PC/SA, PC/DOTAP, PC/$RPRC_{18}$; and (b) PC/SA/PEG, PC/PEG and PC/DSPE. Grey: initial surface pressure $\pi_i = 12$ mN/m, white: initial surface pressure $\pi_i = 25$ mN/m.

4. Discussion

We have previously shown that triglyceride-based cationic emulsions may be efficient systems for the delivery of ON to cancer cells (submitted). However, it was observed that ON stability and the release profile were most dramatically influenced by the nature of the ON/lipid interactions [10], which must be investigated. This has been done in this paper by FRET, surface tension measurements and cryomicroscopy.

4.1. FRET experiments

FRET experiments clearly evidenced that energy transfer between the two probes (pdT_{16}-rho and NBD-PE) could only occur with the cationic emulsions and not with the single PC-formulation. The presence of a positive charge on the droplet surface clearly contributed to bring the polyanionic ON molecules closer to the interface. Thus, ON/SA ON/DOTAP and ON/$RPRC_{18}$ ion-pairs should be localized between the interfacial film. The addition of 2.5 mol.% DSPE-PEG to the composition of the film led, however, to the absence of transfer because of the steric hindrance induced by the PEG chains, reducing ON/SA interactions. In these emulsions (PC/SA/PEG), ON molecules will interact with both SA and PEG chains, as evidenced by surface tension measurements at low initial surface pressure (Fig. 7b). Results obtained at a high initial surface pressure even suggested that ON interacted strongly with PEG and led to a very hydrophilic complex which tended to desorb from the interface. Thus, it results that in the final PC/SA/PEG emulsion, ON localize less close to the interface than in single PC/SA emulsions. All the experiments were carried out at 12.5 M ON so that the results obtained could be correlated to the ON release kinetics described previously [10]. In the case of PC/SA/PEG, ON was indeed immediately released after the dilution of the emulsion by 1/25 in PBS or in culture medium. This observation is consistent with the fact that ON molecules only partially interact with SA and remain partially free in the vicinity of the PEG chains, thus being less tightly associated to the droplets than in PC/SA emulsions.

4.2. Surface tension measurements: case of SA and DOTAP

The investigation of the interfacial behavior of pdT_{16} at the free air/solution interface has evidenced the weak surface properties of this homopoly (nucleic acid). The same feature has already been reported by Blanc et al. [21] with a slightly longer ON sequence.

Whereas no significant increase in surface pressure was observed after ON injection beneath a monolayer solely composed of a zwitterionic lipid (PC), an important and almost immediate increase in $\Delta\pi$ occurred when the monolayer was formed by a monocationic lipid, such as SA or DOTAP. The increase in $\Delta\pi$ was attributed to the adsorption of the ON into the monolayer, favored by electrostatic interactions between the positively charged lipid head groups and the negatively charged ON. Evidences of such electrostatic interactions have been largely described in the literature [12–14]. A comparison between Figs. 6 and 7 shows that the dilution of these lipids by PC molecules (mixed monolayers) at low initial surface pressure ($\Delta\pi\iota = 12$ mN/m) led to slightly lower $\Delta\pi$ values, which may be explained by the decrease in charged interacting sites for ON at the interface. The results obtained at a higher initial pressure (25 mN/m) account for a still weaker ON adsorption due to the reduction of the available space between lipid molecules at the interface, which demonstrate that pdT_{16} interaction with monocationic lipid monolayers is controlled by an adsorption/penetration mechanism.

However, we must point out the occurrence of another contribution to ON interaction in the case of DOTAP (see Fig. 6) or PC/DOTAP (see Fig. 7) monolayers. The higher $\Delta\pi_{max}$ values observed could be explained by the existence of additional hydrophobic interactions due to the presence of a double acyl chain in DOTAP compared to SA. Nevertheless, we cannot exclude the fact that it might also be due to the nature of the

acyl chains themselves: unsaturated for DOTAP, saturated for SA. Egg PC is in a liquid state at ambiant temperature and it may be suggested that SA will decrease the interface fluidity, even leading to some phase separation in extreme conditions. On the contrary, DOTAP will probably have a lesser influence on fluidity and lateral phase separation.

4.3. Surface tension measurements: case of $RPRC_{18}$

The interaction of the ON with the polycationic $RPRC_{18}$ (see Fig. 6) produced only a slight increase in the surface pressure followed by its gradual decrease. If the increase in surface pressure may be due to electrostatic attraction between the ON and the polycationic lipid, the subsequent decrease in $\Delta\pi$ values seems to indicate a progressive desorption of the ON or the ON/lipid complexes from the interface.

In the presence of a mixed $PC/RPRC_{18}$ monolayer (Fig. 7), higher $\Delta\pi_{max}$ values were found compared to the pure $RPRC_{18}$ monolayer. This comportment is consistent with the previously reported hydrophilic character of this lipid [22]. The $RPRC_{18}$ molecules that have a tendency to rapidly exchange between the interface and the bulk phase should be stabilized at the interface by hydrophobic interactions with the neighboring phospholipids (PC). The complexes formed with ON molecules will, therefore, also have a longer residence time at the interface in the case of the mixed $PC/RPRC_{18}$ monolayer compared to a pure $RPRC_{18}$ one. At a high surface pressure, the decrease in π led to negative values, which implies that ON desorption was accompanied by the sweeping of lipid molecules away from the interface into the subphase.

Finally, two opposite trends can be found depending on the lipids. In the case of DOTAP and SA, pdT_{16} interacted with positively charged lipid polar headgroups and this interaction drove the ON molecules towards the interface and stabilized them into the monolayer. In the case of $RPRC_{18}$, ON also reached the interface. However, since the positive charges of $RPRC_{18}$ were all localized into the subphase, perpendicularly to the surface, ON did not penetrate the interface and rapidly desorbed.

If we extrapolate this comportment to the emulsion itself, we must suggest that, in the case of $PC/RPRC_{18}$, the ON molecules would not be associated with the emulsion droplets but with lipid aggregates co-existing with the emulsion. These aggregates would be composed of cationic lipids, but also of the zwitterionic lipids (PC), since FRET experiments have shown a close proximity between the ON molecules and the interfacial lipids. In addition, the size of these aggregates should be very close to the size of the emulsion since no bimodal distribution could be seen in photon correlation spectroscopy, and also because no diffusion of pdT_{16} molecules could be measured across a 0.1-μm porosity membrane in the ultrafiltration/centrifugation procedure [10]. In order to assess the existence or not of such lipidic aggregates, an additional cryomicroscopy study has been carried out.

4.4. CryoTEM (cryotransmission electron microscopy)

No extra structures could be found among the oil droplets, but interesting comportments of the emulsions in the presence of ON were revealed. Indeed, whereas PC/SA and PC/DOTAP droplets remained morphologically unchanged after ON addition, $PC/RPRC_{18}$ droplets organized in strings of various lengths. This observation could be correlated with an obvious increase in the sample viscosity, also increasing with the ON concentration. If the droplets tend to aggregate, we must admit that some weak bridges may form. We previously mentioned that the conformation of the polycationic amphiphile at the interface will induce a peculiar organization of the ON molecules, with some part exposed to the water environment. It is not out of question that some phosphate groups along the pdT_{16} chain remained free, able to interact with neighboring cationic droplets.

The more ON molecules will be distant from the interface, the more numerous phosphate groups will remain free, and the more the droplets

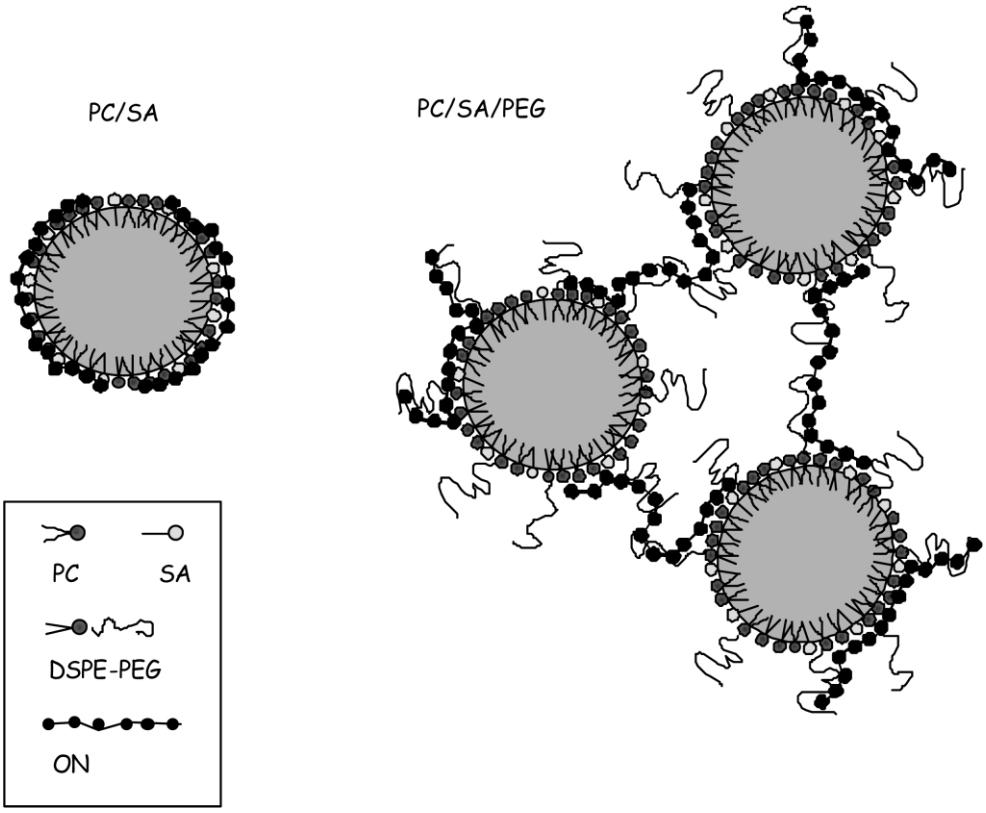

Fig. 8. Schematic representation of the arrangement between droplets in the case of ON-containing PC/SA and PC/SA/PEG emulsions (for the purpose of the cartoon, ON molecules were drawn at an excessive length compared to the droplet size).

will be able to aggregate, as it can be observed in the case of PC/SA/PEG (Fig. 8). In these emulsions indeed and as already mentioned previously, ON only partially interact with SA at the interface and many phosphate groups must be free in the vicinity of PEG chains, favoring the formation of strong bridges between neighboring droplets as seen in cryomicroscopic profiles and attested by size determinations.

This study emphasizes the interest of physicochemical surface studies in elucidating the nature of the ON/lipid interactions at the molecular level. Indeed, monolayers are an appropriate model in the case of emulsions since the droplets are stabilized by a monomolecular interfacial film. However, the presence of oil in the emulsions in which the phospholipids can anchor instead of the air in the surface tension experiments may explain the observed discrepancies when extrapolating to supramolecular structures. This reinforces the interest of FRET and cryomicroscopy in order to have a better insight into the supramolecular assemblages formed between ON and the constituting lipids.

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