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Research paper

# Short- and long-term stability study of lyophilized solid lipid nanoparticles for gene therapy

A. del Pozo-Rodríguez, M.A. Solinís, A.R. Gascón, J.L. Pedraz *

*Pharmacy and Pharmaceutical Technology Laboratory, University of the Basque Country (UPV-EHU), Vitoria-Gasteiz, Spain*

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ABSTRACT

Most studies in gene therapy are focused on developing more efficient non-viral vectors, ignoring their stability, even though physically and chemically stable vectors are necessary to achieve large easily shipped and stored batches. In the present work, the effect of lyophilization on the morphological characteristics and transfection capacity of solid lipid nanoparticles (LyoSLN) and SLN-DNA vectors (Lyo(SLN-DNA)) has been evaluated. The lyophilized preparations were stored under three different sets of temperature and humidity ICH conditions: 25 °C/60%RH, 30 °C/65%RH and 40 °C/75%RH. After lyophilization we found an increase in particle size which did not imply a reduction of "in vitro" transfection capacity. Stability studies of formulations lyophilized with trehalose showed that SLNs were physically stable during 9 months at 25 °C/60%RH and 6 months at 30 °C/65%RH. This stability was lost when harder conditions were employed (40 °C/75%RH). LyoSLNs maintained or increased the transfection efficacy (from 19% to approximately 40% EGFP positive cells) over time only at 25 °C/60%RH and 30 °C/65%RH. Lyo(SLN-DNA) resulted in almost no transfection under all conditions. LyoSLNs showed less DNA condensation capacity, whereas in Lyo(SLN-DNA) the plasmid became strongly bound, hampering the transfection. Furthermore, the storage of lyophilized lipoplexes stabilized with the disaccharide trehalose did not affect cell viability.



## 1. Introduction

The development of gene therapy has boosted the use of a new group of pharmaceutical agents for the treatment of human diseases [1–3]: gene delivery systems. Non-viral vectors are being extensively studied because of their greater security as compared to viral vectors. Non-viral vectors can be composed by polymers, lipids, peptides or mixtures of them. The typically employed lipidic systems are cationic liposomes, but it has been proved that cationic solid lipid nanoparticles (SLNs) can also provide good transfection levels [4–6]. Although the most well-known problem of non-viral systems is their low transfection efficiency, the poor physical stability of these systems in aqueous suspensions is also a barrier to their development as medicaments [7]. Lipoplexes tend to form aggregates which decrease their transfection capacity. In order to avoid this limitation, clinical trials using lipoplexes have traditionally used systems freshly prepared at the bedside prior to injection [8,9]. However, the quality and the particle size of these extemporaneous preparations are hardly ever controlled. Most studies in gene therapy are focused on developing more efficient non-viral vectors, ignoring their stability, even though physically and chemically stable vectors are necessary to achieve large easily shipped and stored batches.

Different authors [9–12] have demonstrated that frozen DNA formulations maintain transfection rates, but they require strict storage and shipping temperatures, which imply a substantial increase in costs. This fact has generated an increasing interest in developing dehydrated formulations, which can be stored and shipped at room temperatures. Lyophilization is one of the most employed techniques to generate dried pharmaceuticals in general, and DNA-based formulations in particular [9,12–17].

Lyophilization subjects formulations to two important stresses, freezing and drying [7], which can damage biomolecules unless appropriate stabilizers, such as sugars, are used. Different sugars have been employed to stabilize lyophilized SLNs and non-viral vectors composed by cationic lipids and DNA [13,18–21]: monosaccharides (glucose), disaccharides (trehalose, sucrose), oligosaccharides (inuline) or polysaccharides (hydroxyethyl starch, high molecular weight dextrans). Among these, trehalose is one of the most commonly employed sugars providing positive results.

Although there are some studies about the long-term stability of SLNs for poorly water-soluble pharmaceutical drugs [22–24], we have not found any work about the long-term stability of lyophilized cationic SLNs for gene therapy, and only a few studies

* Corresponding author. Pharmacy and Pharmaceutical Technology Laboratory, Pharmacy Faculty, University of the Basque Country (UPV-EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain. Tel.: +34 945013091; fax: +34 945013040.
*E-mail addresses:* ana.delpozo@ehu.es (A. del Pozo-Rodríguez), knppemuj@vc.ehu.es (J.L. Pedraz).

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about lyophilized liposomes [12,19,24–26]. The aim of our work was to evaluate the short- and long-term stability of lyophilized SLNs containing the pCMS-EGFP plasmid. The lyophilized preparations were stored under three different sets of temperature and humidity conditions, according to ICH guidelines (CPMP/ICH/2736/99): 25 ± 2 °C/60% RH ± 5% RH (long-term study), 30 ± 2 °C/65% RH ± 5% RH (intermediate study) and 40 ± 2 °C/75% RH ± 5% RH (accelerated or short-term study). Parameters such as size, zeta potential, DNA protection and conformation, transfection capacity and cell viability were studied over time.

## 2. Materials and methods

### 2.1. Materials

Precirol® ATO 5 was provided by Gattefossé (Madrid, Spain). *N*-[1-(2,3-Dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTAP), lauryl sulfate sodium (SDS), glucose, antibiotic/antimycotic and D-trehalose were purchased from Sigma-Aldrich (Madrid, Spain). Tween-80 was provided by Vencaser (Bilbao, Spain) and dichloromethane by Panreac (Barcelona, Spain).

Plasmid pCMS-EGFP encoding the enhanced green fluorescent protein (EGFP) was purchased from BD Biosciences Clontech (Palo Alto, US) and amplified by Dro Biosystems S.L. (San Sebastián, Spain).

The materials employed for the electrophoresis on agarose gel were acquired from Bio-Rad (Madrid, Spain). The cell culture reagents were purchased from LGC Promochem (Barcelona, Spain). The BD Viaprobe kit was provided by BD Biosciences (Belgium).

### 2.2. SLNs and SLN-DNA vectors production

The SLNs were produced by a solvent emulsification/evaporation technique [27]. Precirol® ATO 5 was dissolved in dichloromethane (5% w/v), and then emulsified in an aqueous phase that contained DOTAP (0.4% w/v) and Tween-80 (0.1% w/v). The emulsion was obtained by sonication (Branson Sonifier 250, Danbury) during 30 s at 50 W. The organic phase/aqueous phase ratio was 1/5. Dichloromethane was then removed from the emulsion by evaporation using a magnetic agitator for 45 min followed by vacuum conditions for 15 min. A SLNs suspension was formed upon precipitation of the Precirol® ATO 5 in the aqueous medium. Finally, the SLNs were washed by centrifugation (3000 rpm, 20 min, ×3) using Millipore (Madrid, Spain) Amicon® Ultra centrifugal filters (100,000 MWCO).

SLN-DNA vectors were prepared by mixing a pCMS-EGFP plasmid DNA solution at 2 μg/μL concentration and a SLNs suspension, at DOTAP/DNA ratio 5/1 (w/w) for 30 min at 25 °C.

The formulations studied were the following: LyoSLNs, (LyoSLN)-DNA and Lyo(SLN-DNA). Table 1 summarizes how those formulations were prepared. In all cases, three batches were studied.

### 2.3. Lyophilization procedure

SLNs (without DNA) or SLN-DNA samples were transferred to poly-propilene tubes and diluted with the sugar solutions. Samples were frozen at −80 °C. After 24 h, frozen samples were lyophilized at −55 °C and 0.2 mbar for 48 h (Telstar Cryodos freeze-dryer). Lyophilized samples were reconstituted in HBS (Hepes Buffer Saline) by mixing, and the external appearance of the resulting formulations was observed.

### 2.4. Stability study conditions

LyoSLNs were packed in amber-colored borosilicate vials and the Lyo(SLN-DNA) in poly-propylene cryovials in order to prevent the DNA from binding to glass [28,29]. The samples were stored in environmental simulation chambers for constant climatic conditions (Binder, Tuttlingen, Germany). Table 2 shows the storage conditions used in the stability study, as well as the times when the samples were tested.

### 2.5. Size and zeta potential measurements

The sizes of SLNs and SLN-DNA vectors were determined by photon correlation spectroscopy (PCS). Zeta potential was measured by laser doppler velocimetry (LDV). Both measurements were performed on a Malvern Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). All samples were diluted in 0.1 mM NaCl.

### 2.6. Agarose gel electrophoresis

SLN-DNA vectors were diluted in water Milli-Q™ to a final concentration of 0.1 μg DNA/μL and subjected to electrophoresis on an agarose gel (1% ethidium bromide included for visualization) for 30 min at 120 V. The bands were observed with a model TFX-20M transilluminator (Vilber-Lourmat). Images were captured using a digital camera from Bio-Rad, DigiDoc model.

### 2.7. SDS-induced release of DNA from SLN-DNA vectors

A 2% SDS solution was added to the samples to a final concentration of 1% to release DNA from SLNs. Samples were then analysed by electrophoresis on agarose gel, and the integrity of the DNA in each sample was compared with untreated DNA as control.

### 2.8. DNase I protection study

DNase I was added to SLN-DNA systems to a final concentration of 1 U DNase I/2.5 μg DNA, and the mixtures were incubated at 37 °C for 30 min. Afterwards, 2% SDS solution was added to the samples to a final concentration of 1% to release DNA from SLN. Samples were then analysed by electrophoresis on agarose gel and the integrity of the DNA in each sample was compared with untreated DNA as control.

### 2.9. Cell culture and transfection protocol

In vitro assays were performed with the Human Embryonic Kidney (HEK293) cell line, obtained from the American Type Culture Collection (ATCC). Cells were maintained in Eaglés Minimal Essen-

**Table 1**
Names of the assayed formulations and summary of their preparation.

| Name | Preparation |
|---|---|
| LyoSLNs | Free DNA lyophilized SLNs and later reconstituted |
| (LyoSLN)-DNA | LyoSLNs later reconstituted and bound to DNA |
| Lyo(SLN-DNA) | Lyophilized SLN-DNA vectors and later reconstituted |

**Table 2**
Storage conditions of the different groups included in the study (RH, relative humidity).

| Group | Storage conditions | Time points |
|---|---|---|
| Time 0 | – | 0 |
| Long-term | 25 ± 2 °C/60% RH ± 5% RH | 3, 6, 9 and 12 months |
| Intermediate | 30 ± 2 °C/65% RH ± 5% RH | 3, 6, 9 and 12 months |
| Accelerated | 40 ± 2 °C/75% RH ± 5% RH | 1, 3 and 6 months |

tial Medium with Earlés BSS and 2 mM L-glutamine (EMEM) supplemented with 10% heat-inactivated horse serum and 1% antibiotic/antimycotic. Cells were incubated at 37 °C with 5% $CO_2$ in air and subcultured every 2–3 days using trypsin/EDTA.

For transfection, HEK293 cells were seeded on 24 well plates at a density of 150,000 cells per well and allowed to adhere overnight. 75 μL of the vectors solution diluted in HBS buffer (2.5 μg DNA) was added, and cells were incubated with the non-viral systems for 4 h at 37 °C. The medium containing the vectors in the wells was diluted with 1 mL of complete medium, and cells were allowed to grow for 72 h.

### 2.10. Flow cytometry-mediated analysis of transfection efficacy and cytotoxicity

At the end of the incubation period, cells were washed once with 300 μL of PBS and detached with 300 μL of trypsin/EDTA. Then the cells were centrifuged at 1500*g*, and the supernatant was discarded. The cells were resuspended with PBS and directly introduced to a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, USA). For each sample, 10,000 events were collected.

For transfection, efficacy quantitation fluorescence of EGFP was collected at 525 nm (FL1). For cytotoxicity measurements, BD ViaProbe kit was employed. 5 μL of the kit was added to each sample and after 10 min of incubation fluorescence correspondent to dead cells was measured at 650 nm (FL3).

### 2.11. Statistical analysis

Results are reported as means (S.D. = standard deviation). The statistical analysis was carried out with SPSS 14.0 for Windows® (SPSS®, Chicago, USA). Normal distribution of samples was assessed by Shapiro–Wilk's test, and homogeneity of the variance by Levene's test. The statistical analysis between non-lyophilized and lyophilized samples or between lyophilized samples at different times was determined with a Student's *t*-test. Differences were considered statistically significant if $p < 0.05$.

## 3. Results

### 3.1. Characterization of lyophilized samples at time 0

#### 3.1.1. Physical appearance

Lyophilization of samples without stabilizers resulted in the aggregation of particles forming a cake with rubbery aspect. When LyoSLNs were lyophilized with a solution of glucose at 5%, a powered product was obtained, but after 2 days at room temperature it became rubber. The use of the other glucose concentrations led to rubbery samples from the beginning. But when trehalose was used as stabilizer at 10 and 5% concentrations, lyophilized samples showed a powdery aspect. However, when trehalose solutions at 40 and 20% were used, samples showed a fibrous aspect. Redispersion in HBS buffer by vortex agitation was rapid in all cases, although those stabilized with 5% trehalose presented microparticles.

#### 3.1.2. Size and zeta potential

Fig. 1 shows the effect of trehalose concentration on the particle size and zeta potential of free DNA LyoSLN (Fig. 1A) and Lyo(SLN-DNA) (Fig. 1B) formulations.

Lyophilization of SLNs (LyoSLNs) caused an increase in the particle size (bars in Fig. 1A) of approximately 100 nm with all trehalose concentrations ($p < 0.05$). Zeta potential (line in Fig. 1A) decreased from +44 mV to approximately +35 mV in all cases ($p < 0.05$). When (LyoSLN)-DNA vectors were prepared, the size was higher than 1 μm, and the zeta potential significantly decreased to values lower than +10 mV.

Concerning Lyo(SLN-DNA), lyophilization induced a 2-fold increase in size (bars in Fig. 1B). Zeta potential (line in Fig. 1B) decreased to around +15 mV with all the trehalose concentrations ($p < 0.05$).

#### 3.1.3. Binding of DNA to LyoSLN

The agarose gel electrophoresis in Fig. 2 (time 0) shows that LyoSLNs lyophilized in the presence of trehalose solutions at 10 and 5% (lanes 2 and 3, respectively) had the ability to bind all DNA, as no band was detected in those lanes.

#### 3.1.4. SDS-induced release of DNA from lyophilized samples

Fig. 3 shows the agarose gel electrophoresis corresponding to the DNA released from lyophilized formulations in the presence of trehalose 5 and 10% at time 0 (lanes 2–5). The DNA released from (LyoSLN)-DNA (lanes 2 and 3) showed two bands, which correspond to the open circular conformation (OC) and the supercoiled conformation (SC). Those bands fit with the bands of the free DNA (lane 1). However, the DNA released from Lyo(SLN-DNA) showed three bands (lanes 4 and 5), with the intermediate band as the linear DNA (L).

#### 3.1.5. Transfection "in vitro" and cell viability

Fig. 4 shows transfection and cell viability of non-lyophilized and lyophilized formulations in HEK293 culture cells. Assays were carried out with lyophilized samples stabilized with 5 or 10% trehalose solutions. No differences in transfection levels (bars) were detected between non-lyophilized and lyophilized samples ($p > 0.05$). Furthermore, cell viability (line) was not modified by the lyophilization process ($p > 0.05$).

### 3.2. Stability of lyophilized samples over time

For the stability assay, 10% trehalose solution was chosen as stabilizer. Samples were stored at different temperature and humidity conditions and assayed at different times, as it is summarized in Table 2.

#### 3.2.1. Physical appearance over time

LyoSLNs showed a powdered aspect and were easily dispersed in HBS buffer until 6 months at 30 °C/65%RH and until 9 months at 25 °C/60%RH. The samples stored at 40 °C/75%RH maintained that powered aspect but after redispersion they showed aggregates. Lyo(SLN-DNA) showed a similar behaviour maintaining the powered aspect and the dispersion capacity during 6 and 12 months at 30 °C/65%RH and 25 °C/60%RH, respectively. After storage at 40 °C/75%RH, Lyo(SLN-DNA) presented a rubbery aspect and inadequate redispersion from the first month.

#### 3.2.2. Size and zeta potential over time

Particle size and zeta potential of the LyoSLNs and Lyo(SLN-DNA) were measured at different time points. Fig. 5 features the results.

LyoSLNs stored at 40 °C/75%RH for 1 and 3 months showed sizes (Fig. 5A) twice greater than that at time 0 ($p < 0.01$), and at 6 months we visually detected aggregates, unmeasured by PCS. At 30 °C/65%RH and 25 °C/60%RH, particle size hardly varied during the first 6 and 9 months, respectively. From these times, redispersion was not complete and aggregates appeared. Zeta potential (Fig. 5B) was stable when formulations were stored at 25 °C/60%RH, although a slight but significant increase in surface charge (about 10 mV) was found when storing at 30 °C/65%RH. At 40 °C/75%RH, zeta potential increased during 1 and 3 months, but when measured after 6 months it decreased to −2.3 mV.

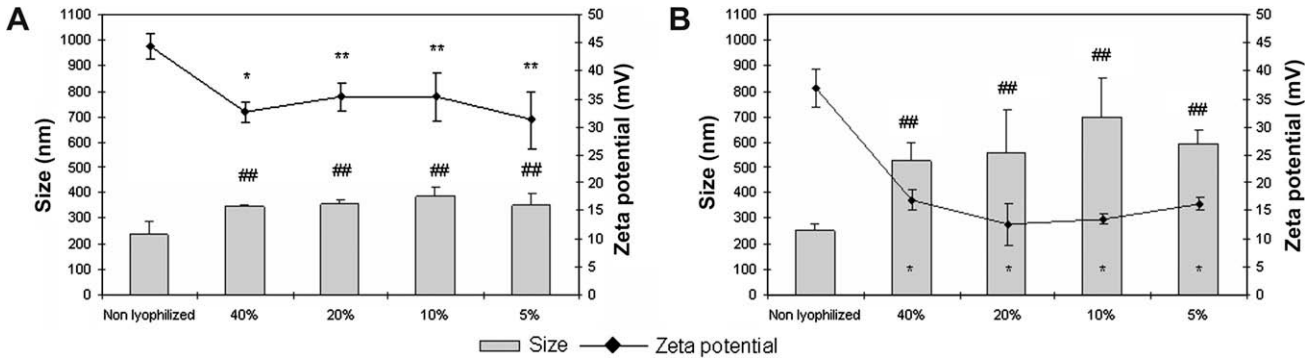


**Fig. 1.** Effect of trehalose concentration on size and zeta potential of LyoSLNs (A) and Lyo(SLN-DNA) (B). X axe indicates the concentration of trehalose solutions employed (w/v). Error bars represent S.D. ($n = 3$). *: $p < 0.01$ in zeta potential against non-lyophilized samples; **: $p < 0.05$ in zeta potential against non-lyophilized samples; ##: $p < 0.05$ in size against non-lyophilized samples.

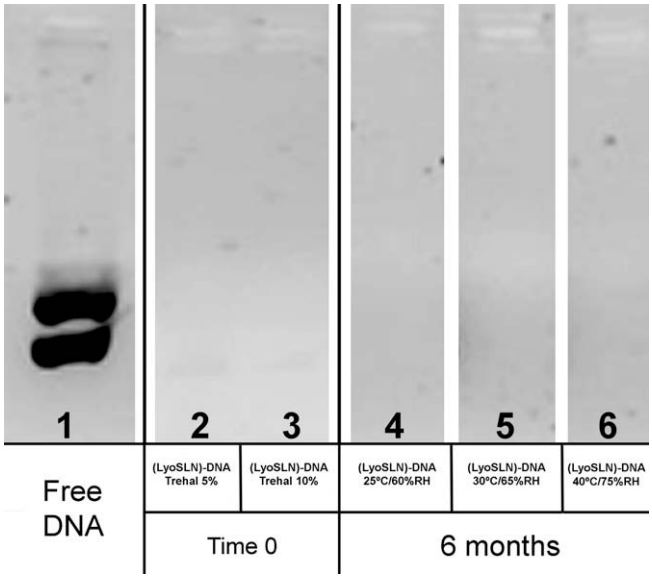


**Fig. 2.** Agarose gel electrophoresis of DNA in contact with LyoSLNs at time 0 and after 6 months of storage. DOTAP/DNA ratio was 5/1 (w/w). Lane 1: free DNA, lane 2: DNA bound to LyoSLNs lyophilized in the presence of a trehalose 5% solution at time 0, lane 3: DNA bound to LyoSLNs lyophilized in the presence of a trehalose 10% solution at time 0, lane 4: DNA bound to LyoSLNs lyophilized in the presence of a trehalose 10% solution at 6 months of storage at 25°C/60%RH, lane 5: DNA bound to LyoSLNs lyophilized in the presence of a trehalose 10% solution at 6 months of storage at 30°C/65%RH, lane 6: DNA bound to LyoSLNs lyophilized in the presence of a trehalose 10% solution at 6 months of storage at 40°C/75%RH.

As to Lyo(SLN-DNA) formulations (Fig. 5C), during the 12-month storage period, a decrease in size from 700 to 450 nm was observed at all storage conditions ($p < 0.05$). Nevertheless, this reduction was progressive at 25 °C/60%RH, but at 30 °C/65%RH and 40 °C/75%RH maximum reduction was obtained at 3 months. Zeta potential (Fig. 5D) decreased from +12 mV to negative values in all cases ($p < 0.05$).

*3.2.3. Binding of DNA to stored LyoSLN*

Fig. 2 (lanes 4–6) features the results of the gel electrophoresis carried out after a 6-month storage period. The absence of bands indicates that LyoSLNs maintained the DNA binding capacity during the storage at the three temperature and humidity conditions. That capacity was maintained throughout the study.

*3.2.4. SDS-induced release of DNA from stored lyophilized samples*

Fig. 3 shows the agarose gel electrophoresis of (LyoSLN)-DNA and Lyo(SLN-DNA) treated with SDS 1% after 6 months at the three conditions of temperature and humidity. Only lanes 6, 8 and 10, corresponding to (LyoSLN)-DNA, showed free DNA. Lyo(SLN-DNA) formulations did not release the DNA (lanes 7, 9 and 11). This behaviour was observed during all the study.

*3.2.5. "In vitro" protection against DNase I*

Fig. 6 shows the agarose gel electrophoresis of the (LyoSLN)-DNA lipoplexes treated with DNase I. Lane 1 represents not-treated free DNA. Lanes 2, 3 and 4 correspond to DNA released from (LyoSLN)-DNA stored at the three conditions of temperature and humidity at 6 months, treated with DNase I, and lane 5 shows the DNA released from LyoSLNs when stored at 40 °C/75%RH for 3 months, after the treatment with DNase I. Lanes 2, 3 and 5 show a less intense SC band, the appearance of the L band and DNA fragmentation. These results indicate that LyoSLNs were still able to partially protect the DNA; this behaviour was observed throughout the study at 25 °C/60%RH and 30 °C/65%RH. Nevertheless, the DNA protection was totally lost after a 6-month storage period at 40 °C/75%RH, as can be seen in lane 4, where all DNA is fragmented.

*3.2.6. "In vitro" transfection and cell viability over time*

Fig. 7 presents the percentage of transfected cells and cell viability measured by flow cytometry.

After 6 months, the percentage of transfected cells with (LyoSLN)-DNA increased from 19% to 40% when stored at 25 °C/60%RH and to 37% when stored at 30 °C/65%RH, decreasing to initial values after 12 months. However, transfection levels suffered an important decrease to 1.5% EGFP positive cells at 6 months ($p < 0.01$), when (LyoSLN)-DNA formulations were stored at 40 °C/75%RH.

Lyo(SLN-DNA) resulted in a dramatical reduction of transfection efficiency in all storage conditions ($p < 0.01$), reaching percentages of EGFP positive cells under 3% (Fig. 7C).

The lyophilization and posterior storage did not have an effect on cell viability in any case, (Fig. 7B and D).

## 4. Discussion

An important limitation of aqueous suspensions of non-viral vectors is their poor stability [7,30–32]. SLNs suspensions are not an exception. In a previous work, Freitas and Müller [22] showed an increase in the particle size of SLNs in a short period of time; although formulations prepared by these authors were not used for gene therapy, the same behaviour should be expected for our SLNs. In fact, the SLNs suspensions employed in the present study, composed by Precirol® ATO 5, DOTAP and Tween-80, were stable only for 1 week at room temperature and for 1 month at 4 °C (data not shown).

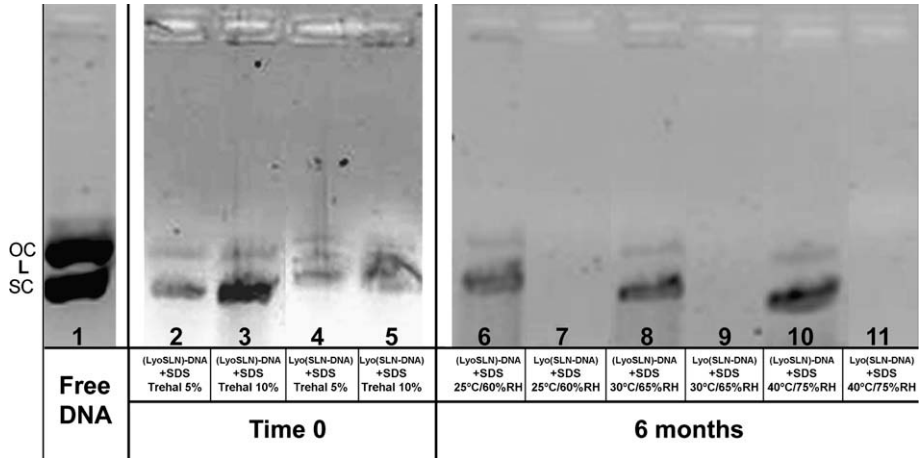


**Fig. 3.** Agarose gel electrophoresis of (LyoSLN)-DNA and Lyo(SLN-DNA) treated with SDS 1%. Lane 1: free DNA, lane 2: DNA released from LyoSLNs lyophilized in the presence of a trehalose 5% solution at time 0, lane 3: DNA released from LyoSLNs lyophilized in the presence of a trehalose 10% solution at time 0, lane 4: DNA released from Lyo(SLN-DNA) lyophilized in the presence of a trehalose 5% solution at time 0, lane 5: DNA released from Lyo(SLN-DNA) lyophilized in the presence of a trehalose 10% solution at time 0, lane 6: DNA released from LyoSLNs lyophilized in the presence of a trehalose 10% solution at 6 months of storage at 25°C/60%RH, lane 7: DNA released from Lyo(SLN-DNA) lyophilized in the presence of a trehalose 10% solution at 6 months of storage at 25°C/60%RH, lane 8: DNA released from LyoSLNs lyophilized in the presence of a trehalose 10% solution at 6 months of storage at 30°C/65%RH, lane 9: DNA released from Lyo(SLN-DNA) lyophilized in the presence of a trehalose 10% solution at 6 months of storage at 30°C/65%RH, lane 10: DNA released from LyoSLNs lyophilized in the presence of a trehalose 10% solution at 6 months of storage at 40°C/75%RH, lane 11: DNA released from Lyo(SLN-DNA) lyophilized in the presence of a trehalose 10% solution at 6 months of storage at 40°C/75%RH. OC, open circular DNA; L, lineal DNA; SC, supercoiled DNA.

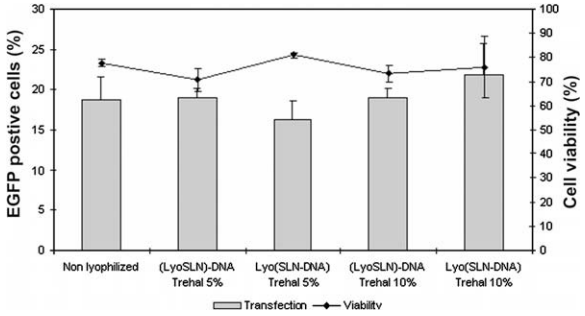


**Fig. 4.** Transfection and cell viability of non-lyophilized and lyophilized formulations in the presence of trehalose solutions at 5% or 10% concentrations. Error bars represent S.D. ($n = 3$). DOTAP/DNA 5/1 (w/w).

Lyophilization is one of the most employed methods to obtain dehydrated formulations which can be stored and shipped at room temperatures. However, it subjects samples to two main transformations which results in additional stability problems. First, the aqueous dispersion is freezing, prior to the evaporation of water under vacuum. Then, and before the administration, redispersion must be done. The process of lyophilization of SLNs, with or without DNA and in the absence of any stabilizer samples, resulted in the aggregation of particles forming a rubbery-looked cake. The freezing and the redispersion of the samples may be responsible for the aggregation because in both cases the water amount where particles are suspended decreases, which favours their concentration [13,27,33]. In the present work, the effect of lyophilization of SLNs and SLN-DNA vectors on their morphological characteristics and transfection capacity has been evaluated. The first strategy was to lyophilize the SLNs and later reconstitute them to finally prepare the lipoplexes with the plasmid. The second strategy was to lyophilize the lipoplexes, which would be much more convenient because it only requires the reconstitution prior to clinical use. As lyophilization can damage DNA [34], resulting in a loss of activity, an exhaustive evaluation of the behaviour of the lyophilized products is necessary. In order to improve stability, carbohydrates as lyoprotectants are frequently used, avoiding particle aggregation and denaturation of macromolecules.

Different studies have demonstrated that disaccharides are good stabilizers for lipoplexes [13,14,18], and one of them, trehalose, has been extensively demonstrated to provide good stabilization of lyophilized SLNs [20,21,35,36]. The stabilizer sugar must possess high glass transition temperature ($T_g$) to maintain the temperature during lyophilization below it. Otherwise, the freeze concentrated fraction would be in the liquid or in the rubbery state during the process [37]. Furthermore, the samples lyophilized in the presence of sugars with high $T_g$ (trehalose) show less tendency to aggregate during storage as compared to sugars with lower $T_g$, such as sucrose or glucose [38]. When we used the monosaccharide glucose as stabilizer, the lyophilized samples showed a rubbery aspect. Only when LyoSLNs were lyophilized with a solution of glucose at 5% a powered product was obtained, but after 2 days at room temperature it became rubber. However, the lyophilization with the stabilizer trehalose provided fibrous or powdery samples. On the basis of these observations, we have chosen trehalose as stabilizer of SLN-based non-viral vectors.

Our results indicate that the stabilizer effect of trehalose on the physical appearance of LyoSLNs and Lyo(SLN-DNA) was similar. In both cases, the lyophilized samples showed a powdery aspect when 10 or 5% trehalose solutions were used. Moreover, redispersion by vortex agitation was rapid for all the trehalose concentrations studied, except for formulations stabilized with 5% trehalose, in which microparticles appeared.

When comparing the particle size and the zeta potential of the SLNs (Fig. 1A), lyophilization induced a size increase (about 100 nm) and a slight decrease of zeta potential, regardless of the trehalose concentration. When those LyoSLNs were bound to DNA, vectors showed sizes bigger than 1 μm and superficial charge lower than +10 mV. When lipoplexes were lyophilized, Lyo(SLN-DNA), lyophilization caused a 2-fold or even higher increase in particle size. Moreover, a significant decrease in the zeta potential was observed. Therefore, in both cases lyophilization induces changes on particles leading to a decrease in the zeta potential and to an increase in the size. The decrease in superficial charge could be attributed to two phenomena: Schiff's base [19] or the water replacement hypothesis [39,40]. Schiff's base phenomenon refers to the reaction between reducing groups of sugars and amine groups of DOTAP. This is possible when reducing sugars are em-

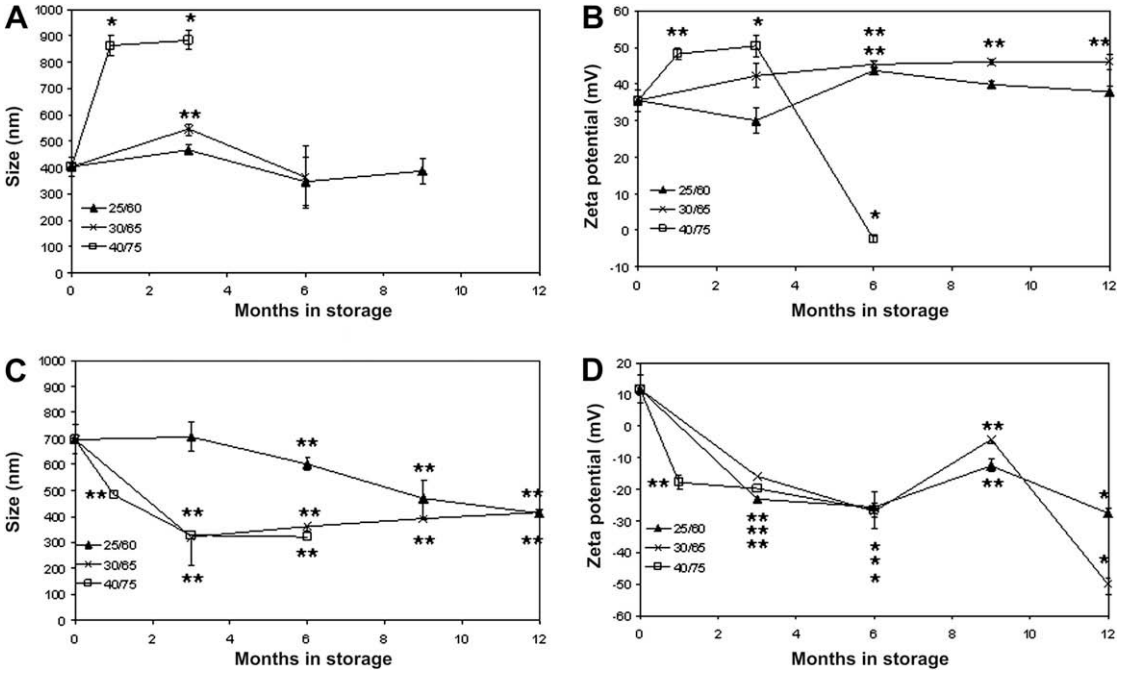


**Fig. 5.** Size and zeta potential of LyoSLNs (A, B) and Lyo(SLN-DNA) (C, D) stored at different conditions during different times from 1 to 12 months ($n = 3$). *X*-axis indicates time in storage (months). Error bars represent SD ($n = 3$). *: $p < 0.01$ against time 0; **: $p < 0.05$ against time 0.

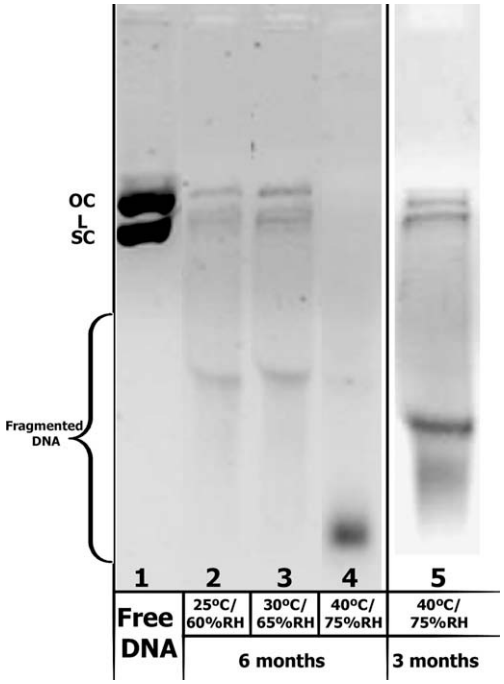


**Fig. 6.** Agarose gel electrophoresis of the (LyoSLN)-DNA complexes treated with DNase I. Lane 1: free DNA, lane 2: (LyoSLN)-DNA lyophilized in the presence of a trehalose 10% solution at 6 months of storage at 25°C/60%RH, lane 3: (LyoSLN)-DNA lyophilized in the presence of a trehalose 10% solution at 6 months of storage at 30°C/65%RH, lane 4: (LyoSLN)-DNA lyophilized in the presence of a trehalose 10% solution at 6 months of storage at 40°C/75%RH, lane 5: (LyoSLN)-DNA lyophilized in the presence of a trehalose 10% solution at 3 months of storage at 40°C/75%RH. OC, open circular DNA; L, lineal DNA; SC, supercoiled DNA.

ployed, or when non-reducing sugars are hydrolyzed resulting in the formation of reducing ones. Nevertheless, trehalose is a non-reducing disaccharide which is strongly resistant to hydrolysis, so the Schiff's base would not be responsible for the reduction on the superficial charge. Therefore, the decrease in the zeta potential may be better explained by the formation of hydrogen bonds between trehalose and SLN-DNA vectors (water replacement hypothesis). Those bonds can happen with cationic lipid head groups or with DNA. The cationic lipid head groups have the ammonium groups, which are responsible for the positive charge. When sugars form hydrogen bonds with those groups, the positive charge is partially neutralized, the superficial charge decreases and the binding of DNA by electrostatic interactions is weaker, keeping less condensed. The smaller condensation results in an increase in the size and a decrease in the zeta potential, as the negative charges of DNA are more exposed.

As the decrease in the zeta potential may reduce the DNA binding capacity of the LyoSLNs, we carried out an agarose gel electrophoresis with (LyoSLN)-DNA lipoplexes (Fig. 2), and we demonstrated that those formulations maintained the binding capacity, necessary for transfection. DNA topology is another factor which needs to be evaluated during the development of non-viral vectors. The agarose gel electrophoresis in Fig. 3 shows the bands corresponding to the DNA released from the lyophilized formulations. When DNA was released from LyoSLNs (lanes 2 and 3 in Fig. 3), the original bands of free DNA appeared. However, the DNA released from Lyo(SLN-DNA) showed three bands (lanes 4 and 5 in Fig. 3); an intermediate band corresponding to the linear DNA (L) appeared. The OC isoform is formed by a reversible change from SC DNA, and the L isoform appears when the OC isoform is degraded; that conversion is not reversible. Accordingly, lyophilization partially damaged DNA, although the most bioactive isoform [41,42], the SC DNA, did not completely disappear.

We also studied the transfection capacity of both (LyoSLN)-DNA and Lyo(SLN-DNA) formulations. Fig. 4 shows that all formulations maintained transfection rates and cell viability. Several studies have correlated the maintenance of lipoplex size and transfection rate. In our study, lyophilization did not produce changes in cell transfection in spite of the increase in size; however, it is important to consider that in all these studies [14,16,18,33,38,43] lipo-

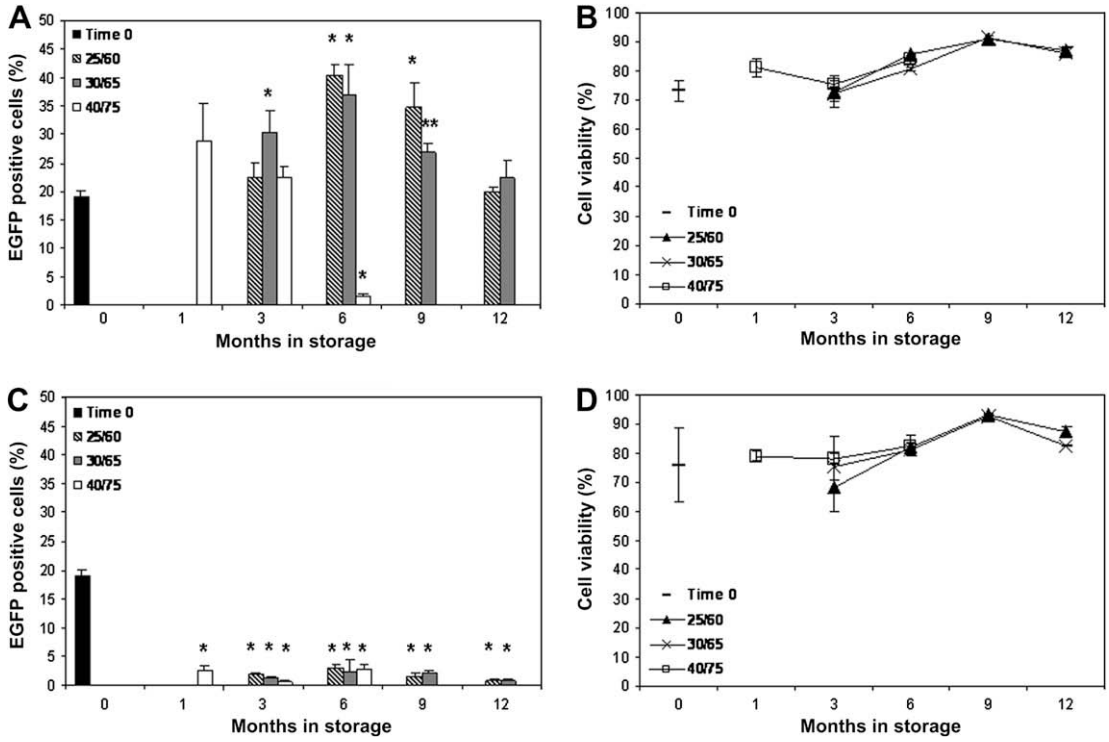


**Fig. 7.** Transfection (graphics in the left) and cell viability (graphics in the right) of (LyoSLN)-DNA (A and B) and Lyo(SLN-DNA) (C and D) stored at different conditions along time ($n = 3$). X axe indicates the months of storage. Error bars represent S.D. ($n = 3$). *: $p < 0.01$ against time 0. **: $p < 0.05$ against time 0.

somes but not SLNs were evaluated. Therefore, changes in the particle size do not necessary imply changes in the "in vitro" transfection efficacy. It is important to take into account that the change in particle size after lyophilization is an important factor to be considered when administering the vectors "in vivo" because it may compromise the administration route, the biodistribution and the efficacy. Size influences the targeting of the intravenously administered gene delivery systems, because the structure of the blood capillary wall varies greatly in different organs and tissues [44]. Furthermore, after oral administration the size of the particles determines the uptake by Peyer's patches and passage to blood [45]. Superficial charge of the gene delivery systems is also important, because it does not only determine the physical aspect, but it may also influence the "in vivo" behaviour. We have also observed in this work that despite the differences in the net charge of the SLN-DNA vectors before and after lyophilization, "in vitro" transfection levels did not undergo changes. However, an excess of positive charges when vectors are administered to living animals induces the binding to serum or plasma components such as proteins or erythrocytes, resulting in the elimination from bloodstream and a reduction in the efficacy [46].

Once it was demonstrated that the lyophilized formulations maintained the transfection capacity, a stability study was performed. Trehalose solution at 10% was chosen as lyoprotectant, because powdery samples were obtained, without the presence of microparticles after redispersion. The conditions and time of storage are summarized in Table 2.

During storage, stability of LyoSLNs depended on the conditions of temperature and humidity. The formulations maintained the particle size (Fig. 5A) during 6 and 9 months, at 30 °C/65%RH and 25 °C/60%RH, respectively. From those times, LyoSLNs suspensions became heterogeneous, with particles of 400 nm but also smaller nanoparticles of about 40 nm, indicating changes in SLNs. Zeta potential (Fig. 5B) did not change at 25 °C/60%RH, but at 30 °C/65%RH it suffered a slight and progressive increase over time. The physical appearance of the samples stored at 40 °C/75%RH was rubbery from the first month and dispersion was difficult. The presence of particles with size twice higher than at time 0 resulted in unmeasured aggregates at 6 months, fitting with a sudden decrease in the zeta potential to negative values (−2 mV). As we have mentioned above, the samples lyophilized in the presence of sugars with high $T_g$, such as trehalose, show less tendency to aggregate during storage at temperatures quite below the $T_g$, as vectors possess more restricted mobility [47]. Therefore, in our opinion the physical changes in the vectors rather than aggregation should be responsible for increase in the size. Fig. 7A shows that after 6 months, the percentage of transfected cells with (LyoSLN)-DNA increased from 19 to 40% when stored at 25 °C/60%RH and to 37% when stored at 30 °C/65%RH, decreasing to initial values after 12 months. The size does not seem to be the responsible for these differences, because when the formulations were stored at 25 °C/60%RH the increase in the transfection levels was observed at 6 and 9 months, and the differences in size were detected after 9 months of storage. Moreover, when samples were stored at 30 °C/65%RH, the improvement in transfection was observed from 3 to 9 months, and the changes in size distribution occurred after 6 months of storage.

In spite of the changes observed during the study, the DNA binding capacity was not lost (lanes 4–6 in Fig. 2), although protection was reduced. The gel electrophoresis of the samples treated with DNase I (Fig. 6) shows that the protection of DNA by LyoSLNs stored at 25 °C/60%RH and 30 °C/65%RH decreased, as the SC band was less intense and the L band and fragmented DNA appeared. We had previously observed [4] that the decrease in the protection is probably due to a lower DNA condensation, which favours the release of the plasmid improving transfection "in vitro". This lower DNA condensation, better than size, may be responsible for the higher transfection levels mentioned above. This could be an important drawback when using the formulations "in vivo", since a higher DNA release could facilitate the plasmid degradation

and thus a reduction in transfection efficacy. Furthermore, variation in transfection efficacy over time (Fig. 7A) could imply an erratic pharmacological response.

Formulations stored at 40 °C/75%RH lost their transfection capacity at 6 months probably due to the low DNA condensation, which is not only necessary to protect DNA but also to facilitate the mobility of DNA molecules through the cytoplasm to the nucleus. These formulations showed a too weak DNA condensation capacity (lane 4 in Fig. 6) necessary to protect and transport it through the cytoplasm. If condensation is not enough to direct DNA to the nucleus, posterior entry is hampered and consequently transfection decreases.

The storage of Lyo(SLN-DNA) in all conditions studied led to an important loss of transfection. In the SDS-induced DNA release study, no bands were observed in the gel electrophoresis (lanes 7, 9 and 11 in Fig. 3). The decrease in the size and the absence of free DNA after treatment with SDS indicate that the bounds between SLNs and DNA become stronger during storage. The release of DNA from vectors is necessary in the cytoplasmic compartment for the posterior entry to the nucleus and synthesis of the encoded protein [4]. Therefore, the strong DNA binding to the SLNs seems to be responsible for the lack of transfection. Moreover, the negative values of zeta potential indicate that the charges of DNA are more exposed, in spite of the high condensation degree, indicating a different DNA disposition.

This work also shows that the storage of lyophilized lipoplexes stabilized with the disaccharide trehalose did not affect cell viability (Fig. 7B and D).

## 5. Conclusions

On the basis of these results, we can conclude that lyophilization with trehalose may provide physically stable dried SLNs during 6 months at 30 °C/65%RH and 9 months at 25 °C/60%RH. This stability was lost when harder conditions are employed (40 °C/75%RH). LyoSLNs maintained or increased the transfection efficacy over time at 25 °C/60%RH and 30 °C/65%RH, but not if they were stored at 40 °C/75%RH, and Lyo(SLN-DNA) resulted in almost no transfection in all conditions. Differences in DNA condensation between the two kinds of formulations explain the differences observed in the transfection efficacy. LyoSLNs showed less DNA condensation capacity, whereas in Lyo(SLN-DNA) the plasmid became strongly bound, hampering transfection. Unlike liposomes, the increase in size occurring during the lyophilization of SLNs does not imply a reduction in the "in vitro" transfection capacity. This study shows that it is possible to prepare lyophilized SLNs easily stored in the controlled conditions, although their "in vivo" application still requires some improvements in order to extend the stability period and to control variability in transfection efficacy.

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