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Research paper

Novel cationic liposome formulation for the delivery of an oligonucleotide decoy to NF-κB into activated macrophages

Giuseppe De Rosa [a,*,1], Daniela De Stefano [b,1], Valeria Laguardia [a], Silvia Arpicco [c], Vittorio Simeon [b], Rosa Carnuccio [b], Elias Fattal [d]

[a] *Dipartimento di Chimica Farmaceutica e Tossicologica, Università degli Studi di Napoli Federico II, Naples, Italy*
[b] *Dipartimento di Farmacologia Sperimentale, Università degli Studi di Napoli Federico II, Naples, Italy*
[c] *Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Turin, Italy*
[d] *UMR CNRS 8612, Faculté de Pharmacie, Université Paris-Sud, Châtenay-Malabry, France*

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Abstract

Nuclear factor-κB (NF-κB) is involved in several pathological processes, such as inflammation. Pro-inflammatory genes expression can be down-regulated by using an oligonucleotide (ODN) decoy to NF-κB. Cationic liposomes are largely used to improve ODN uptake into cells, although a higher transfection efficiency and a lower toxicity are required to use them in therapy. In this work, we investigated the potential of a novel liposome formulation, based on the recently synthesised cationic lipid (2,3-didodecyloxypropyl) (2-hydroxyethyl) dimethylammonium bromide (DE), as the delivery system for a double stranded ODN decoy to NF-κB. Liposomes composed of DE or DE mixed with 1,2-dioleyl-sn-glycero-3-phosphoethanolamine or cholesterol as *helper lipids* were complexed with ODN at different +/− charge ratios. *In vitro* uptake and the effect of ODN, naked or complexed with DE-containing liposomes, were evaluated in lipopolysaccharide-stimulated RAW 264.7 macrophages. The use of *helper lipids* increased liposome physical stability up to 1 year at 4 °C. ODN complexed with DE/cholesterol liposomes, at the +/− charge ratio of 8, showed a limited cytotoxicity and the highest inhibition of nitrite production, inducible nitric oxide synthase protein expression and NF-κB/DNA binding activity. Confocal microscopy confirmed a high ODN cell uptake obtained with DE/cholesterol liposomes at the highest +/− charge ratio.

Keywords: Nuclear factor-κB; Cationic liposomes; Decoy oligonucleotide; RAW 264.7 macrophages

1. Introduction

NF-κB plays a critical role in regulating the expression of genes responsible for a wide range of cellular processes, including inflammatory responses, innate and adoptive immunity, and pathways related to apoptosis and cell growth [1]. In quiescent cells, NF-κB resides in the cytosol in a latent form bound to inhibitory proteins, IκBs. Stimulation of different type of cells with lipopolysaccharide (LPS), cytokines or oxidants agents triggers a series of signalling events that ultimately converge to the activation of one or more redox-sensitive kinases which specifically phosphorylate IκB, resulting in their polyubiquitination and subsequent degradation [2]. Activated NF-κB is then free to translocate into the nucleus and stimulate transcription by binding to cognate κB sites in the promoter regions of various target genes such as inducible nitric oxide synthase (iNOS) [3]. NF-κB has been considered as a good therapeutic target for the treatment of several pathologies. Studies related to NF-κB activation pathway have led to the discovery of inhibitors of its transcriptional activity [4]. The decoy

* Corresponding author. Dipartimento di Chimica Farmaceutica e Tossicologica, Università degli Studi di Napoli Federico II, Via D. Montesano 49, 80131 Naples, Italy. Tel.: +39 81 678666; fax: +39 81 678630.
E-mail address: gderosa@unina.it (G. De Rosa).
[1] These authors equally contributed to the work.

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strategy is considered very powerful to down-regulate NF-κB-related gene expression [5,6]. Indeed, synthetic double stranded ODN as decoy *cis*-elements blocks the binding of NF-κB to promoter regions of its targeted genes, resulting in the inhibition of transactivation *in vitro* [7,8] and in *vivo* [5,6]. Despite their high potentialities, the therapeutic use of decoy ODNs is strongly hampered by the poor transport across cell membranes and their short blood half-life due to a quick degradation by nucleases [9,10]. An approach to increase intracellular penetration of ODN to NF-κB consists in its association to positively charged lipids [11]. However, the efficiency of non-viral delivery systems is so low that it is necessary to use high amounts of these cationic lipids to achieve transfection which might result in toxic and side effects. Therefore, there is a need to design more efficient formulations to reduce the doses necessary for transfection. To gain efficacy, the presence of a short acyl chain on the cationic lipids is one major parameter [12,13]. It has been shown that a new synthesised cationic lipid, (2,3-didodecyloxypropyl) (2-hydroxyethyl) dimethylammonium bromide (DE) (Fig. 1) with a short acyl chain has high potential for transfection of plasmid DNA in different cell lines [14]. Following this preliminary experiment, we believe that optimal formulations of DE liposomes could be of interest for the transport of several types of nucleic acids including the decoy ODN. The aim of this work was to investigate the potential of liposomes containing the cationic lipid DE, a delivery system for a double stranded ODN decoy to NF-κB. Liposomes based on DE, alone or associated with 1,2-dioleyl-sn-glycero-3-phosphoethanolamine (DOPE), or cholesterol (chol), were prepared and characterised. The transfection efficiency of ODN complexed with all liposome formulations, at different +/− charge ratios, was evaluated by measuring the inhibition of NF-κB activation in LPS-stimulated RAW 264.7 macrophages.

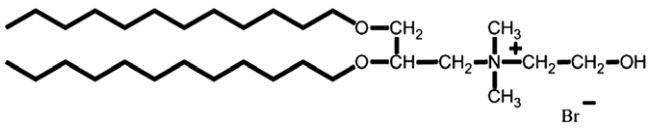

Fig. 1. Chemical structure of the cationic lipid (2,3-didodecyloxypropyl) (2-hydroxyethyl) dimethylammonium bromide (DE).

2. Materials and methods

2.1. Reagents

Cationic lipid DE was synthesised and purified as previously reported [14]. Lipofectamine™ was provided by Invitrogen (Milan, Italy). DMEM, foetal bovine serum, glutamine, penicillin, streptomycin, hepes, sodium pyruvate and PBS were purchased from BioWhittaker (Caravaggio, Italy). DOPE, chol, potassium chloride (KCl), LPS and sodium dodecyl sulphate (SDS) were obtained from Sigma Chemical Co. (Milan, Italy). Analytical grade methylene chloride and methanol were from Carlo Erba Reagenti (Milan, Italy).

2.2. Decoy ODN against NF-κB

Phosphorothioate ODN were customly synthesised by Tib Molbiol (Roche Diagnostics, Italy). Plain double stranded ODN to NF-κB was prepared by annealing of sense and antisense phosphorotioate ODN. Briefly, a mixture of both ODNs was heated at 80 °C for 5 min and allowed to cool slowly at room temperature over night. For each experiment, the ODN was annealed before use. Two double stranded ODNs, one with consensus sequence against NF-κB and the other with mutant NF-κB consensus sequence, were used. The ODNs had the following sequences:

1) wild-type-NF-κB consensus sequence
   5′-GAT CGA GG**G GAC** TTT CCC TAG C-3′
   3′-CTA GCT CC**C CTG** AAA GGG ATC G-5′
2) mutant-NF-κB consensus sequence with a mutation indicated with the bolded bases (GGAC to AAGC) of wild-type NF-κB consensus sequence (Mut ODN)
   5′-GAT CGA GG**A AGC** TTT CCC TAG C-3′
   3′-CTA GCT CC**T TCG** AAA GGG ATC G-5′

2.3. Liposome preparation

Cationic liposomes composed of DE, DE/DOPE (molar ratio 1:1), DE/chol (molar ratio 1:1), DE/DOPE/chol (molar ratio 2:1:1) were prepared in sterile water by the thin lipid film hydration method. The final lipid concentration depended on the formulation as shown in Table 1,

Table 1
Composition of the liposome formulations used to form lipoplexes

Formulation denomination	Lipid composition	Lipid molar ratio	DE final concentration (mg/ml[a])	DOPE final concentration (mg/ml[a])	Chol final concentration (mg/ml[a])
DE	DE	–	2	–	–
DEDO	DE:DOPE	1:1	2	2.56	–
DECHO	DE:chol	1:1	2	–	1.33
DEDOCHO	DE:DOPE:chol	2:1:1	2	1.28	0.66

[a] Volume of liposome suspension.

whilst DE concentration was maintained at 2 mg/ml in all cases. All formulations were extruded using a Thermobarrel Extruder Systems (Northern Lipids Inc., Vancouver, BC, Canada) passing the suspension 10 times under nitrogen through polycarbonate membrane (Nucleopore Track Membrane 25mm, Whatman, Brentford, UK) with progressively decreasing pore size (0.4, 0.2 and 0.1 μm, respectively).

2.4. Liposome characterisation

Liposome diameter was determined by photon correlation spectroscopy (PCS) (N5, Beckman Coulter) soon after preparation, after extrusion and at different times during storage at 4 °C. Zeta potential of all formulations was measured in distilled water using a Zetasizer Nano Z (Malvern, UK). Possible changes in lipid composition resulting from extrusion were investigated by nuclear magnetic resonance (^{1}H NMR) analysis (Bruker AC 300 instrument Karlshrue, Germany). Briefly, liposome suspension was freeze-dried and dissolved in $CDCl_3$. ^{1}HNMR analysis was performed before and after extrusion by determining the ratio between the heights of characteristic peaks in the ^{1}HNMR spectra of the different lipids for each sample. Results were the mean of analysis of three different batches.

2.5. ODN/liposome complexes

After annealing, ODN was diluted in sterile water and complexed with an equal volume of liposome suspension at a final ODN concentration of 0.4 μM. Liposomes concentration was adjusted to obtain complexes with a +/− charge ratios of 0.5, 1, 2, 4 and 8, respectively. Complex size was determined by PCS after incubation at room temperature in water, DMEM and DMEM containing 10% foetal bovine serum (FBS). For each complex, zeta potential in water was also measured as described above.

2.6. Cell culture experiments

The mouse monocyte/macrophage cell line RAW 264.7 was cultured at 37 °C in humidified 5% CO_2/95% air in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM Hepes and 5 mM sodium pyruvate. Cells were plated in 24 culture wells at a density of 2.5×10^5 cells/ml per well or 10 cm diameter culture dishes at a density of 3.10^6 cells/ml in each dish and allowed to adhere for 2 h. Thereafter, the medium was replaced with fresh medium (with or without serum) and cells were stimulated with LPS (1 μg/ml). Naked ODN, naked Mut ODN, ODN/liposome complexes and Mut ODN/liposome complexes were added on the cells at a final ODN concentration of 0.4 μM. After 4 h of cell incubation with the products, the amount of nitrite (NO_2^-, nmol/10^6 cells) corresponding to the accumulation of NO was measured by a colorimetric assay based on the Griess reaction [15]. Briefly, Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine in phosphoric acid) was added to an equal volume of cell culture supernatant and the absorbance was measured after 10 min at 550 nm using a UV/VIS spectrometer (Shimadzu 1204, Japan). The nitrite concentration was determined by reference to a standard curve of sodium nitrite. Cell viability was determined by using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) conversion assay [15]. Briefly, 100 μl MTT (5 mg/ml in complete DMEM) was added to the culture medium and cells were incubated for an additional 3 h. Cells were then lysed and the dark blue crystals solubilised with 500 μl of a solution of water/*N*,*N*-dimethylformamide 1:1 (v/v) containing SDS (20% w/v) adjusted to pH 4.5 with HCl. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCCC/340) equipped with a 620 nm filter. Cell viability in response to the treatment with test compounds was calculated as % dead cells = 100 − (OD treated/OD control) × 100.

2.7. Cytosolic and nuclear extracts

Cytosolic and nuclear extracts from macrophages, previously stimulated for 24 h with LPS (1 μg/ml) in the presence or absence of naked ODN (0.4 μM), naked Mut ODN (0.4 μM), DE/ODN and DE/Mut ODN complexes at the +/− charge:ratios of 0.5 and 8 (0.4 μM), were prepared as previously described with some modifications [15]. Briefly, harvested cells (3×10^6) were washed twice with ice-cold PBS and centrifuged at 180 g for 10 min at 4 °C. Cell pellet was resuspended in 100 μl of ice-cold hypotonic lysis buffer (10 mM hepes, 10 mM KCl, 0.5 mM phenylmethylsulphonyl fluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM DTT) and incubated on ice for 15 min. Cells were lysed by rapid passage five or six times through a syringe needle and the cytoplasmic fraction was then obtained by centrifugation for 1 min at 13 000 g. The supernatant containing the cytosolic fraction was removed and stored at −80 °C. The nuclear pellet was resuspended in 60 μl of high salt extraction buffer (20 mM hepes, pH 7.9, 10 mM NaCl, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM phenylmethylsulphonyl fluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM DTT) and incubated with shaking at 4 °C for 30 min. The nuclear extract was then centrifuged for 15 min at 13 000 g and the supernatant was aliquoted and stored at −80 °C. Protein concentration was determined using a Bio-Rad (Bio-Rad Laboratories S.r.l., Milano, Italy) protein assay kit.

2.8. Western blot analysis

Immunoblotting analysis of anti-iNOS, anti-p50 and anti-p65 was performed on the cytosolic or nuclear fraction. Cytosolic and nuclear fraction proteins were mixed

with gel loading buffer (50 mM tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg/ml of bromophenol) in a ratio of 1:1, boiled and centrifuged at 10 000 g. Equivalent amounts (30 μg) of each sample were submitted to electrophoresis in an 8% discontinuous polyacrylamide minigel. The proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories S.r.l., Milano, Italy) according to the manufacturer's instructions. The membranes were saturated by incubation at room temperature for 2 h with 10% non-fat dry milk in PBS and then incubated with (1:1000) anti-iNOS, anti-p50 and anti-p65 at 4 °C overnight. Membranes were washed with 0.1% Tween 20 in PBS and then incubated with anti-rabbit, anti-mouse or anti-goat immunoglobulins coupled to peroxidase (1:1000) (DAKO, Italy). Immunocomplexes were visualised by the ECL chemiluminescence method (Amersham, Italy). Membranes were stripped and reprobed with β-actin or anti-histone-1 antibodies to verify equal loading of proteins. Subsequently, the relative expression of iNOS, p50 and p65 in cytosolic and nuclear fractions was quantified by densitometric scanning of the X-ray films with a GS-700 imaging densitometer and the Molecular Analyst programme.

2.9. Electrophoretic mobility shift assay

Double stranded ODNs containing the NF-κB (5′-CAACGGCAGGGGAATCTCCCTCTCCTT-3′) recognition sequence were endlabelled with ^{32}P-γ-ATP. Nuclear extracts containing 5 μg protein were incubated for 15 min with radiolabelled oligonucleotides (2.5–5.0 × 10^4 cpm) in 20 μl reaction buffer containing 2 μg poly dI–dC, 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT and 10% (v/v) glycerol. The specificity of the DNA/protein binding was determined by competition reaction in which a 50-fold molar excess of unlabelled wild-type, mutant or Sp-1 oligonucleotide was added to the binding reaction, 15 min before the addition of radiolabelled probe. In the supershift assay, antibodies reactive to p50 or p65 proteins were added to the reaction mixture 15 min before the addition of radiolabelled NF-κB probe. Nuclear protein–oligonucleotide complexes were resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel in 1 × TBE buffer at 150 V for 2 h at 4 °C. The gel was dried and autoradiographed using an intensifying screen at −80 °C for 20 h. Subsequently, the relative bands were quantified by densitometric scanning of the X-ray films with a GS-700 imaging densitometer (Bio-Rad, Italy) and a computer program (Molecular Analyst; IBM).

2.10. ODN cellular uptake studies

To follow ODN intracellular trafficking, a fluorescent ODN containing a 5′ Carboxy-X-rhodamin-labelled antisense filament was used. Briefly, cells were plated in 24 culture wells, containing one cover glass in each well, at a density of 5 × 10^5 cells/ml per well and allowed to adhere for 2 h. Thereafter, the medium was replaced with fresh medium and cells were stimulated with LPS (1 μg/ml). ODN was added to cells, naked or complexed to liposome at the final concentration of 0.4 μM, 5 min after LPS challenge. After 4 h, cells were washed with PBS and fixed with 4% formaldehyde. Cell membranes were stained with *N*-(fluorescein-5-thiocarbamoyl)-1,2-diexadecanoyl-*sn*-glycero-3-phosphoethanolamine (fluoresceine DHPE, Molecular Probes, Netherlands). Cells were washed again with PBS, then treated with 1,4-diazabicyclo [2.2.2] octane (DABCO, Sigma) solution in PBS and mounted with Confocal Matrix® (Micro-Tech-Lab). Cover glasses were mounted on microslides and stored at 4 °C. Observations were carried out with a confocal microscope equipped with a Zeiss 63X/1.4 NA oil immersion objective lens (C Apochromat).

Table 2
^{1}H NMR peak integration of lipid constituent of liposome, before and after extrusion

Formulation	Compared lipids	Ratio between signal height	
		Before extrusion	After extrusion
DEDO	DE vs. DOPE	2.3	2.2
DECHO	DE vs. chol	8.6	8.6
DEDOCHO	DE vs. chol	0.45	0.42
DEDOCHO	DE vs. DOPE	1.6	1.6
DEDOCHO	DOPE vs. chol	0.53	0.52

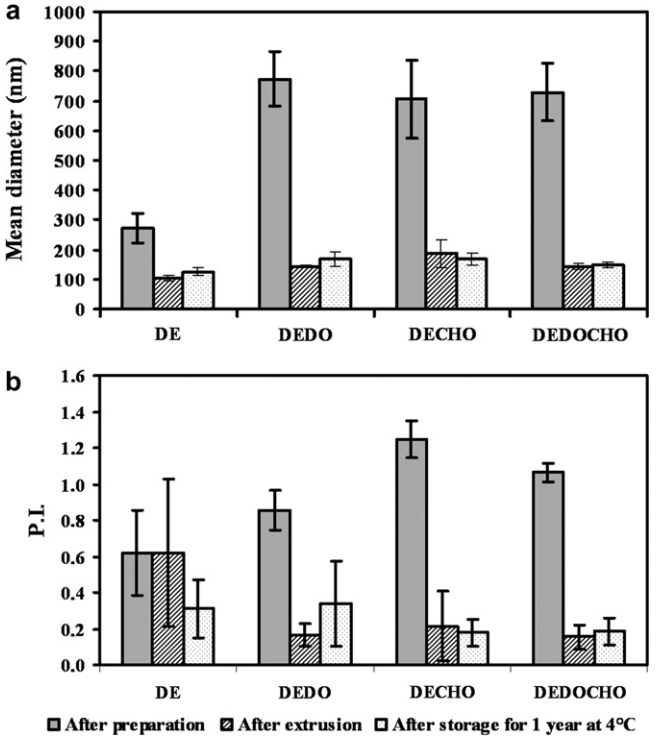

Fig. 2. Mean diameter (a) and polydispersity index (PI) (b) of liposome formulations directly after preparation, after extrusion and after storage for 1 year at 4 °C.

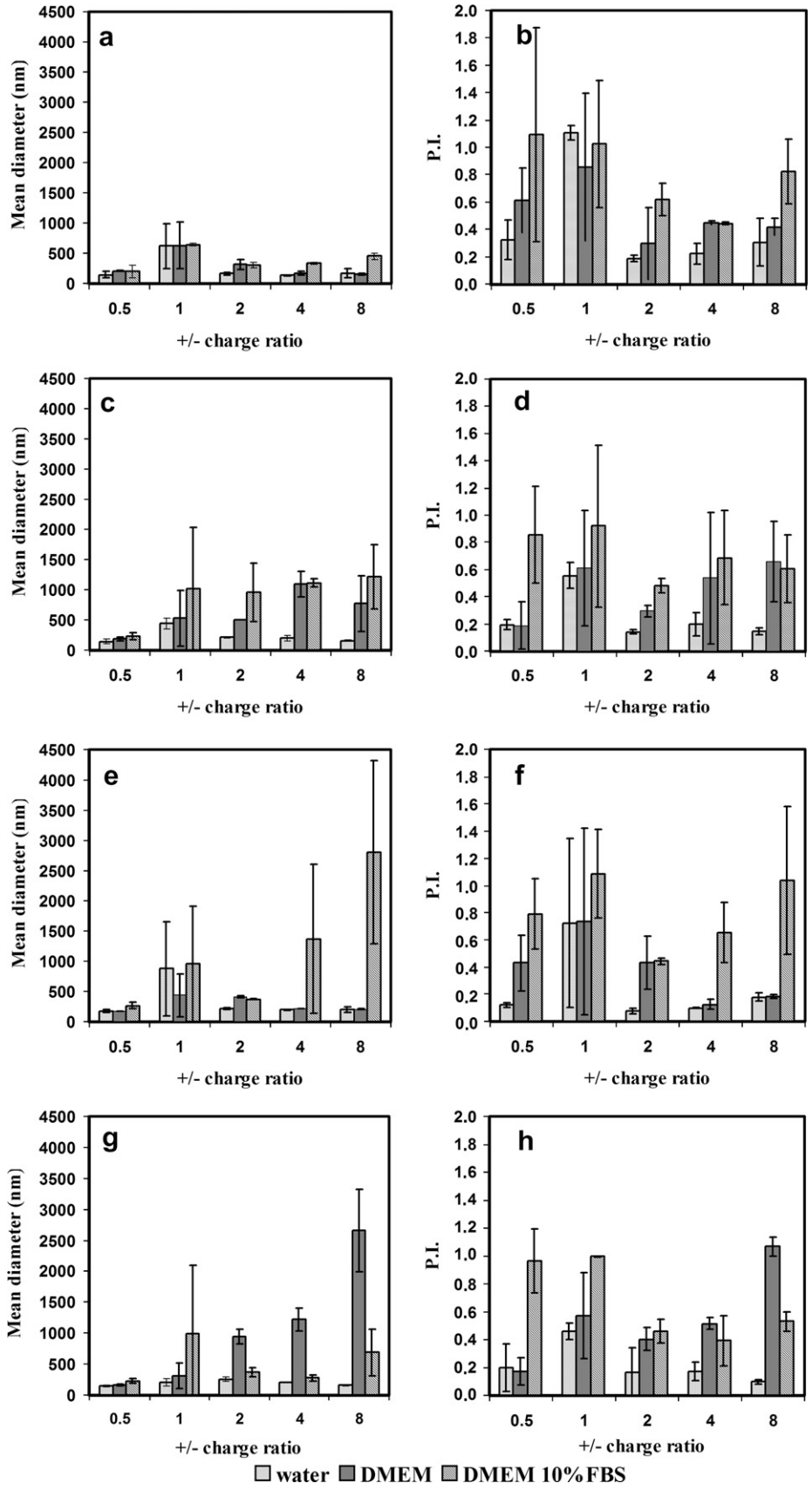

Fig. 3. Mean diameter (a, c, e, g) and PI (b, d, f, h) of lipoplexes in different incubation medium. (a and b) DE/ODN; (c and d) DEDO/ODN; (e and f) DECHO/ODN; (g and h) DEDOCHO/ODN. Results are the mean ± SD of measurements made on three different batches ($n = 3$).

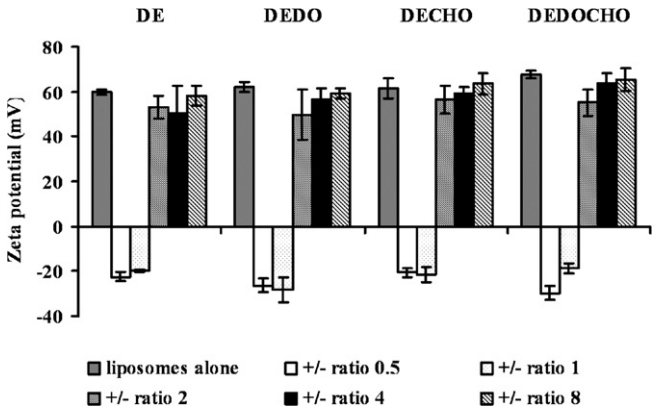

Fig. 4. Zeta potential of liposomes and lipoplexes. Data are expressed as means ± SD of measurements made on three different batches ($n = 3$).

2.11. Statistics

Results are expressed as the means ± SD or SEM of n experiments. Statistical significance was calculated by one-way analysis of variance (ANOVA) and Bonferroni corrected p-value for multiple comparison testing. The level of statistically significant difference was defined as $p < 0.05$.

3. Results and discussion

DE is a recently synthesised cationic lipid which was shown to have interesting properties for the intracellular delivery of nucleic acids [14]. However, no data were available concerning the use of DE for ODN delivery. We investigated the potential of DE-based formulations for the

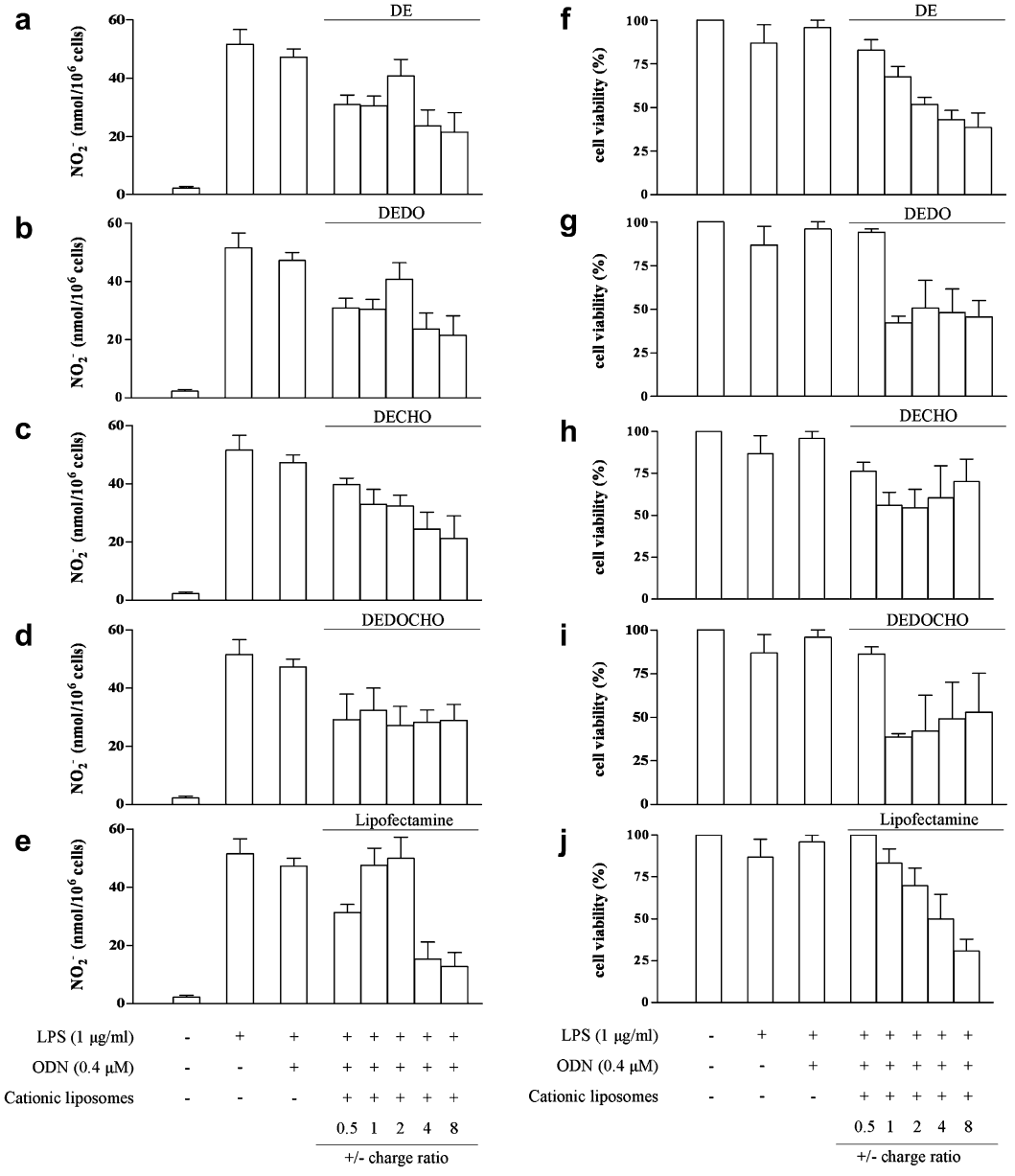

Fig. 5. Nitrite production (a–e) and viability (f–j) in RAW 264.7 macrophages stimulated with LPS (1 μg/ml) for 24 h in the absence of FBS and incubated for 4 h with ODN, as naked or complexed with DE (a and f), DEDO (b and g), DECHO (c and h), DEDOCHO (d and i) and Lipofectamine™ (e and j) at different +/− charge ratios. Data are expressed as means ± SEM of three experiments in triplicate.

intracellular delivery of an ODN against NF-κB. These results were compared with those obtained with Lipofectamine™, a cationic liposome formulation commonly used for transfection of ODN [16]. The composition of liposome formulations is described in Table 1.

Liposomes consisted in vesiscles made of DE, containing or not *helper lipids* such as DOPE (DEDO) or chol (DECHO) or both (DEDOCHO). In a preliminary phase of the study, the eventual modification of the lipid molar ratio, due to the effect of extrusion process, was investigated by ^{1}H NMR analysis. In all cases, the molar ratio between the different lipids was not modified after extrusion, as shown by the integration of the peaks corresponding to each lipid used (Table 2). Then, we investigated the physical stability of all formulations during storage at 4 °C. It has been reported that optimal lipoplexes have to be physically stable with homogeneous size distribution to achieve efficient internalisation of nucleic acids [17,18]. A pre-requisite to obtain homogeneous and stable complexes is the physical stability of the blank liposomes used for their preparation. All liposome formulations were characterised for size and polydispersity index (PI): (i) directly after preparation, (ii) after extrusion and (iii) during storage at 4 °C for one year (Fig. 2). Right after preparation, DE liposomes had a mean diameter of about 250 nm, whilst all formulations containing *helper lipids* displayed

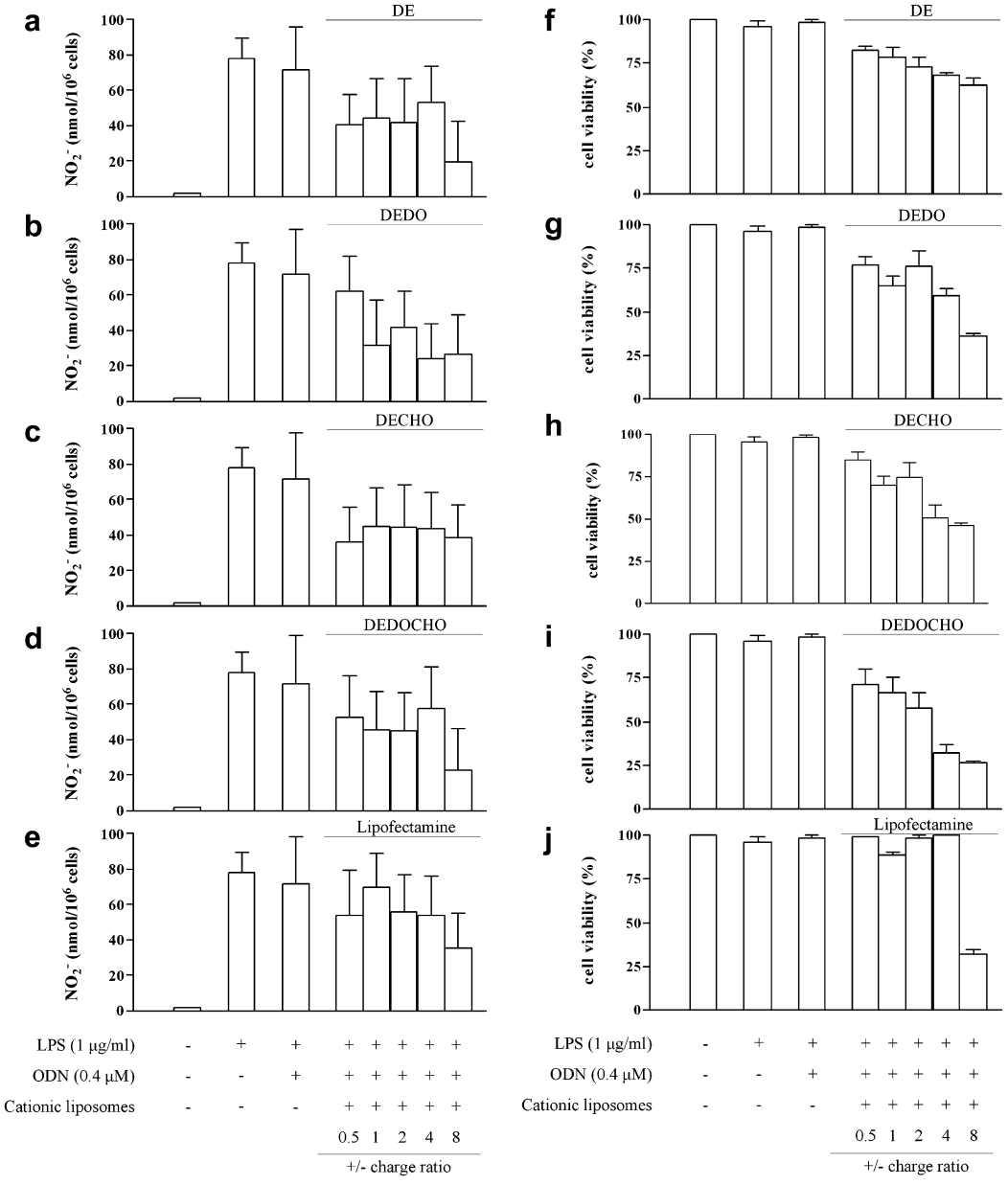

Fig. 6. Nitrite production (a–e) and viability (f–j) in RAW 264.7 macrophages stimulated with LPS (1 μg/ml) for 24 h in the presence of FBS and incubated for 4 h with ODN, as naked or complexed with DE (a and f), DEDO (b and g), DECHO (c and h), DEDOCHO (d and i) and Lipofectamine™ (e and j) at different +/− charge ratios. Data are expressed as means ± SEM of three experiments in triplicate.

a higher mean diameter ranging between 700 and 800 nm (Fig. 2a). After extrusion, for all formulations, the mean size was reduced to values ranging from 100 to 200 nm which did not change even after storage at 4 °C for 1 year (Fig. 2a).

PI of liposomes containing only DE was high after extrusion as well as after storage, whilst PI of *helper lipid*-containing liposomes was low in all cases (Fig. 2b). The higher physical stability of chol-containing liposomes should be attributed to the increased rigidity of the bilayer which contributes to the stabilization of vesicle membrane. Also in the case of DOPE a stabilizing effect on cationic lipid membranes, due to enhancement of the negative curvature of the membrane as well as to the close contact between cationic lipids, has been reported [19]. The double stranded ODN against NF-κB was complexed to the different liposome formulations by mixing an equal volume of ODN aqueous solution and liposome suspension at a final ODN concentration of 0.4 μM. These lipoplexes were characterised for mean diameter and PI after incubation in distilled water, DMEM and DMEM containing 10% FBS (DMEM/FBS). Liposomes composed of DE alone had a high mean diameter at a +/− charge ratio of 1, independently of the incubation medium. For the other ratios, a slight increase in size was observed after incubation in DMEM/FBS (Fig. 3a). When forming lipoplexes with DEDO liposomes, an increase in complex size was observed particularly in DMEM and DMEM/FBS (Fig. 3c). However, in the case of DECHO liposomes, lipoplex size was high only at the +/− charge ratio of 1 for all medium and at the ratios of 4 and 8 in DMEM/FBS (Fig. 3e). Finally, the use of both chol and DOPE in DEDOCHO liposomes did not improve the physical stability of lipoplexes, compared with formulation containing chol alone (Fig. 3g). For all lipoplexes, a larger mean diameter was always accompanied by high values of PI (Fig. 3b, d, f and h). Moreover, in all cases, lipoplexes incubation in DMEM/FBS led to a strong increase of PI. On the contrary, in DMEM without FBS, PI depended on the formulation. In particular, at the higher +/− charge ratios (i.e., 4 and 8), only DECHO lipoplexes had a low PI in this incubation medium. Zeta potential of blank extruded vesicles was positive without significant differences between formulations (Fig. 4). Zeta potential values of lipoplexes were close between formulations and were dependent on the amount of liposomes used (Fig. 4). In particular, zeta potential of lipoplexes was negative for the +/− charge ratio of 0.5 and 1 and became positive starting from a +/− charge ratio of 2 remaining almost constant at higher +/− charge ratios (Fig. 4). Once nucleic acids are mixed with cationic liposomes, characteristics of the resulting lipoplexes depend on different factors, such as chemistry of the lipids, length of DNA and experimental conditions in which complexation occurs [20,21]. It is generally expected that complexes with a +/− charge ratio of about 1 have a neutral zeta potential, with consequent colloidal stability lower than those of complexes exhibiting a net charge [20]. Indeed, in our case, aggregation was observed for complexes with a +/− charge ratio around 1, although zeta potential was found to be negative and not neutral. A further increase of the +/− charge ratio led to a strong zeta potential inversion until a value of about +40 mV. A similar profile, namely a sigmoidal curve obtained by plotting zeta potential *versus* ODN/lipid ratio, has already been reported by other authors [22]. In our case, the inversion of zeta potential at a +/− charge ratio between 1 and 2, which is a light excess of positive charge, could depend on ODN interaction with lipids only present on the monolayer of liposomes surface, as hypothesised by other authors [22]. To investigate the efficiency of DE-containing liposomes for intracellular delivery of decoy ODN against NF-κB, we used RAW 264.7 macrophages stimulated with LPS for 24 h and incubated for 4 h with ODN to NF-κB in free form or complexed with DE-based liposomes, with or without FBS. For comparison purpose, the effect of the same ODN complexed with Lipofectamine™, a successful marketed liposome formulation, was also studied. Firstly, to screen the efficiency of all lipoplexes, at different +/− charge ratios, we investigated the inhibition of nitrites (stable metabolites of NO) production achieved with free or

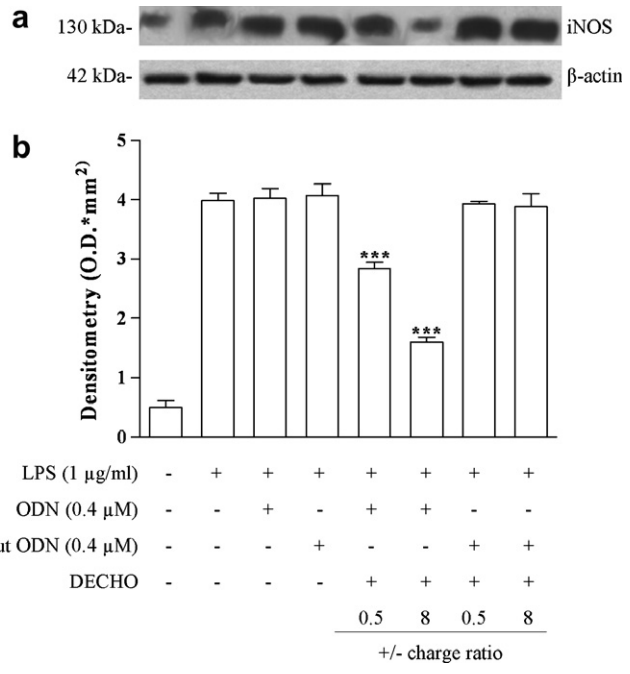

Fig. 7. Western blot (a) as well as the densitometric analysis (b) shows the effect of naked ODN, Mut ODN, ODN/DECHO complexes at +/− charge ratio of 0.5, ODN/DECHO complexes at +/− charge ratio of 8, Mut ODN/DECHO complexes at the +/− charge ratios 0.5 and Mut ODN/DECHO complexes at the +/− charge ratios of 0.5 on iNOS protein expression in LPS-stimulated RAW 264.7 macrophages at 24 h. Cells were treated with ODN, Mut ODN, ODN/DECHO complexes at +/− charge ratio of 0.5, ODN/DECHO complexes at +/− charge ratio of 8, Mut ODN/DECHO complexes at the +/− charge ratios 0.5 and Mut ODN/DECHO complexes at the +/− charge ratios of 0.5. Data are from a single experiment and are representative of three separate experiments. β-actin expression is shown as a control. Densitometric results are expressed as mean ± SEM of three separate experiments. $^{***}p < 0.001$ vs. LPS alone.

complexed ODN in LPS-activated RAW 264.7 macrophages. Moreover, for all lipoplexes, cell viability was properly taken into account. Stimulation of cells with LPS for 24 h resulted in an increase of nitrite production as compared to unstimulated cells. In the absence of serum, the addition of naked ODN (0.4 μM) did not induce any inhibition whereas the addition of lipoplexes, at a final ODN concentration of 0.4 μM, significantly reduced nitrite production and this effect was dependent on liposome formulation as well as on the +/− charge ratio. Moreover, all DE-containing lipoplexes showed a lower cytotoxicity and a higher inhibition of nitrite production, compared to Lipofectamine™ (Fig. 5). Incubation of cells with naked Mut ODN as well as Mut ODN complexed with liposomes did not exhibit any effect (Fig. 1 S). In all cases, cells incubated with lipoplexes, at low +/− ratio (0.5), with or without *helper lipid*, displayed a cell viability always higher than 80%, associated with an inhibition of nitrite production always lower than 40% (Fig. 5). Concerning the effect of the different formulations, in the absence of serum, ODN/DE liposome complexes inhibited nitrite production proportionally to the +/− molar ratio (Fig. 5a). However, this effect was partly due to cell death (Fig. 5f), which was higher as higher was the amount of DE used. Similar results were obtained with the marketed product Lipofectamine™. Due to its properties to facilitate liposome formation and escape from the endosomal compartment, DOPE has been successfully combined with N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and 2,3-dioleyloxy-N-[2(sperminecarboxamido) ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) [23,24]. However, in our work, the use of DOPE did not improve lipoplex performance, if compared to the formulation containing DE alone, suggesting that the escape from endosomal compartment is not the limiting step of ODN/DE liposome complexes. Also, DEDOCHO formulation did not improve the inhibition of nitrite production and cell viability, compared to DEDO lipoplexes. Conversely, when ODN was complexed to DECHO liposomes, at the +/− charge ratio of 8, cell viability was about

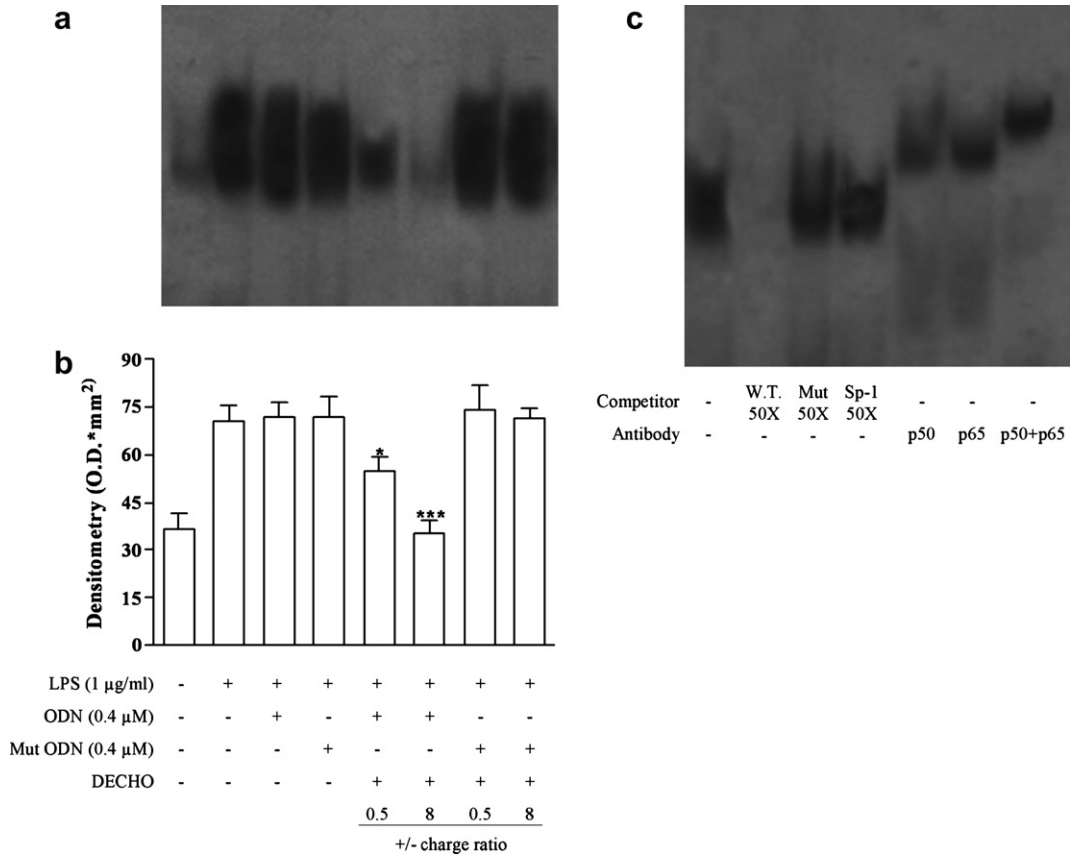

Fig. 8. Representative EMSA (a) as well as the densitometric analysis (b) shows the effect of naked ODN, Mut ODN, ODN/DECHO complexes at +/− charge ratio of 0.5, ODN/DECHO complexes at +/− charge ratio of 8, Mut ODN/DECHO complexes at the +/− charge ratios 0.5 and Mut ODN/DECHO complexes at the +/− charge ratios of 0.5 on NF-κB/DNA binding activity in LPS-stimulated RAW 264.7 macrophages for 24 h. Data in (a) are from a single experiment and representative of three separate experiments. Data in (b) are expressed as means ± SEM of three separate experiments. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. LPS alone. Characterisation of NF-κB/DNA complex was performed on LPS-stimulated cells at 24 h (c). In competition reaction nuclear extracts were incubated with radiolabelled NF-κB probe in the absence or presence of identical but unlabelled oligonucleotide (W.T., 50×), mutated non-functional NF-κB probe (Mut., 50×) or unlabelled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1, 50×). In supershift experiments nuclear extracts were incubated with antibodies against p50 and p65, 15 min before incubation with radiolabelled NF-κB probe. Data in (c) are from a single experiment and are representative of three separate experiments.

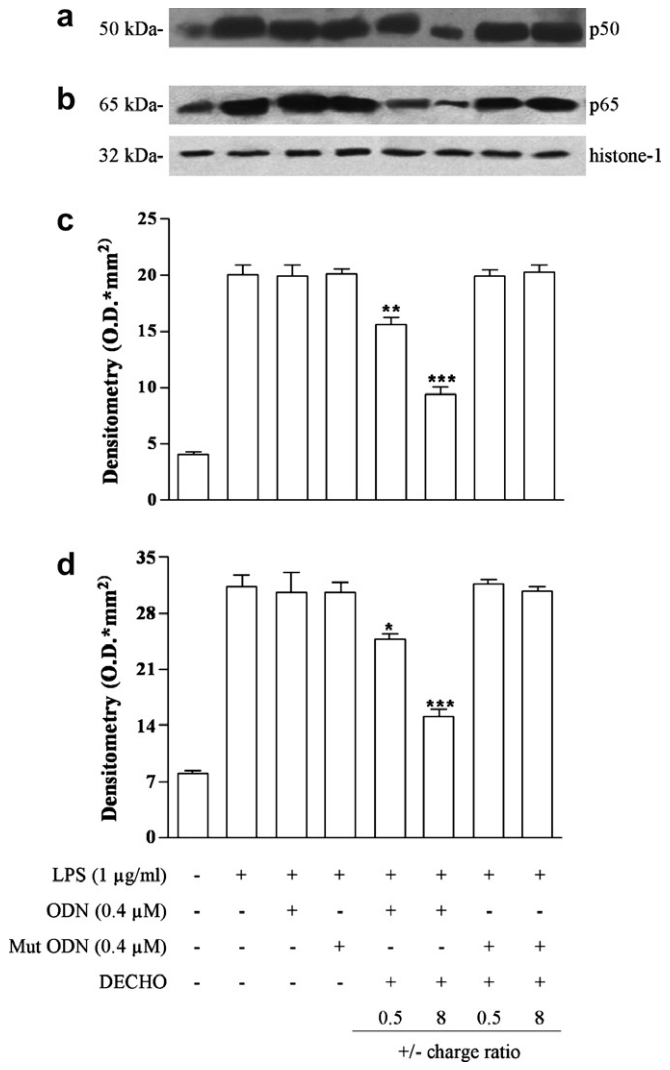

Fig. 9. Representative Western blot (a and b) as well as the densitometric analysis (c and d, respectively) show the effect of ODN, Mut ODN, ODN/DECHO complexes at +/− charge ratio of 0.5, decoy ODN/DECHO complexes at +/− charge ratio of 8, Mut ODN/DECHO complexes at the +/− charge ratios 0.5 and Mut ODN/DECHO complexes at the +/− charge ratios of 0.5 on p50 and p65 nuclear translocation in LPS-stimulated RAW 264.7 macrophages at 24 h. Data in (a and b) are from a single experiment and are representative of three separate experiments. Histone-1 expression is shown as control. Data in (c and d) are expressed as means ± SEM of three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. LPS alone.

70% and nitrite production was inhibited by about 90%. According to our results, chol has been already associated to cationic lipids, leading to a higher transfection efficiency on different cell types [25]. In this case, the stabilization effect of chol on liposome membrane was thought to favour an endocytotic uptake rather than direct membrane fusion. The same experiments were performed in the presence of DMEM/FBS and the results are reported in Fig. 6. In the presence of FBS, ODN complexed to DE-based lipoplex reduced nitrite production (Fig. 6), whilst any effect was observed with Mut ODN/liposome complexes (Fig. 2 S). It is worth to note that higher variability in the inhibition of nitrite production was observed in the presence of serum (as showed by comparing the error bars of Figs. 5 and 6). The highest ODN transfection obtained with DECHO liposomes, compared with the other formulations, could be related to the highest physical stability in DMEM. Indeed, when lipoplexes are incubated in DMEM and especially in DMEM/FBS, other charged molecules, such as salts and proteins, can interact with the liposome surface, thus neutralising the net charge and favouring liposome aggregation [26]. The decreased fluidity of the membrane obtained by using chol in the bilayer could certainly contribute to increase the physical stability of ODN/DECHO complexes. Lipoplex size is known to be one of the main factors determining the *in vitro* delivery of nucleic acid [18]. In this work, we found that the small size of lipoplex, combined with a high net positive charge, corresponded to the highest inhibition of NF-κB activation. In agreement with our finding, Jaaskelainen and co-workers observed a more efficient cellular delivery of phosphorotioate ODN with small sized lipoplexes [27]. Moreover, it has been reported that macrophages take up preferentially chol-rich liposomes [28] and this could significantly contribute to the higher cell uptake of ODN/DECHO lipoplexes. In the same work, according to us, the highest DNA delivery in RAW 264.7 macrophages was achieved at a high content of cationic lipid. Concerning the toxicity of lipoplexes, it has been proposed that cell death occurs through apoptosis that was found to depend on the lipoplex size [28]. According to these findings, we observed a lower toxicity with chol-containing lipoplexes, which maintained low dimensions in DMEM. The results obtained in the presence of FBS showed the inhibitory effect of serum on DNA delivery, as previously reported [29,30]. This effect was attributed either to the reduced amount of lipoplex associating to target cells [31], or to serum protein binding to lipoplexes thereby diminishing their ability to deliver DNA [17]. Recently, lipoproteins have been incriminated as the major components responsible for the inhibitory effect of serum [32]. Moreover, the high variability of the results could be reasonably attributed to the physical instability of lipoplexes in the presence of serum. An heterogeneous size distribution should be the main cause of the high variability of the ODN delivery by DE-based liposomes. This hypothesis is strengthened by the high PI of all lipoplexes after incubation in DMEM with FBS. For this reason, ODN/DECHO complexes at the +/− charge ratio of 8 were selected for further investigation. The same complex, with a lower performance (i.e +/− charge ratio of 0.5), was used for comparison purpose. For both formulations, we investigated, by Western blot, the inhibition of iNOS protein expression in RAW 264.7 stimulated with LPS (1 μg/ml) for 24 h. Upon stimulation with LPS, cells showed higher levels of iNOS protein expression as compared with unstimulated cells (Fig. 7). Incubation of cells with naked ODN or naked Mut ODN did not exhibit any effect. ODN complexed with DECHO, at the +/− charge ratio of 8, significantly reduced iNOS protein

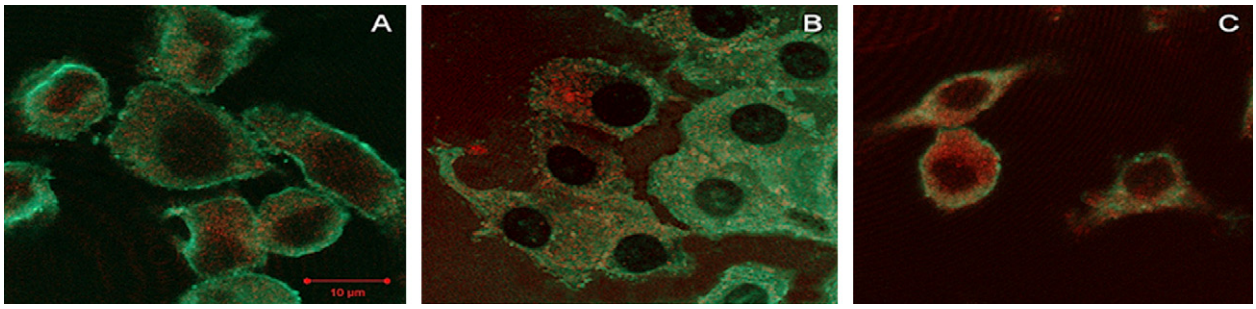

Fig. 10. Confocal images of RAW 264.7 macrophages stimulated with LPS (1 μg/ml) and incubated with the rhodamine-labelled decoy ODN (red) for 4 h: naked ODN (A), ODN/DECHO complexes at the +/− charge ratio of 0.5 (B) and ODN/DECHO complexes at the +/− charge ratio of 8 (C). FITC-DHPE has been used to label the membranes (green). Fields are representative of three separate experiments. (For interpretation of colour mentioned in this figure the reader is referred to the web version of the article.)

expression (by $59.8 \pm 0.1\%$; $n = 3$) whilst ODN complexed with DECHO, at the +/− charge ratio of 0.5, showed a weak effect (by $28.7 \pm 0.1\%$; $n = 3$). Mut ODN complexed with DECHO, at both the +/− charge ratios of 0.5 and 8, did not exhibit any effect (Fig. 7). In order to explore whether the reduced iNOS protein expression was related to a reduced binding of NF-κB to DNA, EMSA was performed on nuclear extracts from RAW 264.7 cells stimulated with LPS (1 μg/ml) for 24 h with ODN in naked form or complexed to DECHO liposomes, at the +/− charge ratios of 0.5 and 8. As shown in Fig. 8, a low basal level of NF-κB/DNA binding activity was detected in nuclear proteins from unstimulated macrophages. Conversely, stimulation of cells with LPS induced an increase of NF-κB/DNA binding activity which was significantly reduced by treatment with ODN/DECHO complex, at the +/− charge ratio of 8, (by $58.5 \pm 7.8\%$ compared to LPS-stimulated cells; $n = 3$). In contrast, ODN/DECHO complex, at the +/− charge ratio of 0.5, determined a weak effect (by $23.0 \pm 2.2\%$ compared to LPS-stimulated cells; $n = 3$). Naked ODN and Mut ODN as well as Mut ODN complexed with DECHO, at both +/− charge ratios, did not exhibit any effect (Fig. 8a). The composition of protein–DNA binding complexes was determined by competition and supershift experiments. In the reaction of competition, the specificity of NF-κB/DNA binding complexes was evidenced by complete displacement of protein–DNA binding in the presence of a 50-fold molar excess of unlabelled NF-κB probe. In contrast a 50-fold molar excess of unlabelled mutated NF-κB probe or Sp-1 ODN had no effect on DNA binding activity (Fig. 8b). In supershift experiments, addition of either anti-p50 or anti-p65 antibodies to the binding reaction clearly gave rise to a characteristic supershift of the retarded complex, suggesting that the NF-κB complex contained p50 and p65 dimers (Fig. 8b). To confirm these results, we investigated, by Western blot, the nuclear translocation of NF-κB subunits p50 and p65 in RAW 264.7 cells stimulated with LPS for 24 h and incubated with ODN to NF-κB in naked form or complexed to DECHO liposomes at both the +/− charge ratios of 0.5 and 8. Upon stimulation with LPS, cells exhibited p50 and p65 higher nuclear levels as compared with unstimulated cells (Fig. 9). Treatment of cells with ODN/DECHO complex, at the +/− charge ratio of 8 caused a significant reduction of nuclear translocation of both NF-κB subunits p50 and p65 (by $53.0 \pm 0.64\%$ and $51.7 \pm 0.9\%$, respectively; $n = 3$) induced by LPS. When cells were incubated with ODN/DECHO complex, at the +/− charge ratio of 0.5, only a weak effect (by $22.2 \pm 0.7\%$ and $21.1 \pm 1.0\%$ for p50 and p65, respectively; $n = 3$) was observed. Naked ODN, Mut ODN as well as Mut ODN complexed with DECHO in the +/− charge ratios of 0.5 and 8 did not exhibit any effect (Fig. 9). The cellular fate of the ODN delivered by DECHO liposomes was also studied by confocal microscopy. Images of cells incubated with naked ODN and DECHO/ODN complexes at the +/− charge ratio of 0.5 and 8, respectively, are reported in Fig. 10. When naked ODN was added to cells, only a weak fluorescence was found in the cytoplasm (Fig. 10a). In the case of DECHO lipoplexes at the +/− ratio of 0.5 a low amount seemed to be internalised (Fig. 10b) whereas in the case of the +/− ratio of 8 a high punctuate fluorescence was observed into the cytoplasm (Fig. 10c). In conclusion, liposomes composed of the cationic lipid DE and associated to chol have shown a high potential for the delivery of a double stranded ODN against NF-κB into LPS-stimulated RAW 264.7 macrophages. This liposome-based system could allow to use ODN to significantly inhibit NF-κB/DNA binding activity and to down-regulate NF-κB-related pro-inflammatory genes. The tendency of DE/chol-based lipoplexes to aggregate in presence of serum suggests their use for local delivery of ODN, i.e. inflammed sites, rather than for i.v. administration. Further studies will follow to investigate the *in vivo* behaviour of these liposomes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2008.03.012.

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