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Counter ions and constituents combination affect DODAX : MO nanocarriers toxicity *in vitro* and *in vivo*†

Ana Cristina Norberto Oliveira,[a,b] Marisa Passos Sárria,[c] Pedro Moreira,[a] Joana Fernandes,[a] Lisandra Castro,[a] Ivo Lopes,[a,b,d] Manuela Côrte-Real,[a] Artur Cavaco-Paulo,[e] Maria Elisabete Cunha Dias Real Oliveira[b] and Andreia Castro Gomes*[a]

Liposomes have received extensive attention as nanocarriers for bioactive compounds due to their good biocompatibility, possibility of targeting and incorporation of hydrophilic and hydrophobic compounds. Although generally considered as safe, detailed knowledge of the effects induced in cells and tissues with which they interact is still underexplored. The aim of this study is to gain insight into the toxicity profile of dioctadecyldimethylammonium (DODAX) : monoolein(MO) liposomes (X is bromide or chloride), previously validated for gene therapy, by evaluating the effect of the counter ions Br^- or Cl^-, and of the cationic : neutral lipid molar fraction, both *in vitro* and *in vivo*. Effects on cellular metabolism and proliferation, plasma membrane integrity, oxidative stress, mitochondrial membrane potential dysfunction and ability to trigger apoptosis and necrosis were evaluated in a dose-/time-dependent manner in normal human skin fibroblasts. Also, newly fertilized zebrafish zygotes were exposed to liposomes, permitting a fast-track evaluation of the morphophysiological modifications. *In vitro* data showed that only very high doses of DODAX : MO induce apoptosis and necrosis, inhibit cell proliferation, and affect the metabolism and plasma membrane integrity of fibroblasts in a dose-/time-dependent manner. Furthermore, liposomes affected mitochondrial function, increasing ROS accumulation and disturbing mitochondrial membrane potential. DODAC-based liposomes were consistently more toxic when compared to DODAB-based formulations; furthermore, the inclusion of MO was found to reduce toxicity, in contrast to liposomes with cationic DODAX only, especially in DODAB : MO (1 : 2) nanocarriers. These results were corroborated, in a holistic approach, by cytotoxicity profiling in five additional human cell lines, and also with the zebrafish embryotoxicity testing, which constitutes a sensitive and informative tool and accurately extends cell-based assays.

[a]CBMA (Center of Molecular and Environmental Biology), Department of Biology, University of Minho, Campus of Gualtar, 4710 057 Braga, Portugal.
E-mail: agomes@bio.uminho.pt; Fax: +351 253 678 980; Tel: +351 253 601 511
[b]CFUM (Center of Physics), Department of Physics, University of Minho, Campus of Gualtar, 4710-057 Braga, Portugal
[c]INL – International Iberian Nanotechnology Laboratory, Av. Mestre José Veiga, 4715-330 Braga, Portugal
[d]Nanodelivery-I&D em Bionanotecnologia Lda., Department of Biology, University of Minho, Campus of Gualtar, 4710-057 Braga, Portugal
[e]CEB, Department of Biological Engineering, University of Minho, Campus of Gualtar, 4710-057 Braga, Portugal
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1. Introduction

The development of nanotechnological solutions for biomedical applications has been a major focus of interest over recent years. New nanocarriers are constantly developed for therapeutic applications, and every year more nanomedicines are approved for human therapy.[1] Nevertheless, potential adverse impact of nanomaterials on human health is yet to be fully understood,[2] and the molecular events involved in nanoparticle toxicity need further in depth studies. While most chemicals are cytotoxic due to interactions with specific biomolecules, one single nanoparticle might induce the production of reactive oxygen species (ROS), morphological alterations, genotoxicity and immunological effects.[3] In fact, although several factors contribute to the toxicity of nanoparticles, their nanometric size is one of the most important

parameters, since the high surface area to mass ratio strongly influences interactions with biological molecules such as proteins and cells. Additionally, binding to/interaction with biological molecules alter nanoparticles' surface properties, influencing the interface with the cellular microenvironment.[4] Other variables apart from size such as type of material, shape, surface charge, hydrophobicity or concentration must also be considered.[5]

Even though "cytotoxicity of nanoparticles" has been the subject of excellent reviews in recent years, these are generally focused on metallic nanoparticles, quantum dots or carbon nanotubes, whilst liposomes have not been systematically evaluated.[6–8] The cytotoxicity mechanism of liposomes is usually reported to be surface charge dependent.[9,10] Liposomes influence several signaling pathways,[11] inducing apoptosis depending on their association with cells and ROS production.[9,12] Moreover, the type of hydrophobic chain, hydrophilic group and linker also have influence on the cytotoxic response.[9,13]

Although *in vitro* cell-based testing is a proficient, informative and fairly cost-effective approach to be considered for initial screening of nanotoxicity,[14] it does not replace the high degree of biological organization that *in vivo* 3D-models represent. Because cell culture systems do not take into consideration the responses at the whole-organism scale, they can lead to misleading conclusions.

To date, many studies addressing the *in vivo* toxicity assessment of nanoparticles are contradictory or have limited scope, mainly due to the unclarity of the regulatory guideline(s),[15] which when combined with the lack of efficient acute/chronic screening protocols and putative standards, limits the preclinical testing of nanotherapeutics. Zebrafish *Danio rerio* Hamilton 1822 is a small vertebrate (Pisces: Teleostei) model that requires less expensive husbandry and accommodation than do mammals, commonly used for toxicity evaluation. Zebrafish offers genetic, physiological and anatomical resemblance with mammals including the blood brain barrier, endothelial cells and immunogenic responses.[16] Moreover, zebrafish biology has other advantageous characteristics: (i) females have the capability to lay around 200–300 eggs per day every 5–7 days; (ii) equals the longevity and generation time of mice (3–5 months); and (iii) transparent embryos develop promptly into larvae, *ex-utero*, within 120 hours postfertilization (hpf)[17,18] allowing *in vivo* real-time imaging of ongoing processes and significant reduction of experiment duration. The zebrafish embryo toxicity (ZET) assay has been extensively used for conservative risk assessment of metallic nanoparticles, carbon-based nanostructures and polymers, screening ecological benchmarks.[19–22]

We have previously observed that liposomes and siRNA-lipoplexes composed of dioctadecyldimethylammonium bromide or chloride (DODAB/DODAC):monoolein (MO) induce dissimilar *in vitro* responses.[23] The counter ion (Cl⁻ or Br⁻) of the cationic lipid not only influences the structure, stability and silencing efficiency of siRNA-lipoplexes, but it also seems to affect their cytotoxicity. There are also indications that the fraction cationic/neutral lipid on the liposomal formulation can play a role in cellular responses. Therefore, in the present study, we investigated the influence of the counter ion (Br⁻ or Cl⁻) and of the cationic/neutral lipid molar fraction on the cytotoxic response of several human cell lines to DODAX:MO liposomes, and the ZET assay was performed to validate the results *in vivo*. Cellular metabolic activity, proliferation and plasma membrane integrity, induction of ROS accumulation, changes of the mitochondrial membrane potential, and cell death mechanisms (apoptosis *versus* necrosis) were evaluated *in vitro*. In the ZET protocol, zebrafish embryos were monitored *via* continuous waterborne exposure to DODAX:MO liposomes, for 80 hpf. The following endpoints were assessed: mortality, developmental delay signals, phenotypical malformations, spontaneous movements and free-swimming patterns, cardiac rhythm and hatching rate – using a "non-animal" (according to European Directive 2010/63/EU on the protection of animals used for scientific purposes) "intermediate" system, positioned amid cellular and mammal models.[24]

We propose that a more complete and reliable approach to assess the effects of exposure to non-metallic particles should integrate both *in vitro* and *in vivo* parameters, suggesting the ZET assay as an informative platform for screening nanotoxicity effects.

2.   Materials and methods

2.1   Chemicals and reagents

Dioctadecyldimethylammonium bromide (DODAB), dioctadecyldimethylammonium chloride (DODAC), and 1-monooleoyl-rac-glycerol (MO) were supplied by Sigma-Aldrich (Bornem, Belgium). DMEM® and M199® cell culture media, L-glutamine (L-Glu), Fetal Bovine Serum (FBS), trypsin–EDTA solution, Penicillin–Streptomycin (P/S) (5000 IU mL⁻¹ penicillin and 5000 μg mL⁻¹ streptomycin) and Hygromycin B® were purchased from Gibco (UK). The MTS assay kit was obtained from Promega (USA), and SRB solution was purchased from Sigma-Aldrich (USA). The Annexin-FITC kit was obtained from BD Biosciences (USA) and MitoTracker Red CMXRos from Molecular Probes (UK).

2.2   Preparation and biophysical characterization of liposomes

Liposomes were prepared by ethanolic injection.[25] Briefly, defined volumes of DODAC, DODAB and MO (20 mM stock solutions in ethanol) were injected into HEPES buffer, 25 mM pH 7.4, under strong vortex and above the transition temperature of the lipids (>50 °C), to a final total lipid concentration of 3 mM. The cationic:neutral lipid mixtures will be referred to as B:M (1:0), B:M (2:1), B:M (1:2), C:M (1:0), C:M (2:1), and C:M (1:2), where B represents DODAB, C represents DODAC and M stands for monoolein. The molar ratios 1:0, 1:2 and 2:1 are represented between brackets

(mol : mol). Liposomes were stabilized at room temperature (RT) before use.

The mean size, polydispersity index (PDI) and zeta (ζ-) potential of liposomes (1 mM) were determined by dynamic light scattering (DLS) in a Zetasizer Nano ZS (Malvern, UK), equipped with Dispersion Technology Software (DTS), at 25 °C. All results were based on intensity distributions.

2.3 *In vitro* nanocytotoxicity evaluation

2.3.1 Culture conditions and incubation with liposomes. The human foreskin fibroblast cell line BJ5-ta (ATCC® CRL-4001™) was grown on a mixture (4 : 1 v:v) of DMEM® (supplemented with 4 mM L-Glu, 4.5 g L^{-1} glucose and 1.5 g L^{-1} sodium bicarbonate) and Medium 199® (supplemented with 0.01 mg mL^{-1} Hygromycin B and 10% FBS) with 1% P/S solution. Cells were kept in a humidified incubator (37 °C, 5% CO_2) and sub-cultured every 3–4 days in order to maintain sub-confluency.

Several cytotoxicity assays were performed after BJ5-ta cells were exposed to B : M (1 : 0), B : M (2 : 1), B : M (1 : 2), C : M (1 : 0), C : M (2 : 1) and C : M (1 : 2) liposomes. For MTS and SRB assays, cells were seeded into 96-multiwell microplates (TPP, Switzerland), at a density of 10×10^3 cells per well; for LDH and DCFH-DA assays, cell were seeded at 100×10^3 cells per well in 24-multiwell microplates (TPP, Switzerland). For the Annexin/PI assay and mitochondrial membrane potential ($\Delta\Psi_m$) determination, BJ5-ta cells were seeded at a density of 100×10^3 cells per well in 6-multiwell microplates (TPP, Switzerland).

In all assays the culture medium was renewed prior to the addition of liposomes (5, 20 and 40 μg mL^{-1} for SBR; 5, 20, 40, 80 and 160 μg mL^{-1} for MTS and LDH; 40 and 80 μg mL^{-1} for DCFH-DA; and 40 μg mL^{-1} for Annexin/PI and $\Delta\Psi_m$ assays). 30% (v:v) DMSO was used as death control.

2.3.2 Assessment of metabolic cytotoxicity with MTS assay. The metabolic cytotoxicity was assessed with the MTS assay after 8, 24 or 48 h incubation with liposomes, and according to the manufacturer's instructions. The percentage of metabolic activity was expressed in relation to non-treated cells (NT).

2.3.3 Determination of cell proliferation by SRB assay. Cellular proliferation was determined by the sulphorhodamine B (SRB) assay after 8, 24 and 48 h contact with liposomes. Briefly, the medium was removed, cells were washed with phosphate-buffered saline (PBS) and a solution of 1% acetic acid in methanol was added to each well. After 1 h at −20 °C, the wells were washed with PBS and dried, and 5% SRB solution (in 1% acetic acid) was added to each well. The microplates were incubated for 90 min at 37 °C. After washing off the excess of SRB, 10 mM Tris was used to dissolve SRB, and absorbance was read at 540 nm using a multiplate reader (SpectraMax Plus 384, Molecular Devices). Timepoint 0 h (before addition of liposomes) was considered as 100% cell proliferation. The percentage of cell proliferation was expressed relative to non-treated cells (NT).

2.3.4 Evaluation of cell membrane integrity by LDH assay. The lactate dehydrogenase (LDH) assay was used to determine cellular membrane integrity after 8, 24 and 48 h incubation with liposomes, as previously described.[25] The percentage of membrane integrity was expressed as a percentage of the intracellular LDH activity relative to the total (extracellular + intracellular) LDH activity.

2.3.5 Evaluation of apoptosis/necrosis by Annexin V/PI assay. The Annexin V/propidium iodide (PI) assay was used to determine apoptosis/necrosis. After 24 and 48 h incubation with liposomes floating and adherent cells were collected and washed with PBS. 1×10^5 cells were suspended in binding buffer, incubated with Annexin V-FITC (green) and PI (red) according to the manufacturer's instructions, and analyzed by flow cytometry.

2.3.6 Determination of intracellular ROS by DCFH-DA assay. The accumulation of reactive species of oxygen (ROS) was determined by the DCFH-DA assay.[26] After a 2, 4 and 8 h-period incubation with liposomes, the cell culture medium was removed and the cells were washed twice with PBS. DCFH-DA (100 μM) was added to each well and the microplates incubated at 37 °C for 30 min, in the dark. Extracellular DCFH-DA was removed by washing the wells twice with PBS, and cells were lysed with 90% DMSO/10% PBS solution. The fluorescence was read with a black 96-well microplate (TPP, Switzerland) in a Fluoroskan Ascent FL (Thermo Scientific), with λ_{exc} = 485 nm and λ_{em} = 538 nm.

2.3.7 Analysis of mitochondrial membrane potential ($\Delta\Psi_m$) alterations. MitoTracker Red CMXRos was used to determine the mitochondrial membrane potential ($\Delta\Psi_m$) after incubation with liposomes. After the 24 and 48 h-period incubation, floating and adherent cells were collected and washed with PBS. 1×10^5 cells were suspended in PBS, incubated with Mito-tracker Red CMXRos according to instructions and analyzed by flow cytometry.

2.3.8 Flow cytometry analysis. Samples double stained with Annexin V/PI or stained with MitoTracker Red CMXRos were analyzed using a flow cytometer (Epics XL, Beckman Coulter, Miami, FL, USA) equipped with an argon-ion laser (488 nm beam, 15 mW). FL-1 (488/525 nm) and FL-4 (488/620 nm) were used to detect green and red fluorescence, respectively. For each sample 20 000 cells were run, and data was analyzed with the Flowing software (version 2.5.1, Turku Centre for Biotechnology).

2.4 *In vivo* nanotoxicity assessment

All experiments were performed in compliance with the guidelines on the protection of experimental animals by the Council of Europe, Directive 86/609/EEC, which allows zebrafish embryos to be used up to the moment of free-living. As the ZET assays were carried out up to 80 hpf (*i.e.* within the regulatory limit of exposure, set at 120 hpf, which corresponds to the end of the embryonic development), no license is required.

2.4.1 ZET protocol and collected data. *In vivo* nanotoxicity and bioactivity of DODAX : MO liposomes were assessed with the zebrafish embryo toxicity (ZET) assay.[20] Fertilized

eggs were randomly allocated into 24-well microplates (≈16-cell stadium, cleavage period; 5 eggs per mL), with continuous waterborne exposure to liposomes at different molar ratios (4 : 1, 2 : 1, 1 : 1, 1 : 2 and 1 : 4 mol : mol), for 80 hpf. For each set of test conditions, all embryos were derived from the same eggs spawn. For experimental validation, viable eggs were obtained only from spawns with $a > 90\%$ fertilization rate. The individual and combined contribution of the nanoformulation components was screened (see Table 1).

The (embryo)toxicity of ethanol (solvent of the lipids' stock solutions) and HEPES buffer (used to prepare the liposomes) was first investigated (ESI, Table S1†) to set the toxicity limits for liposome production. Four replicates were considered for each condition, including experimental and solvent controls. The tested conditions were defined based on in vitro (nano) cytotoxicity profiles. The freshwater system was demonstrated not to affect the chorion and plasma membrane permeability of zebrafish embryos, enhancing their development and hatching rates more than high-calcium standard media, but common protozoan contamination is difficult to avoid.[27] Ultrapure water was used in all treatments to maintain zebrafish embryos mortality below 10%. To ensure optimal incubation temperature of the zebrafish embryos, all the test solutions and suspensions were pre-heated to 28 ± 1 °C.

To analyze the effect of DODAX : MO liposome exposure on zebrafish embryos epiboly, the percentage of the epibolic arc at 8 hpf was estimated. This variable can provide an early warning signal of a developmental delay, given that at this embryonic stage the epibolic arc is known to correspond to 75% for the normal development of zebrafish.[28] The head–trunk angle (HTA) of 32 hpf zebrafish embryos was also measured in order to detect atypical development, given that this index was defined by Kimmel as a function of hours of development.[28] Additionally, 20 embryos from each treatment (10 per quadruplicates) were randomly selected and the number of heart beats was counted during 10 s. In order to avoid bias, "blind" observations were performed by a single person. At 8, 32, 56 and 80 hpf, 20 zebrafish embryos from each condition were photographed using a Leica DM IL LED inverted microscope coupled with an ICC50 HD camera. Measurements of the chorion, yolk, eye, pupil, head–trunk angle and body length (respective to each hpf) were obtained using Image Tool (UTHSCSA, v3.00). The following morphological and combined developmental endpoints were further assessed: mortality, developmental delay signals, phenotypical malformations, spontaneous movements and free-swimming patterns, cardiac rhythm and hatching rate. These variables were selected, given their potential implications in vital processes such as neuro-motor coordination. In all ZET experiments, dead embryos were removed to avoid cross-contamination, a process repeated three times every 24 h.

3. Results

Liposomes were found to have mean sizes <200 nm, a polydispersity index (PDI) < 0.5 and to be positively charged (>+50 mV), with no clear trend between formulations (ESI, Table S2†). These characteristics were consistent with the formulations prepared using a similar methodology in previous studies.[25,29] Nevertheless the structural organization of DODAX : MO liposomes is known to be dependent on the counter ion and MO molar fraction (Fig. 1): the reduced screening capacity of a Cl^- ion when compared to a Br^- ion results in a less ordered polar region for DODAC when compared to DODAB bilayers. These packing characteristics determine how MO is incorporated into the nanosystems: for high cationic lipid contents MO is homogeneously distributed into DODAC bilayers, while it forms DODAB- and MO-enriched domains in DODAB bilayers; when MO is in excess (molar fraction (1 : 2)), DODAB/DODAC-rich bilayers are formed, incorporating MO-rich inverted structures.[23]

3.1 DODAX : MO liposomes decrease cellular viability by more than one mechanism

To obtain a more holistic and integrated perspective on the possible adverse in vitro effects of liposomes, metabolic

Table 1 Nanoformulation components screened with the ZET assay

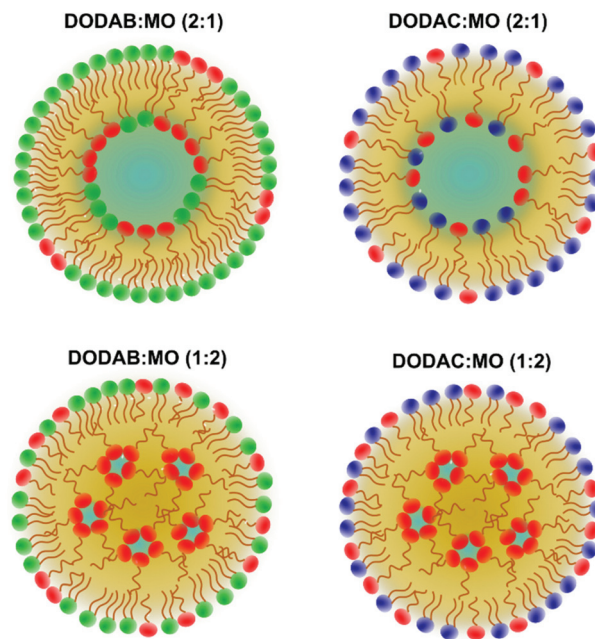| Component	Conditions tested				
DODAX lipids (μM)	0.5	1	3	5	9
MO lipids (μM)	5	15	25	50	75
Ratio of DODAX : MO lipids (mol : mol)	4 : 1	2 : 1	1 : 1	1 : 2	1 : 4
Percentage of ethanol (v/v)	0.25	0.5	1.5	3	5
HEPES buffer (mM)	0.5	1	3	5	15

Fig. 1 Schematic representation of DODAX : MO liposomes' structural organization (in green: DODAB, in blue: DODAC, in red: monoolein).
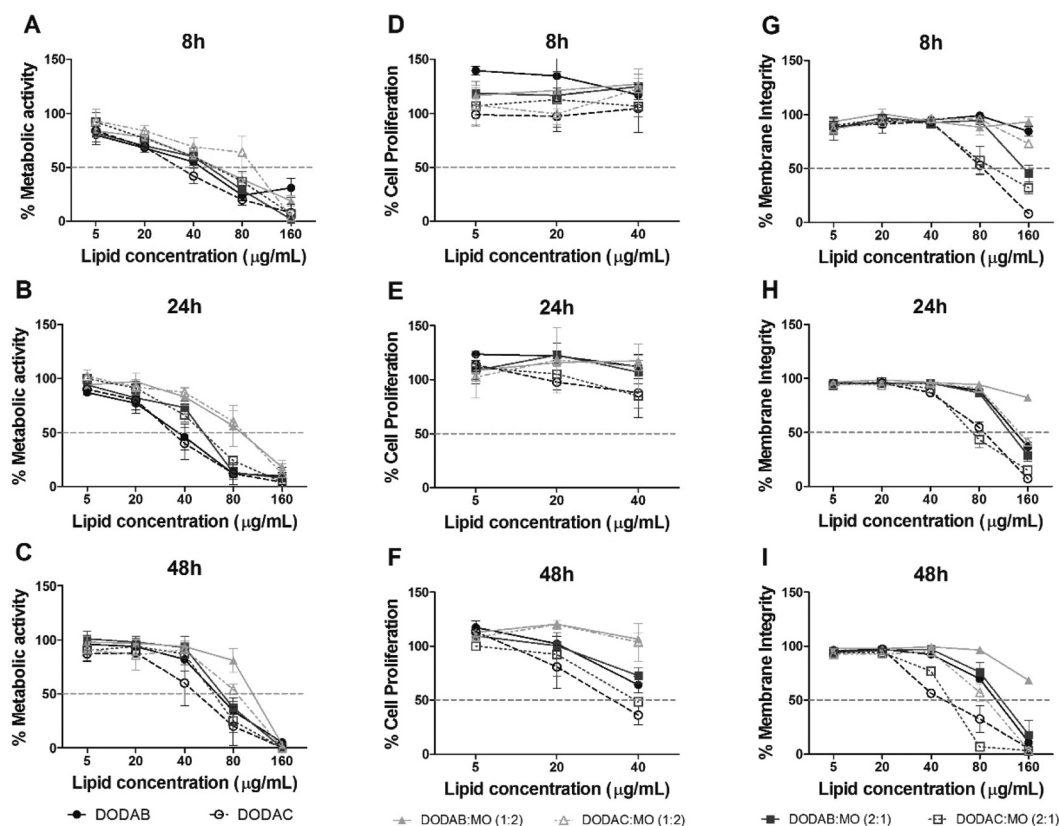
Fig. 2 *In vitro* evaluation of DODAX : MO liposomes' cytotoxicity. Metabolic activity (A−C), cellular proliferation (D−F) and plasma membrane integrity (G−I) of BJ5-ta cells evaluated after 8 h, 24 h and 48 h incubation with DODAX : MO liposomes.

activity, cellular proliferation and plasma membrane integrity were assessed using immortalized normal human fibroblast cells (BJ5-ta) exposed for 8, 24 and 48 h to DODAX : MO liposomes (Fig. 2). Cells were exposed to 5, 20, 40, 80 and 160 μg mL^{-1} of B : M (1 : 0), B : M (2 : 1), B : M (1 : 2), C : M (1 : 0), C : M (2 : 1) and C : M (1 : 2) liposomes, and metabolic activity was determined by the MTS assay, cellular proliferation by the SRB assay and plasma membrane integrity by the LDH assay. For MTS, the percentage of metabolic activity was expressed relative to non-treated cells (NT), set to 100%; for SRB, time-point 0 h (before addition of liposomes) was considered as 100% cell proliferation, and the percentage of cell proliferation was expressed relative to non-treated cells (NT); for LDH, the percentage of plasma membrane integrity was expressed as the percentage of intracellular LDH activity relative to the total (extracellular + intracellular) LDH activity. Results are expressed as mean ± S.E.M. of three independent experiments.

DODAX : MO liposomes induced a concentration- and time-dependent effect on fibroblast viability.

MTS results showed that the cytotoxic effects at the level of cellular metabolism become less evident with longer incubation times (Fig. 2A *versus* 2B *versus* 2C). In general, liposomal formulations with higher MO contents (B : M (1 : 2) and C : M (1 : 2)) caused lower cytotoxicity, supporting the notion that inclusion of MO, apart from improving efficiency, also enhances cytocompatibility. For the same molar fraction and after 48 h incubation, DODAC- were clearly more cytotoxic than DODAB-based formulations. For other timepoints, no clear trend was observed.

DODAX : MO negative effects on BJ5-ta proliferation, assessed by SRB, were only clearly visible for long exposure times (Fig. 2D *versus* 2E *versus* 2F), with DODAC-based liposomes inducing a stronger negative effect on cell proliferation which was attenuated by the inclusion of MO in the formulation. Plasma membrane integrity evaluated by the LDH assay increased with increasing incubation time for all nanoformulations (Fig. 2G–I). After 8 and 24 h, this parameter was not compromised with up to 40 μg mL^{-1} liposomes, while after 48 h incubation with C : M (1 : 0) and C : M (2 : 1) resulted in a significant loss of plasma membrane integrity. The presence of MO also had a positive effect on the cytotoxicity, but only up to 40 μg mL^{-1}. In any case, B : M (1 : 2) prompted less disruptive effects on BJ5-ta plasma membranes and, for the same molar fraction, DODAC-based liposomes lead to more evident toxicity, at all exposure times.

It also became evident that the effects of DODAX : MO liposomes on the metabolic activity, cellular proliferation and plasma membrane integrity were cell line-dependent (ESI,
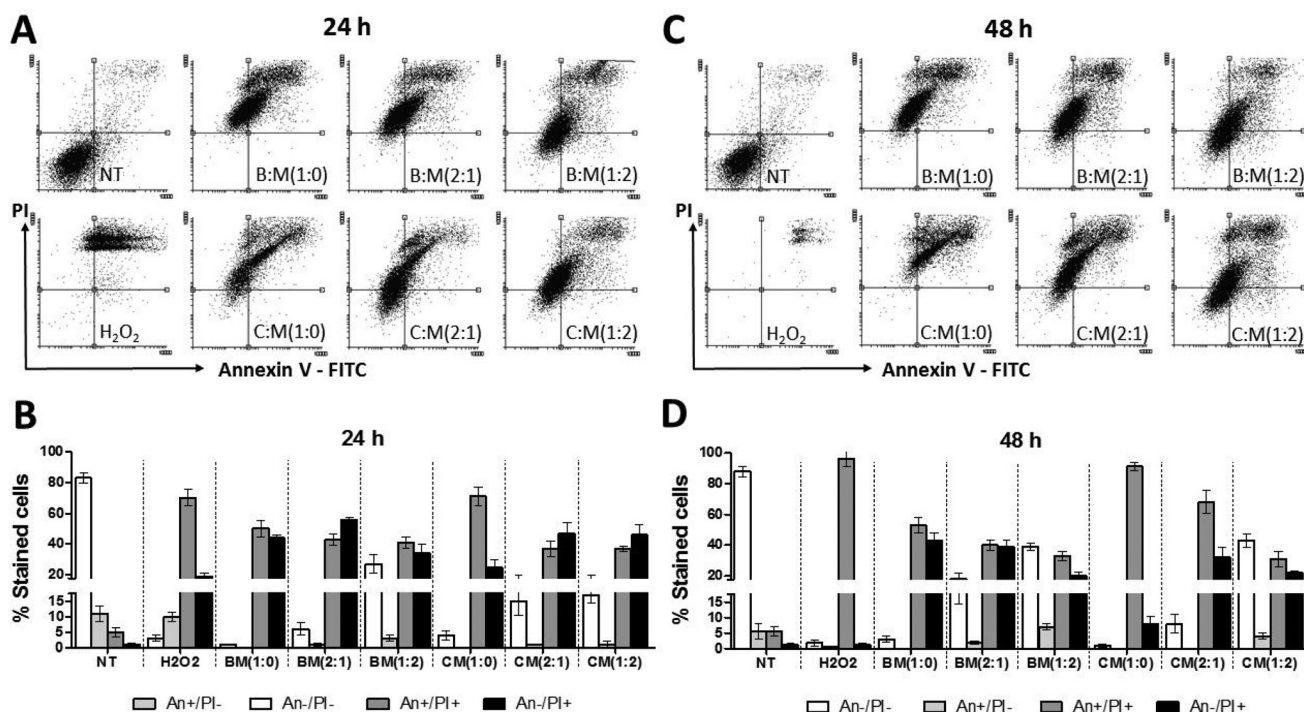
Fig. 3 Apoptosis/necrosis induced by DODAX : MO liposomes on BJ5-ta cells. Cells were incubated with 40 µg mL^{-1} liposomes and Annexin/PI assay was performed after 24 h (A and B) and 48 h (C and D). A and C – Histograms of BJ5-ta cells labeled with Annexin V and PI after incubation with DODAX : MO liposomes. B and D – Quantitative analysis of Annexin/PI staining of BJ5-ta cells. Values represent mean ± S.E.M. of four independent experiments. H$_2$O$_2$ was used as control; NT – non-treated cells; An−/PI−: Annexin negative and PI negative staining (live cells); An+/PI−: Annexin positive and PI negative staining (early apoptotic cells); An+/PI+: Annexin positive and PI positive staining (late apoptotic cells); An−/PI+: Annexin negative and PI positive staining (necrotic cells).

Fig. S1–S3†). A dose-dependent metabolic response was observed for the six tested cell lines (293 T, MDA-MB-435, MDA-MB-468, K562, THP1 and BJ5-ta); for the same molar fraction, DODAC-based liposomes induced higher metabolic cytotoxicity and plasma membrane disruptive effects than DODAB-based liposomes; and no clear correlation between DODAC and DODAB, or with the presence of MO, could be established on cellular proliferation.

Induction of cell death after exposure of BJ5-ta to DODAX : MO liposomes was also assessed with the Annexin V/propidium iodide (PI) assay, in order to evaluate if different liposomal formulations induce the exposure of phosphatidylserine at the outer leaflet of the plasma membrane, a typical marker of apoptotic cells (Fig. 3).

Fig. 3 shows that, for the concentrations and timepoints tested, all DODAX : MO liposomes lead to an increase in the percentage of single (An+/PI−, early apoptotic cells; or An−/PI+, necrotic cells) and double stained cells (An+/PI+ late apoptotic cells), although almost no late apoptotic cells were detected. Overall, the contribution of the death mechanisms was very similar for all DODAX : MO liposomes, where the percentage of live cells (An−/PI−) increased from 24 to 48 h incubation with DODAX : MO liposomes (Fig. 3B *versus* 3D). The exception was noted in C : M (1 : 0) and C : M (2 : 1) liposomes, with which less necrotic (An−/PI+) and more apoptotic

(An+/PI+) cells were observed after 48 h incubation. The presence of MO in the liposomal formulation resulted in a higher number of live cells (An−/PI−): B : M (1 : 2) and C : M (1 : 2) > B : M (2 : 1) and C : M (2 : 1) > B : M (1 : 0) and C : M (1 : 0). This effect was more evident after 48 h of exposure (Fig. 3D).

3.2 DODAX : MO liposomes trigger ROS accumulation and affect the mitochondrial membrane potential

Fig. 4 shows the accumulation of ROS by BJ5-ta cells after short exposure times (2, 4 and 8 h) to liposomes, chosen to limit massive cell death, and consequent reduced cell numbers, which would drive one to misleading conclusions.

Fig. 4A shows time-dependent ROS accumulation after incubation with 40 µg mL^{-1} DODAX : MO liposomes. C : M (2 : 1) liposomes significantly upregulated ROS levels in comparison with endogenous levels in NT cells after only 2 h of contact, while C : M (1 : 0) caused the same effect after 4 h. All other formulations except B : M (1 : 2) led to a similar increase after 8 h of exposure.

Incubation of BJ5-ta cells with higher liposome concentrations (80 µg mL^{-1}, Fig. 4B) showed that, for the same molar fraction, DODAC-based liposomes led to higher ROS accumulation. Nevertheless, at 8 h, existence of cell death interfered with the ROS levels detected (Fig. 2A and G).
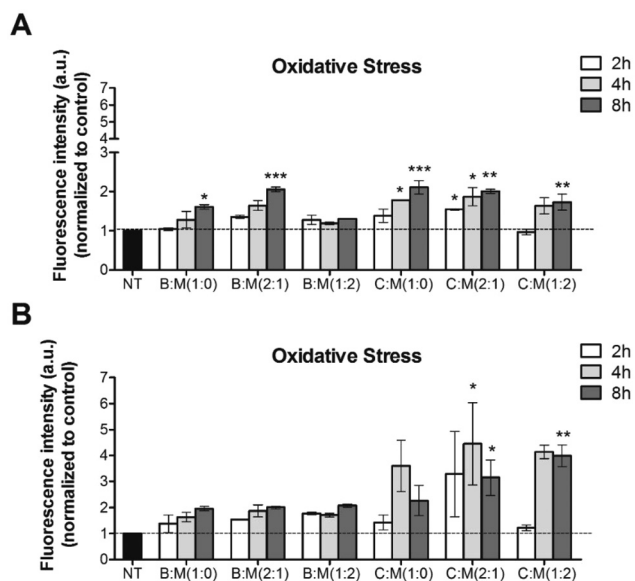
Fig. 4 Induction of ROS production by BJ5-ta cells after exposure to DODAX : MO liposomes. Cells were incubated with 40 µg mL⁻¹ (A) and 80 µg mL⁻¹ (B) liposomes and DCFH-DA assay was performed after 2, 4 and 8 h. Values represent mean ± S.E.M. of three independent experiments. NT – non-treated cells.

It was interesting to notice that, not only did other cell lines (MDA-MB-468 and K562) produce less ROS after incubation with DODAX : MO liposomes, but also that their behavior did not follow the same trend as seen for BJ5-ta cells (ESI, Fig. S4†). This observation reinforces the notion that the effects are cell specific.

Flow cytometry assays were performed in order to evaluate possible alterations in the mitochondrial membrane potential ($\Delta\Psi_m$) after incubation with DODAX : MO liposomes (Fig. 5).

A drop in $\Delta\Psi_m$ generally occurred after incubation with liposomes for 24 and 48 h (Fig. 5), but this was only significantly different from NT cells after a 24 h exposure to C : M (2 : 1) liposomes.
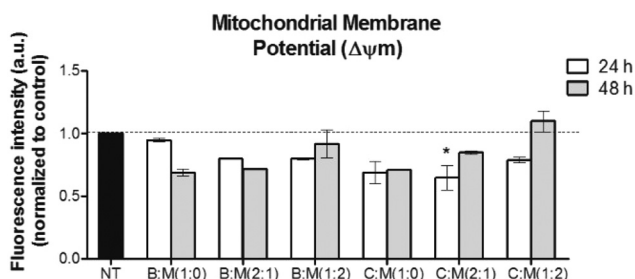

Fig. 5 Induction of alterations in the mitochondrial membrane potential ($\Delta\Psi_m$) of BJ5-ta cells due to the presence of DODAX : MO liposomes. Cells were incubated with 40 µg mL⁻¹ of liposomes and the assay was performed after 24 and 48 h. Values represent mean ± S.E.M. of two independent experiments. NT – non-treated cells.

3.3 *In vivo* evaluation of DODAX : MO liposomes toxicity using the ZET assay

When analyzing the effects of DODAX and MO, as free lipids or formulated in liposomes, on zebrafish embryos survival, a significant interaction was observed for DODAC (Fig. 6).

In line with the data obtained with all *in vitro* experiments, DODAC showed higher embryotoxicity than DODAB, while MO did not cause any apparent toxicity. Moreover, after 8 hpf the
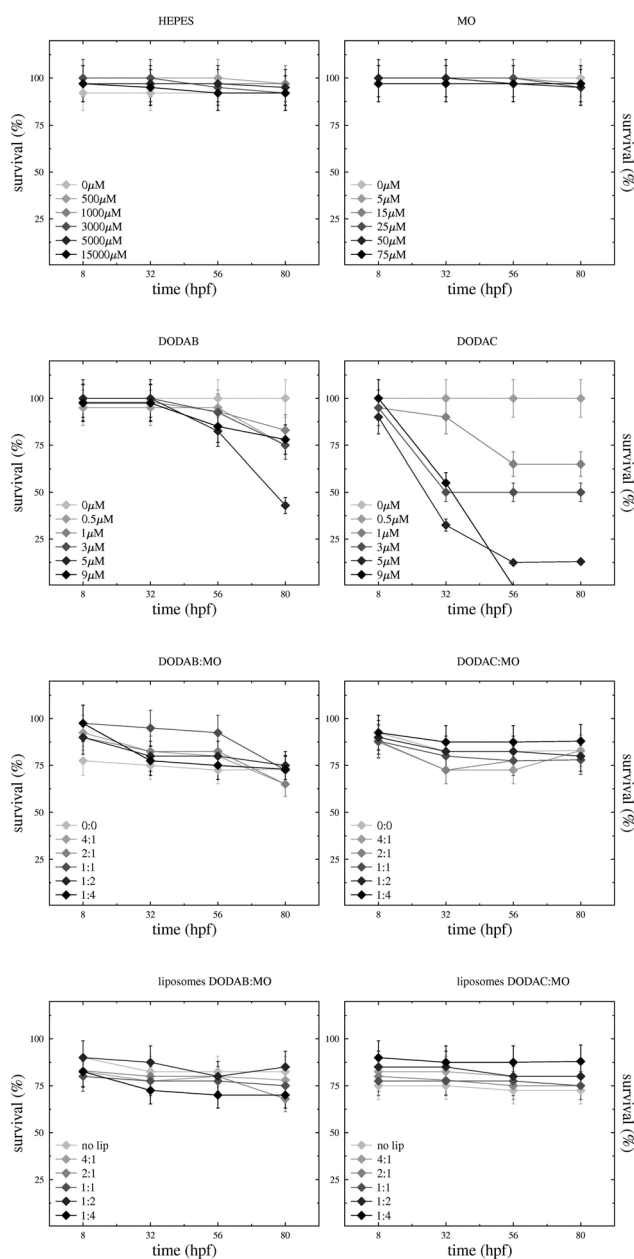

Fig. 6 Effects of exposure to DODAX : MO liposomes on *Danio rerio* embryos survival. Values represent mean ± S.D. of quadruplicate wells. Error bars represent the coefficient of variation for the findings in the replicate wells. 0 : 0 (*i.e.* control group) – no DODAX: zebrafish embryos were exposed to freshwater only. No lip (*i.e.* control group) – no liposomes: zebrafish embryos were exposed to freshwater only.

Table 2 Zebrafish embryotoxicity testing. (+) Corresponds to the significant effect and (−) corresponds to the non-significant effect on tested independent variables

	hpf	Independent variables	Ethanol	HEPES	MO	DODAB	DODAC	DODAB : MO liposomes	DODAC : MO liposomes
Morphometric analysis	8	Epibolic arc	+	−	−	+	+	−	−
	8	Yolk volume	+	−	−	−	+	−	−
	32	Head–trunk angle	+	−	−	+	+	−	−
	56	Eye surface	+	−	−	−	+	−	−
Muscular coordination	32	Cardiac rhythm	+	+	−	−	+	+	+
	32	Spontaneous movements	−	+	−	+	+	+	+
	80	Free-swimming	+	+	−	+	+	−	−
	80	Survival	+	−	−	+	+	−	−

overall survival of zebrafish embryos exposed to DODAC decreased in a concentration-dependent manner. The highest concentration (*i.e.* 9 μM of DODAC) led to 100% mortality of zebrafish eleutheroembryos (embryos post-hatch, but prior to external feeding). Interestingly, when mixed with MO, DODAC embryotoxicity was reduced, independently of the DODAC : MO ratio. Furthermore, the zebrafish embryos exposed to DODAX : MO liposomes, independently of the percentage of MO, showed survival rates ≥75%. These results are coherent with the cytotoxic profile obtained for *in vitro* cell-based tests.

Table 2 summarizes the effect on the different parameters monitored during zebrafish embryogenesis (8–80 hpf) upon exposure to different concentrations of the tested conditions, giving an overall and important perspective of the toxicity of single components, when in a mixture or in liposomal formulations.

4. Discussion

In this study we evaluated the effect of cationic liposomes' composition on the viability of several human cell lines, exploiting both the influence of the counter ion (Br⁻ or Cl⁻) in the cationic surfactant and the fraction of cationic/neutral lipid of DODAX : MO liposomes, previously validated for nucleic acid delivery (Silva *et al.* 2011,[25] 2012,[34] 2014,[29,32,33] Oliveira *et al.* 2014,[23] 2015,[31] Lopes *et al.* 2015[30]). Our aim was to obtain a comprehensive perspective of the toxicity profile of DODAX : MO liposomes and to understand how changing the lipid mixture influenced cells (metabolism, proliferation, cell death), tissues and organisms. Moreover, we set to highlight the potential of *in vivo* nanotoxicity assessment of liposomal nanocarriers in zebrafish embryotoxicity assays. These nanocarriers have been scarcely studied using these intermediate *in vivo* models, in stark contrast with metallic nanocarriers, in recent years.

Results obtained with the *in vitro* assays point to two general conclusions: DODAC-based liposomes induce higher cytotoxicity than DODAB-based liposomes, for the same molar fraction, in normal human fibroblasts; and the inclusion of MO in the formulation reduces cytotoxicity. Although the results were clearly cell-type dependent, the same trend

was found for the other cellular models tested (293 T, MDA-MB-435, MDA-MB-468, K562 and THP1), in terms of metabolic activity and plasma membrane integrity (Fig. S1 and S3†), but not for proliferation assessment (Fig. S2†). Interestingly, *in vivo* ZET assays showed that individual administration of DODAX (*i.e.* as free compounds) significantly disrupted fish development, with DODAC revealing a more toxic profile than DODAB, in a concentration-dependent manner. In contrast, DODAB or DODAC combined with MO in liposomes, in general, did not exert such level of toxicity. Moreover, the overall embryotoxicity profile of liposomes did not vary with the DODAX : MO molar fraction.

We have shown earlier that DODAC : MO and DODAB : MO have a different lipid structural organization due to the presence of the counter ions Cl⁻ or Br⁻: MO is more homogeneously distributed in DODAC than in DODAB bilayers, which results in the formation of lamellar phases with less tight polar headgroups in DODAC : MO liposomes.[23] These characteristics may directly influence the different cytotoxic response observed for DODAC- and DODAB-based liposomes, as it is expectable an easier transference of DODAC to plasma membranes, promoting higher destabilization. In addition to having a less packed headgroup region, DODAC : MO liposomes also form smaller and more curved aggregates than DODAB-based liposomes,[23,35,36] and are more fusogenic due to the more homogeneous MO incorporation.[23] A less ideal packing of lipid molecules promotes an easier escape of cationic lipids when compared with nanoformulations with more tightly packed lipids and lower curvature bilayers.[37] Also, DODAB liposomes were shown to be more stable under physiological conditions,[23] which may limit lipid loss, thereby decreasing cytotoxicity.[38]

MO is considered a safe and biocompatible lipid, with several reports on its use for biomedical applications (Kulkarni *et al.* 2011,[41] Silva *et al.* 2011,[25] 2012,[34] 2014,[29] Oliveira *et al.* 2014,[23] 2015,[31] Carneiro *et al.* 2015,[40] Lopes *et al.* 2015[30]). MO's fusogenicity and capacity to increase the fluidity of the lipid bilayers[39] impelled us to further explore its influence on DODAX : MO formulations when in interface with cells. Our results show that MO presence in the nanoformulation was generally beneficial for the cellular metabolism in all exposure times and for proliferation (48 h) of fibroblasts, with cell survival increasing in the order of DODAX : MO (1 : 0) < (2 : 1) < (1 : 2).

While this trend was not so clear regarding plasma membrane integrity, B : M (1 : 2) liposomes compromised it to a lesser extent when compared to other liposomal formulations. This might be explained by the fact that MO becomes mainly confined to the liposomal core, not imprinting a high fusogenic capacity to the system,[23,42] decreasing the disruption caused at the plasma membrane level. Nevertheless, for long incubation times, as interaction with cell membranes is favored, lipid rearrangements occur and MO might boost the integration of cationic lipids into the plasma membrane, resulting in a synergistic effect between both DODAC and MO lipids in terms of membrane toxicity. For shorter exposure times (4 h) MO always attenuated the negative effects associated with DODAC-based liposomes on plasma membranes, but the same was not true for DODAB-based liposomes.

Cells are heterogeneous entities whose composition of plasma membranes depends on developmental and environmental stimuli, varying significantly between cell types.[43] This strongly affects the cellular response to nanomaterials,[44] as observed by the slightly different response to DODAX : MO liposomes in the six cell lines tested. Therefore, it becomes evident that not only is the selection of the cell type very important for toxicity assessment, but also the integration of these results with data from *in vivo* models.

An important consideration in ZET assays is the presence of the zebrafish chorion, an acellular envelope surrounding mature eggs of teleosts that enables for *e.g.* oxygen/carbon dioxide to pass through *via* passive diffusion.[45] The zebrafish chorion is composed of three intercrossed layers and possesses pores with approximately 0.5–0.7 μm diameter,[46] which should not impair the passage of the tested nanocarriers. In the present study, the clarification of specific and/or nonspecific interactions between dechorionation and nanotoxicity of DODAX : MO liposomes was not addressed. Nevertheless, our data showed that normal patterns of spontaneous movements and heart rhythm of 32 hfp zebrafish embryos varied with the DODAX : MO molar fraction. This differential effect allows one to anticipate that the toxicity of DODAX : MO liposomes was not conditioned by the presence of the chorion. On the other hand, the effect of zebrafish chorion removal has already been demonstrated with Luviquat HM 552, which blocks it due to the cationic polymer molecular weight (~400 000 Da).[47] Interestingly, DODAX surfactants (with a higher molecular weight than Luviquat HM 552) were shown to affect multiple developmental variables of non-dechorionated zebrafish embryos/larvae, in a concentration-dependent manner. In agreement with the results from *in vitro* experiments, MO might favor the interaction of the cationic lipids with the chorion. Yet, the overall permeability contribution of the zebrafish chorion to the embryotoxicity of DODAX and MO, administered alone or combined in liposomal formulations, still needs to be confirmed.

Since very high doses of DODAX : MO liposomes affected BJ5-ta cell metabolism, cellular proliferation and plasma membrane integrity, promoting cell death, we investigated if it was occurring through apoptosis or necrosis. Cationic liposomes might induce apoptosis, often after 24–48 h exposure,[9,12,48,49]

but they can also trigger acute cell necrosis, even before the occurrence of apoptosis, in a positive surface charge-dependent manner.[49] Accordingly, we found that DODAX : MO liposomes induced cell death by both apoptosis and necrosis. Interestingly, the percentage of live cells (An−/PI−) generally increased from 24 h to 48 h of exposure, in line with what was observed with the MTS assay: metabolic activity slightly increased with longer incubation times. This might be explained by a dilution of the cellular nanoparticle content when cells are proliferating, therefore minimizing the negative effects on the metabolism of the surviving cells.[2] The presence of MO in the liposomal formulation resulted in a higher number of live cells. An equivalent outcome was achieved *in vivo*. Yet, the zebrafish embryos survival was independent of the DODAX : MO liposomal molar fraction tested.

When cells are exposed to minor levels of oxidative stress, their antioxidant protection is activated, but when ROS production overcomes the cellular antioxidant capacity, its accumulation can lead to cell death. Thus, ROS play an important role in apoptosis.[50] DODAX : MO liposomes induced accumulation of ROS in a time-, concentration-, liposomal formulation- and cell line-dependent manner. DODAC-based liposomes seemed to prompt higher ROS accumulation in fibroblasts, especially for 80 μg mL^{-1}, while the MO content did not have a clear effect. Since mitochondria is another key player in apoptosis and is related to oxidative stress,[51] alterations on mitochondrial potential induced by DODAX : MO liposomes were evaluated. Although no significant effect was observed, our results suggest that DODAX : MO liposomes affected the mitochondrial membrane potential, especially after 24 h incubation with C : M (2 : 1). Future work will determine the zebrafish chorion permeability effects on embryonic ROS levels upon continuous waterborne exposure to DODAX : MO liposomes and free surfactants.

The future of nanocarriers as therapeutic agents in medicine depends on the validation of multiparameter testing to account for the effects of administration routes, bioavailability, distribution and degradability, induction of developmental defects and activation of the complement and/or immune system. These factors are critical for *in vivo* nanotoxicity assessment and cannot be fully addressed with *in vitro* experiments. The integration of results from the *in vitro* and *in vivo* experiments will contribute to more comprehensive knowledge and allow predictions regarding the interaction of nanocarriers with cells, tissues and organisms. Zebrafish embryogenesis is an attractive and highly-informative *in vivo* model for fast track nanotoxicity assessment, bridging the gap between cellular trials and mammalian experiments[17,24] and facilitating the translation of nanoformulations into a clinical setup.

5. Conclusions

Very high doses of DODAX : MO liposomes induce cytotoxicity in human fibroblasts, associated with cell death markers typical of apoptosis or necrosis, including exposure of phos-

phatidylserine at the outer leaflet of the plasma membrane, accumulation of ROS, alteration of the mitochondrial membrane potential and plasma membrane disruption. The counter ion of the cationic lipid plays a determinant role in the elicited cytotoxicity: DODAC-based liposomes consistently induce more adverse effects, probably as a consequence of stronger cell membrane destabilization. All liposomal formulations have approximately the same size and surface charge, but the presence of MO promotes a general beneficial effect on cell survival, especially in the DODAB : MO mixture (1 : 2). Notably, an identical toxicity profile was obtained *in vivo*. Our results highlight the importance of a dose-dependent evaluation of the toxicity profile of liposomes, since different assays give different sensitivities to DODAX : MO. This concentration/molar fraction dependency was demonstrated to be relevant to the embryotoxicity profile of DODAX and MO, either as free components or in liposome formulations. Five other cell lines give a consistent response to DODAX : MO liposomes in terms of cellular metabolism and plasma membrane integrity. *In vivo*, the monitoring of the early life of zebrafish allows the detection of toxic signals induced by the different nanoformulations tested. The ZET assay reveals itself as highly appealing for application in "intermediate" nanotoxicity profiling, after initial screening in cultured cells and prior to validation in mammalian models.

Conflict of interest

The authors declare the following competing financial interest(s): The University of Minho, M. E. C. D. R. O. and A. C. G. have filed patent applications of Portuguese Patent PT104158 (2011), European Patent EP2335687 A2 (2011), and International Patent WO2010/020935 A2 (2010), respectively.

Abbreviations

DLS	Dynamic light scattering
DODAB	Dioctadecyldimethylammonium bromide
DODAC	Dioctadecyldimethylammonium chloride
hpf	Hours post-fertilization
HTA	Head–trunk angle
MO	1-Monooleoyl-*rac*-glycerol, monoolein
RT	Room temperature
ZET	Zebrafish embryo toxicity
ζ-potential	Zeta potential
3D-models	Three dimensional models

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References

1 C. A. Schütz, L. Juillerat-Jeanneret, H. Mueller, I. Lynch and M. Riediker, Therapeutic nanoparticles in clinics and under clinical evaluation, *Nanomedicine*, 2013, **8**, 449–467.

2 F. Joris, B. B. Manshian, K. Peynshaert, S. C. De Smedt, K. Braeckmans and S. J. Soenen, Assessing nanoparticle toxicity in cell-based assays: influence of cell culture parameters and optimized models for bridging the in vitro-in vivo gap, *Chem. Soc. Rev.*, 2013, **42**, 8339–8359.

3 A. Manke, L. Wang and Y. Rojanasakul, Mechanisms of nanoparticle-induced oxidative stress and toxicity, *Biomed. Res. Int.*, 2013, **2013**, 1–15.

4 I. Lynch and K. A. Dawson, Protein-nanoparticle interactions, *Nano Today*, 2008, **3**, 40–47.

5 R. Simón-Vazquez, M. Peleteiro, T. Lozano, Á. Gonzáles-Fernández and A. Casal, Nanobiotechnology - inorganic nanoparticles vs organic nanoparticles, in *Frontiers of Nanoscience*, Elsevier, 2012, pp. 443–485.

6 P. V. AshaRani, G. Low, K. Mun, M. P. Hande and S. Valiyaveettil, Cytotoxicity and genotoxicity of silver, *ACS Nano*, 2009, **3**, 279–290.

7 H. J. Johnston, G. Hutchison, F. M. Christensen, S. Peters, S. Hankin and V. Stone, A review of the in vivo and in vitro toxicity of silver and gold particulates: particle attributes and biological mechanisms responsible for the observed toxicity, *Crit. Rev. Toxicol.*, 2010, **40**, 328–346.

8 M. Van der Zande, R. Junker, X. F. Walboomers and J. A. Jansen, Carbon nanotubes in animal models: a systematic review on toxic potential, *Tissue Eng., Part B Rev.*, 2011, **17**, 57–69.

9 Y. Aramaki, S. Takano, H. Arima and S. Tsuchiya, Induction of apoptosis in WEHI 231 cells by cationic liposomes, *Pharm. Res.*, 2000, **17**, 515–520.

10 K. B. Knudsen, H. Northeved, P. K. Ek, A. Permin, T. L. Andresen, S. Larsen, K. M. Wegener, H. R. Lam and J. Lykkesfeldt, Differential toxicological response to positively and negatively charged nanoparticles in the rat brain, *Nanotoxicology*, 2014, **8**, 764–774.

11 C. Lonez, M. Vandenbranden and J. M. Ruysschaert, Cationic liposomal lipids: from gene carriers to cell signaling, *Prog. Lipid Res.*, 2008, **47**, 340–347.

12 S. Takano, Y. Aramaki and S. Tsuchiya, Physicochemical properties of liposomes affecting apoptosis induced by cationic liposomes in macrophages, *Pharm. Res.*, 2003, **20**, 962–968.

13 H. Lv, S. Zhang, B. Wang, S. Cui and J. Yan, Toxicity of cationic lipids and cationic polymers in gene delivery, *J. Controlled Release*, 2006, **114**, 100–109.

14 S. Arora, J. M. Rajwade and K. M. Paknikar, Nanotoxicology and in vitro studies: The need of the hour, *Toxicol. Appl. Pharmacol.*, 2012, **258**, 151–165.

15 K. Greish, G. Thiagarajan and H. Ghandehari, In vivo methods of nanotoxicology, *Methods Mol. Biol.*, 2012, **926**, 235–253.

16 C. Santoriello and L. I. Zon, Hooked! Modeling human disease in zebrafish, *J. Clin. Invest.*, 2012, **122**, 2337–2343.

17 J. P. Bohnsack, S. Assemi, J. D. Miller and D. Y. Furgeson, The primacy of physicochemical characterization of nanomaterials for reliable toxicity assessment: a review of the zebrafish nanotoxicology model, *Methods Mol. Biol.*, 2012, **926**, 261–316.

18 J. R. Goldsmith and C. Jobin, Think small: Zebrafish as a model system of human pathology, *J. Biomed. Biotechnol.*, 2012, **2012**, 1–12.

19 S. Scholz, S. Fischer, U. Gündel, E. Küster, T. Luckenbach and D. Voelker, The zebrafish embryo model in environmental risk assessment - applications beyond acute toxicity testing, *Environ. Sci. Pollut. Res.*, 2008, **15**, 394–404.

20 L. Y. Rizzo, S. K. Golombek, M. E. Mertens and Y. Pan, In vivo nanotoxicity testing using the Zebrafish Embryo Assay, *J. Mater. Chem. B Mater. Biol. Med.*, 2013, **1**, 1–13.

21 R. N. Wang, A. Meredith, M. Lee, D. Deutsch, L. Miadzvedskaya, E. Braun, P. Pantano, S. Harper and R. Draper, Toxicity assessment and bioaccumulation in zebrafish embryos exposed to carbon nanotubes suspended in Pluronic® F-108, *Nanotoxicology*, 2015, **5390**, 1–10.

22 O. J. Osborne, B. D. Johnston, J. Moger, M. Balousha, J. R. Lead, T. Kudoh and C. R. Tyler, Effects of particle size and coating on nanoscale Ag and TiO (2) exposure in zebrafish (Danio rerio) embryos, *Nanotoxicology*, 2013, **7**, 1315–1324.

23 A. C. N. Oliveira, T. F. Martens, K. Raemdonck, R. D. Adati, E. Feitosa, C. Botelho, A. C. Gomes, K. Braeckmans and M. E. C. D. Real Oliveira, Dioctadecyldimethylammonium: Monoolein nanocarriers for efficient in vitro gene silencing, *ACS Appl Mater Interfaces*, 2014, **6**, 6977–6989.

24 V. E. Fako and D. Y. Furgeson, Zebrafish as a correlative and predictive model for assessing biomaterial nanotoxicity, *Adv. Drug Delivery Rev.*, 2009, **61**, 478–486.

25 J. P. N. Silva, A. C. N. Oliveira, M. P. P. A. Casal, A. C. Gomes, P. J. G. Coutinho, O. P. Coutinho and M. E. C. D. Real Oliveira, DODAB:monoolein-based lipo-

plexes as non-viral vectors for transfection of mammalian cells, *Biochim. Biophys. Acta*, 2011, **1808**, 2440–2449.

26 G. Wang, Y. Gong, F. J. Burczynski and B. B. Hasinoff, Cell lysis with dimethyl sulphoxide produces stable homogeneous solutions in the dichlorofluorescein oxidative stress assay, *Free Radical Res.*, 2008, **42**, 435–441.

27 S. L. Adams, T. Zhang and D. M. Rawson, The effect of external medium composition on membrane water permeability of zebrafish (Danio rerio) embryos, *Theriogenology*, 2005, **64**, 1591–1602.

28 C. B. Kimmel, W. W. Ballard, S. R. Kimmel, B. Ullmann and T. F. Schilling, Stages of embryonic development of the zebrafish, *Dev. Dyn.*, 1995, **203**, 253–310.

29 J. P. N. Silva, A. C. N. Oliveira, M. Lúcio, A. C. Gomes, P. Coutinho and M. E. C. D. Real Oliveira, Tunable pDNA/DODAB:MO lipoplexes: the effect of incubation temperature on pDNA/DODAB:MO lipoplexes structure and transfection efficiency, *Colloids Surf., B*, 2014, **121**, 371–379.

30 I. Lopes, A. C. N. Oliveira, M. P. Sárria, J. P. N. Silva, O. Goncalves, A. C. Gomes and M. E. C. D. Real Oliveira, Monoolein-based nanocarriers for enhanced folate receptor-mediated RNA delivery to cancer cells, *J. Liposome Res.*, 2015, 1–12.

31 A. C. N. Oliveira, K. Raemdonck, T. Martens, K. Rombouts, R. Simón-Vázquez, C. Botelho, I. Lopes, M. Lúcio, Á. G. Fernández, M. E. C. D. Real Oliveira, A. C. Gomes and K. Braeckmans, Stealth monoolein-based nanocarriers for delivery of siRNA to cancer cells, *Acta Biomater.*, 2015, **25**, 216–229.

32 J. P. N. Silva, I. M. S. C. Oliveira, A. C. N. Oliveira, M. Lúcio, A. C. Gomes, P. J. G. Coutinho and M. E. C. D. Real Oliveira, Structural dynamics and physicochemical properties of pDNA/DODAB:MO lipoplexes: effect of pH and anionic lipids in inverted non-lamellar phases versus lamellar phases, *Biochim. Biophys. Acta*, 2014, **1838**, 2555–2567.

33 J. P. N. Silva, A. C. N. Oliveira, M. Lúcio, A. F. C. Gomes and M. E. C. D. Real Oliveira, How Multi-Step versus One-Step preparation method affects the physicochemical properties and transfection efficiency of DNA/DODAB:MO lipoplexes, *J. Appl. Solution Chem. Model.*, 2014, **3**, 94–105.

34 J. Silva, A. Oliveira, A. Gomes and M. E. C. D. Real Oliveira, Development of dioctadecyldimethylammonium bromide/monoolein liposomes for gene delivery, in *Cell Interaction*, InTech, Rijeka (Croatia), 2012, pp. 245–272.

35 E. Feitosa and F. R. Alves, The role of counterion on the thermotropic phase behavior of DODAB and DODAC vesicles, *Chem. Phys. Lipids*, 2008, **156**, 13–16.

36 E. Feitosa, F. R. Alves, E. M. S. Castanheira and M. E. C. D. Real Oliveira, DODAB and DODAC bilayer-like aggregates in the micromolar surfactant concentration domain, *Colloid Polym. Sci.*, 2009, **287**, 591–599.

37 S. J. H. Soenen, A. R. Brisson and M. De Cuyper, Addressing the problem of cationic lipid-mediated toxicity: the magnetoliposome model, *Biomaterials*, 2009, **30**, 3691–3701.

38 J. Wojewodzka, G. Pazdzior and M. Langner, A method to evaluate the effect of liposome lipid composition on its interaction with the erythrocyte plasma membrane, *Chem. Phys. Lipids*, 2005, **135**, 181–187.

39 A. Ganem-Quintanar, D. Uerrero and P. Buri, Monoolein: a review of the pharmaceutical applications, *Drug Dev. Ind. Pharm.*, 2000, **26**, 809–820.

40 C. Carneiro, A. Correia, T. Collins, M. Vilanova, C. Pais, A. C. Gomes, M. E. C. D. Real Oliveira and P. Sampaio, DODAB:monoolein liposomes containing Candida albicans cell wall surface proteins: a novel adjuvant and delivery system, *Eur. J. Pharm. Biopharm.*, 2015, **89**, 190–200.

41 C. V. Kulkarni, W. Wachter, G. Iglesias-Salto, S. Engelskirchen and S. Ahualli, Monoolein: a magic lipid?, *Phys. Chem. Chem. Phys.*, 2011, **13**, 3004–3021.

42 C. Leal, N. F. Bouxsein, K. K. Ewert and C. R. Safinya, Highly efficient gene silencing activity of siRNA embedded in a nanostructured gyroid cubic lipid matrix, *J. Am. Chem. Soc.*, 2010, **132**, 16841–16847.

43 M. Mahmoudi, J. Meng, X. Xue, X. J. Liang, M. Rahman, C. Pfeiffer, R. Hartmann, P. R. Gil, B. Pelaz, W. J. Parak, P. del Pino, S. Carregal-Romero, A. G. Kanaras and S. T. Selvan, Interaction of stable colloidal nanoparticles with cellular membranes, *Biotechnol. Adv.*, 2014, **32**, 679–692.

44 S. Laurent, C. Burtea, C. Thirifays, F. Rezaee and M. Mahmoudi, Significance of cell 'observer' and protein source in nanobiosciences, *J. Colloid Interface Sci.*, 2013, **392**, 431–445.

45 S. Berghmans, P. Butler, P. Goldsmith, G. Waldron, I. Gardner, Z. Golder, F. M. Richards, G. Kimber, A. Roach, W. Alderton and A. Fleming, Zebrafish based assays for the assessment of cardiacvisual and gut function - potential safety screens for early drug discovery, *J. Pharmacol. Toxicol. Methods*, 2008, **58**, 59–68.

46 D. M. Rawson, T. Zhang, D. Kalicharan and W. L. Jongebloed, Field emission scanning electron microscopy and transmission electron microscopy studies of the chorion, plasma membrane and syncytial layers of the gastrula-stage embryo of the zebra Brachydanio rerio: a consideration of structural and functional relation, *Aquacult. Res.*, 2000, **31**, 325–336.

47 K. Henn and T. Braunbeck, Dechorionation as a tool to improve the fish embryo toxicity test (FET) with the zebrafish (Danio rerio), *Comp. Biochem. Physiol. C Toxicol. Pharmacol.*, 2011, **153**, 91–98.

48 Y. Aramaki, S. Takano and S. Tsuchiya, Cationic liposomes induce macrophage apoptosis through mitochondrial pathway, *Arch. Biochem. Biophys.*, 2001, **392**, 245–250.

49 X. Wei, B. Shao, Z. He, T. Ye, M. Luo, Y. Sang, X. Liang, W. Wang, S. Luo, S. Yang, S. Zhang, C. Gong, M. Gou, H. Deng, Y. Zhao, H. Yang, S. Deng, C. Zhao, L. Yang, Z. Qian, J. Li, X. Sun, J. Han, C. Jiang, M. Wu and Z. Zhang, Cationic nanocarriers induce cell necrosis through impairment of Na+/K+-ATPase and cause subsequent inflammatory response, *Cell Res.*, 2015, **25**, 237–253.

50 H. U. Simon, A. Haj-Yehia and F. Levi-Schaffer, Role of reactive oxygen species (ROS) in apoptosis induction, *Apoptosis*, 2000, **5**, 415–418.

51 K. Sinha, J. Das, P. B. Pal and P. C. Sil, Oxidative stress: the mitochondria-dependent and mitochondria- independent pathways of apoptosis, *Arch. Toxicol.*, 2013, **87**, 1157–1180.