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Submicron Cationic Emulsions as a New Delivery System for Oligonucleotides

H. Teixeira,[1] C. Dubernet,[1,3] F. Puisieux,[1] S. Benita,[2] and P. Couvreur[1]

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Purpose. The main purpose of the present study was to investigate submicron emulsions as potential oligonucleotide (ON) delivery system.

Methods. Submicron emulsions containing various concentrations of stearylamine (SA) were prepared by microfluidization. After association with model oligothymidylates, these emulsions were characterized in terms of particle size, ζ-potential, association efficiency and release upon dilution. The interactions between ON and SA were investigated by partitioning studies between water and oily phases, with ON of three different lengths (pdT_{16}, pdT_{30}, pdT_{50}). The stability of pdT_{16} in the presence of nucleases was evaluated by incubation in cell culture medium supplemented with 10% of foetal calf serum.

Results. The ON association efficiency was much higher with emulsions containing SA (E_{SA}) than with control emulsions (E_0), whatever the ON length. In addition, E_{SA} was shown to protect ON against degradation for up to 3 hours in culture medium. ON and SA were able to form ion-pairs and the resulting complex was found to be insoluble both in water and in oil. Zeta potential was maintained constant when increasing the ON concentration, until flocculation occurred (up to 250μM in the case of pdT_{16} for example). This has been explained by the presence of SA in excess, soluble in the oily core of droplets, able to migrate towards the interface and replacing SA neutralized in ion-pairs.

Conclusions. E_{SA} appears to be a valuable system for delivery of ON and might even be improved by selecting an oily phase in which the SA/ON complex would be soluble.

KEY WORDS: oligonucleotides; cationic emulsions; stearylamine; partitioning.

INTRODUCTION

During the last decade there has been a considerable increase in interest of antisense oligonucleotides (ON) molecules in regards to correction of genetic diseases, suppression of oncogenes and eradication of viral infections (1). However, ON-based therapy is compromised by rapid degradation of ON in biological fluids and by their inability to efficiently cross cellular membranes due to their hydrophilic and polyanionic character and to their large molecular structure (1).

The association of ON to lipid- or polymer-based colloidal carriers as liposomes (2) and nanoparticles (3,4) can circumvent these drawbacks. However, the cellular uptake of these carriers is mainly via endocytosis pathway and, their ultimate fate is therefore to be degraded in the lysosomes. Another promising approach consists in associating ON to cationic liposomes which can form complexes with polyanionic ON. As an example, ON associated to cationic liposomes were shown to be delivered into the cytoplasm at an early stage of the endocytotic pathway with a marked increase of antisense activity (5) and an efficient protection against nuclease attack (6). However, the instability of the ON/liposomes complex, and its interactions with plasma components, as opsonins, limit the cellular delivery of ON *in vivo* (7).

Elbaz *et al.* (8) have described a positively charged submicron emulsion based on lecithin, medium-chain triglycerides, poloxamer 188 and stearylamine. These systems exhibit prolonged stability because of steric stabilization due to poloxamer molecules, and also because of the electrostatic repulsion between the surface charged nanodroplets. In addition, the potential toxicity of these positively-charged emulsions has been assessed: they are accepted as both intravenous and ocular delivery systems (9). For all these reasons, such cationic emulsions appeared to be a good potential carrier for ON delivery.

In this paper, we have used oligothymidylates of various lengths as model oligonucleotides, and we have investigated the potential of these submicron emulsions in terms of association efficiency, ON partitioning and protection in culture medium.

MATERIALS AND METHODS

Chemicals

Oligothymidylates were purchased from Pharmacia (pdT_{16}) (France) and Eurogentec (pdT_n and dT_n with n = 16, 30 and 50) (France). 5′-end labeled $^{33}P\text{-}dT_n$ was synthesized using T4 polynucleotide kinase (Biolabs, U.K.) and ^{33}P ATP (Isotopchim, France). Submicron emulsions were prepared using Medium-chain triglyceride (MCT) (Societé des Oleagineux, St. Laurent, Blangy, France), Lipoid E-80® (Lipoid Ag, Ludwigshafen, Germany), Polyoxyethylene-polyoxypropylene, poloxamer 188® (BASF, Ludwigshafen, Germany), α-tocopherol (Fluka) and Stearylamine (Sigma MO, USA).

Methods

Oligonucleotide Radiolabeling

The 5′- radiolabeled oligothymidylate (n = 16, 30, and 50) were obtained as follows: 5 μl of a solution of dT_n (10 μM), 1 μl of T4 polynucleotide kinase and 1 μl of $\beta\text{-}^{33}P$-ATP (sp act 7.4 Bq/mmol) were incubated for 30 min at 37°C. The reaction was stopped by heating at 80°C for 15 min. $^{33}P\text{-}pdT_n$ was recovered after purification by exclusion chromatography and centrifugation at 2400 rpm for 1 min. The purity of the $^{33}P\text{-}pdT_n$ was evaluated using an automatic TLC-linear analyzer as previously reported (10).

Emulsion Preparation

The submicron emulsions were prepared according to previously described procedures (8). Briefly, the oil and aqueous

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[1] Laboratoire de Physico-chimie, Pharmacotechnie et Biopharmacie, URA CNRS 1218, Faculté de Pharmacie, Université Paris XI, 5, Rue J.B. Clément, 92296 Châtenay-Malabry, France.

[2] Pharmaceutics Department, School of Pharmacy, The Hebrew University of Jerusalem, PO Box 12065, Jerusalem. Affiliated with the David R. Bloom Center for Pharmacy at the Hebrew University of Jerusalem.

[3] To whom correspondence should be addressed. (e-mail: catherine.dubernet@cep.u-psud.fr)

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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 different operating conditions (4-bar pressure, two to ten cycles). The typical formulations (%, w/w) consisted of MCT 8.5, Lipoid E-80 2.0, α-tocopherol 0.02, poloxamer 1.68, glycerol 2.25, stearylamine 0.5 (E_{SA}), 0.25 ($E_{SA/2}$), 0.06 ($E_{SA/8}$) or 0 (E_0) and MilliQ® water up to 100.

Oligonucleotides Association to Submicron Emulsions

Oligonucleotide association was performed at the end of the manufacturing process. Varying volumes of an aqueous solution of ON were added to 1 ml of submicron emulsions and incubated for two hours at room temperature. The concentration of each of the three ON solutions ($pdT_{16,\ 30,\ 50}$) was adjusted in order so the added volume did not exceed 50 μl. Except in the cases where a flocculation occurred, free pdT_n was determined in the clear ultrafiltrate obtained after centrifugation (5 minutes) of the emulsion at 7950 g through a porous membrane (30.000 Da cut off, Ultrafree MC Millipore, Bedford, U.S.A.). The amount of radiolabeled oligonucleotide in the whole emulsions and in the ultrafiltrates was determined by liquid scintillation (Beckman LS 6000TA). In these conditions, membrane binding and rejection were calculated for a pdT_{16} aqueous solution (1 to 100 μM) and the percent recovery was around 90%.

Droplet Size Analysis and ζ-Potential Determinations

The droplet size distribution and the ζ-potential of the emulsions were estimated by photon correlation spectroscopy using a nanosizer (Nanosizer ND4, Coultronics, France) and a Malvern Zetasizer® (Malvern Instruments, U.K.), respectively. The samples were diluted 200-fold in glycerol 2.25% for size determinations or in water for ζ-potential measurements, and analyzed at 20 ± 2°C.

Calculation of the Surface Available for Lipid Molecules at the Interface

The theoretical surface available per stearylamine molecule in non-loaded E_{SA}, $E_{SA/2}$ and $E_{SA/8}$ has been calculated. For this purpose, it has been postulated that all the phospholipids and the stearylamine were localized at the interface and that every molecule occupied the same area, which is of course a rough approximation.

If S_{TOT} is the total area of the interface developed in 1 ml of emulsion:

$$S_{TOT} = (4\pi r^2) \cdot V_{TOT}/(4/3\ \pi r^3)$$

with the mean droplet radius r = 83.5, 78.5 and 74 nm for E_{SA}, $E_{SA/2}$ and $E_{SA/8}$, respectively, and V_{TOT} = 100 μl of oil per ml of emulsion.

If M_{SA} and M_{PC} are the number of molecules of stearylamine and phospholipids respectively, in 1 ml of emulsion, the theoretical surface per lipid molecule (S_{LIP}) will be:

$$S_{LIP} = S_{TOT}/(M_{SA} + M_{PC}) = S_{SA}$$

with the numerical values for M_{PC} and M_{SA} being:

$$M_{PC} = 16.02 \times 10^{18} \text{ and } M_{SA}$$

$$= 11.17 \times 10^{18}, \text{ or } 5.58 \times 10^{18}, \text{ or } 1.34 \times 10^{18}$$

for E_{SA}, $E_{SA/2}$, and $E_{SA/8}$, respectively.

When needed, the SA mole fraction has been calculated as equal to $M_{SA}/(M_{SA} + M_{PC})$, and the values obtained were 0.41, 0.26 and 0.08 for E_{SA}, $E_{SA/2}$, and $E_{SA/8}$, respectively.

Stability over Storage and Dilution

In order to evaluate the ability of ON to be released upon dilution, emulsions (E_0 and E_{SA}) loaded with pdT_{16} (1 μM) were diluted (1:200, 1:500 and 1:1000) either in water containing 2.25% of glycerol or in PBS buffer at 37°C. After 30 min stirring, the medium was filtered off and free pdT_{16} was determined as described above. The effects of storage time were assessed by droplet size measurements, ζ-potential determinations and free pdT_{16} assay, after four months of storage at 4°C.

Partitioning Studies

SA was dissolved in MCT or *n*-octanol at different concentrations (0.01 to 2.5 mg/ml) after a slight heating. 1 ml of the resulting organic phase at room temperature was then added to 1 ml pdT_n aqueous solution (1 μM) (n = 16, 30, and 50). The samples were mixed vigorously by vortexing for 1 minute, and centrifuged at 500 g for 5 minutes at room temperature. Aliquots (100 μl) of the upper *n*-octanol or MCT phase, and of the lower aqueous phase, were removed and the amount of pdT_n in each phase was determined by liquid scintillation as described above.

In Vitro Stability of pdT_{16}

In vitro stability experiments were carried out at 37°C in RPMI 1640 medium supplemented with a final concentration of 10% of foetal calf serum. A volume of sample containing pdT_{16} at 1 μM (either in solution, or associated to emulsions E_0 and E_{SA}) was then incubated in an equal volume of culture medium. After 15, 60, 120, and 360 minutes, the enzymatic reaction was stopped by heating at 80°C for 5 minutes. Oligonucleotides were recovered by phenol extraction and the aqueous phase was directly used for electrophoresis using a 20% polyacrylamide 7M-urea gel. ATP was used as a control of the migration of a mononucleotide. The bands were analyzed by measuring the radioactivity using an automatic TLC-linear analyzer as previously reported (10).

RESULTS

Physico-Chemical Characterization of E_0 and E_{SA}

In a preliminary step, the optimization of the emulsification process led to appropriate selectioning pressure conditions and a number of microfluidization cycles in order to obtain an emulsion of finest and constant size. Both E_0 and E_{SA} emulsions were then prepared at 4-bar pressure and after 4 microfluidization cycles.

Table I shows mean droplet size and zeta potential measured after incubation of E_0 and E_{SA} with increasing amounts of

Table I. Size and ζ Potential of Submicron Emulsions, Loaded or Not with pdT_n

			E_0[a]		E_{SA}[a]	
	[Nucleotides] (mM)	[ON] (μM)	Size[b] (nm)	ζ-Potential[b] (mV)	Size[a] (nm)	ζ-Potential[b] (mV)
	Non-loaded	—	169 ± 8	−26.3 ± 6.4	183 ± 3	28.2 ± 3.2
pdT_{16}	1.5	94	151 ± 8	−32.6 ± 8.3	207 ± 25	33.6 ± 1.9
	3.0	187	180 ± 25	−36.1 ± 3.6	311 ± 39	33.2 ± 0.6
pdT_{30}	1.5	50	169 ± 12	−32.4 ± 5.4	248 ± 83	31.9 ± 0.3
	3.0	100	176 ± 16	−32.3 ± 4.9	355 ± 50	31.9 ± 0.6
pdT_{50}	1.5	30	158 ± 9	−34.4 ± 5.7	228 ± 38	30.8 ± 2.1
	3.0	60	174 ± 24	−36.1 ± 2.0	301 ± 76	31.4 ± 0.2

Note: ON concentrations are expressed either in μM ON or in the corresponding total nucleotide concentration (mM).

[a] E_0: emulsion prepared without stearylamine, E_{SA}: emulsion containing 0.5% stearylamine.

[b] The particle size and ζ potential represent the mean ± S.D. of three independent experiments.

pdT_n. Concentrations have been expressed either as the effective concentration of pdT_n, or as the total concentration of nucleotides (according to the fact that pdT_n contains n nucleotides and n + 1 charges). For clarity, only two particular concentrations have been reported in Table I: 1.5 and 3.0 mM in nucleotides for each of the oligothymidylate studied (16, 30, and 50 mer).

In the case of non-loaded emulsions, it can be verified that E_{SA} beared a positive surface charge, whereas E_0 was negatively charged. Surprisingly, the addition of increasing amounts of oligothymidylate did not significantly change the zeta potential of both emulsions, nor the droplet size of E_0. In the case of E_{SA}, the mean droplet size tended to increase with increasing nucleotides concentration, independently of the length of the ON.

Concerning the stability over storage, E_{SA} loaded with ON remained still stable after at least 4 months in terms of mean droplet size and zeta potential, whereas all the formulations without stearylamine (E_0) separated within one month of storage at 4°C.

pdT_n Association Efficiency

The amount of ON associated with E_0 or E_{SA} emulsions can be found in Fig. 1. Results have been expressed in terms of total nucleotides in order to keep the same scale for the three oligothymidylates with different lengths. The data clearly show that the association efficiency of ON with E_0 was always lower than with E_{SA}, irrespective of the ON length. For a given emulsion (E_0 or E_{SA}), the association efficiency decreased when ON length increased, and this was particularly pronounced in the case of pdT_{50}. Furthermore, it was noteworthy that more than 90% of pdT_{16} and pdT_{30} were found associated to E_{SA}, even for the highest concentrations tested.

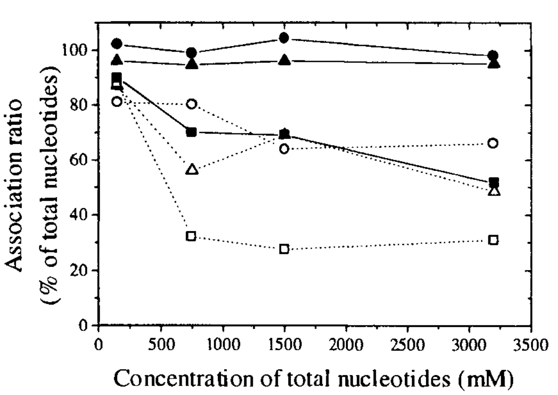

Fig. 1. Amount of nucleotides associated to submicron emulsions E_0 (open symbols) and E_{SA} (dark symbols) versus the total nucleotide concentration for 3 types of ON: 16 mer (○, ●), 30 mer (△, ▲), 50 mer (□, ■). (E_0: emulsion prepared without stearylamine, E_{SA}: emulsion containing 0.5 % stearylamine).

However, it should be emphasized that increasing the concentration of nucleotides over 3.5 mM led in every case to an instantaneous destabilization of E_{SA}.

Table II reports, among the concentrations tested, the maximum concentration of ON for which emulsion E_{SA} has still withstood, and the threshold concentration for which flocculation occurred. It was remarkable that E_{SA} became destabilized in every situation for a positive/negative charge ratio of 4; the positive charges coming from stearylamine and the negative charges from the ON phosphate groups.

Table III describes the behavior of emulsions containing decreasing concentrations of SA as compared to E_{SA}. When SA concentration was decreased by half ($E_{SA/2}$), flocculation occurred for the same ON concentration as in E_{SA}, that means for a +/− ratio of 2. When SA concentration was further decreased (by a factor 8), no flocculation could experimentally be shown. However, Table III clearly shows that zeta potential of $E_{SA/8}$ progressively decreased along with the ON concentration, with an inversion of the net surface charge between 150 and 200 μM. The theoretical surface available for SA has been

Table II. ON Concentration Leading to Flocculation of E_{SA}[a]

ON length	Concentration leading to flocculation [pdT_n] (μM)	Corresponding charge ratio (+/−)
16-mer	250	4
30-mer	133	4
50-mer	80	4

Note: The corresponding (+/−) ratio was calculated from the stearylamine concentration (+) and the phosphate groups beared by each nucleotide (−).

[a] E_{SA}: emulsion containing 0.5% stearylamine.

Table III. Characterization of Emulsions Containing Decreasing Concentrations of SA and Varying Concentrations of pdT_{16}

Type	Size (nm)	S_{LIP} (nm^2)	[pdT_{16}] (μM)	+/− ratio	ζ-potential (mV)
E_{SA}[a]	167 ± 33	0.13	250	4	flocculation
$E_{SA/2}$[a]	157 ± 52	0.17	250	2	flocculation
$E_{SA/8}$[a]	148 ± 59	0.23	125	1	20.7 ± 0.8
$E_{SA/8}$[a]	148 ± 59	0.23	150	0.9	15.8 ± 0.4
$E_{SA/8}$[a]	148 ± 59	0.23	200	0.7	−26.3 ± 0.9
$E_{SA/8}$[a]	148 ± 59	0.23	250	0.5	−32.1 ± 2.4

Note: S_{LIP} is the surface theoretically available for SA at the interface, considering that all SA and phospholipids molecules were present at the interface, and occupied the same area.

[a] E_{SA}, $E_{SA/2}$, $E_{SA/8}$: emulsions containing 0.5%, 0.25 or 0.06% of stearylamine, respectively.

calculated considering that all the SA and the phospholipid molecules were present at the interface and that each SA and phospholipid molecule occupied the same area. These values have to be compared to the minimum surface occupied by these molecules in a mixed SA/phospholipid monolayer (11).

Additional experiments were made, in which E_{SA} was incubated with 200μM pdT_{16}, and then supplemented with further 50 μM ON each 24 hours (Table IV). It was interesting to note that instantaneous flocculation was not observed even up to 300 μM pdT_{16}. This last emulsion however, was destabilized within 4 hours.

Release upon Dilution

E_0 and E_{SA} loaded with pdT_{16} (1 μM) have been diluted in various proportions. Figure 2 exhibits the results obtained at 25°C when dilution was performed in the continuous phase of the emulsion (glycerol 2.25%) or in PBS. As previously described, it could be verified that before any dilution, the amount of free ON reached 20% in the case of E_0, whereas it was insignificant in the case of E_{SA}. Whatever the dilution medium was, low amount of ON was released from E_{SA} with a maximum of 20% for the dilution 1:1000. On the contrary, with E_0 and especially in the case of PBS, the overall amount of adsorbed ON was released on 1:200 dilution.

Partitioning Studies

In order to evaluate the solubility of ON of different lengths in the oil phase of the emulsions, and to identify the role of SA on this parameter, partitioning studies were performed between water and two different organic phases (MCT and *n*-octanol). In each experiment, ON has been assayed in both water and organic phases. Figure 3 shows the results obtained. In the case of MCT, irrespective of the ON length, only a very low amount of ON could be found in the organic phase, whereas it appeared clearly that ON disappeared from the water phase along with the increase in SA concentration. For each SA concentration, the difference between the total amount of ON and the amount of ON present in the water and in the organic phases has been calculated and attributed to ON localized at the interface. As Fig. 3A shows, increasing the SA concentration led to the extraction of quite the whole quantity of ON initially dissolved in the water phase. For the highest concentrations, a white interfacial film was observed to be formed between the water and MCT phases. The 16, 30, and 50 mer ON behaved similarly, but the amount of SA needed to extract 50% of the ON present in water increased with the ON length: 0.09, 0.28, and 0.88 mg/ml for 16, 30, and 50 mer, respectively.

Figure 3B depicts the results obtained with the *n*-octanol/water system. Contrary to what was observed with MCT, the solubilities of the different ON in *n*-octanol were rather high in the presence of SA. As long as the aqueous concentration of ON decreased with increasing SA concentrations, ON appeared in *n*-octanol and consequently, only a low amount of ON is calculated to be present at the interface. As in the case of MCT however, the SA concentration needed to extract 50% of the aqueous ON increased when the ON length increased (0.04 mg/ml for the 16 mer, 0.40 mg/ml for the 30 mer and 0.49 mg/ml for the 50 mer). Finally, it could be noticed that no ON could be found in the organic phase (MCT or *n*-octanol) in the absence of SA.

Table IV. Macroscopic Observations of E_{SA}[a] in Which pdT_{16} Concentration was Progressively Increased

t = 0	[pdT_{16}] (μM)	Observations
t = 0	250	Flocculation
t = 0	200	Stable
t = 24h	+50	Stable
t = 48h	+50	Stable[b]

[a] E_{SA}: emulsion containing 0.5% stearylamine.
[b] Destabilization was observed within 4 hours.

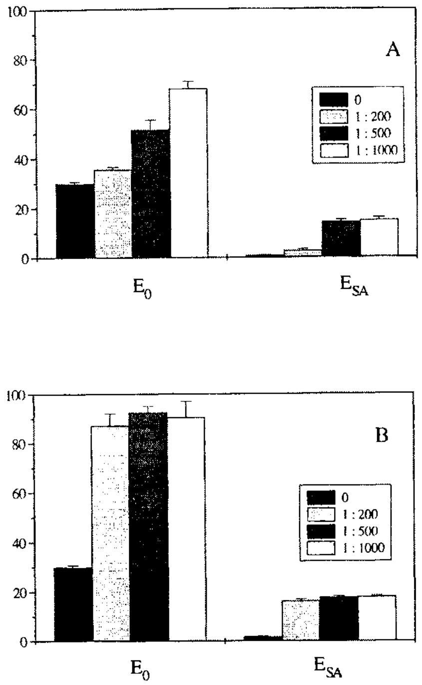

Fig. 2. Percentage of free pdT_{16} measured after dilution (1:200, 1:500, 1:1000) of the emulsions in glycerol 2.25% (A) and in PBS (B) at 25°C. Initial pdT_{16} concentration was 1 μM (E_0: emulsion prepared without stearylamine, E_{SA}: emulsion containing 0.5% stearylamine).

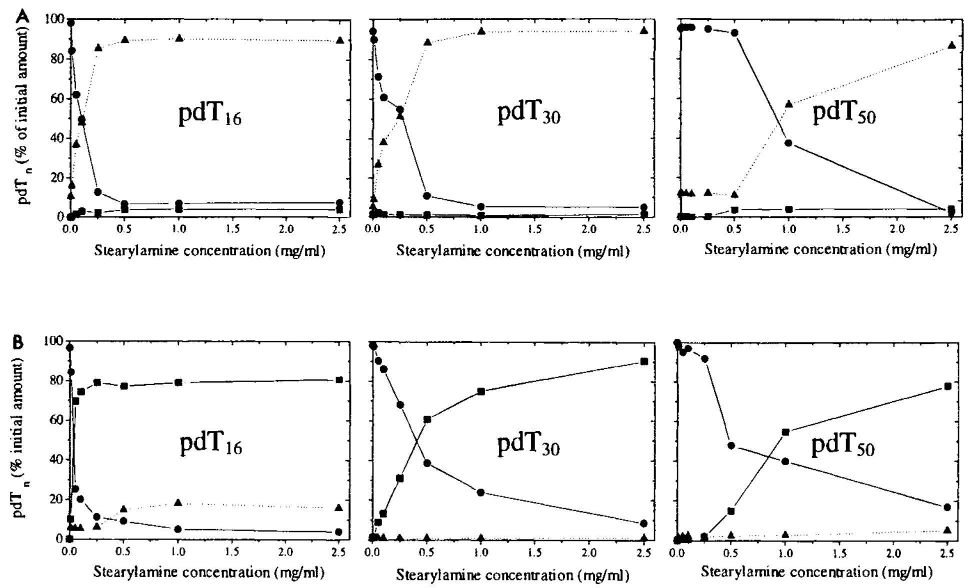

Fig. 3. Effect of increasing amounts of stearylamine on the partition of pdT$_{16}$, pdT$_{30}$, pdT$_{50}$ between water and MCT (A) or water and *n*-octanol (B). (■) Amount of pdT$_n$ in *n*-octanol or MCT, (●) amount of pdT$_n$ in aqueous phase, (▲) calculated by substraction, represents the amount of pdT$_{16}$ which must be present at the interface.

In Vitro Stability in Culture Medium Supplemented with 10% FCS

In vitro pdT$_{16}$ stability experiments were carried out in culture medium supplemented with 10% FCS. pdT$_{16}$ integrity was estimated according to its migration profile in PAGE as described in material and methods. The retention-distance of the peak corresponding to intact pdT$_{16}$ was the control (3.6-cm-long) (Fig. 4A). As a reference, the retention distance for ATP was 18 cm. Longer retention distances corresponded to shorter ON molecules. In the case of a single aqueous solution of pdT$_{16}$ incubated with culture medium + 10% FCS, no intact ON could be detected after 15 minutes (Fig. 4A). On the contrary, when associated with E$_{SA}$, pdT$_{16}$ remained unchanged after 120 minutes incubation in the culture medium, and only began to degrade after 360 minutes (Fig. 4C). Diluting E$_{SA}$ to 1:25 in the culture medium instead of 1:1 led to a slight faster degradation of pdT$_{16}$ (120 min) but it is important to point out that pdT$_{16}$ has, however, been protected for at least 60 min (Fig. 4D).

DISCUSSION

Antisense oligonucleotides have been associated with particulate transport systems in order to protect them from degradation and to improve their diffusion through biological membranes (3,4,5,12). Taking benefit from the polyanionic character of ON, cationic systems have generally been developed to encapsulate these molecules. The choice of submicron emulsion of positive charge was intended to this aim. The positive charges brought by SA were supposed to help ON to associate to the oily droplets of the emulsion, despite the hydrophilic character of these molecules.

The development and validation of a separation method based upon ultrafiltration, allowed to quantify the amount of ON associated to the oily phase of both E$_0$ and E$_{SA}$. It was then found that the association efficiency of oligothymidylates was extremely high with E$_{SA}$, and lower with E$_0$. As expected, because of its positive charge, SA should allow to engage electrostatic interactions between ON and the oily droplets which have in turn favored ON association. However, despite of its negative surface charge, E$_0$ was able to associate with ON. The marked dependence of the association rate with the ON length suggested that rather weak interactions existed.

Partitioning studies showed that ON could not be solubilized in MCT in the absence of SA suggesting that ON associated to E$_0$ were localized onto the oily surface, simply adsorbed. As the phospholipids stabilizing the interface are zwitterionic (phosphatidylcholine), it is supposed that local dipoles created by the coexistence of both positive and negative charges on the same polar head group, were able to attract ON, even if the global surface charge was negative.

Upon dilution, the ON adsorbed onto E$_0$ were immediately released, especially in the presence of competing ions of a saline buffer. It was then not surprising in these conditions, to observe the degradation of pdT$_{16}$ associated to E$_0$ when added to a culture medium supplemented with FCS, as if it were simply in an aqueous solution.

The partitioning studies performed with MCT and increasing SA concentrations clearly showed that a complex between ON and SA could form. However, this complex was insoluble in MCT and precipitated at the interface. It must be noticed that the experiments have been performed in the absence of phospholipids, thus allowing a complete separation of the aqueous and oily phases after centrifugation. Phospholipids might increase the solubility of ON/SA complex in MCT. However, it has been reported, in the case of DNA, that zwitterionic lipids such as those present in E$_{SA}$ did not affect the partitioning of the cationic lipid / DNA complex (13). It can therefore reasonably be assumed that ON associated to E$_{SA}$ are localized onto the surface droplets, interacting with the polar head groups of SA molecules.

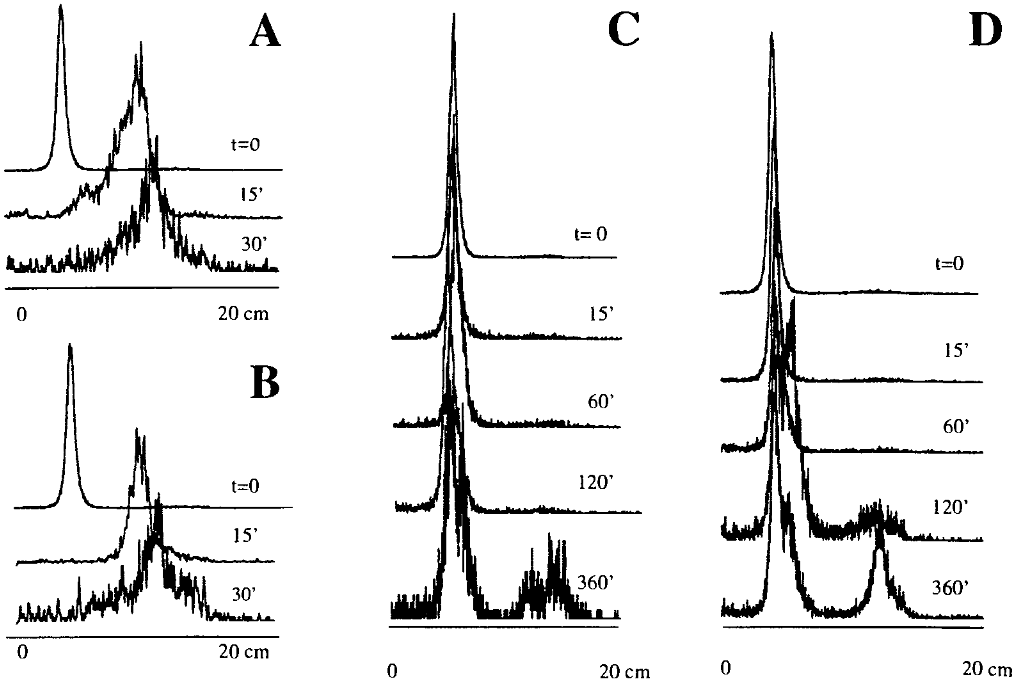

Fig. 4. Stability of pdT$_{16}$ in RPMI supplemented with 10% foetal calf serum as determined by electrophoresis. (A) pdT$_{16}$ (0.5 μM in aqueous solution, (B) pdT$_{16}$ associated to E$_0$, (C) pdT$_{16}$ associated to E$_{SA}$ in the same conditions, or (D) diluted to a higher extent 1:25.

The electrostatic interactions existing between the ON/SA ion-pair were found strong enough to prevent a massive ON release upon dilution in a saline medium, even at a very high dilution ratio (1:1000). In addition, the adsorbed ON were shown to be efficiently protected against degradation in the presence of FCS for a long period of time. The dependence of the duration of the protection with the rate of dilution of E$_{SA}$ in the culture medium suggested that ON might finally be released along with time, in conditions that will further be investigated. Thus, a single surface adsorption of ON was found to be sufficient for protecting ON from degradation as it was already described with polyalkylcyanoacrylate nanoparticles (3). As long as the molecules remained adsorbed onto a surface, they were not sterically available for enzymatic reaction, which could thus only occur after desorption.

It must, however, be mentioned that the first results described in Table I were apparently in contradiction with a surface adsorption of ON, since no change in zeta potential could be noticed. It would have rather been expected that the adsorption of polyanionic compounds onto the droplets surface should bring further negative charges, and then decreased zeta potential of E$_{SA}$ and increased the absolute value of E$_0$. For instance, in the case of polyalkylcyanoacrylate nanoparticles covered with CTAB, a cationic surfactant, the addition of increasing amounts of pdT$_{16}$ led to the progressive inversion of the zeta potential value (14), until a threshold value of −17 mV (increasing even more the ON concentration could not significantly decrease this zeta potential value).

In order to determine whether all the SA molecules could localize at the interface of the emulsions, the theoretical area available per lipid molecule at the interface has been calculated (Table III). This value represents an average surface considering that SA and phospholipids occupy the same area, and can be compared to the values determined experimentally in a previous work (11) with varying mixed monolayers. The values found for the different emulsions were much lower than the expected ones (11). Indeed, in the case of E$_{SA}$, that means for a SA mole fraction of 0.41, the minimal surface per molecule (at the collapse of the interfacial film) must be around 0.35 nm^2, whereas the calculated value was 0.13 nm^2. Again, in the case of E$_{SA/2}$ and E$_{SA/8}$ (SA mole fraction of 0.26 and 0.08, respectively), the expected minimal values should be around 0.45 nm^2 and 0.50 nm^2 against 0.17 nm^2 and 0.23 nm^2 respectively, as calculated from our data.

These observations mean that the emulsions E$_{SA}$, E$_{SA/2}$, and E$_{SA/8}$ all contained lipids (phospholipids and SA), in excess as compared to the area available at the interface. Thus, part of SA molecules should be localized in the core of the oily droplets of the emulsion. The question is whether these SA molecules interact with ON or not.

Tables II and III indicated that flocculation occurred for a given ON concentration, which reasonably means that the added ON molecules had neutralized the positive surface charge of the emulsion, leading to a zeta potential of zero. Flocculation occurred for E$_{SA}$ and E$_{SA/2}$ at the same ON concentration, suggesting that the SA concentration at the interface was at its maximum in both cases, and that 250 μM pdT$_{16}$ were needed to neutralize these positive surface charges. The experimental conditions chosen did not allow to find the exact ON concentration leading to flocculation of E$_{SA/8}$. However, zeta potential determinations suggested that flocculation might occur for an ON concentration comprised between 150 and 200 μM (+/- ratio between 0.9 and 0.7). The decreasing of the +/-ratio at flocculation, when the SA concentration decreased, is to be linked to the decreasing of SA in excess in the oily core in the case of E$_{SA/2}$ and E$_{SA/8}$.

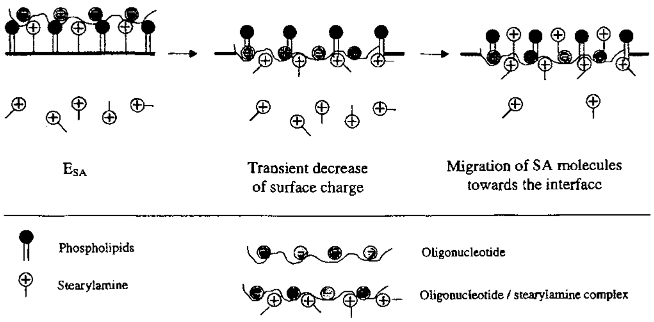

Fig. 5. Schematic representation of the equilibrium existing between SA present at the interface and SA solubilized in MCT of E_{SA}, in the presence of ON.

Since the SA/ON ion-pair was found to be insoluble in MCT, SA in excess present in the oil, was supposed to be unable to participate in the interaction with ON. However, zeta potential of E_{SA} was not affected by the adsorption of ON, even at concentrations close to the flocculation region, as if all the SA molecules at the interface were free. Because zeta potential determinations were carried out in hours following the addition of ON, it can be assumed that SA in excess had time to migrate from the oily core to the interface, replacing the SA molecules engaged in an ion pair with ON (Fig. 5), therefore maintaining constant the surface charge. The existence of such a dynamic process is confirmed by the results shown in Table IV indicating that flocculation could be avoided if ON were progressively added to the emulsion. In this case, after each ON addition, SA in excess had time to migrate to the interface, providing the surface droplets with new positive charges. Flocculation finally occurred when free SA, not neutralized in ion-pair formation with ON, was not sufficient to maintain the zeta potential value above zero.

In the case of $E_{SA/8}$, zeta potential determinations clearly indicated that the amount of SA in the oily core was not sufficient to maintain the zeta potential constant upon the addition of increasing amounts of ON.

In summary, the overall results obtained provide a real evidence that ON molecules combined with SA at the droplets surface, by electrostatic interactions. The oily core plays the role of a reservoir of SA molecules, which migrate towards the interface, replacing SA neutralized through ion-pairs by ON. In such a system, zeta potential can be kept constant as long as significant excess of SA is available.

Interestingly, this study reported finally that the use of *n*-octanol instead of MCT in the partitioning experiments was able to extract ON into the oily phase. The ion-pair ON/SA is then able to be soluble in an appropriate organic phase, of adequate polarity. This observation has to be compared to the dissolution of DNA/cationic lipid complex in chloroform and its further incorporation in reconstituted chylomicrons (15). This result opens very interesting perspectives for dissolving ON molecules into the oily core of the emulsion which could still improve ON protection against degradation.

CONCLUSIONS

This study was the first to demonstrate the potential interest of submicron cationic emulsion as a new ON delivery system. ON in a wide range of size (up to 50 mer) can be associated to this type of emulsion with a high efficiency. In a culture medium supplemented with serum, ON have been protected for a long period of time. The judicious choice of the oily phase of the emulsion might even improve the characteristics of this new system, in terms of release kinetics and protection against degradation. Further studies are in progress in our laboratory, including thorough physico-chemical characterization.

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