How Multi-Step *versus* One-Step Preparation Method Affects the Physicochemical Properties and Transfection Efficiency of DNA/DODAB:MO Lipoplexes

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Abstract: The consequences for the transfection efficiencies of different lipoplexes preparation methods, largely remain to be explored, but the knowledge of how different experimental approaches can affect the physicochemical properties and transfection efficiency is essential for a proper tailoring of transfection complexes to particular applications. Therefore, the influence of the number of mixing steps (one-step addition *versus* multi-step addition of liposomes to plasmid DNA (pDNA)) and lipoplex incubation temperature on the final physicochemical properties and transfection efficiency of pDNA/ Dioctadecyldimethylammonium Bromide (DODAB):1-monooleoyl-rac-glycerol (MO) complexes was studied in three distinct DODAB:MO molar ratios: 4:1, 2:1 and 1:1. Dynamic Light Scattering (DLS), Zeta (ζ) Potential, Ethidium Bromide (EtBr) exclusion assays were used to assess the formation, structure and destabilization of the lipoplexes, whereas *in vitro* transfection assays with pSV-β-gal plasmid DNA were performed to evaluate their transfection efficiency on the 293T mammalian cell line.

Results indicate that the morphology of pDNA/DODAB:MO complexes is dependent on the lipoplex preparation method, resulting in particles of distinct size, surface charge and membrane fluidity. These variations are visible during the complexation dynamics of pDNA and continue throughout the profile of pDNA release from pDNA/DODAB:MO lipoplexes upon incubation with Heparin (HEP), as well as in the *in vitro* transfection assays.

The stepwise addition of DODAB:MO vesicles to pDNA decreases the transfection efficiency of the lipoplexes, while the effect of the lipoplex preparation methods is dependent on the MO content.

Keywords: Monoolein-Based Lipoplexes, Lipoplex Preparation Method, Transfection.

1. INTRODUCTION

Cationic lipid complexes (lipoplexes) have several advantages as nucleic acid delivery systems over viral vectors, which are difficult to prepare, can be mutagenic and have low loading capacity [1-7]. There are several possible methods to prepare lipoplexes, and the implications of the different preparation procedures should be evaluated to reach better tailored gene nanocarriers.

The final transfection efficiency of the lipoplexes has been reported in the literature as dependent of the preparation strategies used in numerous aspects that include: (i) the type and proportions of main lipid and helper lipids chosen (e.g. changing lipoplex composition affects physicochemical properties, such as, size, surface charge and fluidity [8-25]); (ii) the type of aggregates formed (e.g. in comparison to multilamellar vesicles, lowest molecular weighted small unilamellar vesicles require more aggregates to complex the same amount of DNA) [26]; (iii) the chosen addition procedure (e. g. lipoplexes prepared by a titration procedure where cationic liposomes were added stepwise to DNA led to smaller and more fluid lipoplexes than the lipoplexes obtained by the instant mixing procedure [27, 28]); (iv) the order by which DNA and liposomes are mixed (e.g. lipoplexes resulting from the addition of DNA to cationic vesicles had bigger sizes and higher negative charge densities, thus presenting lower cellular binding and transfection efficiencies than lipoplexes obtained from the addition of cationic vesicles to DNA [29]); (v) the ionic strength (e.g. altering ionic strength demonstrated effects in the aggregation, complexation and transfection efficiency [30, 31]).

Given the reported impact that the optimization of experimental procedures (such as the vesicle preparation method or the stepwise addition of cationic liposomes to DNA) may have on the lipoplex

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transfection efficiency we have decided to study the influence of the number of mixing steps (one-step addition *versus* multi-step addition of liposome vesicles to pDNA) on the final physicochemical properties and transfection efficiency of a recently described lipoplex system (pDNA/DODAB:MO complexes) in three distinct DODAB:MO molar ratios: 4:1, 2:1 and 1:1. The physicochemical properties of the lipoplexes (size, charge and structure) prepared by the different methods were studied by Dynamic and Electrophoretic Light Scattering (DLS and ELS) and Cryo-Transmission Electron Microscopy (Cryo-TEM). Ethidium Bromide (EtBr) exclusion fluorescence assays and electrophoretic mobility were used to study the complexation dynamics of pDNA after addition of DODAB:MO vesicles, and also the extent of pDNA release from pDNA/DODAB:MO systems upon incubation with Heparin (HEP) or Fetal Bovine Serum (FBS). Finally, *in vitro* transfection assays were performed in 293T human cell line using the pSV-β-gal plasmid, in order to investigate the correlation between these different lipoplex preparation methods and the final lipofection efficiency.

2. MATERIALS AND METHODS

2.1. Materials

MO, Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin/amphotericin B (10 000 units/10 mg/ 25 µg per mL) solution, agarose gel, and heparin from the porcine intestinal mucosa were purchased from Sigma-Aldrich. DODAB was purchased from Tokyo Kasei (Japan). Opti-MEM I Reduced Serum Medium was purchased from Gibco (UK). The intercalating probe EtBr was purchased from Molecular Probes (UK). FBS was purchased from Invitrogen. The *Wizard Plus Midipreps DNA Purification System* and *β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer* were purchased from Promega (USA). GelRed™ Nucleic Acid Gel Stain was purchased from Biotium (CA).

2.2. Plasmid DNA Preparation

pSV-β-gal plasmid DNA was amplified with *Escherichia coli DHB4* competent cells. The pDNA was isolated and purified with the *Wizard® Plus Midipreps DNA Purification System Extraction Kit*. After purification, pDNA was ressuspended in ultra-pure water at a nucleotidic phosphate group concentration of 0.5 µg µL^{-1} determined by absorption at 260 nm [32] with NanoDrop ND1000 Spectrophotometer. pDNA purity was also verified by determining the ratio of absorbance at 260/280 nm with the same equipment.

2.3. Liposomes Preparation

Defined volumes from the stock solutions of DODAB and MO in ethanol (20 mM) were injected under vigorous vortexing to an aqueous buffer solution of Tris-HCl (30 mM) at 70 ºC, so that the final lipid concentration ([DODAB + MO]) was 1 mM and the different DODAB:MO molar ratios (4:1, 2:1, and 1:1) were obtained.

2.4. Lipoplexes Preparation

pDNA/DODAB:MO (4:1, 2:1 and 1:1) lipoplexes were prepared by incubating pSV-β-gal plasmid DNA and DODAB:MO (4:1, 2.1 and 1:1) vesicles in Opti-MEM I Reduced Serum medium at CRs (+/-) 0.0, 0.25, 0.75, 1.0, 1.5, 2.0 and 4.0, under 5-30 min stirring.

The CR (+/-) is an indicator of balance between positive charges (given by the concentration of ammonium groups present in DODAB) and negative charges (given by the concentration of nucleotidic phosphate groups in pDNA, which corresponds to nucleotide concentration) [12]:

$$CR(+/-) = \frac{[+]}{[-]} = \frac{[Ammonium\ groups\ from\ DODAB]}{[Phosphate\ groups\ from\ DNA]} \quad (1)$$

The different CRs (+/-) prepared resulted from the addition of adequate volumes of cationic vesicles to the pDNA solution for all the molar ratios studied. The incubation procedure previously described was performed in two distinct manners: one-step addition of cationic vesicles to pDNA and multi-step addition of cationic vesicles to pDNA.

2.5. Dynamic Light Scattering (DLS) Assays

pDNA/DODAB:MO (4:1, 2:1, and 1:1) lipoplexes at CRs (+/-) 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 and 4.0 were prepared by different methods and placed in disposable polystyrene cuvettes for DLS measurements in a Malvern ZetaSizer Nano ZS particle analyzer. Malvern Dispersion Technology Software (DTS) was used with multiple narrow mode (high resolution) data processing, and mean diameter (nm) average and error values were considered.

2.6. Electrophoretic Light Scattering (ELS) Assays

pDNA/DODAB:MO (4:1, 2:1, and 1.1) lipoplexes at CRs (+/-) 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 and 4.0 were

prepared by different methods and placed in universal dip cells for ζ-potential measurements by ELS in a Malvern ZetaSizer Nano ZS particle analyzer. Malvern Dispersion Technology Software (DTS) was used with monomodal mode data processing, and ζ-potential (mV) average and error values were considered.

2.7 Ethidium Bromide Exclusion Fluorescence Assays

Ethidium bromide exclusion assay is a commonly used technique to access the lipoplex formation by conjugation of polycationic lipossomes and anionic nucleic acids. The principle behind this assay is that ethidium bromide (EtBr) is a fluorescent probe that, when excited at 510 nm in an aqueous solution, gives a weak fluorescence emission; when EtBr intercalates into the DNA helix, the fluorescence emission increases. However, when the polycationic lipossomes are conjugated with DNA, EtBr molecules are displaced from the DNA strand to the water media, and a reduction in the fluorescence emission is seen [13].

In this assay, lipoplexes were prepared by adding the desired volume of cationic liposome suspensions (1 mM) to 2.5 mL of pSV-*β*-gal plasmid DNA solution (20 μg mL^{-1}) diluted in Opti-MEM I medium. EtBr was added to the lipoplexes at a concentration (7.0×10^{-6} M) six times lower than that of the pDNA, to assure that the decrease in the probe fluorescence is directly proportional to the amount of cationic lipid at a given nucleotide base concentration [33]. Suitable control experiments were performed (solution of DNA + EtBr, and solution of cationic vesicles + EtBr).

The steady-state fluorescence measurements were performed in a Horiba Jobin Yvon Spex Fluorolog-3 spectrofluorimeter for each CR (+/-) analyzed, after a 5 min agitation period with a magnetic stirrer. The fluorescence intensities were determined at λ_{exc} = 510 nm, because this wavelength is known to be an isosbestic point for EtBr/DNA solutions [34]. All emission spectra were integrated, and the ratio of the areas was determined, after subtraction of the solvent background. Each fluorescence emission spectrum was fitted to a sum of two log-normal functions [35], corresponding to different environment states (DNA and H_2O). Assuming that the quantum yield of EtBr in the lipoplex remains constant for all the CRs (+/-), the percentage of complexed pDNA (α) at $CR(+/-)x$ can be determined from the spectral decomposition previously made [35]:

$$\alpha_{at\ C.R.(+/-)x} = \left(\frac{\int I^{DNA}_{F\ at\ C.R.(+/-)0.0} - \int I^{25°C}_{F\ at\ C.R.(+/-)x}}{\int I^{DNA}_{F\ at\ C.R.(+/-)0.0}} \right) \times 100 \quad (2)$$

Lipoplex stability was also analyzed after addition of endogenous glycosaminoglycan (GAG) negative polyelectrolytes (heparin) to the pre-formed lipid/pDNA complexes at CR (+/-) 4.0 and 37 ºC. EtBr emission spectra were recorded for each heparin addition until CR (+/-) reverted to 0.125 ([Heparin] = 278 μM).

2.9. Cell Transfection Assays

The 293T human cell line was cultured in DMEM medium supplemented with 10 % (v/v) heat-inactivated FBS and penicillin/streptomycin/amphotericin B (10 000 units/10 mg/ 25 μg per mL) solution. Cells were subcultured every two days in order to maintain sub-confluency.

For cell transfection assays, 293T cells were seeded into 24-wells plates 12-16 h prior to the addition of the different lipoplex solutions. Individual 100 μL lipoplex solutions were prepared by one-step/multi-step addition of adequate volumes of 1 mM liposome solutions (DODAB:MO (4:1, 2:1 or 1:1) to Opti-MEM I medium containing 0.5 μg of pDNA. The solutions were left to stabilize for 30 min under constant stirring and finally added to each well after culture medium replacement. Transfection of pDNA was also carried out with the Lipofectamine™ LTX Reagent according to manufacturer's instructions.

β-galactosidase activity was evaluated 48 h later with the β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer, according to standard protocol. Data from three independent experiments were log transformed to obtain a normal distribution and one-way analysis of variance statistical test (ANOVA) was applied to identify differences across the various groups, followed by a Turkey's post test. $P < 0.05$ was considered to be statistically significant.

3. RESULTS & DISCUSSION

In excess of water and above the main phase transition temperature (T_m), the synthetic cationic surfactant DODAB tends to form lamellar aggregates (T_m = 45 ºC [36]), whereas the amphiphilic neutral lipid of natural origin MO (T_m = 35 ºC [37]) forms inverted aggregates with negative curvature [38]. The conjugation of these two different surfactants results in a structurally rich polymorphic system, highly

dependent on physicochemical parameters such as temperature and DODAB:MO molar ratios, as recently reported [39]. Moreover, it was found that the addition of cationic liposomes to pDNA leads to a dual-lipoplex phase diagram, with prevalence of lamellar structures at DODAB molar fractions above 0.5 and inverted bicontinuous cubic mesophases at DODAB molar fractions below 0.5 [40]. The fluidizing effect of MO improves the complexation efficiency of pDNA, accelerating lipoplex formation and enhancing the transfection efficiency in 293T cell line [40-42]. Additionally, it has also been shown that MO promotes the existence of inverted bicontinuous cubic mesophases, resulting in non-lamellar aggregates that are more resistant to destabilization by proteoglycans [40].

All these evidences suggest that pDNA/DODAB:MO lipoplexes are strongly affected by the same physicochemical parameters that influence DODAB:MO lipid phase equilibrium, with possible consequences for the lipofection success of the system. Therefore, we have decided to study the influence of lipoplex preparation method (one-step addition, OS, *versus* multi-step addition, MS, of liposomes to pDNA) on the final physicochemical properties and transfection efficiency of pDNA/DODAB:MO systems at DODAB:MO molar ratios: 4:1, 2:1 and 1:1.

3.2. Influence of the Lipoplex Preparation Method on pDNA Complexation, Lipoplex Size and Lipoplex Surface Charge

EtBr is a fluorescent probe that is excited at 510 nm and emits at: 610 nm, when it is intercalated in DNA (DNA band); or at 630 nm with low Φ_F, when DNA suffers condensation releasing the probe to the aqueous media (H_2O band). By fitting each EtBr fluorescence emission spectrum into a sum of 2 log-normal functions corresponding to the different environment states (DNA and H_2O), it is possible to follow the pDNA condensation by the cationic vesicles (Supplementary Material 1). EtBr exclusion assays

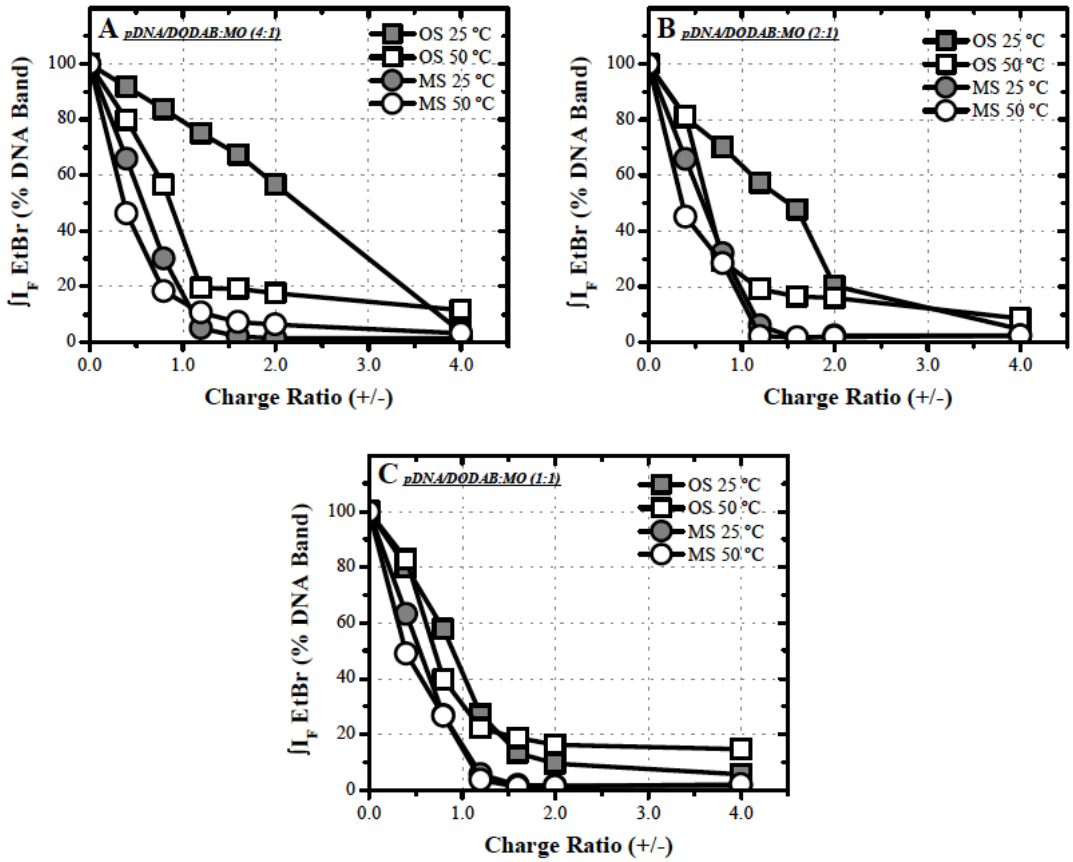

Figure 1: pDNA complexation efficiency upon addition of increasing amounts of different DODAB:MO formulations (4:1, 2:1 and 1:1). Variation of the decomposed fluorescence intensities of EtBr in pDNA band for different pDNA/DODAB:MO systems prepared by different lipoplex preparation methods (OS 25 ºC-gray square, OS 50 ºC-white square, MS 25 ºC – gray circle, MS 50 ºC – white circle) at different CRs (+/-). **A** – pDNA complexation for pDNA/DODAB:MO (4:1); **B** – pDNA complexation for pDNA/DODAB:MO (2:1); and **C** – pDNA complexation for pDNA/DODAB:MO (1:1).

were used to monitor pDNA complexation by DODAB:MO liposomes. Figure **1** shows the pDNA complexation efficiency upon addition of DODAB:MO (4:1; 2:1 and 1:1), at different lipid phases (25 ºC – L_β and 50 ºC – L_α) and using a different number of mixing steps (OS *versus* MS).

The effect of the temperature or the number of mixing steps is more pronounced for lipoplexes with higher DODAB content (4:1 > 2:1 > 1:1). The decrease in EtBr fluorescence emission indicates that lipoplexes are formed at lower CRs for liposomes richer in DODAB (DODAB:MO (4:1 and 2:1) (Figures **1A** and **1B**).

Lipoplex formation is a highly dynamic event involving two main steps: first, the electrostatic binding of DNA to the liposome surface (DNA coating) and then, the fusion and rearrangement of liposomes to produce the final aggregates [43]. Since the dominant factor of lipoplex formation is the DNA coating step [43], and different DNA complexation dynamics were found on Figure **1**, it can be inferred that the OS and MS procedures will mainly affect the DNA coating step when lipoplexes are being formed. In MS procedure, the stepwise addition of cationic aggregates to pDNA makes the condensation process more cooperative and sequenced, facilitating lipoplexes formation, that is practically not affected by the temperature. This is in agreement with other reported MS procedures [28], where the multiple interactions of one component with the other fastened the nucleation process, thus turning the lipoplex formation into a highly cooperative step. Contrastingly, when the OS procedure is used, the condensation of pDNA is slower when compared to MS procedure at the same temperature, because the complexation is neither cooperatively facilitated, nor sequenced from a previously metastable intermediate structure. Furthermore, the OS mixing procedure is temperature dependent. Therefore, as observed through EtBr fluorescence emission decrease on Figure **1A** and **1B**, the formation of lipoplexes by the OS procedure occurs at lower CRs for higher temperatures (higher than T_m of the liposomes). This can also be inferred by the smaller isoelectric points obtained at higher temperatures (isoelectric point occurs at CR (+/-) 1.5 and CR (+/-) 1.0, for 25 and 50 °C, respectively) particularly evident in the case of lipoplex prepared by one-step preparation method (OS).

Figure **1C** shows that the differences between OS and MS procedures, as well as temperature, are attenuated, and the lipoplex formation follows a similar dynamics for all the conditions, being practically complete at CR (+/-) ≥ 2. This result can be explained by the presence of a high content of the *helper* lipid MO, which has a fluidizing effect on DODAB:MO liposome bilayers, promoting the lipoplex assembly and increasing the pDNA cooperative collapse.

The effect of the different lipoplex preparation methods on pDNA condensation efficiency was further evaluated by DLS. Results are shown in Figure **2**.

Upon liposomal addition to pDNA and, consequently, after the DNA coating of the liposomes, two processes occur for the formation of cationic lipoplexes: the DNA-induced membrane fusion and the liposomes-induced cooperative DNA collapse [43]. Both processes can be observed on Figure **2**: the peaks correspond to the big structures obtained during lipoplex assembly driven by DNA-induced membrane fusion, and the decrease in size observed after this point corresponds to the latter DNA collapse process. This is a key event, and consists on the entrapment of DNA molecules between the lipid lamellas, in clusters of condensed structures. These condensed structures correspond to the smaller sizes reached during the lipoplex assembly, and are seen in Figure **2** for higher CRs (+/-).

When the OS procedure is used to prepare pDNA/DODAB:MO lipoplexes, at higher CRs (+/-), the rapid mixing caused by the excess of cationic vesicles leads to instant vesicle-vesicle adhesion and rupture, originating highly organized lipoplexes with big particle sizes [45]. When the temperature is increased from 25 to 50 ºC, the fluidity of the liposomes is enhanced, which dictates a higher compaction level of pDNA, forming lipoplexes which are up to ¼ smaller than the lipoplexes prepared at 25 ºC. This result was also observed for cryo-TEM imaging studies [46].

The MS addition of DODAB:MO cationic vesicles to pDNA produces opposite effects on the final size of the lipoplexes: at 25 ºC the size of the lipoplexes decreases and at 50 ºC the size of the lipoplexes increases, regardless of the MO content. The MS titration method implies that the component that is being titrated (pDNA) is in excess until the last stages of the DODAB:MO liposomes addition (assuming formation of a final complex that is near surface charge neutrality) therefore, pDNA coated vesicles with low diameters are formed, that persist metastably at a lower CR (+/-) range (Figure **2**) [45]. Nevertheless,

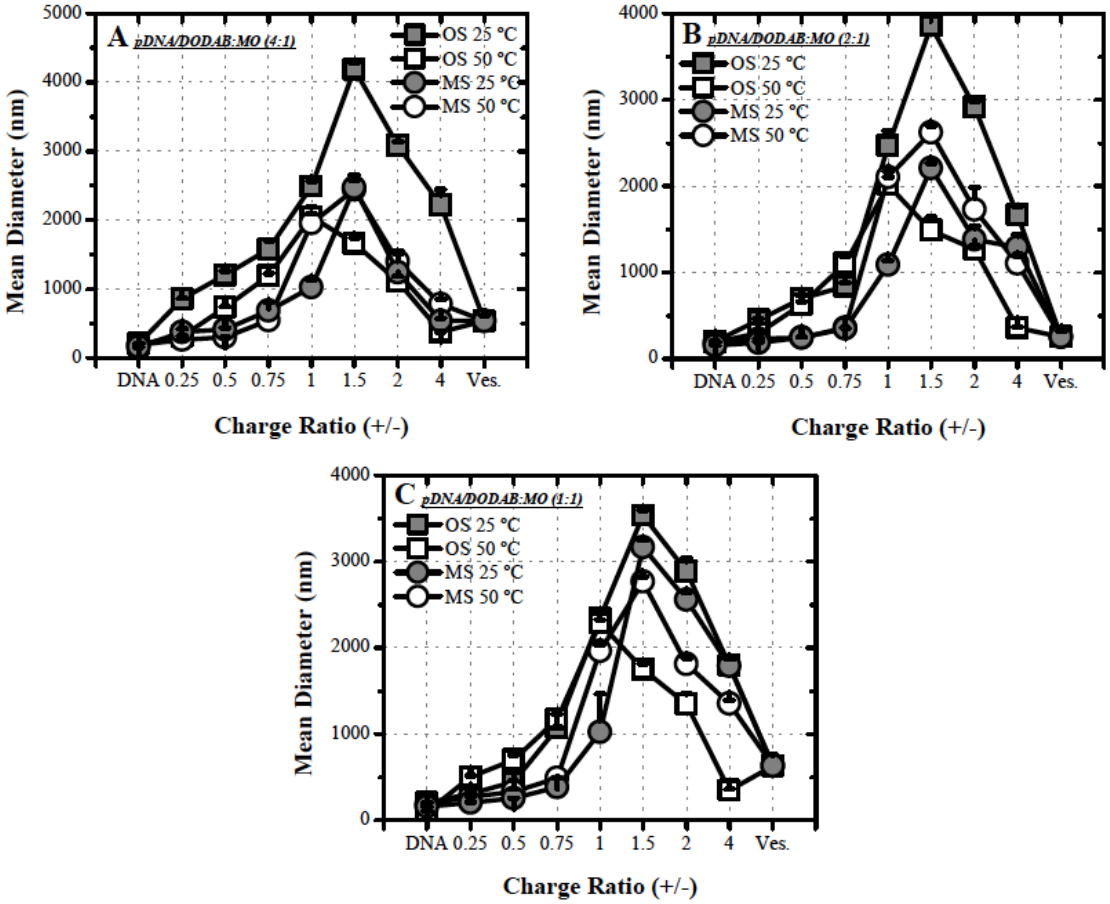

Figure 2: Mean diameter (nm) of free pDNA, free DODAB:MO vesicles and pDNA/DODAB:MO lipoplexes prepared by different methods (OS 25 ºC-gray square, OS 50 ºC-white square, MS 25 ºC – gray circle, MS 50 ºC – white circle) at different CRs (+/-). **A** – pDNA/DODAB:MO (4:1); **B** – pDNA/DODAB:MO (2:1); and **C** – pDNA/DODAB:MO (1:1).

while at 25 ºC these metastable lipoplexes persist, at 50 ºC the fluidity increases and the extra lipid mobility leads to changes on the lipid bilayers mean curvature. As a result, lipoplex fusion occurs, causing an increase of lipoplexes size (Figure **2**).

Figure **3** presents the zeta (ζ) potential of free pDNA, free DODAB:MO vesicles and pDNA/DODAB:MO lipoplexes prepared by different methods (OS 25 ºC, OS 50 ºC, MS 25 ºC, MS 50 ºC) and at different CRs (+/-).

Preparation of pDNA/DODAB:MO at 50 ºC forms lipoplexes that are clearly more positively charged than lipoplexes prepared at 25 ºC, irrespective of the MO content or the mixing steps employed (OS *versus* MS). As previously explained, raising the temperature has an impact in the liposomes fluidity, improving their binding efficiency to pDNA. However, after the liposomes have been covered by DNA, they fuse and suffer reorganization to assure the best stability of the lipoplex formed. This restructuring consists on the distribution of DODAB lamellar structures around the DNA/lipid aggregates, to avoid lipoplex disaggregation and to maximize favorable interactions with the surrounding water molecules. This restructuring was also reported in other lipoplexes containing DNA [47] and is facilitated by the temperature increase. At 25 ºC and at CR (+/-) 4.0, MS addition of cationic vesicles to pDNA produces metastable lipoplexes that are not fully covered of pDNA, presenting lower zeta potentials. Contrastingly, the lipoplexes formed by OS are highly organized structures that protect the pDNA molecules, leading to higher zeta potentials (Figure **3**).

3.3. Modulation of pDNA Release by the Lipoplex Preparation Method and MO Content

The EtBr fluorescence exclusion assay used to study pDNA complexation can also be also used to evaluate the extent of pDNA release from lipoplexes. Figure **4** shows pDNA release upon addition of the negative polyelectrolyte heparin to lipoplexes, based on the fact that the reintercalation of EtBr on the newly released pDNA will enhance the pDNA emission band.

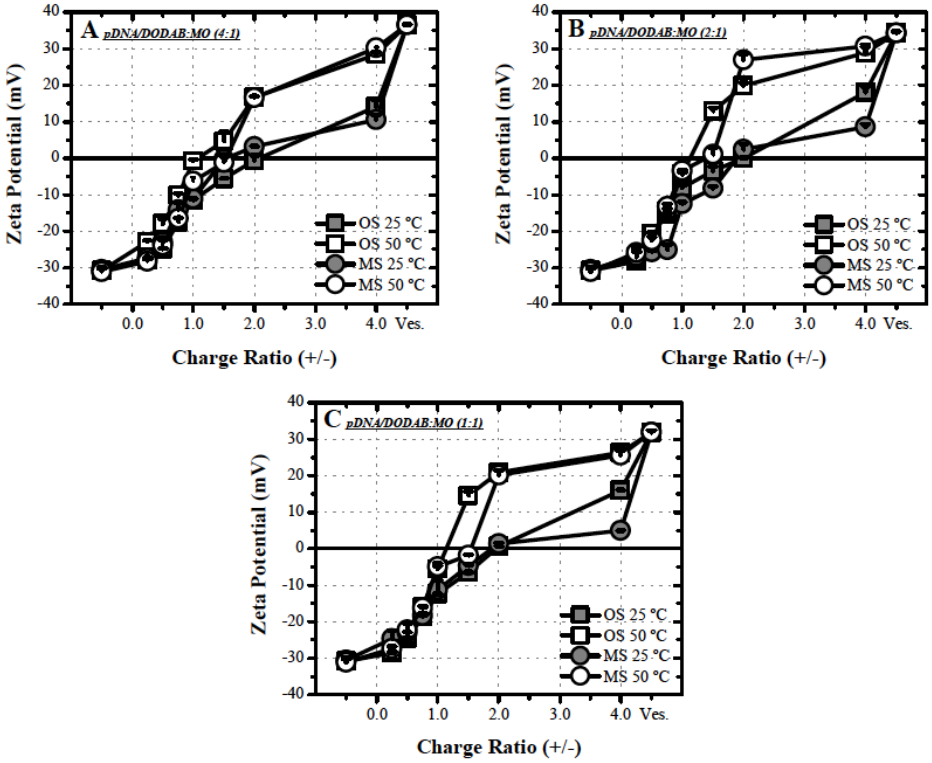

Figure 3: Zeta (ζ) potential (mV) of free pDNA, free DODAB:MO vesicles and pDNA/DODAB:MO lipoplexes prepared by different methods (OS 25 ºC-gray square, OS 50 ºC-white square, MS 25 ºC – gray circle, MS 50 ºC – white circle) at different CRs (+/-). **A** – pDNA/DODAB:MO (4:1); **B** – pDNA/DODAB:MO (2:1); and **C** – pDNA/DODAB:MO (1:1).

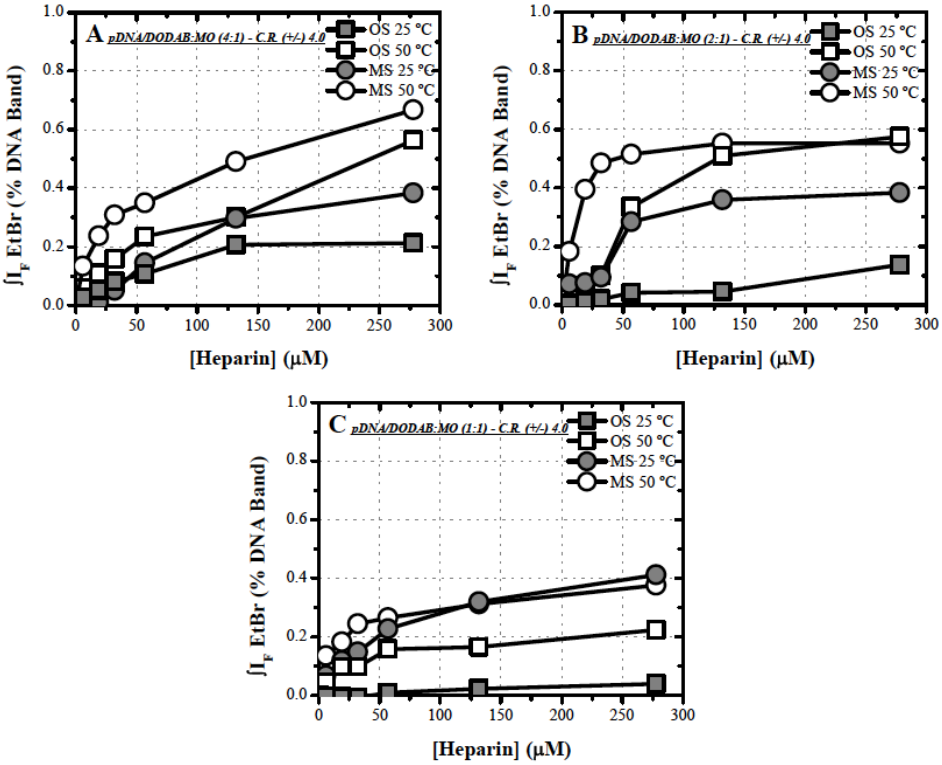

Figure 4: pDNA release from pDNA/DODAB:MO lipoplexes upon addition of increasing amounts of heparin (HEP). Variation of the decomposed fluorescence intensities of EtBr in pDNA band for different pDNA/DODAB:MO lipoplexes prepared by different methods (OS 25 ºC-gray square, OS 50 ºC-white square, MS 25 ºC – gray circle, MS 50 ºC – white circle) at different CRs (+/-). **A** – pDNA release for pDNA/DODAB:MO (4:1); **B** – pDNA release for pDNA/DODAB:MO (2:1); and **C** – pDNA release for pDNA/DODAB:MO (1:1).

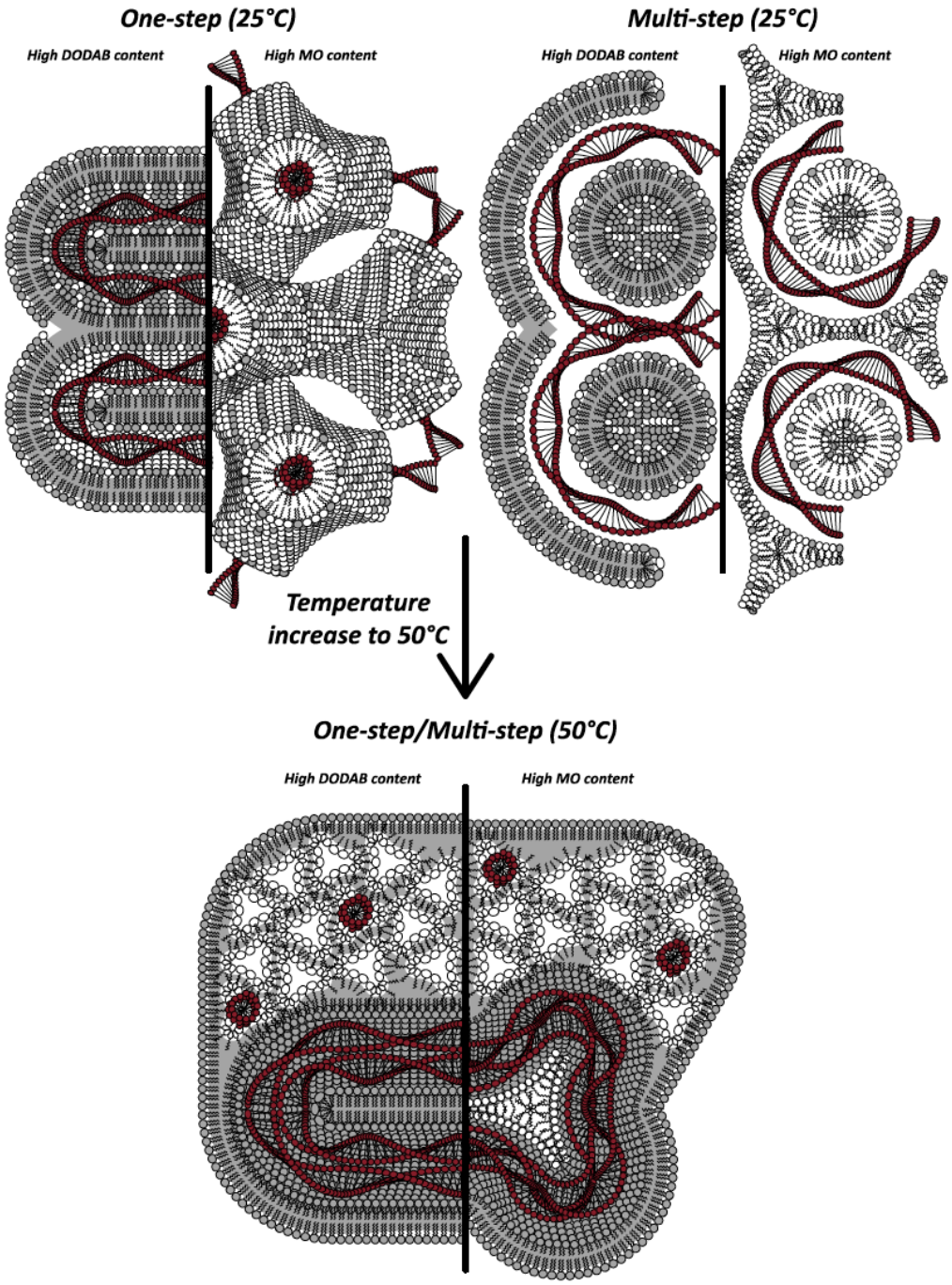

Figure 5: Theoretical model of lipid/DNA structural organization in pDNA/DODAB:MO lipoplexes at different DODAB:MO contents ("high DODAB content" refers to (4:1) and (2:1) formulations and "high MO content" to (1:1) and prepared by the different methods in study: OS (25 ºC), OS (50 ºC), MS (25 ºC) and MS (50 ºC). Double-tailed molecule with grey-headgroup represents DODAB and single-tailed molecule with white-headgroup represents MO. Grey-coloured regions represent DODAB rich-domains and white-coloured regions represent MO rich-domains.

Heparin induces very different patterns of pDNA release from lipoplexes, according to the different DODAB:MO formulations. Upon the final addition of heparin, DODAB enriched lipoplexes (pDNA/DODAB: MO (4:1 and 2:1, Figure **4A** and **4B**, respectively)) release higher pDNA amounts when compared to MO enriched lipoplexes (pDNA/DODAB:MO (1:1) (Figure **4C**)), reinforcing the already reported protective effect of MO on lipoplex stabilization [40]. In general, the lipoplex destabilization seems to be less dependent on the addition procedure (MS or OS) and more related with the lipoplex surface potential. In fact, if the lipoplex surface charge is highly positive, then the interactions with the negatively charged proteoglycans will be

promoted, leading to a more efficient pDNA release. For the same reason, pDNA/DODAB:MO lipoplexes prepared at 50 ºC are associated with a higher percentage of pDNA release, once the lipoplex surface charge is more positive at this temperature due to the distribution of the DODAB lipids in lamellar structures, protecting the pDNA inside the lipoplex.

Additionally, at a given temperature (25 or 50 ºC), the pDNA release is higher for lipoplexes prepared by MS procedure. This is consistent with the DLS (Figure **2**) and zeta potential (Figure **3**) results and might be explained by the MS promotion of metastable lipoplexes that may present regions of exposed pDNA, which will act as packing defects and facilitate destabilization. According to the results obtained by the different techniques, a theoretical model of DNA/lipid aggregation is proposed in Figure **5**. This model summarizes the structural variations observed for the diverse pDNA/DODAB:MO formulations prepared by the different lipoplex preparation methods (OS 25 °C, OS 50 °C, MS 25 °C and MS 50 °C).

3.4. One-Step/Multi-Step Preparation Methods Effect on 293T Cell Transfection Efficiency

We have previously shown that MO-based lipoplexes prepared by OS procedure, at 25 ºC, efficiently transfect 293T cells [40]. It was also found that, with increasing MO content, the appearance of inverted non–lamellar structures at pDNA/DODAB:MO (1:1) lipoplexes results in higher pDNA compaction and higher lipoplex resistance to destabilizing agents [40]. As the temperature may also favor the inverted non-lamellar phases, we have decided to explore how temperature and preparation method affect the transfection efficiency of pDNA/DODAB:MO lipoplexes.

Figure **6** depicts the transfection efficiency of 293T cells incubated with the various lipoplexes tested.

For the three lipoplexes (pDNA/DODAB:MO 4:1, 2:1 and 1:1), OS preparation method promotes the higher transfection efficiencies.

The re-localization of positive charges at 50 ºC in the lipoplex surface (irrespective of OS or MS addition) affects pDNA/DODAB:MO (4:1, 2:1 and 1:1) lipoplexes at different extents, because the initial structures of the liposomes are also different. DODAB enriched liposomes (DODAB:MO (4:1, 2:1)) have a lamellar organization, where MO is mainly distributed in the lamellar phase of DODAB [48]. Encapsulation of pDNA will maintain the lamellar phase of DODAB:MO (4:1 and 2:1), predominantly forming lipoplexes with

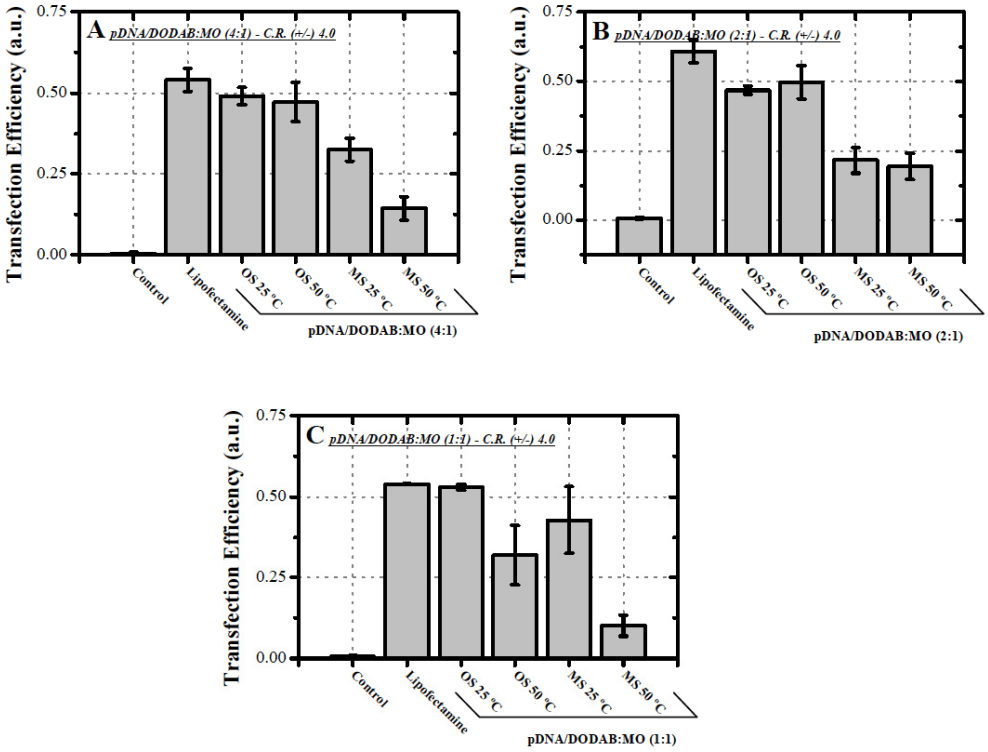

Figure 6: Reporter β-galactosidase activity 48 h after transfection of 293T cells with different cationic lipoplexes (0.5 µg pDNA per well) prepared by different methods (OS 25 ºC; OS 50 ºC; MS 25 ºC; and MS 50 ºC). **Control**: cells incubated with free pDNA. The mean (+/-) SD was obtained from three independent experiments.

multilamellar structures, where the nucleic acid is located between the lipid membranes. In these pDNA/DODAB:MO (4:1 and 2;1) lipoplexes, the effect of charge re-localization is practically negligible in terms of transfection efficiency. In MO enriched liposomes (DODAB:MO (1:1) different vesicles with internal organization co-exist, and DODAB and MO self-assemble, respectively, into lamellar and non-lamellar phases [48]. The encapsulation of pDNA by DODAB:MO (1:1) will originate a DODAB-rich lamellar phase enclosing MO non-lamellar inverted phases, where DNA preferentially localizes. In these pDNA/DODAB:MO (1:1) lipoplexes, the effect of charge re-localization decreases transfection efficiency by 2-fold.

For lipoplexes with higher DODAB content (pDNA/DODAB:MO (4:1, 2:1), the OS procedure generally leads to better transfection efficiencies than the MS procedure. This might be explained by the higher DNA/lipid compaction achieved with the OS preparation method, which leads to smaller lipoplexes that are less destabilized by surface proteoglycans and consequently retain more pDNA.

The lipoplexes prepared with MO enriched liposomes (pDNA/DODAB:MO (1:1)) seem more dependent on the preparation procedure. This behavior is related to the different structure of this type of lipoplex at 25 ºC, and also to the effect of the temperature on promoting phase separation and promoting an increase in lamellar *versus* non-lamellar structures at 50°C (Supplementary Material 2). Lower temperatures imply more ordered lipid structures, which hinder the tight contact during DNA-lipid interaction essential for lipoplex production [17, 44].

The lower transfection efficiency obtained with lipoplexes prepared by MS procedure at 50 ºC may be also related with the packing defects of these lipoplexes structures, which are more sensible to destabilization by surface proteoglycans, consequently prematurely releasing their p-DNA content (Figure **4**).

4. CONCLUSIONS

Optimization of the preparation method is essential to form lipoplexes with the best physicochemical properties to achieve high transfection efficiencies. This work shows that, at lower temperatures (under the lipid transition temperature), the MS addition of DODAB:MO cationic liposomes to pDNA is an adequate method for lipoplex preparation. However, above the lipid phase transition temperature, MS procedure promotes the formation of bigger lipoplexes with metastable structures. Furthermore, MS procedure forms less stable lipoplexes, able to easily release their nucleic acid content. All these characteristics explain the lower transfection efficiency obtained by lipoplexes prepared by MS procedure, regardless the MO content of the lipoplex. Therefore, OS addition of DODAB:MO liposomes to pDNA was found to be the best preparation method to form pDNA/DODAB:MO lipoplexes.

Our results also suggest that MO content must be tuned in the formulation in order to accomplish higher transfection efficiencies. The introduction of a third lipoid component, which may further influence the structural properties of the lipoplex formulations, is currently underway in order to improve the transfection efficiency of this non-viral vector.

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ABBREVIATIONS

DODAB	=	dioctadecyldimethylammonium bromide
MO	=	1-monooleoyl-*rac*-glycerol
DLS	=	dynamic light scattering
ELS	=	electrophoretic light scattering
ζ potential	=	zeta potential
cryo-TEM	=	cryo-Transmission Electron Microscopy
EtBr	=	Ethidium Bromide
HEP	=	heparin
OS	=	One step procedure for lipoplex preparation

MS = Multi-step procedure for lipoplex preparation

SUPPLEMENTAL MATERIALS

The supplemental materials can be downloaded from the journal website along with the article.

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