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Effect of cationic lipid composition on properties of oligonucleotide/emulsion complexes: Physico-chemical and release studies

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Abstract

This paper describes the influence of cationic lipid composition on physico-chemical properties of complexes formed between oligonucleotides (ON) and cationic emulsions. Formulations containing medium chain triglycerides, egg lecithin, increasing amounts of either oleylamine (OA) or 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and water were prepared by a spontaneous emulsification procedure. ON adsorption on emulsions was evidenced by the inversion of the ζ-potential, the increase in droplet size, and the morphology of the oil droplet examined through transmission electron microscopy. Adsorption isotherms showed a higher amount of ON adsorbed on emulsions containing DOTAP when compared to emulsions containing OA. In a final step, the role of the main parameters, which may in fact influence the ON release rate from emulsions, was investigated. ON were progressively released from emulsions with an increase in dilution ratio and remained quite similar for both OA and DOTAP emulsions over time. Conversely, the effect of the cationic lipid composition was observed upon increasing the charge ratio of complexes. ON release at a same charge ratio was lower from emulsions containing DOTAP (bearing dioleyl chains) than from those containing OA (bearing monoleyl chain).

Keywords: Oligonucleotides; Cationic emulsions; DOTAP; Oleylamine; Release; Adsorption isotherms

1. Introduction

Nucleic acid association with cationic lipid or polymer-based colloidal carriers has been considered a promising strategy to their *in vitro* and *in vivo* delivery (Woodle and Scaria, 2001; Clement et al., 2005; Park et al., 2006).

Over the past decade, nucleic acid adsorption on oil-in-water (o/w) cationic emulsions has been investigated at length (Liu et al., 1996; Teixeira et al., 1999, 2001a,b, 2003; Yi et al., 2000; Bivas-Benita et al., 2004; Kim et al., 2005; Min et al., 2005). Cationic emulsions are generally composed of an oily core (from natural or synthetic origin) stabilized by a binary mixture of phospholipids and cationic lipids. Nucleic acids in solution interact spontaneously with oppositely charged cationic lipids to form polyionic complexes. Positively charged oil droplets of emulsions led to an increase in the cellular uptake of nucleic acids because of their electrostatic interactions with negatively charged membranes (Teixeira et al., 2003; Kim et al., 2005; Min et al., 2005). In addition, nucleic acid adsorption on emulsions reduces their enzymatic degradation by nucleases (Yi et al., 2000; Teixeira et al., 2003; Bivas-Benita et al., 2004).

We have recently designed triglyceride-based emulsions stabilized by different phospholipid and cationic lipid combinations as potential delivery systems for single strand oligonucleotides (ON) (Teixeira et al., 1999, 2001a,b, 2003). ON molecules in a wide range of lengths (up to 50-mer) can be associated to oil droplets through ion-pair formation (Teixeira et al., 1999). In addition, the cationic lipid composition of the emulsion (nature of hydrophilic headgroup and acyl chains) has been optimized

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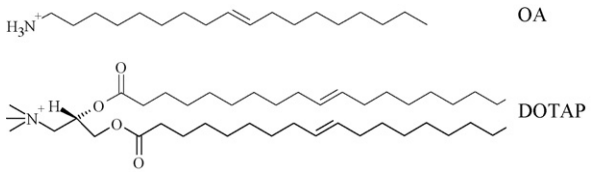

Fig. 1. Chemical structures of the cationic lipids.

in order to obtain the best conditions for ON–emulsion decomplexation (Teixeira et al., 2001a). In final, triglyceride-based emulsions have been shown to efficiently improve the delivery of ON after intratumoral administration to ascite cancer cells (Teixeira et al., 2003).

Despite the great potential of cationic emulsions as carriers for the delivery of ON, the characterization of the complexes formed between ON and emulsions remains poorly investigated and understood. Previous studies have clearly shown that ON adsorption is mainly influenced by the composition of the interface (Teixeira et al., 2001b; Trimaille et al., 2001, 2003). The binding process was found to proceed according to two regimens: at low coverage it was independent of ON concentration, whereas on the reverse at high coverage binding was concentration-dependent (Trimaille et al., 2001). This behavior was considered to be due to the contribution of repulsive interactions with increasing amounts of immobilized ON molecules. Morphologically, the characterization, by cryomicroscopy of ON–cationic emulsions delivery system has revealed the existence of bridges between emulsion droplets. This was attributed to the configuration of the ON at the interface (Teixeira et al., 2001b).

Up to now, however, comparative studies concerning the effect of cationic lipid composition on properties of complexes formed between ON and cationic emulsions have yet to be performed. Thus, the aim of the present study was to evaluate the effect of either oleylamine (a monocationic lipid bearing a monoleyl chain) or DOTAP (a monocationic lipid bearing dioleyl chain) on physico-chemical properties of the ON/cationic emulsion complexes as well as on ON adsorption and release from emulsions. The chemical structures of cationic lipids are presented in Fig. 1.

2. Materials and methods

2.1. Materials

Medium-chain triglycerides (MCT) (Societé des Oleagineux, France), Egg lecithin-Lipoid E-80® (Lipoid, Germany), Oleylamine (Fluka, France), DOTAP (Sigma, USA), and glycerol (Merck, Brazil) were used for emulsion preparation. Oligohexadecathymidylate (pdT_{16}) was purchased from Biogen (São Paulo, Brazil).

2.2. Emulsions preparation

Emulsions were prepared according to a previously described spontaneous emulsification procedure (Trimaille et al., 2001, 2003; Silva et al., 2006). Briefly, the oil phase components (MCT, Lipoid E-80, and cationic lipids) were dissolved in ethanol. This limpid ethanolic solution was slowly added to a water phase containing glycerol, under moderate magnetic stirring. The water phase immediately turned milk as a consequence of the emulsion formation. The organic solvent was then removed under reduced pressure at 50 °C until reaching the desired final volume. The final composition of emulsions was made up of MCT 8.0% (w/w), Lipoid E-80 2.0% (w/w), glycerol 2.25% (w/w), and either oleylamine 0.005–0.5% (w/w) (OA emulsions) or DOTAP 0.0132–1.32% (w/w) (DOTAP emulsions), and MilliQ® water up to 100. Typically, five kinds of formulations were prepared for each cationic lipid, which corresponds to 0.02, 0.2, 1, 2, or 20 mM of OA or DOTAP. A control emulsion obtained in the absence of cationic lipids was also prepared.

2.3. Physico-chemical characterization

The mean droplet size and ζ-potential of the emulsions were determined by photon correlation spectroscopy (PCS) and electrophoretic mobility, respectively, at 25 °C and at an angle of 90° (3000HS Zetasizer, Malvern Instruments, England). The samples were adequately diluted in water for size determinations or in 1 mM NaCl solution for ζ-potential measurements. The bulk pH of the emulsions was recorded using a pH-meter B474 (Micronal, Brazil) in recently prepared emulsions and after having been adjusted to approximately 7.0 using 0.1 M hydrochloridric acid. The mean and standard deviation of three different sets of emulsions are presented for each cationic lipid concentration.

Morphologic examination of emulsions containing either OA or DOTAP at 2 mM was performed by means of transmission electron microscopy (TEM). Emulsions were diluted at 1:10 ratio obtaining an oil phase concentration equal to 1%. Specimens for TEM visualization were prepared by mixing samples with one droplet of 2% (w/v) uranyl acetate solution. The samples were then adsorbed to the 200 mesh formvar-coated copper grids, left to dry, and examined by TEM (JEM-1200 ExII, Jeol, Japan). The mean droplet size, ζ-potential, and TEM studies were also performed for ON/emulsion complexes.

2.4. Adsorption and release studies

Prior the adsorption and release studies, the validation of an analytical method for the determination of pdT_{16} employing UV spectrometry was carried out according to the *International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use* (ICH, 2005). A linear response was obtained in the evaluated concentration range (2.5–25 μg/mL), with a correlation coefficient of 0.9974. In addition, this method showed to have a good precision (R.S.D. < 2.05% for repeatability and <1.70% for intermediate precision). The specificity was tested in the presence of glycerol (external phase of the emulsions). This excipient did not interfere with pdT_{16} quantification.

Studies of adsorption of ON onto nanoemulsions were performed at the end of the spontaneous emulsification process according to a previously described conditions (Teixeira et al., 1999; Trimaille et al., 2003). Briefly, emulsions containing OA or DOTAP at 2 mM were added to water solutions containing increasing amounts of pdT_{16} and incubated for 30 min at room temperature. Free pdT_{16} was quantified in the clear ultrafiltrate obtained after centrifugation (20 min) of the emulsions through a porous membrane (30,000 Da cut off, Ultrafree MC Millipore, U.S.A.). The amount of pdT_{16} in the ultrafiltrate was measured by determination of the optical density at 260 nm (Hewlett-Packard 8452 spectrophotometer, U.S.A.). UV spectra were recorded from 200 to 400 nm in order to check the presence of any interference. In all cases, the final concentration of the oil phase of emulsions in the adsorption media was 2 mg/mL. The amount of adsorbed pdT_{16} per g of the inner phase could be obtained from the following equation:

$$N = \frac{C_i - C_r}{[NE]}$$

where N is the amount of pdT_{16} adsorbed (mg of pdT_{16} per g of oil phase); C_i and C_r represent the initial concentration of pdT_{16} and that recovered in the ultrafiltrate (mg/g), respectively, whereas [NE] is the concentration of the oil phase of emulsions in the adsorption media (g). The mean and standard deviation of three different sets of adsorption isotherms were presented.

Release studies were performed in PBS buffer pH 7.4 as dilution media. Three sets of experiments were performed for OA or DOTAP emulsions (2 mM) containing pdT_{16}: (a) the emulsion/pdT_{16} complex was diluted 20-fold, and the samples were collected after 15, 30, 60, 120, and 240 min; (b) the emulsion/pdT_{16} complex was diluted 5-, 20-, 40-, 80-, and 100-fold, and the samples were collected after 15 min; (c) the emulsion/pdT_{16} complexes presenting different (+/−) charge ratio (+0.2/−, +2/−, +5/−, +10/−, and +20/−) were diluted 20-fold, and the samples were collected after 15 min. In all cases, released pdT_{16} was quantified in the ultrafiltrate as described above.

2.5. Statistical analysis

Results are expressed as mean ± standard deviation of three independent experiments and were analyzed by Student's *t*-test with $p < 0.05$ significance.

3. Results

3.1. Physico-chemical properties

Fig. 2 shows the effect of OA or DOTAP (0.02–20 mM) on physico-chemical properties of cationic emulsions. As can be seen in Fig. 2A, the gradual addition of OA increased the pH of formulations up to nearly 10. Conversely, the pH of DOTAP formulations remained unchanged throughout the progressive addition of this cationic lipid. This finding can be explained due to the presence of quaternary ammonium groups in the molecular structure of DOTAP. It must be pointed out that in the case of emulsions containing OA, the pH was adjusted to approximately 7 in order to produce theoretically fully ionized OA molecules at the interface (Rabinovich-Guilatt et al., 2004).

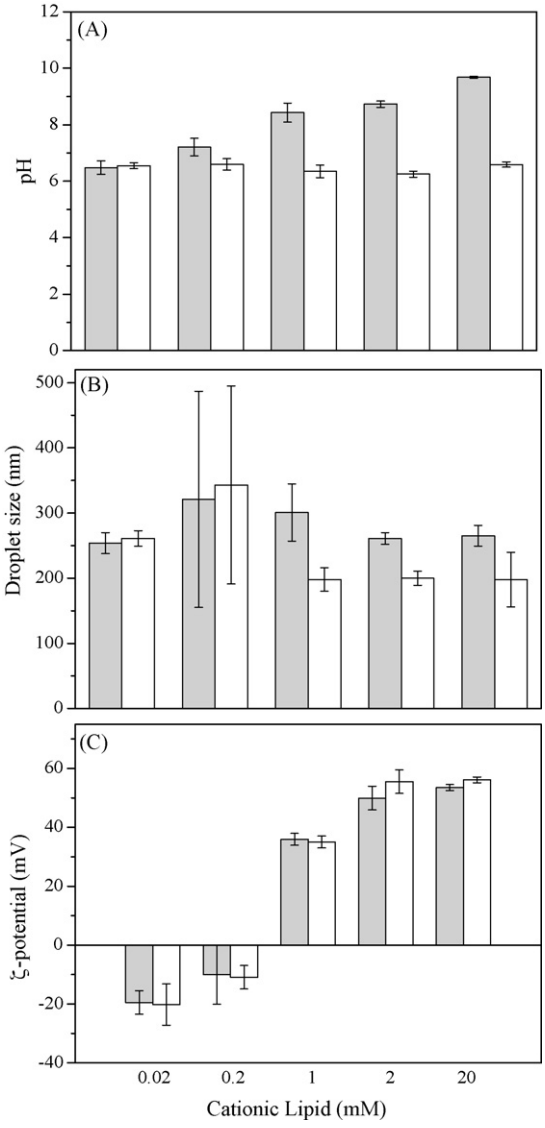

Fig. 2. pH (A), mean droplet size (B) and ζ-potential (C) of emulsions. OA emulsions (grey bars) or DOTAP emulsions (blank bars).

The mean droplet size of emulsions (Fig. 2B) varied from 200 to 350 nm while the polydispersity index (IP) varied from 0.1 to 0.45, in agreement with results previously reported for emulsions obtained by spontaneous emulsification procedures (Trimaille et al., 2001, 2003; Silva et al., 2006). The smallest droplet size values could be detected for formulations containing higher concentrations of DOTAP (about 200 nm). The highest droplet size and IP values were observed for both formulations containing cationic lipids at 0.2 mM. This result would be related to the ζ-potential values (Fig. 2C) since at this concentration the values were closer to neutrality (near −10 mV), whatever the nature of the cationic lipid.

Fig. 2C shows the ζ-potential of the emulsions containing increasing amounts of either OA or DOTAP (0.02–20 mM). A negative value for ζ-potential could be detected for low amounts of both cationic lipids (0.02 and 0.2 mM), which can be attributed to the presence of negatively charged lipids in egg lecithin (Teixeira et al., 1999). Upon further addition of cationic lipids, the emulsions displayed positive values (up to $\zeta = +50$ mV). However, it was observed that beyond 2 mM of cationic lipids, the ζ-potential was not significantly ($p < 0.05$) influenced by further addition of cationic lipids (at 20 mM).

3.2. Adsorption studies

Fig. 3 shows the pdT_{16} adsorption on cationic emulsions containing OA or DOTAP at 2 mM, since they exhibit the highest ζ-potential (about 50 mV). A similar progressive adsorption of pdT_{16} onto both emulsions was observed until reaching a plateau. It was observed a significant ($p < 0.05$) higher adsorption of pdT_{16} on DOTAP emulsions (70 mg/g) compared to OA ones (40 mg/g). Furthermore, the results revealed a low association level for the control formulation obtained in the absence of cationic lipids (up to ~20 mg/g).

Concerning the physico-chemical properties of emulsions after pdT_{16} adsorption (Fig. 4), the adsorption of increasing amounts of pdT_{16} led to a decrease in the ζ-potential values (~−30 mV) for both formulations. Furthermore, it was observed that higher amounts of pdT_{16} caused no significant alterations in the ζ-potential values. On the other hand, an increase in droplet size was detected by PCS after ON adsorption for both formulations.

Transmission electron microscopy investigations (Fig. 5) of the oil droplets showed the typical appearance of an o/w emulsion with droplets displaying a size of nearly 200–300 nm, according to the PCS experiments. OA and DOTAP emulsions containing pdT_{16} revealed a high electronic density at the interface (Fig. 5C and D). It is known that the nucleic acids interact with uranyl acetate resulting in the appearance of darker regions. Thus, these findings suggest that the pdT_{16} is located at the interface of the emulsion.

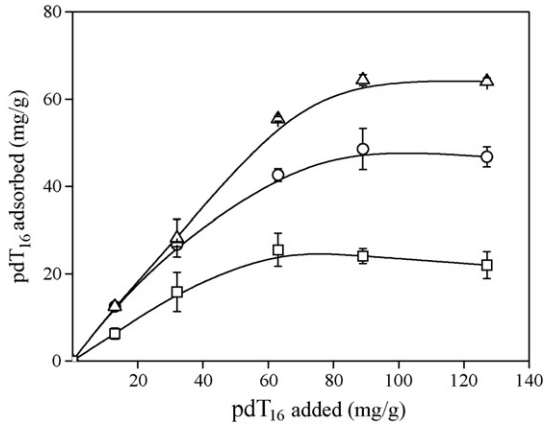

Fig. 3. Adsorption isotherms of pdT_{16} on emulsions. (◯) OA emulsion, (△) DOTAP emulsion and (□) emulsion obtained in the absence of cationic lipids.

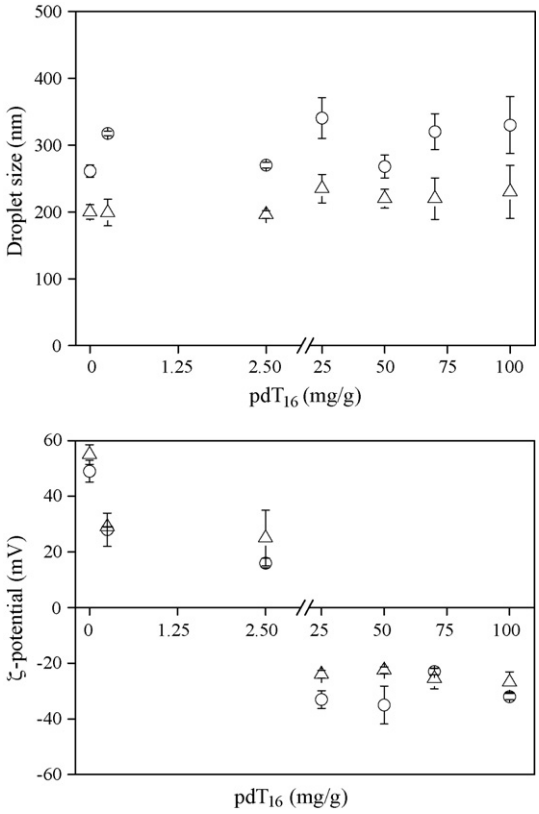

Fig. 4. Mean droplet size (A) and ζ-potential of emulsions (B) as a means to increase amounts of pdT_{16}. (◯) OA emulsion and (△) DOTAP emulsion.

3.3. Release studies

The profile of pdT_{16} release from emulsions over time, dilution, and +/− charge ratios were investigated (Fig. 6). As demonstrated in Fig. 3, the adsorbed amounts of pdT_{16} on OA and DOTAP emulsions were similar at a +2/− charge ratio (about 25 mg/g). Thus, these formulations were chosen for pdT_{16} release evaluation as function of the time and dilution ratio. About 60–70% of the pdT_{16} were released from emulsions 15 min after dilution and remained unchanged for 120 min (Fig. 6A). In contrast, pdT_{16} molecules were completely released from a control formulation obtained in the absence of cationic lipids in 30 min. Concerning the dilution effect over pdT_{16} leakage from emulsions, it was observed that the increase in dilution led to a higher pdT_{16} desorption from the emulsions (Fig. 6B). Whatever the cationic lipid, a progressive amount of pdT_{16} was released from OA or DOTAP emulsions with a maximum of 100% for the dilution 1:100. On the contrary, in the case of emulsion without cationic lipid, approximately 90% of the pdT_{16} adsorbed was released upon 1:10 dilution. In a final step, the pdT_{16} release investigations from complexes formed at +/− charge ratios, varying from a 0.2 to 20 ratio, were performed. As can be seen in Fig. 6C, an increase in the charge ratio reduces significantly ($p < 0.05$) the pdT_{16} release level for OA and DOTAP emulsions. In the case of OA emulsions, this decrease varied from 80 to 40% when the +/− charge ratio was equal to 0.2 and 20, respectively. The reduction of pdT_{16} release was more pronounced for DOTAP emulsion due to the +/− charge ratio. This

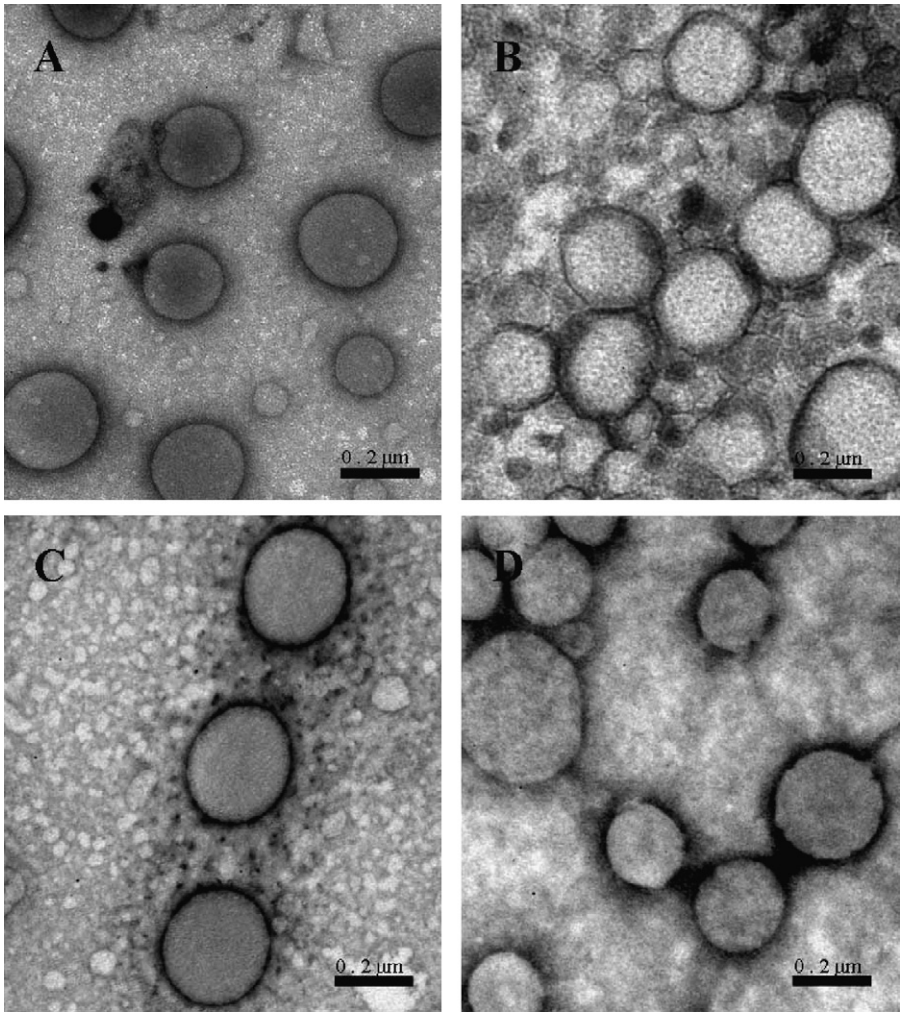

Fig. 5. TEM micrographs of cationic emulsions before and after pdT_{16} association at 25 mg/g. (A) OA emulsion, (B) DOTAP emulsion, (C) +ON and (D) +ON.

system showed 70 and 10% pdT_{16} release for the +/− charge ratio of 0.2 and 20, respectively.

4. Discussion

This paper was aiming to investigate the effect of cationic lipid composition on complexes formed between a model ON (pdT_{16}) and cationic emulsions. In a first step, the influence of increasing amounts of either OA or DOTAP on the ζ-potential of emulsions was evaluated in order to optimize their concentration in the formulations. A higher positive surface charge was found to be related to a greater number of available interaction sites for ON adsorption. ζ-potential increased progressively with the gradual addition of both cationic lipids until reaching a plateau at 2 mM ($\zeta = 50$ mV), suggesting a similar location at the o/w interface (Fig. 2C). However, the ζ-potential remained unchanged with further cation addition. These findings suggest a saturation of the droplet surface caused by the cationic lipids. Recently, Rabinovich-Guilatt et al. (2004) demonstrated that the constant values of ζ-potential obtained for cationic emulsions containing different concentrations of OA is a result stemming from the surface pH. At a bulk pH of 7.4, cationic emulsion containing 9.3 mM of OA showed a surface pH equal to 9.6. This alkaline microenvironment at the droplet interface can prevent the ionization of OA molecules and therefore cancel their contribution to the overall emulsion charge. Most likely, this phenomenon is also occurring in cationic emulsion in the present study. However, the plateau of ζ-potential observed for DOTAP emulsion cannot be explained by the difference between the bulk and surface pH since this cationic lipid presents quaternary ammonium groups. Thus, it can be inferred that another mechanism is in fact responsible for the ζ-potential profile of the DOTAP emulsions. Therefore, in this case, the ζ-potential profile may be due to the surface saturation of emulsions by DOTAP molecules, which has also been reported by other authors (Min-Woo et al., 2001; Kim et al., 2003; Rabinovich-Guilatt et al., 2004).

Regardless of the nature of the cationic lipid, the ζ-potential of emulsions decreased sharply due to the +/− charge ratio, indicating the adsorption of ON on emulsions (Fig. 4B). Electrostatic interactions between positively charged headgroups and negatively charged ON have been considered the main force leading to ON adsorption at o/w interfaces of emulsions (Teixeira et al., 2001a,b; Trimaille et al., 2003). The results showed an inversion in the ζ-potential of OA and DOTAP emulsions in a sigmoidal

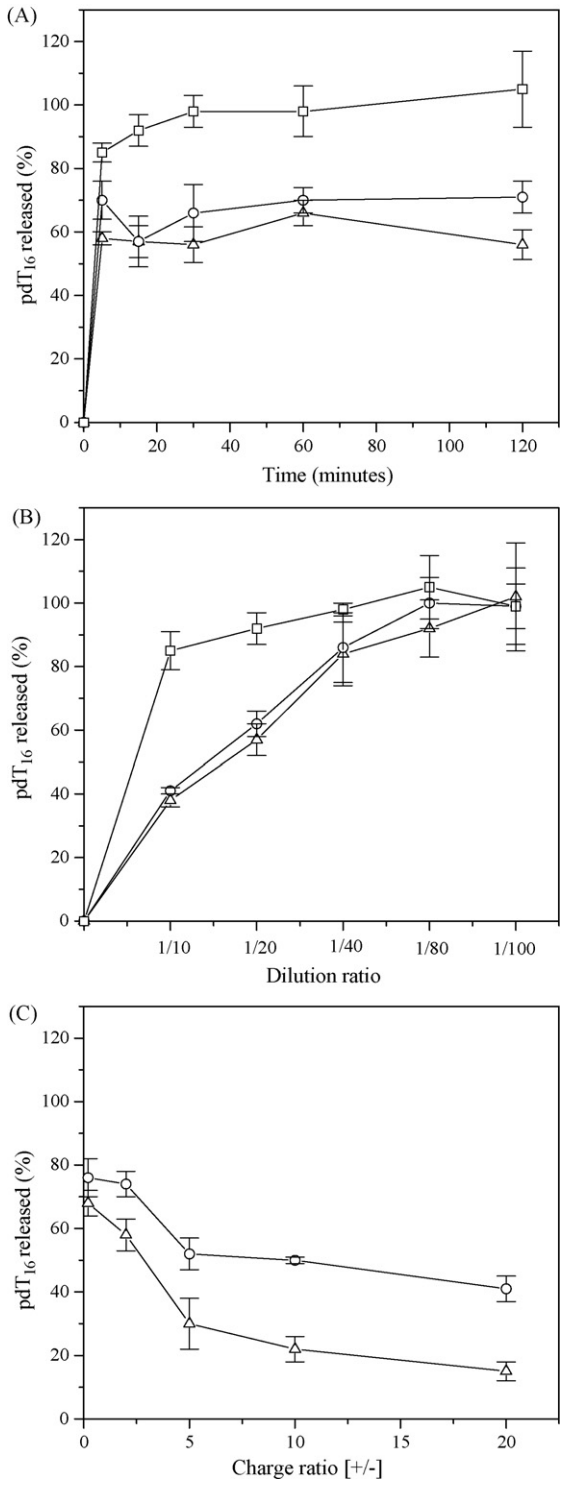

Fig. 6. Release of pdT_{16} from emulsions as affected by time (A), dilution ratio (B) or +/− charge ratio (C). (◯) OA emulsion, (△) DOTAP emulsion and (□) emulsion obtained in the absence of cationic lipids.

shape observed between +0.2/− and +2/− charge ratios. Since ζ-potential tends to neutrality at a +/− charge ratio (equal to 1), a more precise assessment of the inversion point cannot be experimentally recorded due to instability. Even so, the results led us to estimate a ζ-potential inversion at a charge ratio equal to 1. In this case, the overall cationic lipid molecules located at the o/w interface were theoretically involved in ON adsorption. Interestingly, with higher amounts of ON, negative charges incurred were enough to allow the electrostatic stabilization of OA and DOTAP emulsions.

The development and validation of a separation method based upon ultrafiltration, allowed us to estimate the amount of ON associated with OA and DOTAP emulsions. As indicated in the ζ-potential determinations, ON molecules adsorb progressively on positively charged emulsions containing either OA or DOTAP. The isotherms displayed a maximum of ON adsorption which was significantly lower for OA emulsion when compared to DOTAP emulsion (Fig. 3). This result can be attributed to the fact that the OA emulsion presents a more fluid interface since this lipid presents a monoleyl chain, limiting the anchorage of ON molecules at the interface. Furthermore, we have previously reported the occurrence of an additional contribution of hydrophobic interactions between ON and mixed lipid monolayers composed of DOTAP/lecithin, as compared to stearylamine/lecithin, due to the presence of double acyl chains in the DOTAP molecular structure (Teixeira et al., 2001b).

TEM investigation of OA and DOTAP emulsions allowed us to analyze their morphology in the absence and presence of ON (Fig. 5). The morphology of the oil droplets was similar in the presence or absence of ON. In contrast, a high electronic density at the interface was observed after ON addition in both OA and DOTAP emulsions (Fig. 5C and D). This finding suggests the occurrence of adsorption of pdT_{16} at the emulsion interface. A higher contrast at the interface of oil droplets can be related to a higher affinity of ON to uranyl acetate used for negative staining, as described by other authors (Tarahovski et al., 1996; Chevallier, 2004). In the conditions used in this study, we did not detect aggregates or typical bridges formed between neighboring droplets as had been previously observed in microscopy studies (Teixeira et al., 2001b). These observations were supported by PCS through size determinations of oil droplets (Fig. 4A).

Although the emulsion, composed solely of lecithin/MCT, presented a negative surface charge, it was able to associate with ON but to a lesser extent (Fig. 3). Considering that phospholipids located at emulsion interfaces are zwitterionic molecules, it can be suggested that the existence of positive charges on the polar headgroup is able to attract ON, even if the global surface charge is negative. However, upon dilution, the ON adsorbed are immediately released, indicating that the interactions between the ON and interfacial phospholipids are weak (Fig. 6A and B).

The lower pdT_{16} release observed for OA and DOTAP emulsions is probably due to the electrostatic interactions between ON and cationic lipids at the interface of emulsion. Previously performed partitioning studies allowed us to exclude the possibility of ON solubilization in an oil core of emulsion even in the presence of cationic lipids (Teixeira et al., 1999; Hara et al., 1997). The low level of pdT_{16} leakage is most likely related to its retention at the emulsion interface by means of electrostatic forces. It is noteworthy that the steady state was rapidly obtained, which suggests the existence of a partitioning equilibrium (Fig. 6A) as previously reported (Teixeira et al., 2001a). However, this partitioning was displaced throughout dilution in the presence of competing ions of a saline buffer (Fig. 6B). ON

release profiles as function of time and dilution ratio, were quite similar for both OA and DOTAP emulsions at the same +/− charge ratio, indicating that under determined conditions, this parameter is the main factor governing ON release from emulsions. In addition to the +/− charge ratio, this study shows that the nature of interaction can have an effect on pdT_{16} release from emulsions. A significant reduction in the ON release from DOTAP emulsions as compared to that from OA emulsions, was observed upon increasing the +/− charge ratio (Fig. 6C). These findings suggest the contribution of the double anchorage of DOTAP on the pdT_{16} release from emulsions, as mentioned above in adsorption studies.

5. Conclusions

This study first presents the effect of the cationic lipid composition (qualitative and/or quantitative) over physico-chemical properties of cationic emulsions. Despite the foreseeable influence of the nature of the polar group on the bulk pH of emulsions, the oil droplet size and ζ-potential of emulsions remained quite similar, whatever the cationic lipid used. These observations underline the effect of spontaneous emulsification conditions on the physico-chemical properties of emulsions. The ON adsorption and release profiles from the o/w interface of emulsions were clearly influenced by the chemical nature of the cationic lipid used. In addition to electrostatic interactions established between anionic phosphate groups of ON and positively charged amine groups of cationic lipids, the double anchorage of DOTAP at the o/w interface, through hydrophobic interactions with the oil core, seems to directly affect these parameters.

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