*Romanian Biotechnological Letters* *Vol. , No. , 2017*

**ORIGINAL PAPER**

# The effects of lyophilization and cryoprotectants on solid lipid nanoparticle-DNA systems

Received for publication, October, 27, 2015
Accepted, July, 29, 2016

**HASAN AKBABA[1], DEVRİM DEMİR DORA[2], AYŞE GÜLTEN KANTARCI[1*]**
[1]*Ege University, Faculty of Pharmacy, Department of Pharmaceutical Biotechnology, 35100, Izmir, Turkey*
[2]*Akdeniz University, Faculty of Medicine, Department of Pharmacology, 07070, Antalya, Turkey*
**Address for correspondence to: gulten.kantarci@ege.edu.tr*

## Abstract

*The development of optimized plasmid DNA delivery systems is necessary to deliver genetic material for effective gene therapy. To achieve this aim, a novel SLN:pDNA system was developed and the influence of the lyophilization procedure and the role of cryoprotectants in establishing the system's characteristics and stability were examined. SLNs were prepared by modified microemulsion dilution method. Afterward, a green fluorescent protein expression plasmid was loaded into the SLN system via electrostatic interactions. To examine the effects of cryoprotectants on the lyophilization process, sucrose, mannose, and trehalose were added at different ratios into the dilution medium; Tween 80 was also studied as an outer surfactant without cryoprotectants by incorporating it into the dilution medium. The system was characterized before and after the lyophilization procedure and tested with pDNA-loaded and unloaded SLNs. The compact form of the SLN:pDNA complexes were dissociated with cryoprotectants except %5 sucrose. Redispersing lyophilized SLNs and forming complexes with pDNA in an aseptic environment would be suitable at the application stage.*

**Keywords**: solid lipid nanoparticle, cryoprotectant, lyophilization, gene delivery

## 1. Introduction

Gene delivery systems have gained increasing interest due to a variety of functions that helps to introduce genes into cells (N. PEDERSEN & al. [1], G.M. RUBANYI & al. [2] K.F. BUCKLAND & al. [3]). An optimized transfection system is necessary for successfully delivering genetic material for gene therapy (G.M. RUBANYI & al. [2], S.H. CHOI & al. [4]). Non-viral transfection systems such as lipoplexes, polyplexes, liposomes, and nanoparticles are usually composed of cationic peptides, cationic polymers or cationic lipids, although the combinations of these are also possible (C. OLBRICH & al. [5], J.M. DANG & al. [6], S. NIMESH & al. [7], S.Y. WONG & al. [8], K. TABATT & al. [9]). Solid lipid nanoparticles (SLNs) usually consist of physiologically well-tolerated ingredients that are already approved for pharmaceutical applications in humans, are amenable to large-scale production, have good storage capabilities including freeze-drying, can be sterilized and have low cytotoxicity ( C. FREITAS & al. [10], A. HEYDENREICH & al. [11], E. MARENGO & al. [12], Y. LUO & al. [13]). In addition, one of the advantages of SLNs is that the charge of the particles can be modulated by SLN composition, thus allowing the binding of oppositely-charged molecules via electrostatic interactions (A. del POZO-RODRIGUEZ & al. [14]). Since SLNs can be produced in nano-scale sizes (10–300 nm), the particles are sufficiently small to traverse the microvascular system and prevent macrophage uptake; therefore, SLNs are suitable particularly for systemic delivery (M. GAUMET & al. [15]).

The aim of this study is to develop a novel SLN:pDNA system and evaluate the influence of the lyophilization procedure and the role of cryoprotectants on the system's characteristics

and stability. SLNs were prepared with a modified microemulsion dilution method with stearic acid as the solid lipid and Tween 80 as the non-ionic surfactant. Stearylamine and Esterquat1 (EQ1) were added to the system to give the SLNs cationic properties (A. HEYDENREICH & al. [11], W. MEHNERT & al. [16], A. del POZO-RODRIGUEZ & al. [17]). Afterward, a green fluorescent protein expression plasmid (pEGFP-C1) was loaded into the prepared SLN systems. Finally, the SLN:pDNA system was characterized while applying the lyophilization procedure at different steps during the preparation of the SLNs.

## 2. Materials and Methods

### 2.1. Materials

Plasmid which encoding green fluorescent protein (pEGFP-C1) was used at all formulations as genetic material (Invitrogen, California, USA). Ethanol, stearic acid, Tween 80, Sodium dodecyl sulfate (SDS), were provided by Merck-Co. (Hohenbrunn, Germany). Stearylamine, mannose, sucrose and trehalose were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The cationic surfactant N,N-di-(b-stearoylethyl)-N,N-dimethyl-ammonium chloride (EQ1) was provided by Gerbu Biotechnik (Gaiberg, Germany). Materials for agarose gel electrophoresis and cell culture reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ultra-pure distilled water -HPLC grade- (Up W.) was used at all formulations.

### 2.2. Methods

#### 2.2.1. Obtaining Solid Lipid Nanoparticles:

- **Preparation of Oil in Water (O/W) microemulsion systems:**

To form microemulsion systems, the following were used: stearic acid as the oil phase, Tween 80 as the non-ionic surfactant, and ethanol as the co-surfactant. Pseudoternary phase diagrams were drawn by water titration with components of the microemulsion over the lipid melting temperature ($80^oC$). The transparent regions that belong to the o/w microemulsion area in phase diagrams were determined and some transparent systems were selected for forming SLNs. Afterwards, SLNs were prepared.

- **Preparation of SLN formulations by microemulsion dilution technique:**

SLNs were developed by modified microemulsion dilution technique (E. MARENGO & al. [12]. Stearylamine and/or EQ1 were used to give the SLNs cationic properties. All microemulsion components and cationic lipids were mixed in a sealed test tube and kept in a water bath at $80^oC$ until they reached a state of equilibrium. One mL of hot microemulsion was placed in an injector, which was previously heated and dropped from 4 cm high, at a $45^o$ angle, into 9 mL of cold distilled water (at 0-2 $^oC$) in an ice water bath by stirring at 1000 rpm (R.C. DOIJAD & al. [18]). SLNs were formed when microemulsion droplets met with cold water. Therefore, the o/w microemulsion formulation was dispersed in cold ultra-pure distilled water at a ratio of 1:10 (v/v) (A. HEYDENREICH & al. [11], A. del POZO-RODRIGUEZ & al. [19], E. VIGHI & al. [20]).

To examine the effect of cryoprotectants on the lyophilization process, sucrose, mannose, and trehalose were added into the aqueous dilution medium to final concentrations of 2%, 5%, or 10% (w/v) (W. ABDELWAHED & al. [21], M. GARCIA-FUENTES & al. [22], P. SHAHGALDIAN & al. [23]). Afterward, characterization studies were carried out before and after the lyophilization process. To determine the effect of a hydrophilic surfactant in the outer membrane on SLN particle size, 2% (w/v) Tween 80 was also added to the dilution medium (R. CAVALLI & al. [24]).

### 2.2.2. Obtaining the genetic material:

pEGFP-C1 (Invitrogen, California, USA) was transformed into Escherichia coli DH5α cells and amplified (J. SAMBROOK & al. [25]). Plasmid DNA (pDNA) purification was done via a MidiPrep Plasmid DNA Isolation Kit (Thermo, California, USA).

### 2.2.3. Preparation of SLN-pDNA complexes:

pDNA (100 µg/mL) and SLNs were mixed, at ratios that ranged from 1:1 to 7:1 (SLN:pDNA) (v/v), at 150 rpm for 30 min by an orbital shaker at 25°C (IKA, Germany). To make a comparison, three different procedures were carried out as follows: 1. Freshly prepared SLNs (FreSLN) were complexed with pDNA (FreSLN:pDNA); 2. SLN:pDNA complexes were lyophilized and then redispersed [Lyo(SLN:pDNA)]; and 3. Lyophilized SLNs (LyoSLN) were redispersed and then complexed with pDNA [(LyoSLN):pDNA].

### 2.2.4. SDS Release Study:

10% (w/v) SDS solution was added to the samples at final concentrations of 1% (w/v). Samples were then analyzed by electrophoresis on agarose gels and the integrity of the pDNA in each sample was compared to untreated pDNA and unloaded SLNs as controls (A. del POZO-RODRIGUEZ & al. [14,17]).

### 2.2.5. Characterization Studies:

- **Particle Size and Zeta Potential Measurements**

The particle size of SLNs and SLN:pDNA complexes were determined by dynamic light scattering (DLS) technique with a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.). Particle size and zeta potential were measured three times and the mean values were calculated.

- **Agarose Gel Electrophoresis**

The interaction of nanoparticles with the plasmid pDNA was studied via the electrophoretic mobility of the samples in agarose gel. SLN-pDNA complexes were diluted in 25% (v/v) glycerol solution to retain them at the bottom of the agarose gel wells. Loading dye was not used because of possible interactions that would affect mobility. For each well, 0.2 µg pDNA were subjected to electrophoresis on a 0.8% (w/v) agarose gel with Tris-acetate-EDTA (TAE) 1X (Tris acetate 40 mM, EDTA 1 mM) and 0.5 µg/ml of ethidium bromide for 60 minutes at 100 V (J. SAMBROOK & al. [25]).

- **Morphology**

The morphology of the selected SLNs and SLN:DNA complexes were assessed by transmission electron microscopy (TEM) using a Philips CM110 (Eindhoven, Netherland). For measurements, one drop of diluted SLN dispersion was placed onto a 300 mesh copper grid coated with a palladium film and allowed to dry under ambient conditions for 5 minutes. The samples were visualized at an accelerating voltage of 80 kV.

### 2.2.6. Lyophilization Procedure:

Samples were frozen at -80°C. After 24 hours, frozen samples were lyophilized at -55°C and 0.07 mBar for 48 hours (Christ Alpha 1-2 LD Plus freeze-dryer, Osterode am Harz, Germany) (W. ABDELWAHED & al. [21], C. SCHWARZ & al. [26]). Lyophilized samples were redispersed in ultra-pure distilled water by gentle shaking (A. del POZO-RODRIGUEZ & al. [19], E. ZIMMERMANN & al. [27]).

## 3. Results and discussion

As shown in Figure 1, the o/w microemulsion area was determined by the aid of a triangle phase diagram, and transparent oil in water systems were formed in a particularly small area of it. Then, the selected formulation, which is coded as F1, was in the center of the transparent systems area and diluted in cold water, as mentioned in the Methods, and FreSLNs were formed and characterized.

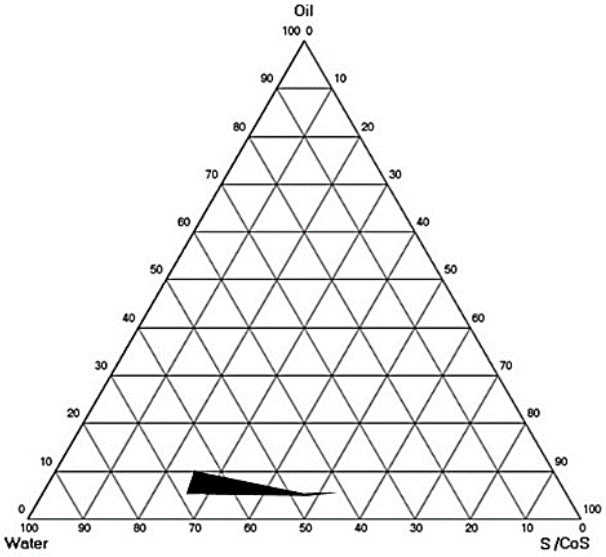


**Figure 1.** The black area shows the o/w microemulsion area with the ingredients stearic acid, Tween 80, and ethanol on a pseudoternary phase diagram.

**Table 1.** Characterization of FreSLNs obtained at different ratios of surfactant, oil, and cationic lipids.

| Formulations | Stearic acid (%) | Stearyl amine (%) | EQ 1 (%) | T80 (%) | Ethanol (%) | UP W. (%) | Z. Ave. ±SD (nm) | PDI ±SD | Zeta P. ±SD (mV) |
|---|---|---|---|---|---|---|---|---|---|
| F1 | 3 | - | - | 15 | 12 | 70 | 251 ±12.7 | 0.257 ±0.022 | -14.9 ±0.77 |
| F2 | 2 | 2 | 1 | 15 | 12 | 68 | 30.8 ±0.38 | 0.285 ±0.006 | 30.9 ±3.88 |
| F3 | 2 | 2 | 2 | 15 | 12 | 67 | 34.7 ±0.38 | 0,239 ±0,006 | 35.2 ±5,65 |
| F4 | 2 | 2 | 3 | 15 | 12 | 66 | 39.7 ±0.69 | 0.223 ±0.004 | 29.5 ±1.06 |
| F5 | 3 | 1 | - | 15 | 12 | 69 | 46.6 ±0.49 | 0.174 ±0.006 | -12.1 ±1.25 |
| F6 | 3 | 2 | - | 15 | 12 | 68 | 51.7 ±0.95 | 0.194 ±0.003 | 2.08 ±0.34 |
| F7 | 3 | 3 | - | 15 | 12 | 67 | 34.7 ±0.46 | 0.177 ±0.006 | 7.35 ±0.93 |
| F8 | 3 | - | 2 | 15 | 12 | 68 | 36,68 ±4,19 | 0,431 ±0,044 | 8,99 ±0,67 |

To add cationic properties to the SLNs, complex formation with pDNA is necessary; therefore, stearylamine and/or EQ1 were added to the F1 formulation as part of the lipid phase. After adding the cationic substance, the particle size became approximately ten times smaller (24.2-51.7 nm), which is more suitable for pDNA loading (N. ANTON & al. [28]). Zeta potential measurements showed that low values were obtained when using a single cationic lipid, while appropriate values for pDNA loading were found in formulations where stearylamine and EQ1 were used together. Therefore, formulation F3 was prepared with stearylamine and EQ1 at 1:1 (w/w) ratios, with the highest zeta potential of +35.2 mV, and selected for further studies (Table 1).

### 3.1. Lyophilization of FreSLNs

The lyophilization procedure applied to both FreSLNs and FreSLN:pDNA complexes that had either various cryoprotectants or Tween 80 as the outer surfactant. Characterization studies were performed by adding sucrose, mannose, and trehalose in the amounts shown in Table 2 and using Tween 80 in the indicated proportions at the last dispersion stage (Table 2) (W. ABDELWAHED & al. [21], R. CAVALLI & al. [24], C. SCHWARZ & al. [26]).

**Table 2.** Characterization of redispersed LyoSLNs prepared with different cryoprotectants and cryoprotectant ratios

| Cryoprotectant | % Ratio | Z. Ave. ±SD (nm) | PDI ±SD | Zeta P. ±SD (mV) |
|---|---|---|---|---|
| **Sucrose** | %10 | 262 ±15.3 | 0.62 ±0.142 | 48.8±0.49 |
| | %5 | 283.2 ±23.62 | 0.583 ±0.061 | 47 ±0.75 |
| | %2 | 451.4 ±72.4 | 0.604 ±0.152 | 50.5 ±0.71 |
| **Mannose** | %10 | 861.1 ±285.5 | 0.873 ±0.047 | 46.8 ±0.6 |
| | %5 | 559.9 ±135.9 | 0.884 ±0.106 | 47.8 ±1.8 |
| | %2 | 184.9 ±15.41 | 0.503 ±0.051 | 48.5 ±2.4 |
| **Trehalose** | %10 | 29.62 ±0.763 | 0.337 ±0.044 | 43.1 ±3.05 |
| | %5 | 37.67 ±1.295 | 0.334 ±0.043 | 49.1 ±1.77 |
| | %2 | 55.52 ±10.52 | 0.439 ±0.162 | 50.6 ±0.86 |
| **Tween 80** | %10 | 894.9 ±232.7 | 0.404 ±0.2 | 20.1 ±0.4 |
| | %5 | 941 ±49.1 | 0.693 ±0.098 | 22.8 ±0.6 |
| | %2 | 1292 ±64.05 | 0.878 ±0.043 | 29.6 ±0.49 |

When sucrose is used as the cryoprotectant, the SLN particle size decreased as the sucrose concentration increased. Particles were rather large with 2% sucrose, whereas particles were smaller than 300 nm at 5% and 10% sucrose. As a result, these systems were designed as pDNA carrier systems and the particle size will further increase after complex formation with pDNA, up to a size that may reduce its membrane penetration (K. REMAUT & al. [29]). Therefore, %5 and %10 sucrose is more appropriate for lyophilization procedure. The PDI values of formulations indicated that the particle distribution was not as homogeneous as desired with sucrose as cryoprotectant (S. KASHANIAN & al. [30]). When using mannose, the protective effect on particle size was reduced, contrary to the increasing ratios of cryoprotectant concentration. The use of 2% mannose was the only mannose amount that was adequate for further study, with a particle size of 184.9 nm (Table 2). Tween 80 was examined as an outer phase surfactant and it was found inadequate for lyophilization. Tween 80 considerably increased SLN size and the redispersion of lyophilized samples was more difficult due to the presence of Tween 80 in the medium. When trehalose was used as a cryoprotectant, the particle size of SLNs changed very little with respect to freshly prepared nanoparticles; these systems provided full protection (C. SCHWARZ & al. [26]). The PDI was within acceptable limits due to with all formulations (S. KASHANIAN & al. [30]). Where 2% trehalose was used, both particle size and PDI increased a little; therefore, 5% trehalose was considered sufficient to protect particle size and zeta potential values. In previous studies, trehalose was examined as a cryoprotectant for SLN systems at different ratios. A 10% ratio of trehalose was considered appropriate for lyophilization. del Pozo et al. [2009] observed an approximately 100nm increase in the particle size of SLNs with all applied trehalose concentrations. In another study, it was reported that 10% trehalose led to

particle sizes that were identical to those of the initial samples (R.C. DOIJAD & al. [18], C. CARRILLO & al. [31]). This study shows that 10% and 5% trehalose maintain the initial SLN particle size after lyophilization.

### 3.2. Agarose Gel Electrophoresis of FreSLNs and LyoSLNs

The complex formation ratio of FreSLN with pDNA was evaluated via the DNA pattern that depended on the quantity of plasmids that remained free in the agarose gel. The complexes were formed by mixing a fixed amount of pDNA with increasing amounts of FreSLNs and LyoSLNs, corresponding to the v/v ratio indicated in Figure 2.

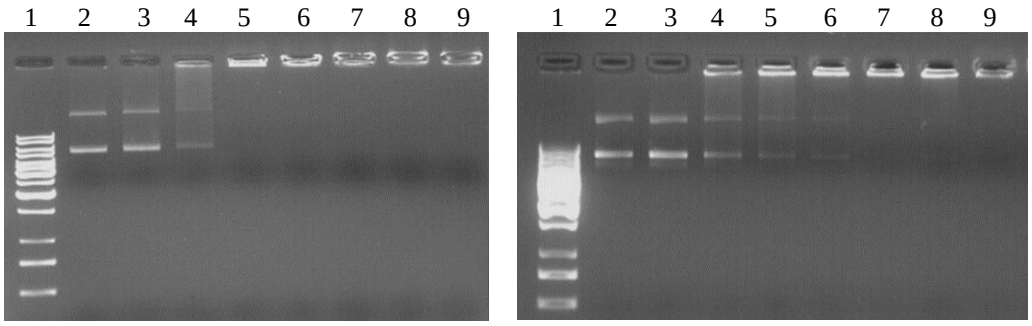


**Figure 2.** Binding of pDNA to F3 formulation. Lanes from left: 1. M.W.M. (1kb pDNA ladder) 2. pDNA, 3-9.SLN:pDNA at a ratio of 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1 (v/v). Figure 2-A. represents the FreSLNs:pDNA complexes and Figure 2-B represents (LyoSLNs):pDNA complexes without any cryoprotectant.

The efficiency of SLN:pDNA complex formation was evaluated by the amount of FreSLNs and LyoSLNs required to retard the migration of the pDNA during agarose gel electrophoresis; optimal complexation of pDNA with SLNs led to pDNA immobilization. As can be seen in Figure 2-A, the absence of pDNA in lane 6 confirms that pDNA was completely immobilized inside the FreSLN:pDNA complexes. For (LyoSLN):pDNA complexes, the absence of pDNA in the agarose gel was observed starting from lane 7 to lane 9 (Figure 2-B).

pDNA complexed with the FreSLNs and LyoSLNs at a 5:1 (v/v) ratio, which was determined by agarose gel electrophoresis; these were adequate for further studies, as shown in Figure 2. Cryoprotectants that were characterized in an earlier study and found to be effective with nanoparticles were then used with SLN:pDNA (5:1, v/v) complexes.

### 3.3. Lyophilization of FreSLN:pDNA complexes

The lyophilization of nanoparticles before complex formation with pDNA is necessary due to issues with stability and storage. The unfavorable effects of lyophilization on pDNA are already known, but the effect of lyophilization on SLN:pDNA complexes remains unclear. For this aim, some cryoprotectants were examined for their effects on nanoparticle lyophilization. The same conditions were applied to FreSLN:pDNA systems and the particle size and PDI were measured in redispersed samples (Table 3).

**Table 3.** Characterization of redispersed Lyo(SLN:pDNA) complexes prepared with different cryoprotectants and cryoprotectant ratios

| Cryoprotectant | % Ratio | Z. Ave. ±SD (nm) | PDI ±SD |
|---|---|---|---|
| **Sucrose** | 5% | 115.5 ±9.82 | 0.42 ±0.029 |
| | 2% | 1408 ±1638 | 0.962 ±0.065 |
| **Mannose** | 5% | 899.9 ±514.8 | 0.859 ±0.131 |
| | 2% | 320 ±71.52 | 0.99 ±0.017 |
| **Trehalose** | 10% | 229.6 ±57.1 | 0.935 ±0.062 |
| | 5% | 328.4 ±167.2 | 0.715 ±0.09 |
| | 2% | 696 ±119.5 | 0.596 ±0.09 |
| **None** | - | 471.8 ±183.8 | 0.796 ±0.212 |

Particle size increased with all cryoprotectants, therefore sample sizes were found smaller than 400 nm with 2% mannose, 5% sucrose, 5% and 10% trehalose (Table 3). While trehalose did provide the best cryoprotection for LyoSLNs, the lowest particle size enlargement was obtained when using 5% sucrose as the cryoprotectant for Lyo(SLN:pDNA). This study indicated that trehalose is not an appropriate cryoprotectant for the lyophilization of Lyo(SLN:pDNA) systems because it induces increases in particle size. Concerning the Lyo(SLN:pDNA) systems with 10% trehalose in the previous study, the lyophilization procedure induced an eight-fold increase in particle size (R.C. DOIJAD & al. [18]). In a previous study, the integration of trehalose to the nanoparticle was also approved with differential scanning colorimetry (DSC) (C. CARRILLO & al. [31], E. VIGHI & al. [32]).

### 3.4. Agarose Gel Electrophoresis of Lyo(SLN:pDNA)

Following the lyophilization procedure, the effects of the complex on pDNA mobility were also followed up by agarose gel electrophoresis (Figure 4).

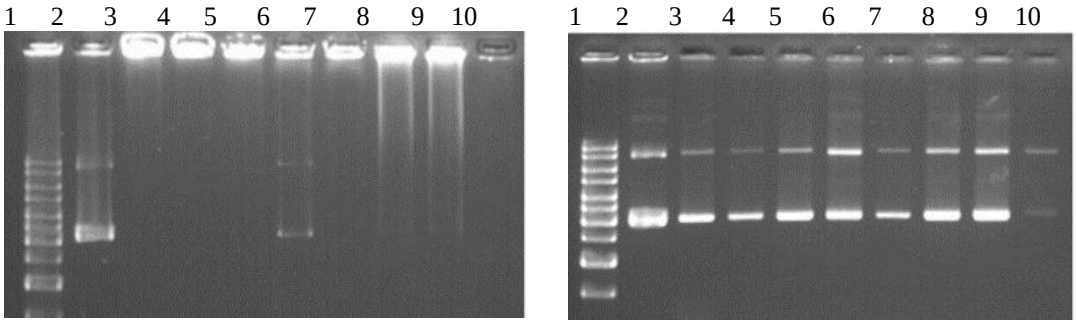


**Figure 4.** Agarose gel images of Lyo(SLN:pDNA). **A.** Lanes from left: 1. M.W.M. (1kb pDNA ladder); 2. pDNA; 3. Lyo(SLN:pDNA) with 2% mannose; 4. Lyo(SLN:pDNA) with 5% mannose; 5. Lyo(SLN:pDNA) with 2% sucrose; 6. Lyo(SLN:pDNA) with 5% sucrose; 7, 8, and 9, respectively; Lyo(SLN:pDNA) with 2%, 5%, and 10% trehalose; and 10. Lyo(SLN:pDNA) without any cryoprotectant. **B.** Figure B represents the same samples as Figure 4.A but includes the pDNA release induced by 1% SDS.

A study of the interactions with pDNA and the agarose gel mobility of the SLN:pDNA complexes should provide a better understanding of this compact structure. Figure 4 shows

agarose gel images of Lyo(SLN:pDNA) complexes with different cryoprotectants. Dissociation of the SLN:pDNA compact structure and the mobility of pDNA that left the carrier system were observed on the gel through the fluorescence of Lyo(SLN:pDNA) complexes, except in the systems prepared with 5% mannose, 2% sucrose, or 2% trehalose. When examining the gel image of the Lyo(SLN:pDNA) complex prepared with 5% sucrose, a small amount of free pDNA was observed in the gel. As these nanoparticles (prepared with 5% sucrose) continue their interaction with pDNA, though, the compact structure is maintained. This situation may be inferred from the fluorescence during pDNA movement in the gel, similar to that of the controls in well 2, and from particle size measurements. This movement is not a gradual separation, as seen clearly formulations with the 5% and 10% trehalose solutions; this movement takes the form of either total dissociation of the complex or retaining, as is, the compact structure of the complex.

pDNA was converted to its free form by adding SDS (to reach a final concentration of 1%) to the samples, as shown in the lower row of the gel images (Figure 4). In all samples, pDNA recovered in free form had a slight increase in nicks, as compared to the supercoiled forms.

To make a comparison on particle size FreSLN, FreSLN:pDNA, and (LyoSLN):pDNA were also prepared without cryoprotectants. PDI, and zeta potential values of FreSLN, LyoSLN, FreSLN:pDNA, Lyo(SLN:pDNA), and (LyoSLN):pDNA] without cryoprotectants are shown in the same table (Table 4).

**Table 4.** Particle size, PDI and zeta potential values of FreSLN, LyoSLN, [SLN:pDNA], [lyo(SLN:pDNA)], and [(LyoSLN):pDNA] without cryoprotectants.

| **Formulation 3** | **Z. Ave. ±SD (nm)** | **PDI ±SD** | **Zeta P. ±SD (mV)** |
|---|---|---|---|
| FreSLN | 34.7 ±0.38 | 0.239 ±0.006 | 35.2 ±5.65 |
| LyoSLN | 103.2 ±0.74 | 0.222 ±0.006 | 42.1 ±2.10 |
| FreSLN:pDNA | 78 ±0.70 | 0.190 ±0.008 | 7.98 ±0.59 |
| (LyoSLN):pDNA | 144.5 ±0.52 | 0.199 ±0.01 | 8.78 ±0.33 |
| Lyo(SLN:pDNA) | 471.8 ±183.8 | 0.796 ±0.212 | 7.32 ±0.65 |

### 3.5. Transmission electron microscopy of LyoSLN and (LyoSLN):pDNA complexes

To observe the structure of the SLN:pDNA complexes that were found relatively adequate for gene delivery, transmission electron microscopy was performed (Figure 5).

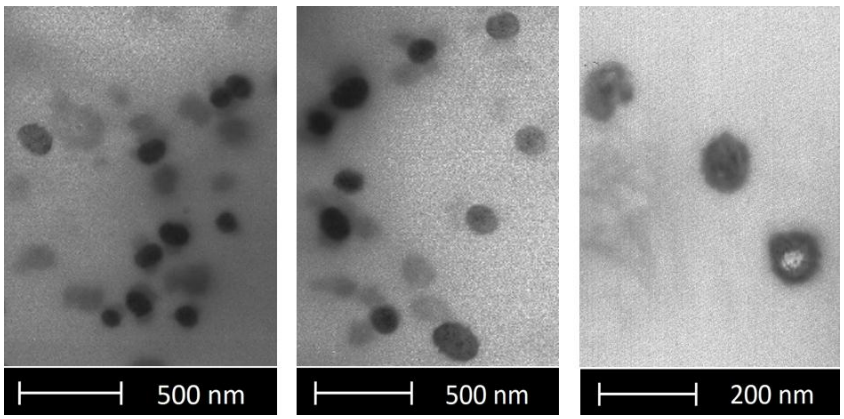


**Figure 5.** TEM observation of lyophilized nanoparticles. Image A is (LyoSLN):pDNA complexes with 10% of trehalose and images B and C are Lyo(SLN:pDNA) complexes with 5% of sucrose.

The approximate particle sizes for the images and the mean diameters of the SLNs and SLN:pDNA complexes determined by TEM were in agreement with the results obtained with DLS. Both SLN:pDNA complexes were spherical with correct dimensions. The compact structure of the SLN:pDNA complexes was maintained with the use of 5% sucrose as a cryoprotectant for the Lyo(SLN:pDNA) complex.

## 4. Conclusion

The particle sizes of LyoSLN systems with 10% trehalose as a cryoprotectant were suitable and the particle size distribution was homogeneous according to the obtained PDI values. No significant change in particle size distribution was observed after lyophilization.

Since, trehalose or mannose were used as cryoprotectants for lyophilizing FreSLN:pDNA complexes, particle size significantly increased and the compact form of the SLN:pDNA complexes was dissociated. Lyo(SLN:pDNA) complexes with 10% and 5% trehalose were enlarged, up to eight-fold of the initial particle size and PDI values were considerably high. On the other hand, using 5% sucrose as a cryoprotectant maintained the compact structure of the Lyo(SLN:pDNA) complexes, compared to other cryoprotectants.

Trehalose was found the most suitable cryoprotectant for the lyophilization step for SLNs, whereas, at the application stage, pDNA loading to redispersed SLNs (LyoSLN):pDNA in an aseptic environment would be convenient. Therefore, 5% sucrose may be preferable for lyophilization in Lyo(SLN:pDNA) systems among all cryoprotectants when the lyophilization procedure is necessary and aseptic environment is not accessible.

## 5. Acknowledgements

We are thankful to the Research Fund of Ege University, Turkey for financial support (Project No: 09-ECZ-011).

## References

1. N. PEDERSEN, S. HANSEN, A.V. HEYDENREICH, H.G. KRISTENSEN, H.S. POULSEN, Solid lipid nanoparticles can effectively bind pDNA, streptavidin and biotinylated ligands. *Eur. J. Pharm. Biopharm..*, 62:155 (2006).
2. K.F, BUCKLAND, H. BOBBY GASPAR, Gene and cell therapy for children - New medicines, new challenges? *Adv. Drug Deliv. Rev.*, 30:162 (2014).
3. G.M. RUBANYI, The future of human gene therapy. *Mol. Aspects Med.*, 22:113 (2001).
4. S.H. CHOI, S.E. JIN, M.K. LEE, S.J. LIM, J.S. PARK, B.G. KIM, Novel cationic solid lipid nanoparticles enhanced p53 gene transfer to lung cancer cells. *Eur. J. Pharm. Biopharm.*, 68:545 (2008).
5. C. OLBRICH, U. BAKOWSKY, C.M. LEHR, R.H. MULLER, C. KNEUER, Cationic solid-lipid nanoparticles can efficiently bind and transfect plasmid pDNA. *J. Control. Release*, 77:345, 355 (2001).
6. J.M. DANG, K.W. LEONG, Natural polymers for gene delivery and tissue engineering. *Adv. Drug Deliv. Rev.*, 58:487 (2006).
7. S. NIMESH, A. GOYAL, V. PAWAR, S. JAYARAMAN, P. KUMAR, R. CHANDRA, Polyethylenimine nanoparticles as efficient transfecting agents for mammalian cells. *J. Control. Release*, 110:457 (2006).
8. S.Y. WONG, J.M. PELET, D. PUTNAM, Polymer systems for gene delivery-Past, present, and future. *Prog. Polym. Sci.*, 32:799 (2007).
9. K. TABATT, C. KNEUER, M. SAMETI, C. OLBRICH, R.H. MULLER, C.M. LEHR, Transfection with different colloidal systems: comparison of solid lipid nanoparticles and liposomes. *J. Control. Release*, 97:321 (2004).

10. C. FREITAS, R.H. MÜLLER, Effect of light and temperature on zeta potential and physical stability in solid lipid nanoparticle (SLN™) dispersions. *Int. J. Pharm.*, 168:221 (1998).
11. A. HEYDENREICH, Preparation and purification of cationic solid lipid nanospheres-effects on particle size, physical stability and cell toxicity. *Int. J. Pharm.*, 254:83 (2003).
12. E. MARENGO, R. CAVALLI, O. CAPUTO, L. RODRIGUEZ, M.R. GASCO, Scale-up of the preparation process of solid lipid nanospheres. *Int. J. Pharm.*, 205:3 (2000).
13. Y. LUO, D. CHEN, L. REN, X. ZHAO, J. QIN, Solid lipid nanoparticles for enhancing vinpocetine's oral bioavailability. *J. Control. Release*, 114:53 (2006).
14. A. del POZO-RODRIGUEZ, D. DELGADO, M.A. SOLINIS, A.R. GASCON, J.L. PEDRAZ, Solid lipid nanoparticles: formulation factors affecting cell transfection capacity. *Int. J. Pharm.*, 339:261 (2007).
15. M. GAUMET, A. VARGAS, R. GURNY, F. DELIE, Nanoparticles for drug delivery: the need for precision in reporting particle size parameters. *Eur. J. Pharm. Biopharm.*, 69:1 (2008).
16. W. MEHNERT, K. MADER, Solid lipid nanoparticles Production, characterization and applications. *Adv. Drug Deliv. Rev.*, 47:165 (2001).
17. A. del POZO-RODRIGUEZ, D. DELGADO, M.A. SOLINIS, J.L. PEDRAZ, E. ECHEVARRIA, J.M. RODRIGUEZ, Solid lipid nanoparticles as potential tools for gene therapy: in vivo protein expression after intravenous administration. *Int. J. Pharm.*, 385:157 (2010).
18. R.C. DOIJAD, F.V. MANVI, D.M. GODHWANI, R. JOSEPH, N.V. DESHMUKH, Formulation and targeting efficiency of cisplatin engineered solid lipid nanoparticles. *Indian J. Pharm. Sci.*, 2008:203 (2008).
19. A. del POZO-RODRÍGUEZ, M.A. SOLINÍS, A.R. GASCÓN, J.L. PEDRAZ, Short- and long-term stability study of lyophilized solid lipid nanoparticles for gene therapy. *Eur. J. Pharm. Biopharm.*, 71:181 (2009).
20. E. VIGHI, B. RUOZI, M. MONTANARI, R. BATTINI, E. LEO, Re-dispersible cationic solid lipid nanoparticles (SLNs) freeze-dried without cryoprotectors: characterization and ability to bind the pEGFP-plasmid. *Eur. J. Pharm. Biopharm.*, 67:320 (2007).
21. W. ABDELWAHED, G. DEGOBERT, S. STAINMESSE, H. FESSI, Freeze-drying of nanoparticles: formulation, process and storage considerations. *Adv. Drug Deliv. Rev.*, 58:1688 (2006).
22. M. GARCIA-FUENTES, D. TORRES, M.J. ALONSO, New surface-modified lipid nanoparticles as delivery vehicles for salmon calcitonin. *Int. J. Pharm.*, 296:122 (2005).
23. P. SHAHGALDIAN, A study of the freeze-drying conditions of calixarene based solid lipid nanoparticles. *Eur. J. Pharm. Biopharm.*, 55:181 (2003).
24. R. CAVALLI, O. CAPUTO, M.E. CARLOTTI, M. TROTTA, C. SCARNECCHIA, M.R. GASCO, Sterilization and freeze-drying of drug-free and drug-loaded solid lipid nanoparticles. *Int. J. Pharm.*, 148:47 (1997).
25. J. SAMBROOK, D.W. RUSSELL, Molecular cloning - a laboratory manual, 3rd ed., *Cold Spring Harbor Laboratory Press, Cold Spring Harbor*, 2001.
26. C. SCHWARZ, W. MEHNERT, Freeze-drying of drug-free and drug-loaded solid lipid nanoparticles (SLN). *Int. J. Pharm.*, 157:171 (1997).
27. E. ZIMMERMANN, R.H. MÜLLER, K. MADER, Influence of different parameters on reconstitution of lyophilized SLN. *Int. J. Pharm.*, 196:211 (2000).
28. N. ANTON, J.P. BENOIT, P. SAULNIER, Design and production of nanoparticles formulated from nano-emulsion templates-a review. *J. Control. Release*, 128:185 (2008).
29. K. REMAUT, N.N. SANDERS, B.G. De GEEST, K. BRAECKMANS, J. DEMEESTER, S.C. De SMEDT, Nucleic Acid Delivery: Where material sciences and bio-sciences meet. *Mater. Sci. Eng: R.*, 58:117 (2007).
30. S. KASHANIAN, A.H. AZANDARYANI, K. DERAKHSHANDEH, New surface-modified solid lipid nanoparticles using N-glutaryl phosphatidylethanolamine as the outer shell. *Int. J. Nanomed.*, 6: 2393 (2011).
31. C. CARRILLO, N. SANCHEZ-HERNANDEZ, E. GARCIA-MONTOYA, P. PEREZ-LOZANO, J.M. SUNE-NEGRE, J.R. TICO, pDNA delivery via cationic solid lipid nanoparticles (SLNs). *Eur. J. Pharm. Sci.*, 49:157 (2013).
32. E. VIGHI, B. RUOZI, M. MONTANARI, R. BATTINI, E. LEO, pDNA condensation capacity and in vitro gene delivery properties of cationic solid lipid nanoparticles. *Int. J. Pharm.*, 389:254 (2010).
33. S.W. POXON, J.A. HUGHES, The effect of lyophilization on plasmid pDNA activity. *Pharm. Dev. Technol.*, 5:115 (2000).