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Research paper

The influence of cationic lipid type on *in-vitro* release kinetic profiles of antisense oligonucleotide from cationic nanoemulsions

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Abstract

Novel formulations of cationic nanoemulsions based on three different lipids were developed to strengthen the attraction of the polyanionic oligonucleotide (ODN) macromolecules to the cationic moieties on the oil nanodroplets. These formulations were developed to prolong the release of the ODN from the nanoemulsion under appropriate physiological dilutions as encountered in the eye following topical application. Increasing the concentration of the new cationic lipid exhibiting two cationic amine groups (AOA) in the emulsion from 0.05% to 0.4% did not alter markedly the particle size or zeta potential value of the blank cationic nanoemulsion. The extent of ODN association did not vary significantly when the initial concentration of ODN remained constant at 10 μM irrespective of the cationic lipid nature. However, the zeta potential value dropped consistently with the low concentrations of 0.05% and 0.1% of AOA in the emulsions suggesting that an electrostatic attraction occurred between the cationic lipids and the polyanionic ODN molecules at the o/w interface. Only the nanoemulsion prepared with *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium salts (DOTAP) remained physically stable over time. DOTAP cationic lipid nanoemulsion was the most efficient formulation capable of retaining the ODN despite the high dilution of 1:100 with simulated tear solution (STS). Less than 10% of the ODN was exchanged in contrast to 40–50% with the other cationic nanoemulsions. The *in-vitro* release kinetic behavior of ODN exchange with physiological anions present in the STS appears to be complex and difficult to characterize using mathematical fitting model equations. Further pharmacokinetic studies are needed to verify our kinetic assumptions and confirm the *in-vitro* ODN release profile from DOTAP cationic nanoemulsions.

Keywords: Cationic; Nanoemulsions; AMD; Oligonucleotide; Stability; *In-vitro* release; Kinetic profile; Ion-exchange process

1. Introduction

Antisense oligonucleotides have been tested widely in the past few years for the treatment of cancer, inflammations, viral diseases and neovascular macular degeneration [1–4]. However, poor stability mainly in biological fluids [5–7] and low intracellular penetration of these ODNs have limited their therapeutic use. Chemical modifications of the ODN phosphodiester backbones into phosphorothioate partially enhanced the chemical stability to enzymatic degradation [8,9] but did not improve the intracellular penetration. An antisense ODN with anti-angiogenic activity has been developed and can be used for age-related macular degeneration (AMD) treatment [10,11] preferably following topical instillation. The main limitation for the clinical application of an ODN strategy in eye diseases is the lack of appropriate topical dosage forms to the targeted intraocular tissues. Indeed, frequent intravitreal injections of ODN as currently performed are associated with risks and side effects [12,13]. Unfortunately, topical application of ODN does not result in efficient intraocular penetration. An effective delivery system is needed to improve the ODN surface ocular

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absorption. Thus, some authors have attempted to overcome the negative effect of ODNs by associating the natural phosphodiester macromolecules with liposomes [14] to provide a better stability and transport of ODNs. Bochot and coll. [15] formulated a model oligonucleotide in various formulations including liposomes dispersed within a 27% poloxamer 407 gel. Following topical instillation to the rabbit eye, the concentration of oligonucleotide model in tissues of the anterior segment and the vitreous was measured. The rank order of the best tissue availability was: solution > gel > liposomes (+gel). The authors concluded that topical liposomes may not be an effective delivery system for the administration of ODNs to the superficial ocular tissues. Furthermore, drug delivery systems based on liposomes still suffer from inherent limitations that restrict their potential as a topical ocular drug delivery system. These limitations include instability and short shelf life, low drug-loading capacity, limited drug retention on ocular surface, sensitivity to sterilization and an expensive large-scale manufacturing process. Recently, cationic emulsions were shown to be well tolerated by the eye as compared to cationic liposomes and exhibited promising ocular delivery application [16]. It was shown, irrespective of the lipophilic active molecule nature, that the cationic emulsion was the most effective formulation in increasing the uptake of the drugs in the various ocular tissues following topical ocular administration as compared to the control solution and anionic emulsion [17]. The contact angle and the spreading coefficient of the different formulations on the isolated freshly excised rabbit cornea were studied to elucidate the effect of the formulation charge on its interaction with the corneal surface. Lower contact angle and higher spreading coefficient were exhibited by the positively charged submicron emulsion indicating better wettability properties on the cornea than either the saline or the negatively charged submicron emulsion [18]. These findings can be attributed to the electrostatic attraction between the cationic charges on the oil nanodroplet surfaces and the anionic moieties on the ocular surface. It can be hypothesized that an association of ODN molecules with cationic oil nanodroplets will reduce significantly the polyanionic character of the ODN molecules and will promote their passage through the ocular surface. It was previously reported that model ODN pdT_{16} molecules can be efficiently associated with cationic oil droplets by an ion-pairing formation at the o/w interface. However, a dramatic burst release of ODN in diluted serum occurred [19]. It was then reported that polycationic lipid molecules are needed to reduce the ODN release in physiological fluids under infinite dilution. This deduction was based on the hypothesis that the electrostatic interactions leading to ion-pairing formation should be strong enough to prevent massive ODN release in the presence of competitive anions or proteins from PBS and serum, respectively. It was shown that ODN can associate efficiently to a primary cationic lipid strearylamine [19,5]. However, this single primary amine lipid molecule was not able to retain ODN molecules in the presence of anions or proteins. This was further confirmed in a recent paper showing that pdT16 associated to cationic nanoemulsion prepared with oleylamine (single primary amine with monoleyl chain) could not be retained within the emulsion upon dilution with PBS while DOTAP (single quaternary ammonium with dioleyl chains) slowly released the ODN upon PBS dilution. This effect could be attributed to the stronger basic nature of the quaternary ammonium that exhibits a pK_a value above 12 while the primary amine has a pK_a value of 10.5 [20]. Thus, it was decided to design a novel cationic lipid bearing two amine moieties with a monoleyl chain. Such a molecule should be able to associate and even anchor ODN molecules at two different sites provided a sufficient ionization of the primary amines occurred. It is anticipated that the ion pairing formation should be strong enough to allow controlled release of the ODN from the nanoemulsion under appropriate physiological dilutions as encountered in the eye following topical application. Novel formulations of cationic nanoemulsions are used to enhance the electrostatic ODN molecular interactions with the cationic moieties of the oil nanodroplets in order to reduce the dosing regimen frequency while increasing the ocular permeation. For this purpose a new bi-functional cationic lipid was synthesized and incorporated at the oil/water interface of the nanoemulsions. The present study examines the physicochemical properties and *in-vitro* release kinetic profiles of these nanoemulsions under various dilutions of simulated tear solution as a function of the cationic lipid type.

2. Materials

Seventeen bases oligonucleotide (sequence: 5′GsCsAsT sCTCCTTTTCsTsGsAsC3′) was purchased from Proligo, Boulder, CO, USA. This is a 17-mer partially phosphorothioated oligonucleotide with a MW of 5217 Da and called ODN in the present study. T4 polynucleotide kinase was purchased from New England Biolabs Ltd., Hitchin, Hertfordshire, UK. [GAMMA-^{33}P]–ATP 9.25MBq, 250 μCi, was purchased from Amersham Biosciences, Buckinghamshire, UK. Mid-chain triglycerides (MCT) was purchased from Societé des Oleagineux, Bougival, France. Lipoid E-80 was purchased from Lipoid AG, Ludwigshafen, Germany. Pluronic F-68 was purchased from BASF, Ludwigshafen, Germany. Vitamin E was purchased from Fluka, Taufkirchen, Germany. (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium salts) DOTAP and stearylamine (SA) were purchased from Sigma, St. Louis, MO, USA. Oleylamine (OA) was kindly supplied by Novagali, Evry, France.

3. Methods

3.1. Synthesis of arginine octadecyl amide (AOA)

1. Coupling of SA with Arginine: A solution of Fmoc-Arg(Mtr)-OH (Sigma, Israel 1 g, 1.65 mM) in Dichloromethane 50 ml, was added drop wise to a solution of Stearylamine (0.5 g, 1.86 mM) in dichloromethane

(50 ml) followed by the addition of dicyclohexylcarboiimide (Sigma, Israel 0.35 g, 1.65 mM). A precipitate, *N*,*N*-dicyclohexylurea, formed from the in situ coupling reaction of SA with the Fmoc-Arg(Mtr)-OH started to separate almost immediately and its amount gradually increased. After 6 h of reaction at room temperature the urea derivative was removed by filtration and washed with dichloromethane (20 ml). The excess of un-reacted Fmoc-Arg(Mtr)-OH in the combined filtrate was removed by $KHCO_3$ 1 N (50 ml). The organic phase was separated from the aqueous phase, dried over anhydrous $MgSO_4$ and evaporated to dryness in vacuum. The yield of the reaction was 80%.

2. Removal of N-terminal F-moc Group: The protected product was dissolved in a mixture of dimethylformamide (18 ml) and piperidine (2 ml). The solution was allowed to stand at room temperature over 4 h. The piperidine and dimethylformamide were removed in vacuum on a water bath not exceeding 60 °C. The residual solid mass was triturated with a mixture of ethylacetate:hexane (70:30) to dissolve the new compound (SA-Arg[Mtr]). The organic dispersion was filtered and the filtrate was evaporated under vacuum on a water bath at 60 °C. The yield of SA-Arg(Mtr) was 90%.
3. The side chain protecting groups (Mtr) were cleaved and removed with a mixture of 88% of TFA, 5% of phenol, 2% of triisopropylsilane and 5% H_2O over 6 h at room temperature. The mixture was removed by vacuum and the residue was washed with 0.1 N NaOH and finally dissolved in hot methanolic solution (100 ml), dried over anhydrous $MgSO_4$ and filtered. The product was crystallized while cooling the solution resulting in a final yield of 40% of pure AOA.

3.2. Nanoemulsion preparation

The blank cationic nanoemulsion was prepared according to the method previously described [17]. Glycerol and Poloxamer-188 were dissolved in the aqueous phase and adjusted to pH 7.4. The Lipoid E-80, α-tocopherol and the various cationic lipids were dissolved in the MCT oil phase. Both phases were heated separately to 70 °C, after which the two phases were mixed and stirred with a magnetic stirrer and further heated to a temperature of 80 °C. The resulting emulsion was then mixed by a high shear mixer Polytron™ (Kinematica, Luzern, Switzerland) at 16,000 rpm over 5 min and rapidly cooled to below 20 °C. After cooling, the emulsion was homogenized using a Rannie (APV Gaulin, Hilversum, The Netherlands) at 10,000 psi for 5 min. The pH of the emulsion was adjusted to 7.4 by titration with hydrochloric acid (0.1 N). The emulsion was then filtered through a TE membrane filter (Schleicher & Schuell, Dassel, Germany) with a pore size of 0.2 μm. The emulsion was filled under nitrogen atmosphere into siliconized glass bottles and then sterilized by steam autoclave at 121 °C for 15 min. A typical formulation consisted of (w/w%): MCT (1.66), poloxamer 188 (0.425), glycerol (2.25), lipoid E-80 (0.5), oleylamine (0.125) or DOTAP (0.33) or AOA (0.2), α-tocopherol (0.01) and doubled distilled water (DDW) up to 100. Equimolar concentrations of cationic lipids were used in each nanoemulsion formulation. In addition, various cationic nanoemulsions with increasing AOA concentrations varying from 0.05 up to 0.4% were prepared and characterized. Each formulation was prepared at least in triplicate.

3.3. Nanoemulsion characterization

Droplet size measurements were carried out utilizing an ALV Noninvasive Back Scattering High Performance Particle Sizer (ALV-NIBS HPPS, Langen, Germany) at 25 °C using water as the solvent. The sensitivity range was 0.5–5 μm.

Zeta potential measurements of the nanoemulsions were performed with the Malvern zetasizer (Malvern, UK) diluted in DDW (150 mV).

3.4. Oligonucleotide radiolabeling

The radiolabeling method used in this study is a well-established and validated process that has been widely reported in the literature [5,19]. The 5′-radiolabeled ODN was obtained as follows: 25 μl of 10 μM ODN solution, 5 μl of T4 polynucleotide kinase (PNK), 5 μl of T4 buffer, 10 μl DDW and 5 μl of ^{33}P–ATP (50 μCi) were incubated for 90 min at 37 °C after a short spin down and a brief vortexing. The reaction was stopped by heating at 80 °C for 15 min. The radiolabeled ODN was recovered after purification by exclusion chromatography (Bio-Spin® 6 chromatography columns) and centrifugation at 2400 rpm for 2 min. (The chromatography column was cleared first from the preserving buffer and washed three times with DDW using centrifugation at 2400 rpm to clear the preserving buffer and the DDW).

3.5. Oligonucleotide association to the blank cationic nanoemulsions

Oligonucleotide association was performed at the end of the manufacturing process. Final ODN concentrations ranging from 1 to 50 μM either in aqueous solutions or cationic nanoemulsions were prepared from ODN stock solution containing as an example 3.634 μg/μl of 17 bases ODN, the molecular weight of which is 5217 g/mole. All ODN-associated cationic nanoemulsions were incubated over 12 h at room temperature and combined with an appropriate amount of ^{33}P-ODN. Free ODN was determined in the clear ultra filtrate obtained following a 4 min centrifugation of the nanoemulsion at 4000 rpm through a porous membrane (300 k Nanosep centrifugal devices, Pall Life Sciences, Ann Arbor, Mi, USA). The amounts of radiolabeled oligonucleotide and respective calculated concentrations in the ultrafiltrates and intact nano-

emulsions were determined by radioactivity counting (Beckman LS 6000TA) against appropriate calibration curves following total dissolution of the formulations in Liquid scintillation (Ultima Gold®, Packard Bioscience B.V., Groningen, The Netherlands). The percent of ODN association was calculated from the difference of the total ODN in the nanoemulsion to the free ODN in the ultrafiltrate. It should be emphasized that the lack of adsorption of ODN on the Nanosep porous membranes was verified by filtrating ODN aqueous solutions at various concentrations and determining the ODN concentration in the supernatants and filtrates. No adsorption was observed validating the use of these membranes for ODN association and future *in-vitro* release experiments. In addition the lack of permeation of intact nanodroplets through the same membranes was tested by incorporating ^{14}C-cholesteryl oleate within the nanodroplets. No radioactivity was detected in the filtrates indicating that no intact nanodroplets permeated through the membrane under the given experimental conditions.

3.6. Influence of ODN association extent on nanoemulsion zeta potential

Different ODN concentrations up to 50 μM were added to the selected equimolar cationic lipid nanoemulsions [oleylamine (0.125%), DOTAP (0.33%), and AOA (0.2%)]. The samples were incubated at room temperature overnight under constant rotation movement. Zeta potential was then determined in DDW.

3.7. Stability assessment

The stability of ODN was validated using the gel electrophoresis method. 1 ml of 10 μM ODN cationic nanoemulsion was incubated under constant mild stirring in a water bath at 37 °C over 24, 48 and 72 h. It should be emphasized that ODN molecules could not be eluted in the presence of the intact nanoemulsion. Thus prior to gel electrophoresis procedure, 10 μl of ODN-cationic nanoemulsion formulations were lysed in 20 μl of 10% Triton X-100 (Aldrich, Rehovot, Israel) solution following a 15-min incubation at 50 °C. A 10 bp DNA ladder (Invitrogen, Karlsruhe, Germany) was used for sizing the oligonucleotide itself and its fragments. The ladder had to be denaturized in order to produce a set of single-stranded oligonucleotides increasing in length by 10-nucleotide increments. To a 5 μl of 10 bp DNA ladder an equal volume of denaturing solution [25 ml 95% (v/v) formamide, 93.06 mg EDTA, 25 mg bromophenol and 25 mg xylene cyanol] was added. After 5 min of incubation at 70 °C the single-stranded DNA ladder was ready to use. 5 μl of sample buffer (Invitrogen, Karlsruhe, Germany) were added to 10 μl of each sample of the various ODN cationic nanoemulsion formulations, fresh 10 μM ODN aqueous solution sample and single-stranded DNA ladder solution and then loaded directly into the TBE urea gel cassette wells. The gel was electrophoresed under the running conditions of 150 V and 500 mA over approximately 75 min (APELEX electrophoresis power supply unit). Finally the gel was stained with a DNA silver staining kit (Amersham Bioscience, Uppsala, Sweden).

3.8. In-vitro release of ODN

The *in-vitro* release kinetic study of ODN (10 μM) from the nanoemulsion was carried out by determining ^{33}P-labeled ODN release from the nanoemulsion following appropriate dilution into either simulated tear solution or DDW as a function of time. The composition of simulated tear solution [21] consisted of 192.4 mg $NaHCO_3$, 111.0 mg KCl, 2.29 mg $CaCl_2$, 672.8 mg NaCl, 669.0 mg albumin, 2.5 mg glucose and DDW up to 100 ml, while pH was adjusted to 7.4.

1600, 800, 200 μl and 80 μl of 10 μM ^{33}P-labeled ODN [oleylamine (0.125%), DOTAP (0.33%), AOA (0.1%) and AOA (0.05%)] nanoemulsions were diluted in 8 ml of simulated tear solution and DDW to elicit 1:5, 1:10, 1:40 and 1:100 dilution ratios, respectively. The samples were placed in a glass vial and incubated at 37 °C on a water bath throughout the experiment. At every time point (1, 16, 36, 56 and 86 min corresponding to the equivalent time intervals of 5, 20, 40, 60 and 90 min including centrifugation time), 400 μl samples were withdrawn from the vial and filtered through a 300 K Nanosep filter over 4 min at 4000 rpm. Aliquots of 10 μl from the filtrate were dissolved in 10 ml of Ultima Gold scintillation liquid and assayed for ODN content as previously described. 10 μl aliquots from the originally diluted nanoemulsion were also assayed for ODN content and were used as the total reference content. All kinetic experiments were done in triplicate.

4. Results and discussion

4.1. Cationic lipid synthesis

The synthesis of AOA was carried out according to the reaction described in Fig. 1.

The formation of AOA molecules as depicted in Fig. 1 was confirmed by LC-MS analysis showing the molecular peak at 425 Da, whereas the HPLC chromatogram data showed a purity level of 97% (data not shown). The chemical structures of the three cationic lipids are presented in Fig. 2.

Various cationic nanoemulsions were prepared with different cationic lipids. The rationale was to enhance the electrostatic molecular interactions between the cationic lipid and the negatively charged moieties of the ODN molecules. Thus, three cationic lipids were selected; OA with a single fatty acid chain and one primary amine, DOTAP, a double acyl chain with a single quaternary ammonium cationic charge and finally AOA, a single acyl chain with two primary amines.

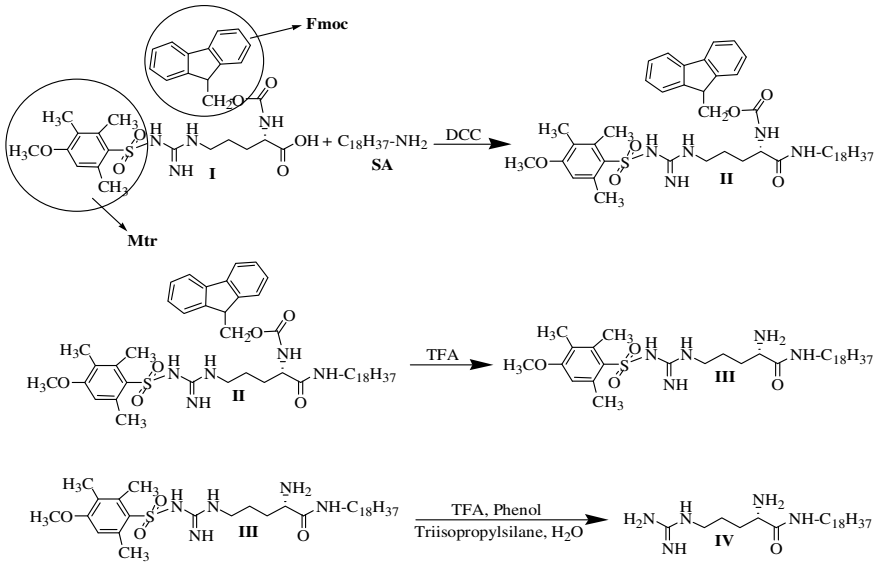

Fig. 1. Schematic description of arginine octadecyl amide (AOA) synthesis from stearylamine (SA) and Fmoc-Arg(Mtr)-OH. [I = Fmoc-Arg(Mtr)-OH, II = Fmoc-Arg(Mtr)-SA, III = Arg(Mtr)-SA, IV = arginine octadecyl amide, whereas Fmoc = Fluorenylmethyloxycarbonyl, Mtr = N_{α}-Fmoc-*N*-(4-methoxy-2,3,6-trimethylbenzenesulfonyl)-L-arginine].

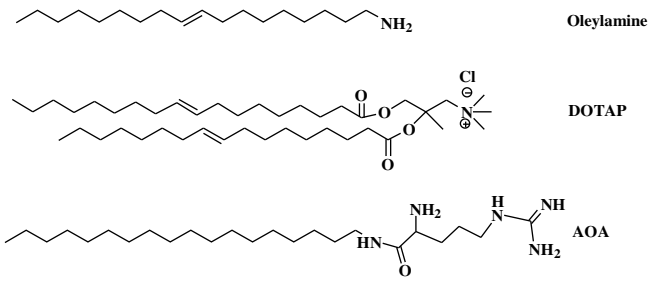

Fig. 2. Chemical structure of the cationic lipids used in this study.

4.2. Nanoemulsion characterization following ODN association

Different nanoemulsions comprising identical excipient formulations were prepared with the three available cationic lipids. The selected concentrations of the three cationic lipids were based on the calculation of an identical equivalent molar fraction of each cationic lipid in the formulation (4.67×10^{-4} mole/100 ml of nanoemulsion equivalent to 4.67 mM).

The physicochemical properties of the blank cationic nanoemulsions were measured at the end of the manufacturing process. It can be noted from the data presented in Table 1 that the average droplet size of the cationic nanoemulsions did not vary significantly with the cationic lipid change. A marked increase in zeta potential value of DOTAP nanoemulsion as compared to the zeta potential value elicited by OA cationic nanoemulsion was observed (Table 1). However, this increase became moderate and was not significant compared to the zeta potential value yielded by the AOA cationic nanoemulsion. This is consistent with the molecular structure of each cationic lipid as depicted in Fig. 2. DOTAP cationic moiety being a quaternary ammonium exhibited a stronger cationic charge than OA with its single primary amine. It should be emphasized that the nanoemulsion formulation without the cationic lipid, which is negatively charged in nature owing to the presence of the phospholipids at the o/w interface, was able to associate up to 66% of ODN (Table 1). Apparently, the net negative charge does not hamper the ODN association. A plausible explanation for such an association extent has already been reported [19,5] and attributed to the zwitter-

Table 1
Physicochemical properties and ODN association extent of various nanoemulsion formulations prepared with various cationic lipids using the experimental conditions described in the methods section following addition of 10 μM ODN

Nanoemulsion type	Average blank nanoemuls. diameter (nm)	Zeta potential blank nanoemuls. (mV)	Extent of association %	Average ODN loaded nanoemuls. diameter (nm)	Zeta potential ODN loaded nanoemuls. (mV)
Without cationic lipid	169 ± 15	−30.0 ± 0.2	66 ± 3.5	162 ± 11	−30.1 ± 2
OA 0.125	122 ± 4	40 ± 0.3	83.6 ± 1.1	117 ± 6	26.7 ± 1
AOA 0.2	148 ± 13	44.5 ± 4.9	93.6 ± 0.9	167 ± 14	46.3 ± 1.9
DOTAP 0.33	112 ± 2	52.4 ± 2.6	96.0 ± 0.4	123 ± 5	38.2 ± 2.3

ionic positive moieties of the phospholipids mainly of phosphatidylcholine which is the main constituent of the Lipoid E-80 used to stabilize the nanoemulsion. It should be pointed out that the electrostatic attraction between the polyanionic ODN groups and the positively charged phospholipid moieties was shown to be weak and unable to retain the associated ODN upon infinite dilution in physiological fluids [19]. More significant ODN associations were achieved with the cationic lipids as expected. It can be observed from the data presented in Table 1 that the extent of association increased with the type of cationic lipid even though equimolar concentrations were used. The ODN association with AOA and DOTAP was markedly higher than with OA. The increase in ODN association with AOA and DOTAP can be attributed either to the presence of two cationic amines/molecule or a quaternary ammonium moiety at the o/w interface in the nanoemulsion resulting in a more enhanced attraction with the anionic ODN groups. No marked change in droplet size was noted with the ODN associated nanoemulsion as compared to blank nanoemulsion, whereas as expected, the zeta potential value diminished with the OA and DOTAP cationic emulsions but remained highly positive irrespective of the cationic lipid used at the given ODN concentration of 10 μM (Table 1). No change in zeta potential was noted with the AOA cationic emulsion. This may be attributed to the presence of two amine groups on each cationic molecule. It should be emphasized that each formulation was done at least in triplicate and the results did not deviate more than a few percent indicating that the experimental conditions were well controlled and elicited reproducible nanoemulsions with similar properties.

4.3. Effect of increasing ODN and AOA concentration

It was interesting to examine whether the positive charge on the surface of the oil droplets would change following association of increasing ODN concentrations. It is well known that the ODN backbones are negatively charged and an important adsorption of ODN on cationic lipid nanoemulsion may reverse the charge. The above mentioned hypothesis was confirmed in the following experiment where the concentration of AOA was kept constant and low (0.05%) while the concentration of ODN was increased up to 25 μM (Fig. 3). Furthermore, when the concentration of ODN was increased to 50 μM, a rapid phase-separation phenomena was observed. It should be added that a large number of cationic nanoemulsion formulations with different oils (MCT, soybean oil, castor oil, sesame oil, mineral oil and respective combinations in 1:1 ratio) were prepared in an attempt to increase the ODN concentrations adsorbed and extend the physical stability of the nanoemulsions. None of the formulations prepared with other oils exhibited a marked advantage over the MCT-based cationic nanoemulsions even when the oil concentration was varied from 1.25 up to 5%. Thus, it

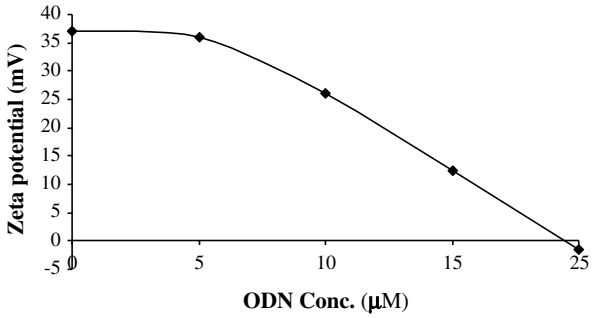

Fig. 3. The effect of increasing ODN concentration on the zeta potential of typical cationic nanoemulsion formulation prepared with 0.05% arginine octadecyl amide (AOA).

was decided to keep the 1.66% MCT as the preferred oil phase for the nanoemulsion.

It can be observed from the data presented in Table 2 that increasing the concentration of AOA from 0.05% to 0.4% did not alter markedly the particle size value of the blank cationic nanoemulsion. However, the zeta potential value decreased moderately with increases in concentration up to 0.2%. This could be attributed to the molecular structure of the cationic lipid bearing two cationic moieties. At low concentration of the AOA in the emulsion, the density at the o/w interface should be moderately lower than with the increased concentrations of AOA although all the concentrations led to sufficient surface droplet coverage. Thus, a better spreading and ionization of the cationic moieties occurred leading to a moderately higher zeta potential for the 0.05% AOA concentration. This is also confirmed indirectly by the resulting zeta potential values following ODN association (Table 2). The extent of ODN association did not vary significantly when the initial concentration of ODN remained constant at 10 μM. However, although the droplet size did not change significantly following ODN association with the exception of the cationic emulsion prepared with 0.05% AOA, the zeta potential value dropped consistently from 56.5 ± 2.8 and 51.3 ± 3.7 to 8.0 ± 0.5 and 38.8 ± 3.3 mV with low AOA concentrations of 0.05% and 0.1% in the cationic emulsions, respectively, as a result of cationic lipid moiety depletion at the o/w interface (Table 2). The increase in droplet size from 208 ± 27 to 297 ± 25 for the 0.05% AOA nanoemulsion following association with 10 μM ODN can be attributed to the marked decrease in zeta potential that can no longer prevent droplet coalescence upon random collisions. As previously reported [19], upon an ion-pairing formation between the cationic lipid and ODN, the lipophilic complex is internalized in the oil droplets and the cationic lipid in excess in the oil droplet core moves toward the interface keeping the net charge positive and zeta potential almost unchanged. This assumption is further supported by the data depicted in Fig. 3 where the concentration of AOA in the cationic emulsion was only 0.05%. It can be noted that the zeta potential is reduced with increasing ODN concentrations and is even reversed to negative from positive

Table 2
Physicochemical parameters and ODN association (10 μM) as a function of cationic lipid concentration

AOA nanoemulsion at various cationic conc. %	Average blank nanoemuls. diameter (nm)	Zeta potential blank nanoemuls. (mV)	Extent of association %	Average ODN loaded nanoemuls. diameter (nm)	Zeta potential ODN loaded nanoemuls. (mV)
0.05	208 ± 27	56.5 ± 2.8	87.0 ± 0.2	297 ± 25	8.0 ± 0.5
0.1	144 ± 17	51.3 ± 3.7	90.5 ± 0.6	180 ± 20	38.8 ± 3.3
0.2	148 ± 9	44.5 ± 4.9	93.6 ± 0.7	167 ± 14	46.3 ± 1.9
0.4	197 ± 17	57.9 ± 1.9	92 ± 0.4	211 ± 9	54.88 ± 0.8

The cationic nanoemulsion composition was MCT (1.66), poloxamer 188 (0.425) glycerol (2.25), lipoid E-80 (0.5), AOA, α-tocopherol (0.01) and DDW (up to 100) (w/w%).

as a result of the ODN association. It can further be observed from the data presented in Fig. 4 that cationic nanoemulsions of AOA, OA and DOTAP at an equimolar concentration of 4.67 mM are able to associate up to 25 μM ODN without losing the cationic charge on the oil droplets. Whereas no zeta potential diminution was observed with the AOA cationic emulsion, a moderate but almost progressive decrease in zeta potential value was detected with increasing ODN concentrations for the OA and DOTAP nanoemulsions with the exception of the 15 μM ODN concentration (Fig. 4). The standard deviation ranges are not larger than the observed variation in the zeta potential values between 10 and 15 μM ODN concentrations. This difference although minor indicates that the higher zeta potential values elicited by the 15 μM concentration irrespective of the cationic lipid are probably related to the density and ionization extent of the cationic lipid molecules at the droplet surface. At a concentration of 4.67 mM cationic lipid, a saturation density at the surface of the droplets was reached as noted as well by other authors with the excess of the cationic lipid molecules being dissolved in the oil cores [22–24]. A plausible explanation for the moderate increase in zeta potential value can be that at the point of 15 μM ODN, most of the available and excess ionized cationic molecules at the o/w interface reacted with the polyanionic ODN molecules. Thus, the density of the cationic lipid at the o/w interface although still high, was diminished and thus led to a better ionization of the remaining cationic lipid moieties. This resulted in a moderate increase in zeta potential value. Further addition of ODN molecules at 25 μM decreased significantly the zeta potential of the OA and DOTAP emulsions. In contrast, such a behavior was not noted with the AOA emulsion (Fig. 4). This effect could be attributed to the molecular structure of AOA that exhibited two cationic moieties per molecule. An additional decrease in surface density led to an increase in zeta potential value owing probably to an increase in cationic moieties ionization (Fig. 4). The overall data (Table 2, Figs. 3 and 4) clearly suggest that an electrostatic attraction occurred between the cationic lipids and the polyanionic ODN molecules at the o/w interface. Indeed, at low AOA concentrations (0.05 and 0.1%) most of the cationic lipid molecules reacted with the polyanionic groups of ODN and the ion-pairing complexes which probably exhibited poor aqueous solubility penetrated within the oil droplets. These molecular electrostatic attractions depleted the reservoir of AOA resulting in a neutralization of the cationic lipid moieties and a marked decrease in zeta potential especially for the lowest AOA concentration (Table 2).

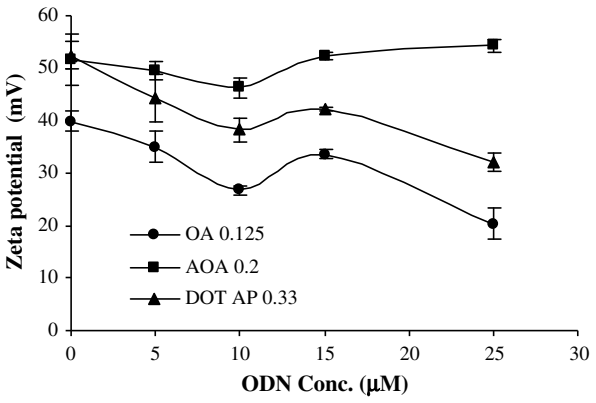

Fig. 4. The effect of increasing ODN concentration on the zeta potential of typical cationic nanoemulsion formulations prepared with various cationic lipids, oleylamine (OA), arginine octadecyl amide (AOA) and DOTAP, at identical equimolar concentrations of 4.67×10^{-4} mole/100 ml nanoemulsion.

4.4. Stability study

ODN was strongly attached to the oil droplets in the nanoemulsion and could not be eluted in the gel. To overcome this drawback and to ascertain the potential of the nanoemulsion to protect ODN from degradation when associated to the cationic oil droplets, it was decided to induce nanoemulsion phase separation prior to gel elution by the addition of 20 μl of 10% Triton X-100 solution to the nanoemulsion samples following 15 min incubation at 50 °C. ODN was released from the nanoemulsion following this phase-separation process. If the degradation of ODN occurred when associated to the nanoemulsion, it could then be assessed.

It can be deduced from the observations depicted in Fig. 5 that the ODN remained qualitatively intact in the nanoemulsion following up to 72 h of incubation at 37 °C. No fragment was detected in the gel electrophoresis chromatogram of the nanoemulsion samples although it appeared that the intensity of the band color declined with

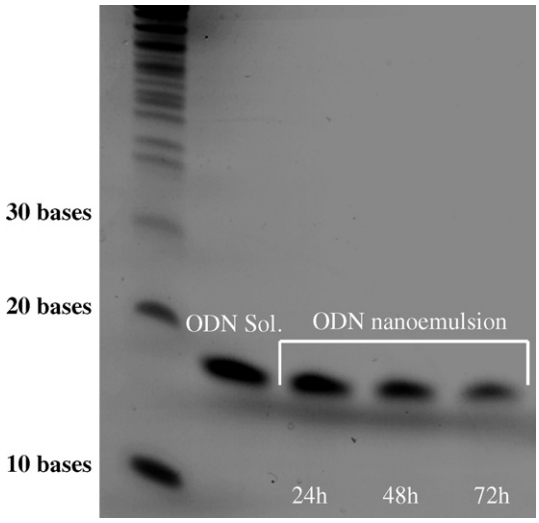

Fig. 5. Gel electrophoresis chromatogram of associated ODN following 24, 48 and 72 h of incubation under constant mild stirring in a water bath at 37 °C. The left lane contains a single-stranded 10 nucleotide ladder for sizing.

incubation time. Furthermore, in preliminary experiments it was found that TritonX-100 had no effect on ODN molecules under the given experimental conditions. It can therefore be deduced that the electrostatic attraction of ODN to cationic nanoemulsions does not alter the chemical integrity of the ODN macromolecules even following 72 h of incubation at 37 °C. It should be stressed that the association of ODN to cationic nanoemulsions did affect the physicochemical stability of the nanoemulsions as could be noted from the results presented in Table 3. The average droplet size of the ODN-associated OA and AOA cationic nanoemulsions increased following shaking at a constant rate of 100 rpm over 48 h at 37 °C while the DOTAP nanoemulsion remained physically stable. This is probably related to the high zeta potential value exhibited by the DOTAP nanoemulsion. The inconsistency in the zeta potential behavior of the various nanoemulsions should be attributed to the chemical structure of the cationic lipids. Oleylamine exhibits one single primary amine, thus any partial degradation of the molecule decreases the number of the cationic moieties resulting in a marked decrease in the positive zeta potential value. Whereas AOA exhibits two cationic moieties per molecule and despite the partial depletion due to the molecule degradation, its local concentration remains sufficiently high to maintain the initial zeta potential value. The DOTAP emulsion physical stability was not affected indicating that the quaternary ammonium DOTAP molecules remained stable. Thus the marked increase in zeta potential may be due only to a better spreading of the cationic moieties on the surface of the nanodroplets as a result of the shaking and increased storage temperature (Table 3).

In view of the stability data, it was decided to work in the future only with DOTAP nanoemulsion for further animal evaluation. Nevertheless, it is expected that if such a dosage form reaches the clinic, it will be prepared immediately prior to administration by incubating the appropriate ODN with the cationic nanoemulsion to prevent any additional degradation.

4.5. In-vitro kinetic experiments

To define optimal experimental conditions for the preparation of associated nanoemulsions that can retain their adsorbed ODN in the presence of electrolytes under the physiological conditions existing in the lachrymal sac in the presence of tear, ODN release profiles from cationic nanoemulsions diluted in different ratios of DDW and STS ranging from 1:5 up to 1:100 were determined. It was noted in the kinetic experiments that less than 5% of ODN was released from the ODN-associated nanoemulsions prepared with a low AOA concentration of 0.05% following various DDW dilutions up to 1:40 (Fig. 6). However, when DDW was changed by a simulated tear solution, a rapid release up to 45% of ODN from the nanoemulsions was observed (Fig. 6) indicating that the presence of electrolytes in the simulated tear solutions induced rapid release. It should be emphasized that proteins, and specifically albumin, can displace ODN from cationic lipids as reported by Zelphati and coll. [25]. However, the concentration of albumin in STS is only 0.1 mM while the concentration of chlorides and carbonates is 130 and 7.6 mM, respectively. Thus, the displacement effect should be mainly mediated by the anions in the STS. Cl^- and CO_3^{-2} probably enabled the rapid release of the ODN over a 40-min period, indicating that an anion-exchange process was probably involved. It is noteworthy that increasing the

Table 3
Physicochemical properties of various cationic nanoemulsions following ODN association and shaking at constant rate of 100 rpm over 48 h at 37 °C

Nanoemulsion	ODN associated cationic nanoemulsion			
	Before accelerated test		After accelerated test	
	Zeta potential (mV)	Particle size (nm)	Zeta potential (mV)	Particle size (nm)
Oleylamine 0.125	26.7 ± 1	117 ± 6	14.9 ± 0.6	178 ± 10
DOTAP 0.33	38.2 ± 2.3	123 ± 5	50.2 ± 1.7	126 ± 7
AOA 0.1	22.1 ± 0.8	161 ± 17	21.8 ± 0.7	209 ± 21

The cationic nanoemulsion composition was MCT (1.66), poloxamer 188 (0.425) glycerol (2.25), lipoid E-80 (0.5), oleylamine (0.125) or DOTAP (0.33), or AOA (0.1), α-tocopherol (0.01) and DDW (up to 100) (w/w%).

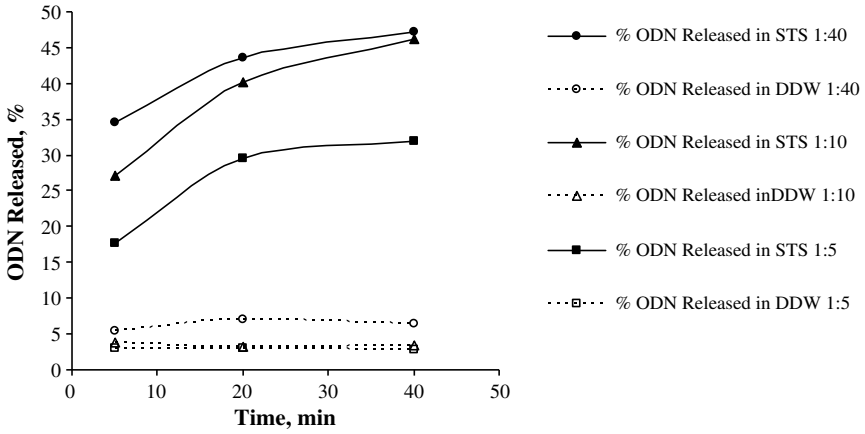

Fig. 6. ODN *in-vitro* release profiles from a typical cationic nanoemulsion formulation prepared using 0.05% AOA as a function of increasing dilution ratios (1:5–1:40) of double-distilled water (DDW) and simulated tear solution (STS).

dilution ratio from 1:5 to 1:10 and more particularly to 1:40 increased the initial fraction released at 5 min from 18% to 27% and 35% following dilution to 1:10 and 1:40, respectively (Fig. 6). However the highest fractions released at 40 min for both of the higher dilutions are similar at around 45% and almost reached the plateau which was always less than 50%. This is also confirmed by the kinetic experiment results in (Fig. 7a–c). It was again observed that the kinetic behavior in DDW was not markedly altered by the type of cationic lipid used and was not consistent with the chemical structure although the DOTAP cationic nanoemulsion retained more than 97% of the ODN even at a dilution of 1:100 (Fig. 7c). The main difference in the profiles was noted immediately following the dilution evidencing a rapid anion exchange between the ODN and the electrolytes. However, DOTAP cationic lipid nanoemulsion was the most efficient formulation capable of retaining the ODN despite the high dilution with simulated tear solution since even at 1:100 dilution less than 10% of the ODN was exchanged. Most recently, other authors investigated the *in-vitro* kinetic release of a model ODN, pdT_{16} from OA and DOTAP nanoemulsions in PBS, buffer pH 7.4 as the dilution media. They reported a marked decrease in ODN release from the DOTAP emulsion as compared to the OA emulsion in agreement with the present kinetic results [24]. The authors reported that the percent of ODN released was dependent on the cationic/ODN charge ratio. The smaller ODN fraction released was observed with a charge ratio of 20 in favor of the cationic lipid. A similar behavior was also noted in the present study since under the given experimental conditions, at the concentration of 4.67 mM (4.67×10^{-4} moles/100 ml) cationic lipid and 10 μM ODN, the molecular cationic lipid/ODN charge ratio (+/−) was 26 for OA and DOTAP and 52 for AOA, well in favor of the cationic charges.

It was interesting to note that irrespective of the dilution with either DDW or STS, no release above 50% was observed. These findings apparently support the previously reported hypothesis that when ODN is associated with cationic lipid at the o/w interface of the emulsion, a lipophilic complex is formed which is internalized with the oil core of the nanoemulsion [5]. Thus, in the presence of anions, there is an exchange only with the ODN located at the interface. The incorporated ODN-cationic lipid complex remains in the nanoemulsions despite the infinite dilution with STS. This amount will probably serve as a drug reservoir upon local administration to the eye. The slow release effect of ODN elicited by DOTAP as compared to OA and AOA could also be attributed to the molecular structure of DOTAP comprising two oleyl chains that allow a double anchoring in the interfacial film and a more prolonged residence time at the o/w interface.

It should be noted that various kinetic equation models based on passive diffusion and derived from Fick law have been used to describe overall release from micro and nanoparticulate delivery systems. However, they are based on the assumption that no ion exchange or erosion occurs. None of these kinetic equations can be used in the present case since the release of ODN from the cationic nanoemulsions is mediated by anion exchange [26,27]. It was decided to analyze kinetic data obtained from the ODN-associated nanoemulsions and carry fittings of cumulative release profiles to specific kinetic models prevailing in anion-exchange processes, using the appropriate possible kinetic equations.

Most of the knowledge on the mechanism of ion-exchange is chiefly based on studies carried out by Boyd and coll. [28] who thoroughly analyzed the ion-exchange kinetics and applied the Nernst concept of a liquid diffusion layer.

Two possible rate-determining steps should be considered:

1. Interdiffusion of counter ions within the ion-exchange oil nanodroplets (particle diffusion);
2. Interdiffusion of counter ions in the adherent liquid film (film diffusion).

In cases where particle diffusion prevails, the nanoemulsions are uniform spheres of radius r and sink conditions

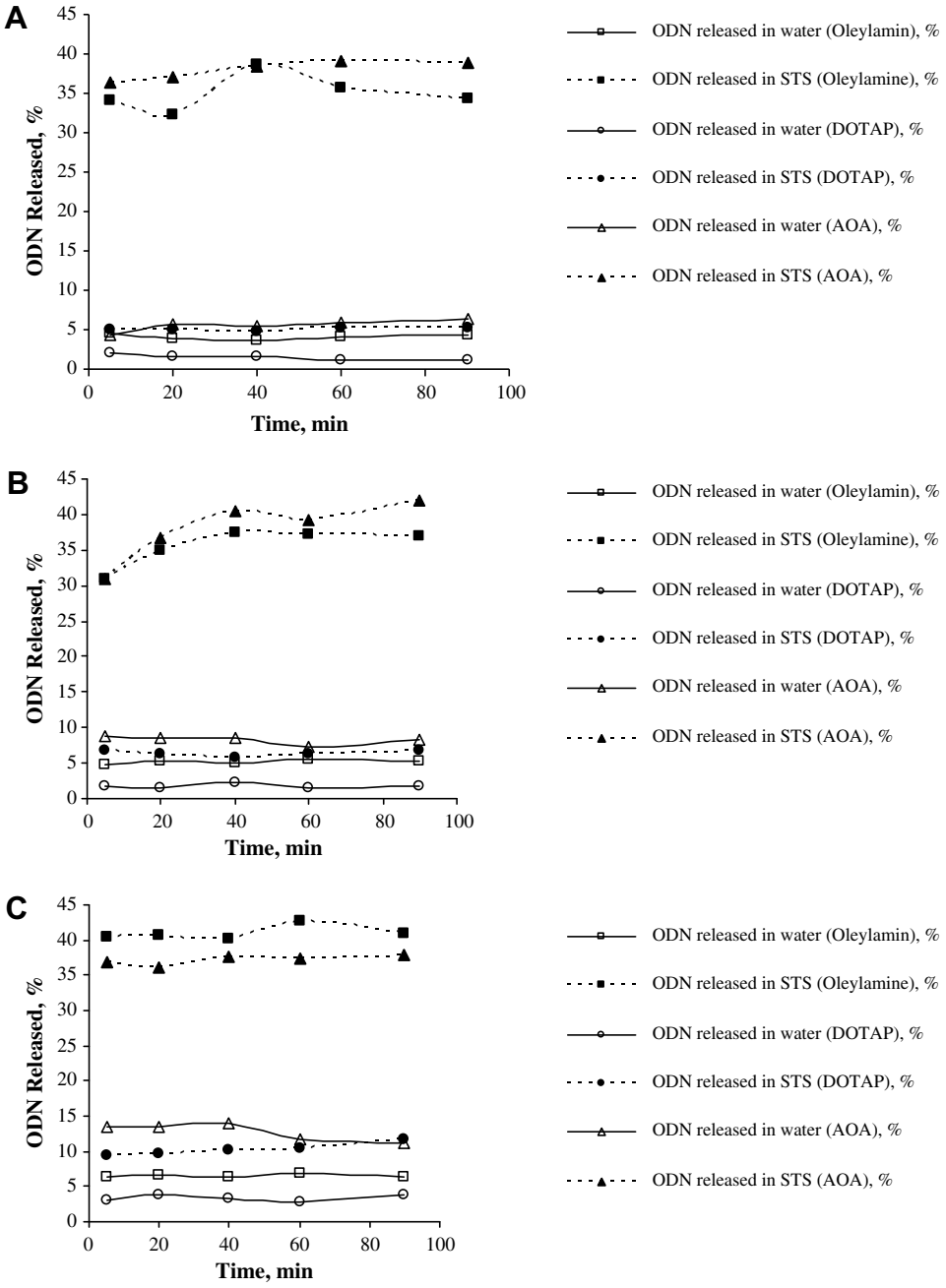

Fig. 7. ODN *in-vitro* release profiles from various typical cationic nanoemulsions prepared with different cationic lipids following dilutions 1:10 (A), 1:40 (B) and 1:100 (C) in double-distilled water (DDW) and in simulated tear solution (STS) at 37 °C. [The cationic nanoemulsion composition was MCT (1.66), poloxamer 188 (0.425) glycerol (2.25), lipoid E-80 (0.5), oleylamine (0.125) or DOTAP (0.33), or AOA (0.2), α-tocopherol (0.01) and DDW (up to 100) (w/w%)].

are respected, the following expression for drug exchange or release should hold [28]:

$$F = \frac{Q_t}{Q_\infty} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{e^{-n^2Bt}}{n^2} \quad (1)$$

where $B = \pi^2 D_i / r^2$

Q_t is the amount of drug released or exchanged at time t, Q_∞ is the amount of drug exchanged at infinite time, F is therefore the fractional attainment of equilibrium and D_i is the effective diffusion coefficient of the two exchanging ions in the nanodroplets.

Obviously it is not possible to estimate the B values from measured F using Eq. (1) because infinity terms are involved. Reichenberg [29] therefore sought a simpler approximate expression for Eq. (1). The main disadvantage of his method is that his standard table needs to be consulted each time [29], and the procedure is cumbersome and inelegant. Bhaskar and coll. [30] proposed an elegant method to test the particle-diffusion controlled release of

drug. Extensive experimental results were used to validate their method based on the following equation.

$$-\mathrm{Ln}(1-F) = 1.59\left(\frac{3}{r}\right)^{1.3} D_i^{0.65} t^{0.65} \quad (2)$$

This suggests that particle diffusion control can be tested by simply testing for linearity between $\mathrm{Ln}(1-F)$ and $t^{0.65}$.

In the majority of cases studied so far, the rate-determining step of the ion-exchange process was established to be diffusion of the counter ions rather than an actual chemical exchange reaction at the fixed ionic groups. Thus, in the present study, the ODN-release experiments were tested for counter ion diffusion as suggested by Eq. (2). Plots of $\mathrm{Ln}(1-F)$ versus $t^{0.65}$ for ODN exchange are shown in Fig. 8. It can be seen from Fig. 8 that the kinetic data do not conform with counter ion diffusion since the R^2 values are not close to 1. It was not possible to apply any mathematical fitting to the *in-vitro* release kinetic data of ODN from DOTAP nanoemulsions because no release above 10% was observed. This marked deviation is probably due to the large ODN molecules, which apparently are attached to the interface of the nanodroplets in more than one active site. The exchange with anions from STS should not be a simple exchange process leading to significant deviations from the expected mathematical model. The *in-vitro* release kinetic behavior of ODN exchange with physiological anions appears to be complex and difficult to characterize using mathematical fitting equations. The non-conformity does not exclude potential therapeutic efficacy since it is expected that despite infinite dilutions and the presence of anions a majority of the associated ODN molecules which remain within the nanodroplets would elicit the desired pharmacological action once the oil nanoproplets or the lipophilic ion-pairing complexes reach the target cells following topical application. It can be deduced that DOTAP cationic nanoemulsion can maintain the initial physicochemical properties, associate ODN molecules and release them under appropriate physiological conditions. Furthermore, the ODN release kinetic process will not be governed by a film diffusion process but rather by low oil degradation at the tissue of target. Further pharmacokinetic and pharmacological animal studies are needed to validate our assumptions and verify the *in-vitro* ODN release kinetic hypothesis.

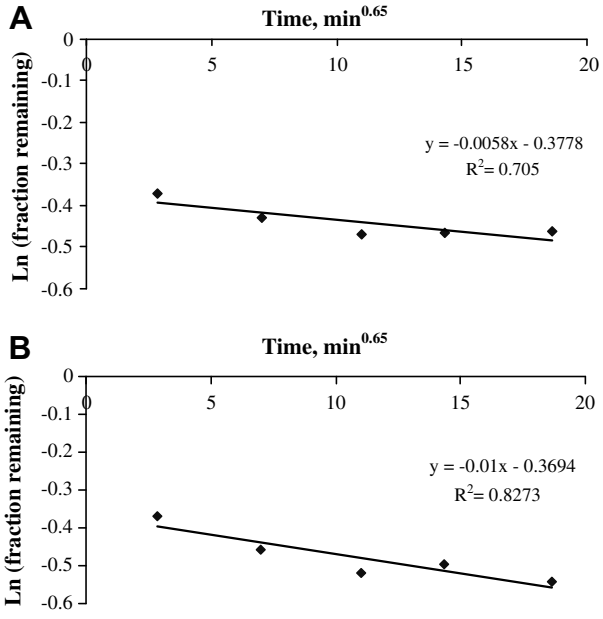

Fig. 8. Ion exchange equation release plots of ODN from typical cationic nanoemulsion formulation prepared using 0.2% arginine octadecyl amide (A) and 0.125% oleylamine (B) in 1:40 dilution in STS.

5. Conclusion

The increase in ODN association with AOA and DOTAP as compared to OA cationic emulsions can be attributed either to the presence of two primary amines/molecule or a quaternary ammonium moiety, respectively, at the o/w interface of the nanoemulsion resulting in a more enhanced attraction with the anionic ODN moieties. No marked change in droplet size was noted with the ODN-associated nanoemulsions as compared to blank nanoemulsion; whereas as expected the zeta potential value diminished but remained highly positive irrespective of the cationic lipid type used at the given ODN concentration of 10 μM. All the comprehensive ODN-associated experimental results suggest that an electrostatic attraction occurred between the cationic lipids and the polyanionic ODN molecules at the o/w interface of the cationic nanoemulsions.

The average droplet size of the ODN associated OA and AOA cationic nanoemulsions increased following shaking at a constant rate of 100 rpm over 48 h at 37 °C while the DOTAP nanoemulsion remained physically stable. This is probably related to the high zeta potential value exhibited by the DOTAP nanoemulsion.

It was noted in the kinetic experiments that less than 10% of ODN was released from the ODN-associated nanoemulsions following various DDW dilutions. However, when DDW was changed by a simulated tear solution, a rapid release up to 45% of ODN from the OA and AOA nanoemulsions was observed but not with the DOTAP nanoemulsion that was able to retain the ODN indicating that the presence of electrolytes in the simulated tear solutions induced a rapid release mediated by an anion-exchange process.

In view of the stability data and the ODN *in-vitro* release kinetic profile observations, it was decided to work only with DOTAP nanoemulsions for further animal evaluation.

Acknowledgement

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