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Niosomes-based gene delivery systems for effective transfection of human mesenchymal stem cells

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ABSTRACT

Gene transfer to mesenchymal stem cells (MSCs) has arisen as a powerful approach to increase the therapeutic potential of this effective cell population. Over recent years, niosomes have emerged as self-assembled carriers with promising performance for gene delivery. The aim of our work was to develop effective niosomes-based DNA delivery platforms for targeting MSCs. Niosomes based on 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA; 0, 7 or 15%) as cationic lipid, cholesterol as helper lipid, and polysorbate 60 as non-ionic surfactant, were prepared using a reverse phase evaporation technique. Niosomes dispersions (filtered or not) and their corresponding nioplexes with a *lacZ* plasmid were characterized in terms of size, charge, protection, and complexation abilities. DOTMA concentration had a large influence on the physicochemical properties of resulting nioplexes. Transfection efficiency and cytotoxic profiles were assessed in two immortalized cell lines of MSCs. Niosomes formulated with 15% DOTMA provided the highest values of β-galactosidase activity, being similar to those achieved with Lipofectamine®, but showed less cytotoxicity. Filtration of niosomes dispersions before adding to the cells resulted in a loss of their biological activities. Storage of niosomes formulations (for 30 days at room temperature) caused minor modification of their physicochemical properties but also attenuated the transfection capability of the nioplexes. Differently, addition of the lysosomotropic agent sucrose into the culture medium during transfection or to the formulation itself improved the transfection performance of non-filtered niosomes. Indeed, steam heat-sterilized niosomes prepared in sucrose medium demonstrated transfection capability.

1. Introduction

Mesenchymal stem cells (MSCs) constitute a pivotal cell population for cell therapy in regenerative medicine. Key attributes of MSCs as their capacity to differentiate into various lineages, feasibility of isolation and expansion *in vitro*, homing and immunomodulatory properties, make them an appealing cell source for tissue repair [1,2]. These properties also point out MSCs as an excellent target for gene therapy approaches as a way to reinforce their therapeutic potential for tissue repair [3]. Genetic modification of MSCs has emerged as a promising tool for the treatment of different tissue pathologies as those affecting musculoskeletal [4–7], cardio-vascular [8–10] or retinal tissues [8].

Among the different types of gene vectors tested to genetically modify MSCs, nonviral carriers represent the safest tools, overcoming immunogenicity and tumorigenic risks associated with viral vectors [9]. Moreover, nonviral systems can transfer longer genes and be produced in larger amounts at lower cost [10]. Current nonviral gene therapy approaches focus on the complexation of negatively charged DNA molecules with cationic polymers (*e.g.*, poly-L-lysine, polyethyleneimine, poly(amidoamine), chitosan) [11] or lipids (*e.g.*, liposomes) [12–14] in order to facilitate their entry in the cell. Notwithstanding, gene transfer efficacy *via* nonviral vectors is still lower when compared with that of their viral counterparts [9,10]. Therefore, improved nonviral vectors are being actively researched.

Niosomes are self-assembled vesicular nanocarriers composed of non-ionic surfactants, helper lipids and charge modifiers [15] that have

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recently emerged as promising nonviral gene delivery systems [16–18]. Replacement of phospholipids by non-ionic surfactants notably improves the stability of niosomes compared to liposomes while still preserving adequate cytocompatibility [19]. Niosomes have been formulated by combination of commercial cationic lipids [20–24] or chemically modified ones [18,20,21,25] with non-ionic surfactants including polysorbate 20 [23], polysorbate 60 [20–22,24] or polysorbate 80, either solely [18,20,21,23,25] or blended with poloxamer [20,21]. Helper lipids as squalene [20,21,23,25], cholesterol [25] or lycopene [20–22] have been shown to improve niosomes physical properties. Most of these niosomes formulations have been tested to target retina [23–25] or central nervous system [20,22] cells. Nonetheless, only a few recent studies have revealed the feasibility of niosomes to genetically modify MSCs, which were from mouse origin [18,21].

The aim of this work was to elucidate whether niosomes could be effective DNA delivery platforms for targeting immortalized human MSCs cell lines (iMSCs) obtained from aged donors [26]. Selected iMSCs cell lines have previously shown to avoid senescence and proliferation problems associated to primary cultures while maintaining their multipotency, therefore being potential tools for regenerative medicine research [26,27]. The hypothesis of this study is that a good balance between DNA condensation capability, gene transfection efficiency and cytocompatibility can be achieved by tuning the content in cationic lipid 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA). Thus, to carry out the work, niosomes based on polysorbate 60 as non-ionic surfactant and cholesterol as helper lipid were prepared covering a wide range in DOTMA content (0, 7 and 15%) using a reverse phase evaporation technique [15]. Since nanocarrier purification is a required step for pre-clinical translation of developed formulations the effects of filtration and heat sterilization under isotonic conditions on the ability of the niosomes to genetically modify the iMSCs were investigated. Both filtration and autoclaving have been mentioned in previous reports to compromise vesicles stability and further performance as non-viral vectors [28]. Niosomes and corresponding nioplexes with a *lacZ* encoding plasmid were characterized in terms of size, polydispersity index (PDI), zeta potential, protection and complexation abilities, and transfection efficiency of iMSCs cultures. The addition of the lysosomotropic agent sucrose firstly to the cells medium and lastly to the formulation itself were explored as a way to enhance the performance of the developed gene delivery systems. Finally, the physicochemical stability of the niosomes and the biological properties of the nioplexes upon long-term storage were monitored.

2. Materials and methods

2.1. Materials

Polysorbate 60 (P60, Tween 60, MW 1311.7 g/mol, HLB 14.9), paraformaldehyde (37% *v*/v), Dulbecco's Modified Eagle's Medium (DMEM) and cholesterol (MW 386.65 g/mol) were purchased from Sigma Aldrich (St Louis, MO, USA). 1,2-Di-O-octadecenyl-3-trimethylammonium propane (chloride salt) (DOTMA, MW 670.575 g/mol) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Fetal bovine serum (FBS), penicillin/streptomycin (P/S), Tris-borate-EDTA (TBE), Lipofectamine® Stem (LPF), Opti-MEM™ reduced serum medium, SYBR Green Gold (10,000 X), DNase I (1000 U), sodium dodecyl sulfate (SDS, MW 288.38 g/mol), dichloromethane, agarose, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), Triton X-100 (MW 646.86 g/mol) and pCMV-SPORT-βgal (p*lacZ*, bp 7853), were from Gibco-ThermoFisher Scientific (Madrid, Spain). WST-1 was purchased from Roche (Mannheim, Germany). β-glo was from Promega (Madison, WI, USA). Label IT® Nucleic Acid Labeling kit Cy3 was from Mirus Bio (Madison, WI, USA). The 18-well μ-chamber slides were from Ibidi (Gräfelfing, Germany).

2.2. Niosome preparation

P60 (35.3 mg), cholesterol (4.4 mg) (P60: cholesterol 1:0.42 mol ratio) and DOTMA (0, 1.8 or 3.8 mg to have 0, 7 and 15 mol%) were dissolved together in dichloromethane (2 mL). The solvent was then evaporated in a rotary evaporator under 400 mbar pressure at 40 °C to form a thin film on the bottom of the flask. The film was desiccated for 30 min under vacuum to remove traces of solvent, hydrated with 5 mL Opti-MEM and sonified for 90 s at 20% amplitude with a probe (1/8″ diameter tapered horn) fitted to a Branson Digital Sonifier 450 (Marshall Scientific, Hampton, NH, USA). Half of the niosomes dispersion was filtered using 0.45 μm polyethersulfone (PES) membranes (Filter-Lab, Filtros Anoia, Barcelona, Spain) and designed as filtered dispersions, F. The other half niosome dispersion was directly used without filtration and designed as non-filtered dispersion, wF.

An additional batch of niosomes was prepared as above but the film was hydrated with 300 mM sucrose aq. solution, subsequent sterilized by steam heat (sh) and used without filtration. This batch was designed as sh.

2.3. Plasmid propagation and formation of nioplexes

The pCMV-SPORT-βgal reporter plasmid (p*lacZ*) was propagated, purified, and quantified following the instructions from manufacturer. Labelling of p*lacZ* with Cy3 was performed using a Label IT Nucleic Acid Labeling kit following the guidelines from the supplier.

Nioplexes were formed by mixing an appropriate volume (always 100 ng of plasmid) of a stock solution of a p*lacZ* (1 mg/mL) with different volumes of niosomes (with 7 and 15% DOTMA) in Opti-MEM medium to get cationic lipid/DNA mass ratios (*w*/*w*) of 2.5/1, 5/1, 10/1, 15/1 and 20/1, which were equivalent to cationic amino groups (N) to nucleic acid anionic phosphate groups (P) ratio of 1, 2, 5, 7 and 10, respectively. Nioplexes formulated with 0% DOTMA were prepared by mixing the same amount of p*lacZ* with the appropriate volumes of 0% DOTMA niosomes to get P60/DNA mass ratios (*w*/*w*) of 2.5/1, 5/1, 10/1, 15/1 and 20/1. The mixtures were allowed to stand for 30 min at room temperature to promote the electrostatic interactions between the niosomes and the negatively charged plasmid [24].

2.4. Size and zeta potential

Niosomes and nioplexes (cationic lipid/DNA mass ratios of 2.5/1, 5/1, 10/1, 15/1 and 20/1) were prepared following the same procedures described in Sections 2.2 and 2.3. The hydrodynamic diameter, polydispersity index (PDI) ($n = 3$) and zeta potential ($n = 2$) of both niosomes and nioplexes were measured in water and in Opti-MEM medium at 20 ℃ by Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS) using a NanoBrook 90Plus Zeta (Brookhaven Instruments Corporation, Holtsville, NY, USA). Folded capillary cuvettes (DTS1070) were used for the measurements.

2.5. Agarose gel electrophoresis

The capacity of niosomes to condense DNA was evaluated by agarose gel electrophoresis assay, at the same cationic lipid/DNA (7% and 15% DOTMA) or P60/DNA (0% DOTMA) mass ratio described in Section 2.3. Naked p*lacZ* (control) or niosome-complexed p*lacZ* samples (always 100 ng of plasmid) were run on a 0.8% *w*/*v* agarose gel after adding DNase I at a final concentration of 1 U DNase per 2.5 μg DNA [18]. The mixtures were incubated at 37 °C for 30 min. Finally, 7% SDS solution was added to release DNA from the nioplexes. The agarose gel was immersed in TBE buffer and exposed for 30 min to 100 V. DNA bands were stained with SYBR Green, and images were observed under a digital Chemi-Doc™ MP Imaging System (Bio-Rad, Madrid, Spain).

2.6. Evaluation of niosomes complexation ability

The ability of niosomes to bind and complex DNA was evaluated by means of a fluorescence-exclusion titration assay [29,30]. Briefly, nioplexes (0, 7 and 15% DOTMA) were invariably prepared by mixing 0.5 μg of pDNA with the corresponding amount of niosome dispersions as described in Section 2.3. Afterwards, nioplexes were incubated for 30 min at RT, then SYBR Green (200×; 3 μL) was added, and the mixture was incubated for 10 min protected from light. Finally, 10 mM HEPES was added to obtain a total volume of 300 μL. Fluorescence measurements were performed with a Synergy HTX Plate Reader (Biotek, Winooski, VT, USA) in 96 black well-plates (λ_{exc} = 485 nm, $\lambda_{em.}$ = 528 nm). The complexation efficiency (%) was expressed as relative fluorescence, normalized to the fluorescence of uncomplexed (naked) pDNA according to the following equation [10].

$$Complexation\ efficiency\ (\%) = \frac{F_{sample} - F_{blank}}{F_{naked\ pDNA} - F_{blank}} \times 100$$

In this equation, F is the recorded fluorescence and F_{blank} represents the fluorescence values recorded from the blank, performed by using same dilution of SYBR Green (200×) in 10 mM HEPES, but in absence of plasmid or niosomes. Each condition was assessed in triplicate.

2.7. Transmission electron microscopy (TEM)

Drops (5 μL) of niosome dispersions were placed on carbon coated grids. The excess was carefully removed with a filter paper. Samples were dyed with uranyl acetate (2% in water), dried, and observed using a high resolution JEM-1011 TEM (JEOL USA Inc., Peabody, MA, USA).

2.8. In vitro cell studies

Immortalized mesenchymal stem cells (iMSCs; iMSCs#6 and iMSCs#13) were kindly donated by Prof. S.M. Diaz-Prado [26]. Cells were grown in DMEM supplemented with 10% FBS and 1% P/S and kept at 37 °C in a humidified atmosphere containing 5% CO_2.

iMSCs cells were seeded in 96 well-plates at an initial density of 10^4 cells/well and allowed to attach for 24 h at 37 °C before the experiments. Cells were exposed to different nioplexes formed upon complexation of niosome formulations (0, 7 and 15% DOTMA; wF and F) with p*lacZ* (always 100 ng of plasmid; cationic lipid or P60/DNA 5/1, 10/1, 15/1 and 20/1 *w/ w* mass ratios) in Opti-MEM medium following the procedure described in Section 2.3. Cells cultured in Opti-MEM without nioplexes and cells transfected with the commercial reagent LPF (1 μL/well) were used as negative and positive controls, respectively. Cells were incubated with nioplexes or LPF lipoplexes for 3 h at 37 °C and 5% CO_2. After this time, the medium was removed and refreshed with growth medium, and cells were allowed to grow for 48 h at 37 °C, until analysis.

In parallel, some of the formulations (7 and 15% DOTMA both wF) were complexed with p*lacZ* (100 ng plasmid; cationic lipid/DNA 5/1 and 10/1 mass ratio, equivalent to 2 and 5 N/P ratios) and tested under the same conditions (cell density and growth time). After adding the nioplexes solutions (10 μL), Opti-MEM or Opti-MEM with sucrose (40 mM) were added to complete a final volume of 100 μL per well. All conditions were assessed in triplicate in each cell line (iMSCs#6 and iMSCs#13) in two independent experiments. Additionally, a batch of niosomes dispersions prepared in 300 mM sucrose and heat sterilized (sh) was evaluated under same conditions as described above (100 ng plasmid; cationic lipid/DNA 5/1, 10/1 and 15/1 mass ratio, equivalent to 2, 5 and 7 N/P ratios).

2.9. Cell viability assay

Viability of iMSCs monolayers at 48 h post-transfection with the different nioplexes (same formulations and *w/w* ratios described in Section 2.3) was monitored using the tetrazolium salt (WST-1) method [31]. Absorbance (A) at 450 nm was measured using a Synergy HTX Plate Reader (Biotek, Winooski, VT, USA) and the percent of cell viability (%) was calculated as follows [31]:

$$Viability\ (\%) = \frac{A_{sample}}{A_{negative\ control}} \times 100$$

Cell numbers upon contact with nioplexes or lipoplexes were estimated from a calibration curve constructed using cultures with known number of cells from each cell line (iMSCs#6 and iMSCs#13).

2.10. Transfection efficiency

The transfection efficiency achieved with the different nioplexes (same formulations and w/w ratios described in Section 2.3) was evaluated by using the β-glo reagent [32]. Luminescence measurements were performed in white 96 well-plates using a plate reader and β-galactosidase activity was expressed as Relative Luminescence Units (RLU). Endogenous β-galactosidase activity recorded for the negative control was used as blank and subtracted for each condition and group of study.

$$\beta - galactosidase\ activity = RLU_{sample} - RLU_{negative\ control}$$

2.11. Cellular and nuclear uptake of placZ

iMSCs (10^4 cells/well) were seeded in sterile 18-well μ-chamber slides and transfected with nioplexes formed with Cy3-labeled p*lacZ* (100 ng pDNA; cationic lipid/DNA 5/1 and 10/1 *w/w* mass ratios, equivalent to 2 and 5 N/P ratios) at the same conditions described in Section 2.7. After 4 h of incubation at 37 °C, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and the membrane was permeabilized by adding Triton X-100 (0.2% in PBS) for 8 min at RT. Then, DAPI (300 nM in PBS) was added to the monolayers in darkness as a nuclear counterstain, and incubated for 5 min at room temperature [33]. Finally, cells were washed 2 times with PBS and observed under a fluorescence inverted microscope Olympus CKX53 (Olympus, Barcelona, Spain) with rhodamine (Cy3) and UV (DAPI) filters sets.

2.12. Stability assays

Niosomes and nioplexes characterization and *in vitro* transfection studies were repeated after storing the niosomes dispersions for 30 days at room temperature protected from light. The nioplexes were prepared immediately before testing using the stored niosomes containing 7 or 15% DOTMA both wF, for cationic lipid/DOTMA 5/1 and 10/1 *w/w* mass ratios, equivalent to 2 and 5 N/P ratios (as described in Section 2.8).

2.13. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using *IBM SPSS Statistics version 23* by parametric tests (one-way ANOVA; Student's *t*-test) and non-parametric ones (Kruskal-Wallis, Multiple Range, *U*-Mann Whitney), when appropriate in each case. A $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Preparation and physicochemical characterization of niosomes and nioplexes

Niosomes were prepared using a quite hydrophilic surfactant (P60) as main component, cholesterol as helper lipid, and DOTMA as lipid that may confer cationic charges. Cholesterol was included at the minimum

proportion required to form niosomes in the 100 nm range, according to preliminary experiments. DOTMA was included at three levels: 0, 7 and 15 mol% in order to elucidate the effect of the cationic charges on the transfection efficiency. Niosomes were prepared using the reverse phase evaporation method and characterized in terms of particle size, zeta potential and polydispersity index (PDI). Table 1 summarizes main physicochemical features from freshly prepared niosomes before (wF) and after (F) filtration.

Freshly prepared niosomes (non-filtered, wF) showed a decrease in size as the content in DOTMA increased. Relevantly, the mean size of the niosomes prepared with 0% and 7% DOTMA decreased in the first few days after preparation (Fig. 1). Statistical analysis revealed that niosomes without DOTMA at time 0, 24, 96 and 120 h were significantly larger (ANOVA, $p < 0.001$; multiple range test) than niosomes stored beyond that time. The mean size of niosomes with 7% DOTMA was also greater in the first few days, but the data dispersion was very broad and no statistical differences could be seen. Nevertheless, homogeneity in size was clearly observed as a function of time, with values converging toward 100 nm. The polydispersity index (PDI) (Fig. S1 in Supporting Information) showed highly variable values in the first few days after preparation and then levelled in 0.5–0.6 range. Differently, the zeta potential was stable over the whole 30-day period. Niosomes prepared with 15% DOTMA (non-filtered) as well as any filtered formulation exhibited more homogeneous and stable values of size, PDI and Z-potential (no statistically significant differences in the 30 days of storage). This finding suggests that both an increase in DOTMA proportion and filtration facilitate the attaining of self-assembly equilibrium during niosome formation. A representative TEM image of non-filtered 15% DOTMA niosomes is shown in Fig. S2 (Supporting Information).

Nioplexes formed upon complexation of p*lacZ* with niosomes prepared with 0% (Fig. 2A), 7% (Fig. 2B) and 15% (Fig. 2C) DOTMA subjected (F) or not (wF) to filtration were characterized regarding size and zeta potential (Fig. 2 upper panels) and agarose gel electrophoresis assays in presence of DNase (Fig. 2 lower panels). Nioplexes formulated with 0% DOTMA exhibited the biggest sizes, being around 360 nm in the filtered dispersions, and larger than 600 nm in the non-filtered ones ($p < 0.001$) (Fig. 2A). Nioplexes prepared with 7 and 15% DOTMA dispersions had sizes around 180 nm before filtration (wF) and 140 nm after filtration (F) ($p < 0.05$) (Fig. 2B and C). These differences were more evident at lower DOTMA/DNA mass ratios; nioplexes formed from F dispersions had smaller sizes ($p < 0.05$ at DOTMA/DNA ratios of 2.5/1 and 10/1). In general, PDI was also lower in all filtered niosomes formulations (0, 7 and 15% DOTMA); nevertheless, no statistical significant differences in this parameter were observed when compared with wF dispersions. Irrespective of the DOTMA/DNA *w/w* ratio considered, the size of nioplexes was similar for the two filtered 7% and 15% DOTMA formulations tested. Similar trend was observed in nioplexes formed using same formulations (7% and 15%) from wF dispersions. Conversely, niosomes formulated without DOTMA had the highest sizes of nioplexes in both wF and F dispersions ($p < 0.001$) when compared with 7 or 15% DOTMA formulations (Fig. 2A–C). Focusing on DOTMA/DNA mass ratios, all formulations regardless whether they were filtered or not, showed a decrease in nioplexes sizes between the lowest (2.5/1) and the highest (20/1) ratio tested ($p < 0.05$).

Table 1
Physical features of non-filtered (wF) and filtered (F) niosomes after 24 h preparation. Mean values ± standard deviation ($n = 3$).

Parameters	Without filtration (wF)			Filtered (F)		
DOTMA (mol%)	0	7	15	0	7	15
Size (nm)	532 ± 41	295 ± 246	118 ± 20	93 ± 6	101 ± 7	103 ± 16
PDI	0.73 ± 0.15	0.47 ± 0.09	0.29 ± 0.01	0.57 ± 0.06	0.30 ± 0.03	0.26 ± 0.01
Z-potential (mV)	−7.3 ± 2.1	+38.7 ± 5.7	+56.2 ± 3.1	−0.5 ± 4.0	+40.8 ± 3.8	+54.4 ± 5.2

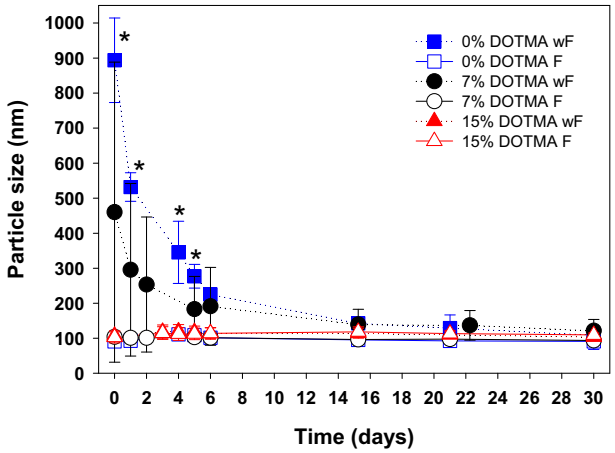

Fig. 1. Evolution of the size of niosomes during storage at room temperature protected from light. * depicts $p < 0.001$ for particle size differences between 0% DOTMA niosomes before and after filtration.

The zeta potential of nioplexes showed also a DOTMA-concentration dependence effect. All nioplexes prepared with 0% DOTMA exhibited a negative zeta potential, which was significantly lower to that achieved with 7 or 15% DOTMA formulations from wF niosomes ($p < 0.05$). Nioplexes formulated with 7% DOTMA showed an increase in electropositivity with the raise of DOTMA/DNA ratio, from −10.0 ± 1.1 mV up to +25.9 ± 2.1 mV for the wF dispersions ($p < 0.01$), and from −6.3 ± 0.0 mV up to +9.4 ± 4.4 mV for the filtered ones. The same trend was observed for 15% DOTMA nioplexes, reaching potential values up to +28.8 ± 0.9 mV in wF dispersions and up to +35.6 ± 0.1 mV in F ones ($p < 0.01$) at 20/1 DOTMA/DNA ratio. No differences in size and zeta potential values were observed when nioplexes formed from 7% or 15% DOTMA niosomes were prepared in Opti-MEM medium (data not shown).

Agarose gel electrophoresis assays in presence of DNase I are summarized in Fig. 2A–C; lower panels. 0% DOTMA niosomes failed to condense and therefore to protect the DNA against enzymatic digestion, as confirmed by the absence of bands, except for nioplexes formed at the highest P60/DNA *w/w* ratio (20/1) (Fig. 2A). Conversely, an enhanced capacity of protection was noticed by increasing DOTMA/DNA ratio in 7% DOTMA and 15% DOTMA formulations (Fig. 2B and C). After the addition of SDS, both 7% DOTMA and 15% DOTMA nioplexes formulations showed SC (supercoiled) and OC (open circular) bands corresponding to the released DNA. Moreover, non-filtered formulations demonstrated enhanced protection of complexed DNA from lower DOTMA/DNA ratios in 7% DOTMA formulations (Fig. 2B).

3.2. Complexation of DNA with the niosome formulations

The ability of the different niosome formulations to efficiently condense p*lacZ* was then evaluated applying the SYBR green dye exclusion assay (Fig. 3). Filtered 7% DOTMA (black line) and 15% DOTMA (red line) niosome formulations showed lower fluorescence values compared with 0% DOTMA niosomes (blue line) ($p < 0.05$). The data confirmed that 0% DOTMA niosomes were not able to make a complex with DNA, except when there was a large excess of P60 to DNA. A reduction in fluorescence values was also observed in wF dispersions of 7% DOTMA (black dot line) and 15% DOTMA (red dot line) niosomes when compared to those prepared without DOTMA (blue dot line) ($p < 0.01$).

Specifically, nioplexes prepared with 7% DOTMA and 15% DOTMA showed a reduction in relative fluorescence units with the increase in DOTMA/DNA ratios. An increment in complexation ability from

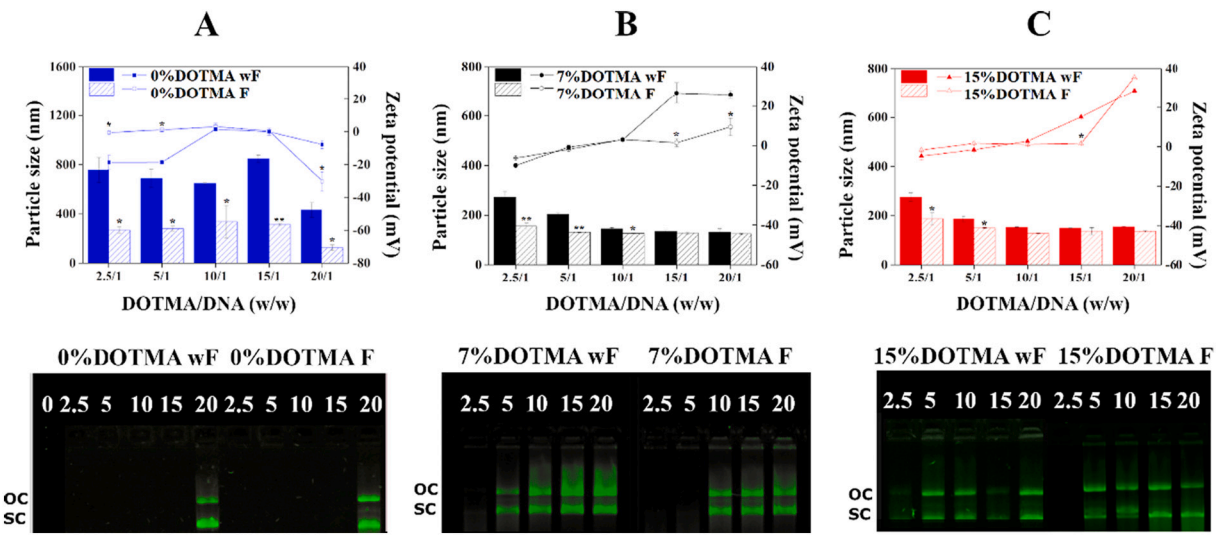

Fig. 2. Particle size, zeta potential and DNase protection ability of A) 0% DOTMA (P60/DNA mass ratios of 2.5/1, 5/1, 10/1, 15/1 and 20/1) B) 7% DOTMA (DOTMA/DNA mass ratios of 2.5/1, 5/1, 10/1, 15/1 and 20/1, equivalent to N/P ratios of 1, 2, 5, 7 and 10). and C) 15% DOTMA (DOTMA/DNA mass ratios of 2.5/1, 5/1, 10/1, 15/1 and 20/1, equivalent to N/P ratios of 1,2, 5, 7 and 10) nioplexes prepared in water. Effect of filtration through PES-membrane (F) and cationic lipid or P60/DNA mass ratio on particle size (bars) and zeta potential (lines) (upper panels), and SDS-induced release and DNA protection at different niosome formulations visualized by agarose electrophoresis (lower panels).OC: open circular; SC: supercoiled; 0: naked p*lacZ*. * depicts $p < 0.05$ and **$p < 0.01$ when compared particle size or zeta potential values obtained with nioplexes formed at the same cationic lipid/DNA mass ratio from filtered or non-filtered formulations.

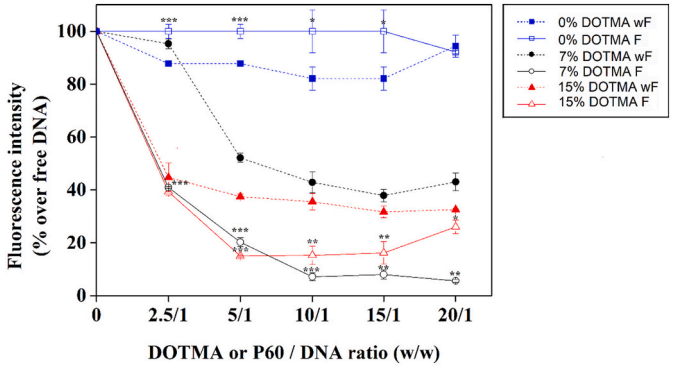

Fig. 3. DNA complexation efficiency of filtered (F; continuous line) and non-filtered (wF; dot line) dispersions of 0% DOTMA- (blue) (P60/DNA mass ratios of 2.5/1, 5/1, 10/1, 15/1 and 20/1), 7% DOTMA- (black) (DOTMA/DNA mass ratios of 2.5/1, 5/1, 10/1, 15/1 and 20/1, equivalent to N/P ratios of 1, 2, 5, 7 and 10) and 15% DOTMA (red) (DOTMA/DNA mass ratios of 2.5/1, 5/1, 10/1, 15/1 and 20/1, equivalent to N/P ratios of 1, 2, 5, 7 and 10) based niosomes formulations. *Depicts $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ when comparing fluorescence intensity obtained with nioplexes formed at the same cationic lipid/DNA mass ratio from filtered or non-filtered formulations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

cationic lipid/DNA ratio 2.5/1 was evidenced in all filtered niosomes formulations ($p < 0.01$). Nioplexes formulated with non-filtered niosomes showed a more gradual decrease of fluorescence values ($p < 0.05$ when compared with 5/1 and 20/1 cationic lipid/DNA ratios. Interestingly, filtered 7% DOTMA and 15% DOTMA formulations exhibited higher ability for making complexes with the plasmid (60–80% complexation) than the non-filtered ones (40–50% complexation) ($p < 0.001$).

3.3. *In vitro transfection of iMSCs*

Based on the data referred above, *in vitro* transfection assays of iMSCs were carried out using dispersions (F and wF) of both 7% and 15% DOTMA niosomes, at cationic lipid/DNA 5/1, 10/1, 15/1 and 20/1 *w/w* ratios. Assays were performed in two different cell lines from iMSCs (iMSCs#6 and iMSCs#13) [38].

The β-galactosidase activity and cell viability of iMSCs are summarized in Fig. 4. A preliminary screening on transfection efficiency (Fig. 4A) in iMSCs#6 (upper panel) and iMSCs#13 (lower panel) cell lines, showed significantly higher values of transfection when non-filtered dispersions were used compared with filtered formulations ($p < 0.01$). These latter formulations exhibited RLU values close to those recorded for the negative control. Focusing on wF niosome dispersions, no differences in RLU values were observed between 7% DOTMA and 15% DOTMA formulations, in none of the two cell lines studied. When comparing the transfection efficiency of wF niosomes to that achieved with the commercial golden standard Lipofectamine (LPF; positive control), no differences were noticed in the iMSCs#13 cell line (Fig. 4B lower panel). 15% DOTMA niosomes also attained comparable values in the iMSCs#6 cell line (Fig. 4B upper panel). Interestingly, the number of β-galactosidase expressing cells was always higher upon transfection with 7% DOTMA or 15% DOTMA niosomes formulations when compared with those exposed to Lipofectamine ($p < 0.001$) (Fig. 4B blue and green lines).

Regarding DOTMA/DNA mass ratios, while 7% DOTMA nioplexes were more efficient when used at the lowest ratio tested (5/1), the 15% DOTMA ones achieved the highest efficiency mean value at an intermediate ratio (10/1), although no statistical differences were observed when compared to other mass ratios studied, in none of the two iMSCs cell lines studied (Fig. 4B).

High percentages of cell survival were noted in both iMSCs#6 (~66%) and iMSCs#13(~69%) cell lines when incubated with 7% DOTMA and 15% DOTMA nioplexes (Fig. 4C), without differences between both formulations. Notably, viability of cells transfected with Lipofectamine was always significantly lower (~25%) than that observed with 7% DOTMA or 15% DOTMA nioplexes at any mass ratio tested, in both cell lines ($p < 0.05$).

In a next step, cell studies were repeated in the presence of the lysosomotropic agent sucrose to elucidate whether the transfection efficiency of nioplexes may be enhanced due to a favored endosomal escape [34]. Selected formulations (non-filtered wF 7% DOTMA and 15% DOTMA niosomes; to simplify, hereafter referred to 7% and 15%

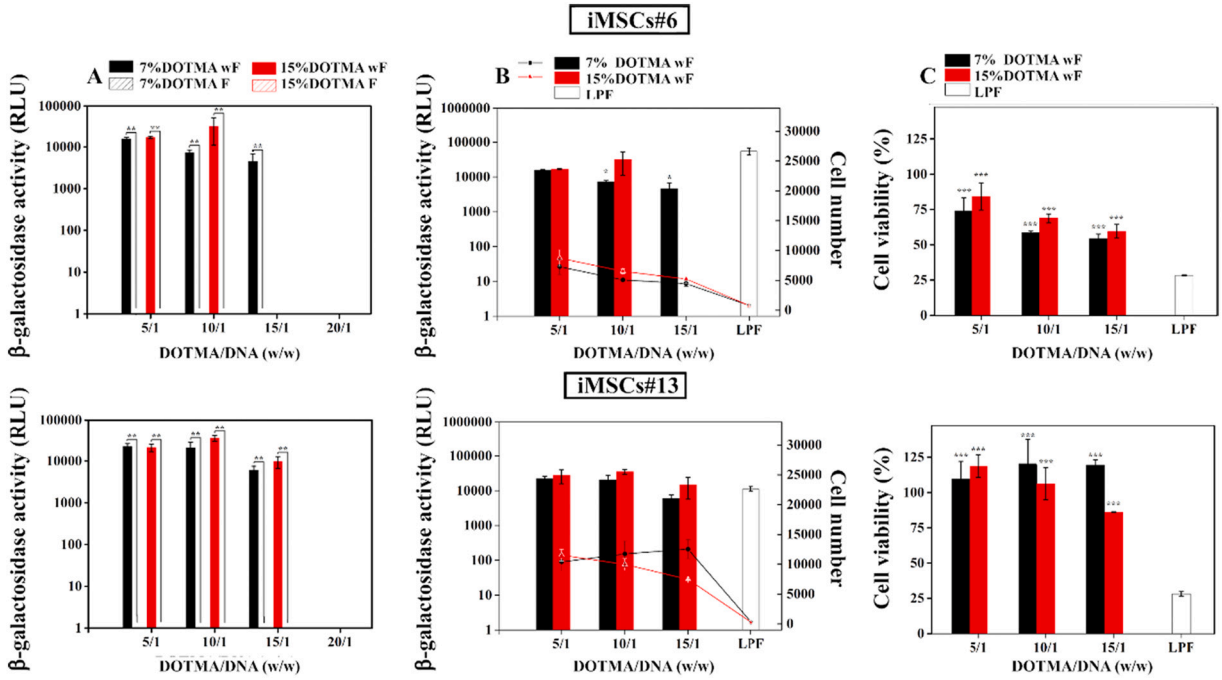

Fig. 4. *In vitro* studies performed in iMSCs#6 (upper panels) and iMSCs#13 (lower panels) cell lines. A) β-galactosidase activity preliminary screening of filtered (F, striped bars; values close to 0) and non-filtered (wF, full bars) 7% DOTMA and 15% DOTMA nioplexes (DOTMA/DNA mass ratios of 5/1, 10/1, 15/1 and 20/1, equivalent to N/P ratios of 2, 5, 7 and 10). B) β-galactosidase activity of non-filtered (wF) 7% DOTMA (black bars), 15% DOTMA (red bars) nioplexes (DOTMA/DNA mass ratios of 5/1, 10/1 and 15/1, equivalent to N/P ratios of 2, 5 and 7) or Lipofectamine (LPF; white bars). Lines represent cell numbers (7% DOTMA nioplexes: black line; 15% DOTMA nioplexes: red line; LPF: intersection of both lines). C) Cell viability in the presence of 7% DOTMA or 15% DOTMA nioplexes (DOTMA/DNA mass ratios of 5/1, 10/1 and 15/1, equivalent to N/P ratios of 2, 5 and 7) and LPF. ** depicts $p < 0.01$ upon comparison of the denoted groups (A) and * depicts $p < 0.05$ or ***$p < 0.001$ when compared the different nioplexes with LPF group (B and C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

DOTMA), were formed at cationic lipid/DNA mass ratios of 5/1 and 10/1, and tested under the same conditions and in the same lines described before, but adding sucrose 40 mM to the culture medium. Results showed an increase of β-galactosidase activity up to 2.6-fold with 7% DOTMA nioplexes at 5/1 ratio in iMSCs#6 cell line ($p < 0.05$), and up to 3.8-fold with 15% DOTMA nioplexes at the same DOTMA/DNA ratio in iMSCs#13 cell line ($p < 0.05$) (Fig. 5). To further investigate the cellular uptake of nioplexes, Cy3-labeled p*lacZ* was used for transfection. A higher internalization of Cy3-labeled p*lacZ* was observed in presence of sucrose, as evidenced by the greater intensity of Cy3 fluorescence around the nucleus (Fig. S3 in Supporting Information).

Transfection of iMSCs with nioplexes in the presence of sucrose did not show cytotoxic effects in any of the iMSCs cell lines. Interestingly, a significant increase in cell viability was noted in presence of sucrose upon transfection with nioplexes formed at DOTMA/DNA of 10/1 (7% DOTMA) and 5/1 (15% DOTMA) in the iMSCs#6 cell line ($p < 0.05$). Once again, treatment with Lipofectamine resulted in a significant decrease of cell survival ($p < 0.05$).

3.4. Performance of stored niosomes to form nioplexes

Physicochemical and transfection features of nioplexes formed from 7% DOTMA and 15% DOTMA niosomes dispersions that had been stored for 30 days was investigated. The non-filtered (wF) niosomes dispersions were kept at room temperature and protected from light in order to check whether they could maintain their capability to form nioplexes after storage. Despite 7% DOTMA and 15% DOTMA niosomes did not significantly changed the size during storage (as reported in Fig. 1), nioplexes formed from stored niosomes dispersions were larger than those formed with fresh niosomes for all DOTMA/DNA ratios tested (Table 2) ($p < 0.001$). PDI values did not change. Also zeta potential values remained stable at 30 days except for those nioplexes formed at cationic lipid/DNA of 10/1, which showed a slight increase ($p < 0.05$). However, niosome capacity to protect DNA against DNase digestion was reduced, as noted by the absence of bands at DOTMA/DNA lower ratios (Fig. 6). A reduction on DNA complexation efficiency was also evidenced for niosomes stored for 30 days, always revealing higher percents of free DNA when compared with niosomes formulated at day 1 ($p < 0.05$) (Table 3). The changes in DNA binding suggest that niosome conformation could have evolved along time.

The transfection efficiencies were also reevaluated for nioplexes prepared using niosomes dispersions kept for 30 days at RT. The transfection efficiencies of iMSCs (iMSCs#6 cell line in upper panels, and iMSCs#13 cell lines in lower panels) attained with niosomes stored for 1 day or 30 days, either in presence or absence of sucrose in the culture medium, are compared in Fig. 7. No differences in β-galactosidase activities were observed upon transfection when using fresh or long-term stored niosomes to form nioplexes with a DOTMA/DNA ratio of 10/1 in the iMSCs#6 cell line (either in presence or in absence of sucrose) (Fig. 7 upper panels). In contrast, a significant decrease in transfection efficiency was noted when using nioplexes prepared with the lowest DOTMA/DNA ratio tested (5/1), either in absence or presence of the lysosomotropic agent ($p < 0.05$). Conversely, when testing in iMSCs#13 cell line, nioplexes prepared with long-term stored niosomes showed a marked reduction in transfection values at DOTMA/DNA mass ratio of 10/1, either in absence ($p < 0.05$) or presence of sucrose ($p < 0.05$).

3.5. Performance of nioplexes from niosomes sterilized in sucrose medium

Since most biomedical applications of niosomes would require sterile formulations and steam heat sterilization is the approach of first choice, processing of niosomes in autoclave was investigated. To avoid the widely reported physical and chemical instability problems of vesicle-

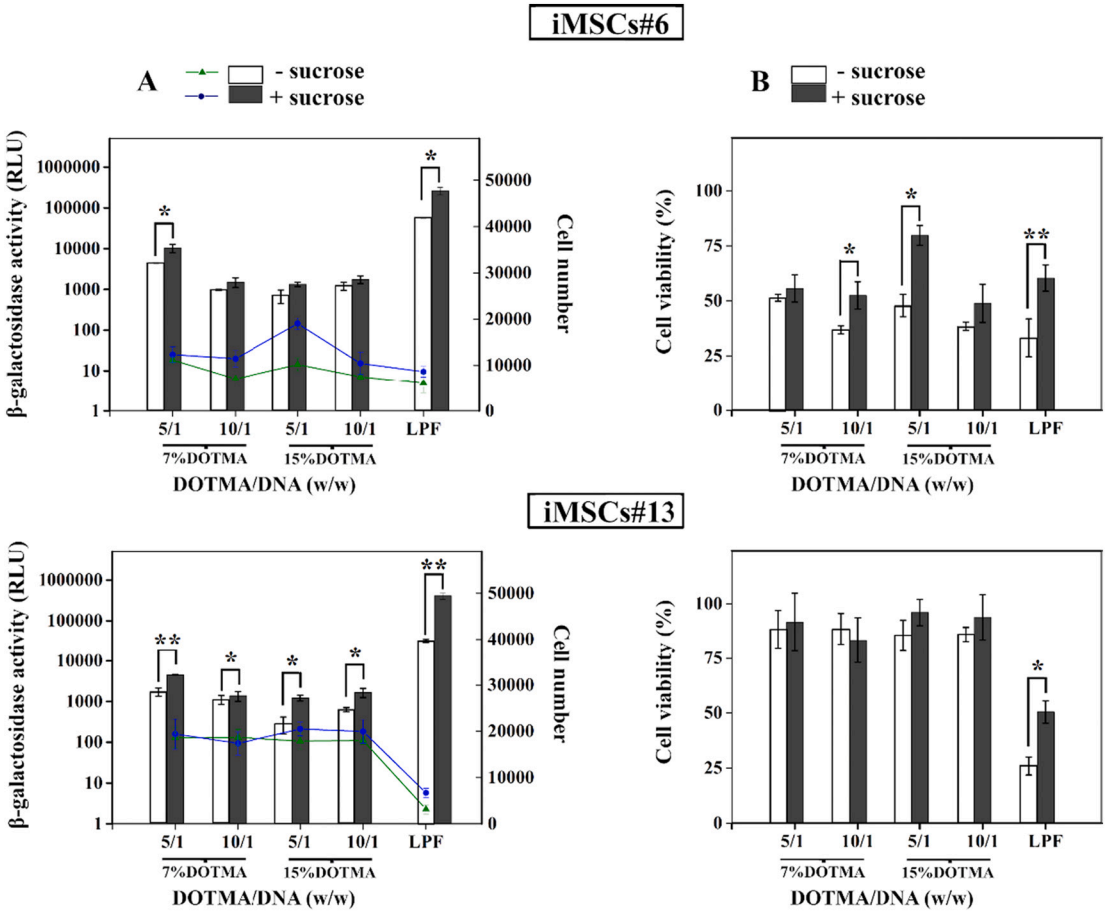

Fig. 5. *In vitro* studies performed in iMSCs#6 (upper panels) and iMSCs#13 (lower panels) cell lines in the presence of sucrose (added to the cell culture medium) A) β-galactosidase activity of 7% DOTMA and 15% DOTMA nioplexes (DOTMA/DNA mass ratios of 5/1 and 10/1, equivalent to N/P ratios of 2 and 5) obtained in absence (grey bars) and in the presence (black bars) of sucrose. LPF (white bars) was used as positive control of transfection. Lines represent cell number in absence (green lines) or presence (blue lines) of sucrose, and after being transfected with LPF (confluence of both lines) B) Cell viability upon contact with LPF (white bars) or with nioplexes (DOTMA/DNA mass ratios of 5/1 and 10/1, equivalent to N/P ratios of 2 and 5) formulated with 7% DOTMA or 15% DOTMA, in absence (grey bars) or presence (black bars) of sucrose. * depicts $p < 0.05$ and **$p < 0.01$ upon comparison of the denoted groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Particle size, PDI and zeta potential of nioplexes prepared with 7% and 15% DOTMA niosomes (DOTMA/DNA mass ratios of 2.5/1, 5/1, 10/1, 15/1 and 20/1, equivalent to N/P ratios of 1, 2, 5, 7 and 10). Niosomes were stored for 1 day or 30 days at room temperature and protected from light before nioplexes preparation. Mean values ± standard deviation ($n = 3$).

	DOTMA/DNA ratio	Day 1			Day 30		
		Size (nm)	PDI	Z-potential (mV)	Size (nm)	PDI	Z-potential (mV)
7% DOTMA	2.5/1	272 ± 23	0.28 ± 0.02	−10.0 ± 1.1	530 ± 11	0.28 ± 0.01	−5.6 ± 0.6
	5/1	210 ± 12	0.35 ± 0.15	−0.8 ± 1.1	464 ± 38	0.33 ± 0.04	1.3 ± 1.8
	10/1	145 ± 5	0.23 ± 0.04	2.9 ± 0.0	329 ± 9	0.27 ± 0.03	4.4 ± 0.0
	15/1	134 ± 7	0.26 ± 0.03	26.8 ± 5.2	345 ± 15	0.29 ± 0.04	6.5 ± 3.0
	20/1	131 ± 13	0.26 ± 0.06	25.9 ± 2.1	200 ± 9	0.29 ± 0.01	12.4 ± 0.6
15% DOTMA	2.5/1	275 ± 17	0.28 ± 0.03	−4.7 ± 2.1	544 ± 35	0.30 ± 0.06	0.9 ± 0.0
	5/1	187 ± 11	0.30 ± 0.02	−1.5 ± 0.0	394 ± 10	0.29 ± 0.00	2.0 ± 1.6
	10/1	154 ± 1	0.27 ± 0.01	2.8 ± 0.0	330 ± 9	0.27 ± 0.03	7.3 ± 0.6
	15/1	150 ± 1	0.27 ± 0.01	15.2 ± 0.5	321 ± 6	0.29 ± 0.00	12.5 ± 5.5
	20/1	155 ± 1	0.26 ± 0.01	28.8 ± 0.9	223 ± 3	0.30 ± 0.00	27.4 ± 8.3

based drug carriers, sucrose was added to the niosomes with the two-fold aim of preserving niosome stability during sterilization [28] and acting as lysosomotropic agent. Niosomes were prepared by hydration of the components in 300 mM sucrose aqueous solution (isotonic) and then steam heat sterilized at 121 °C for 20 min. The niosomes were stored at room temperature for at least 24 h before subsequent evaluation of physical properties and transfection capability. The size of sterilized 7% and 15% DOTMA niosomes was 92.7 and 100.6 nm, with PDI of 0.594 and 0.309, respectively. The *Z*-potential values were +31.8 (±7.6) and +40.7 (±7.5) mV, respectively. Size and PDI values were in the range of those recorded for non-filtered niosomes when stabilized during storage and to those of filtered niosomes. Only for sterilized 15% DOTMA niosomes the Z-potential was slightly lower ($p < 0.05$). A representative TEM image of 7 and 15% DOTMA niosomes after steam heat sterilization

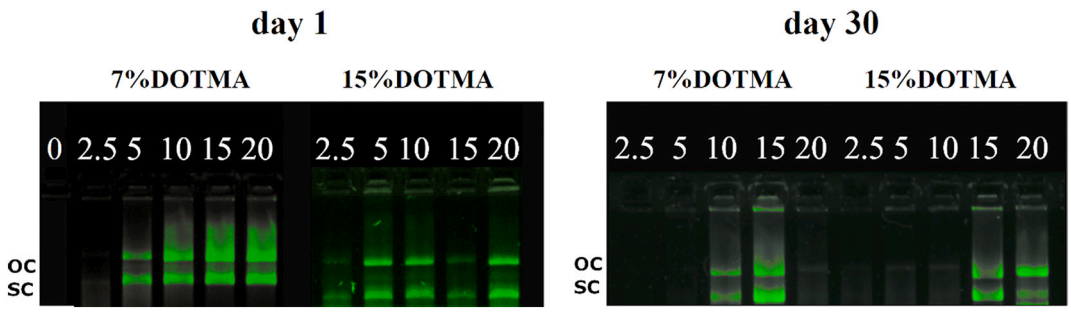

Fig. 6. SDS-induced release and DNA protection ability of different niosomes formulations visualized by agarose electrophoresis. The nioplexes (DOTMA/DNA mass ratios of 2.5/1, 5/1, 10/1, 15/1 and 20/1, equivalent to N/P ratios of 1, 2, 5, 7 and 10) were prepared with niosomes stored for 1 day (left) or for 30 days (right) at room temperature and protected from light. 0: naked p*lacZ*; OC: open circular; SC: supercoiled.

Table 3
DNA complexation efficiency of 7% DOTMA and 15% DOTMA nioplexes (DOTMA/DNA mass ratios of 2.5/1, 5/1, 10/1, 15/1 and 20/1, equivalent to N/P ratios of 1, 2, 5, 7 and 10) when prepared with niosomes stored for 1 day or for 30 days at room temperature and protected from light. Mean values ± standard deviation (n = 3).

	DOTMA/DNA ratio	Day 1	Day 30
		Fluorescence intensity (%)	Fluorescence intensity (%)
7% DOTMA	2.5/1	100.0 ± 0.8	88.3 ± 2.4
	5/1	59.4 ± 1.9	68.9 ± 1.4
	10/1	49.4 ± 4.2	49.0 ± 7.9
	15/1	44.0 ± 3.6	48.1 ± 1.6
	20/1	49.5 ± 5.1	41.9 ± 1.9
15% DOTMA	2.5/1	51.4 ± 5.9	85.6 ± 2.4
	5/1	43.5 ± 1.0	81.7 ± 1.4
	10/1	41.5 ± 3.4	69.2 ± 7.9
	15/1	37.3 ± 2.4	51.2 ± 1.6
	20/1	38.3 ± 0.4	47.3 ± 1.9

in sucrose medium is shown in Fig. S4 (Supporting Information).

When studying β-galactosidase activity of sh niosomes no differences on RLU values were observed between 7% DOTMA and 15% DOTMA formulations, in none of the two cell lines studied ($p > 0.05$) (Fig. 8). Interestingly, incorporation of sucrose to sh niosomes formulations resulted in a higher increment of β-galactosidase values ($p < 0.05$; up to 31.3-fold compared with wF niosomes) than those achieved by direct addition of sucrose into the culture medium (Fig. 5). Formulation of the positive control LPF in sucrose also resulted in an enhancement of transfection values compared with niosomes formulations ($p < 0.001$). Likewise in good concordance with previous results (Fig. 5), viability of cells transfected with Lipofectamine was always significantly lower than that reached with 7% DOTMA and 15% DOTMA nioplexes ($p < 0.001$).

4. Discussion

Transfection efficiency of nonviral vectors has been shown to strongly depend on multiple parameters including particle size, zeta potential and capacity to protect DNA from the cellular environment

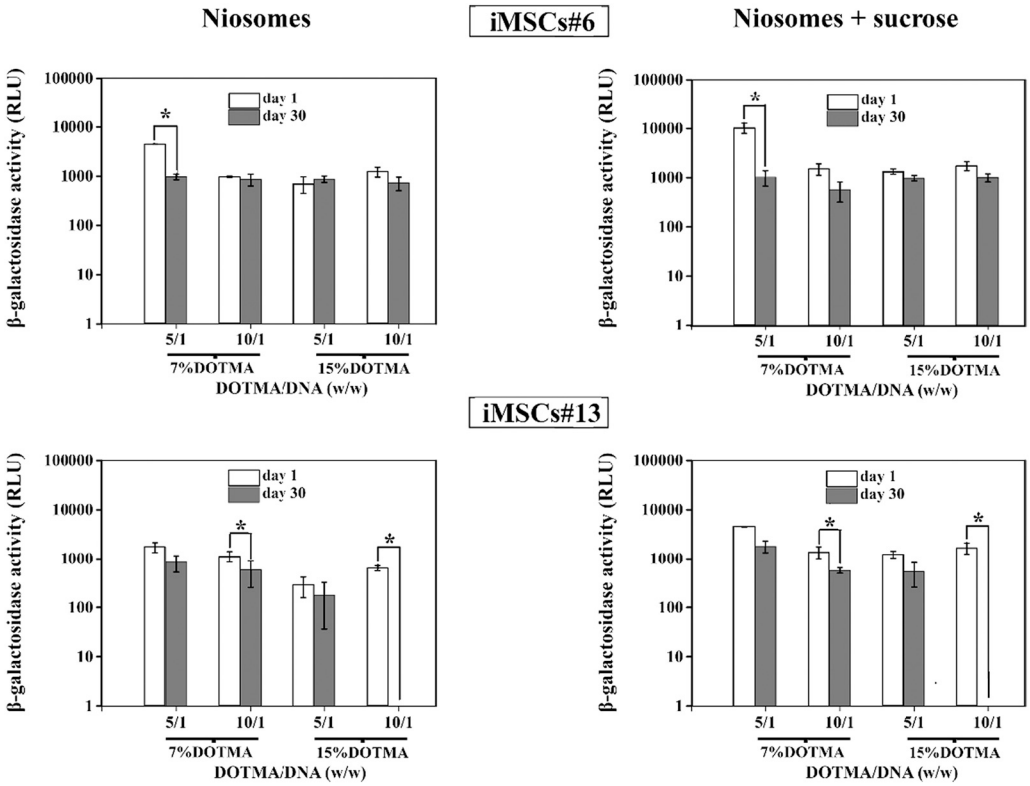

Fig. 7. *In vitro* transfection studies performed in iMSCs#6 (upper panels) and iMSCs#13 (lower panels) cell lines. β-galactosidase activities of nioplexes (DOTMA/DNA mass ratios of 5/1 and 10/1, equivalent to N/P ratios of 2 and 5) formed from 7% DOTMA and 15% DOTMA based niosomes formulations stored for 1 day (white bars) or 30 days (black bars), in absence (left panels) or in presence (right panels) of sucrose. * depicts $p < 0.05$ when compared β-galactosidase activity between day 1 and day 30.

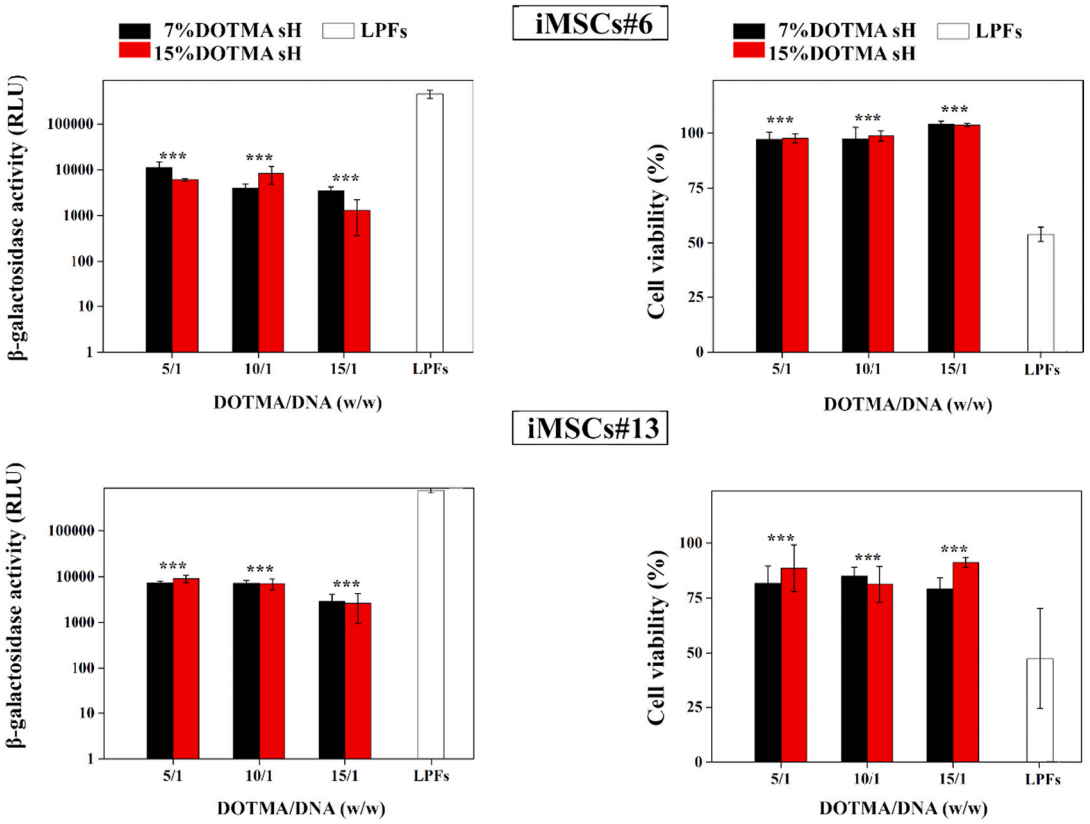

Fig. 8. *In vitro* transfection studies performed in iMSCs#6 and iMSCs#13 cell lines. β-Galactosidase activities of nioplexes (DOTMA/DNA mass ratios of 5/1, 10/1 and 15/1, equivalent to N/P ratios of 2, 5 and 7) formed from 7% DOTMA and 15% DOTMA based niosomes formulations prepared in sucrose 300 mM and heat sterilized (sh). Lipoplexes formulated with the positive control Lipofectamine (LPF) were prepared under the same conditions as nioplexes. *** depicts $p < 0.001$ when compared the different cationic lipid/DNA mass ratios of both nioplexes with LPF group.

[10,15]. Various niosomes formulations based on the combination of the cationic lipid DOTMA with different non-ionic surfactants and helper lipids have demonstrated successful transfection of retina pigment epithelial cells [24,35] or primary neuronal cells [20]. However, up to date very little is known about the use of niosomes as delivery systems to genetically modify MSCs, which is a target cell population in different regenerative medicine approaches [3]. Based on this, the main goal of the present study was to design effective niosomes-based DNA delivery platforms for targeting immortalized human MSCs cell lines obtained from aged donors. To this end, a set of niosomes dispersions based on polysorbate 60 as non-ionic surfactant, cholesterol as helper lipid, and DOTMA as cationic lipid were prepared. To analyze the effect of cationic lipid content on physicochemical and biological properties of nioplexes, the niosomes dispersions were prepared tuning the content in DOTMA (0, 7 and 15 mol%). Likewise, the effect of niosomes filtration on the physicochemical properties and the gene transfer efficiency of the resulting nioplexes was evaluated.

The data from the present study first revealed an influence of DOTMA concentration on physicochemical properties and complexation ability. Niosomes formulated without DOTMA exhibited the highest sizes and the lowest zeta potential values, failing to condense and protect p*lacZ* against DNAase degradation. These observations evidenced the role of the cationic lipid in complexing and condensing the DNA molecule [15]. The ability of niosomes formulated without DOTMA to protect p*lacZ* against DNase degradation at the highest P60/DNA ratio tested (20/1) may be due to hydrophobic interactions between the non-ionic surfactant and the DNA molecule [13] playing a protective role by the creation of a steric barrier [36] that reduces the interaction with nucleases [37].

In good agreement with previous works, 7% and 15% DOTMA nioplexes showed discrete oscillations in particle size upon the addition of p*lacZ* at the different cationic lipid/DNA (2.5/1, 5/1, 10/1, 15/1 and 20/1) ratios or N/P ratios (1, 2, 5, 7 and 10) studied [23]. These observations may be due to the balance between larger space demanded by the cationic lipid and the higher DNA condensation when increasing mass ratio [38] as confirmed in DNA complexation assays. Therefore, both 7% and 15% DOTMA niosomes showed ability to complex p*lacZ*, which was more evident for nioplexes formed from niosomes dispersions containing the highest concentration of cationic lipid (15%). It is known that positive zeta potential values promote electrostatic interactions with negatively charged DNA molecules [39]. Niosomes formulated with either 7% or 15% DOTMA concentration showed zeta potential values around +40 mV, being suitable for transfection assays according to previous works [23,25]. Likewise, surface charge increased with the raise of cationic lipid/DNA ratio [40].

Since filtration is an extensively used procedure for nanocarrier purification, the effect of niosomes dispersions filtering on the physicochemical properties of resulting nioplexes was investigated. A modification of niosomes properties as nonviral gene delivery systems was noticed upon initial filtration of niosomes dispersions, resulting in a reduction of both the size and PDI of resulting nioplexes. Moreover, while filtration of 7% DOTMA dispersions did not show evident changes in zeta potential values at lower DOTMA/DNA ratios, a decrease in the electro-positivity was evidenced at the highest DOTMA/DNA ratios (15/1 and 20/1). The effect of filtration on surface charge was not so evident for nioplexes formulated with higher percent of cationic lipid (15%), showing still higher complexation ability than those nioplexes formulated with non-filtered niosomes dispersions. The discrepancies observed here in physicochemical features of niosomes may help to support previous observations indicating that membrane filtration is not

a suitable procedure for niosomes purification [41].

Agarose gel retardation assays revealed that both 7% and 15% DOTMA niosomes formulations were able to condense, release and protect DNA from enzymatic digestion, as confirmed by the presence of SC bands [22]. Interestingly, formulations with lower cationic lipid content (7%) enhanced protection of complexed DNA at lower DOTMA/DNA ratios in non-filtered dispersions.

In order to test the performance of developed formulations for gene delivery purposes, transfection assays were carried out in two different immortalized MSCs cell lines obtained from bone marrow samples of aged donors. These cell lines have previously shown to prevent senescence and proliferation concerns associated to primary cultures while maintaining their multipotency; therefore, iMSCs are considered as potential means for regenerative medicine research [26]. Strikingly, despite their lower size and higher p*lacZ* complexation ability, nioplexes formed from filtered niosomes dispersions (7 and 15%) failed to transfer p*lacZ* in both iMSCs lines tested. Conversely, nioplexes based on non-filtered 7 and 15% DOTMA formulations led to an effective transfection of both iMSCs cell lines with values similar to that achieved with the commercial reagent Lipofectamine. These observations are in agreement with previous studies noticing a higher transfection efficiency with larger liposomes when compared with smaller and monodisperse ones obtained by filtration [42]. Hence, and similarly to that observed with lipoplexes, larger nioplexes may facilitate cell surface contact by sedimentation resulting in a higher gene transfer [42,43]. Moreover, these results highlight the need of an adequate balance between complexes stability and their ability to deliver the DNA molecules inside the cell to achieve an effective transfection [30]. Of note, while 7% DOTMA nioplexes may be more efficient when used at the lowest DOTMA/DNA ratio (5/1), 15% DOTMA nioplexes achieved the highest efficiency at an intermediate ratio (10/1). Along these lines, the electroneutral to slightly positive zeta potential values detected for these nioplexes were sufficient for condensing and protecting the DNA molecule, which in turn favored its uptake into the cell and prevented cell membrane disruption [44]. Finally, viability of cells transfected with 7% or 15% DOTMA nioplexes was always higher than that observed for the gold standard Lipofectamine, at any mass ratio tested in both cell lines. These results are in agreement with previous reports showing a reduction of MSCs viability in the presence of this commercial reagent [45].

It is known that endosomal escape constitutes one of the least understood steps precluding the effectiveness of nonviral gene delivery [46]. Thereupon, improving endosomal escape might significantly improve transfection efficiency. Based on this, cell studies were repeated in the presence of the lysosomotropic agent sucrose, due to its ability to cause cytoplasmatic vesicular swelling within endosomes and lysosomes [34]. Results showed an increase of β-galactosidase activity in both cell lines at all DOTMA/DNA ratio tested, mostly at the lowest DOTMA/DNA ratio tested. Likewise, in good agreement with previous studies performed in fibroblasts [34], transfection of iMSCs with nioplexes in the presence of sucrose at 40 mM concentration did not show cytotoxic effects in any of the iMSCs cell lines. Thus, sucrose could be used as an excipient in the formulation of niosomes based gene delivery systems. Consequently, performance of niosomes formulated in sucrose-added medium was explored as a proof of concept in the last part of the work. Preparation of niosomes by hydration of the components in isotonic sucrose aqueous solution and subsequent sterilization by steam heat resulted in an enhancement of transfection efficiency compared with niosomes prepared in Opti-MEM medium (as explained below).

Storage of niosomes at 25 °C during several weeks may affect main physicochemical parameters influencing gene delivery process [35]. In agreement with previous works [35], nioplexes formed from long-term stored niosomes showed an increase in size. The surface charge resulted only modified in those nioplexes formed at cationic lipid/DNA ratio of 10/1. These results contrast with previous observations using niosomes based on DOTMA, squalene and polysorbate 20 in which a more drastic reduction of zeta potential values was observed [35]. In this sense, the different composition of helper lipid and non-ionic surfactant from these niosomes as well as the size of plasmid used, may explain these discrepancies. In consonance with previous reports, niosomes capacity to protect DNA against DNase digestion was reduced at lower DOTMA/DNA ratios [35]. Similarly, a reduction of DNA complexation efficiency was also evidenced upon storage of niosomes for 30 days, revealing always higher percentages of free DNA when compared with niosomes formulated at day 1. When studying transfection efficiency, no differences in β-galactosidase activity were observed with nioplexes formed from long-term stored niosomes from DOTMA/DNA ratio of 10/1 in one of the iMSCs cell lines tested (iMSCs#6). Conversely, in the other iMSCs cell line (iMSCs#13) long-term stored niosomes showed a marked reduction of transfection values at DOTMA/DNA mass ratio of 10/1 from nioplexes. It is known that niosome composition has a direct influence on its transfection capacity, but other factors such as changes in niosome conformation (oriented components, aggregation) as well as the cell type and the specific cell internalization pathway also determine the transfection efficiency [47]. Hence, it is important to highlight that iMSCs cell lines used in the present study were obtained from bone marrow samples of patients undergoing hip osteoarthritis or fracture, so these differences may be attributed to the variability inextricably linked to biological samples [26]. In this sense, future studies involving a higher number of iMSCs cell lines may help to clarify these observations.

Finally, and regarding niosomes stability and scaling-up toward clinical use, a sterilization protocol using sucrose as stabilizing agent was implemented. The use of sucrose at concentration close to that required for being isotonic to blood serum (300 mM) has been demonstrated suitable to prevent aggregation and membrane permeabilization of liposomes during autoclaving [28]. No report was found for niosomes. Niosomes formulated in 300 mM sucrose medium withstood the steam heat sterilization process and showed adequate physical features. Since the nioplexes were tested under 1/10 dilution, the formulation could still provide 30 mM sucrose to the medium and then took benefit from its lysosomotropic features. Indeed, to the best of our knowledge this is the first time that the transfection capability of steam heat-sterilized niosomes was demonstrated.

5. Conclusions

Niosomes formulated with DOTMA as cationic lipid, cholesterol as helper lipid and polysorbate 60 as non-ionic surfactant are revealed as suitable nonviral gene delivery platforms for the effective gene transfer of immortalized MSCs. Specifically, nioplexes formed from non-filtered 15% DOTMA niosomes dispersions led to an effective DNA protection and complexation, attaining similar transfection values to those achieved with the gold standard reagent Lipofectamine with a marked lower cytotoxicity in the two iMSCs cell lines tested. Addition of sucrose into the culture medium during transfection process or to the formulati+on itself considerably improved the gene transfer performance from these nioplexes. Both filtration during niosomes formulation and storage of niosomes formulations for 30 days at RT modified physicochemical properties from resulting nioplexes leading to a decrease of their gene transfer efficiency. Further experiments will focus in further evaluation of the filtration and sterilization processes that may help niosomes to maintain the transfection capability during long term storage.

CRediT authorship contribution statement

Natalia Carballo-Pedrares: Methodology, formal analysis, investigation.

Axel Kattar: Methodology, investigation.

Angel Concheiro: Conceptualization, Resources, Writing - Original Draft, Writing - Review & Editing, Supervision, Funding acquisition.

Carmen Alvarez-Lorenzo: Conceptualization, Methodology, Resources, Formal analysis, Writing - Original Draft, Writing - Review &

Editing, Supervision, Funding acquisition.

Ana Rey-Rico: Conceptualization, Methodology, Validation, Resources, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.msec.2021.112307.

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