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Gene transfer by histidylated lipopolyplexes: A dehydration method allowing preservation of their physicochemical parameters and transfection efficiency

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ABSTRACT

Lipid-Polycation-DNA complexes (LPD) is a promising non-viral system for nucleic acids delivery. Usually, LPD are prepared just before their use. In the present work, we have examined whether dehydration of a new type of LPD (named LPD100) might be a storage option. LPD100 comprises PEGylated histidylated polylysine/pDNA polyplexes and a liposomal formulation made with lipophosphoramidates containing N-methylimidazolium and histamine polar heads. LPD100 were dehydrated by evaporation, and the physicochemical parameters and transfection efficiency (TE) of reconstituted LPD100 were compared to that of fresh LPD100. LPD100 previously dehydrated in the presence of 20% saccharose, displayed comparable size and surface charge as freshly prepared LPD100 but gave a better TE. CryoTEM experiments showed that the reconstituted LPD100 exhibited a shape similar to fresh ones. Moreover, when LPD100 were prepared with dehydrated pDNA/polymer complexes and fresh liposomes, TE was as efficient as with fresh LPD100 while a small increase of their size were observed. These results demonstrate that evaporation of LPD100 in the presence of saccharose is a powerful method to store them for a long period of time.

1. Introduction

Gene therapy aims to use nucleic acids (DNA and RNA) as medicine for curing genetic and acquired diseases (Edelstein et al., 2007). In parallel with viral vectors, synthetic systems are intensively investigated as nucleic acid carriers (Templeton, 2009; Mintzer and Simanek, 2009). Lipopolyplexes or LPD (Lipid-Polycation-DNA) are ternary complexes made with liposomes, a cationic polymer and nucleic acids. These LPD have been proved to be efficient to transfer plasmid DNA (Gao and Huang, 1996; Li and Huang, 1997; Dileo et al., 2003; Vangasseri et al., 2005), siRNA (Chen et al., 2009a) or mRNA (Hoerr et al., 2000; Weide et al., 2008; Mockey et al., 2007). We have described LPD made with a histidine rich cationic polymer and lipids with histidine or imidazole polar head (Mockey et al., 2007; Perche et al., 2011). The plasmid DNA (pDNA) is first condensed with a PEGylated histidylated polylysine (PEG-HpK – Scheme 1). Subsequently LPD100 are formed by adding a liposomal formulation made with equimolar quantities of *O*,*O*-dioleyl-*N*-(3*N*-(*N*-methylimidazolium iodide)propylene) phosphoramidate (lipid **1** – Scheme 1) and of *O*,*O*-dioleyl-*N*-histamine phosphoramidate (lipid **2** – Scheme 1) (Perche et al., 2011). Lipid **1** possesses an N-methylimidazolium polar head conferring a permanent positive charge for DNA condensation (Mevel et al., 2008a). The imidazole group of lipid **2** can acquire a cationic charge when the pH of the medium drops bellow pH 6 (Mevel et al., 2008b). This lipid was used as co-lipid to favour endosome destabilisation and pDNA delivery in the cytosol.

LPD are usually assembled just before use rendering their conservation, handling and transportation risky. Here, we examined whether LPD100 dehydration might be a suitable storage option. Lyophilisation has been reported for lipoplexes (Chen et al., 2009b; Clement et al., 2005; Zhong et al., 2007), polyplexes (Mao et al., 2001; Brus et al., 2004; Werth et al., 2006; Hobel et al., 2008) and LPD (Sun et al., 2009). The presence of cryoprotectant and a rapid freezing of lipoplexes in liquid nitrogen before lyophilisation were found to preserve particles-sizes and the transfection efficiency upon rehydration (Yadava et al., 2008; Li et al., 2000; Anchordoquy and Koe, 2000; Maitani et al., 2008). Usually, a slow freezing of lipoplexes was recommended (Higgins et al., 1986;

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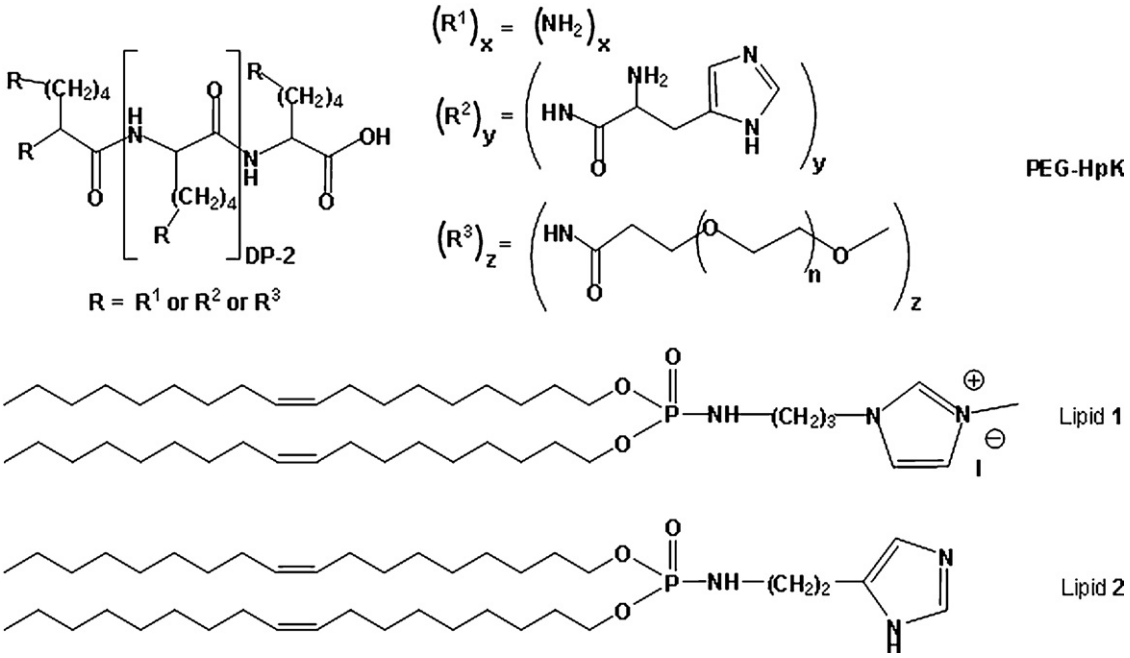

Scheme 1. chemical structures of PEGylated histidylated poly-L-lysine (PEG-HpK; degree of polymerization DP = 190; $n = 113$; $y = 95$; $x = 94$; $z = 1$), *O*,*O*-dioleyl-*N*-[3*N*-(*N*-methylimidazolium iodide)propylene] Phosphoramidate (lipid **1**) and *O*,*O*-dioleyl-*N*-histamine phosphoramidate (lipid **2**).

Mohammed et al., 2006). It was also reported that lyophilization in HEPES buffer prevents acidification during water elimination (Hobel et al., 2008). When LPD100 were slowly freeze-dried in HEPES buffer, in the absence of cryoprotectant or in the presence of either D-mannitol or saccharose, the size of reconstituted LPD100 was always bigger (from 400 nm to 1 μm) than that of the fresh ones and their transfection efficiency was less than 20% of that of fresh LPD100. While rarely employed, the evaporation with a speedvac was used for LPD dehydration (Montanari et al., 2009; Zhong et al., 2007; Sun et al., 2009; Li et al., 2000). Here, we showed that speedvac evaporation of LPD100 in the presence of saccharose is a powerful method to store them for a long period of time.

2. Materials and methods

All reagents were purchased from Sigma (St. Quentin Fallavier, France) unless otherwise stated. PEGylated histidylated poly-L-lysine (named PEG-HpK) was a polylysine of 190 lysine residues substituted with 95 histidine groups and one mPEG molecule of 5 kDa which was synthesized as previously described (Mockey et al., 2007; Midoux and Monsigny, 1999). *O*,*O*-dioleyl-*N*-[3*N*-(*N*-methylimidazolium iodide)propylene] Phosphoramidate (lipid **1**) and *O*,*O*-dioleyl-*N*-histamine phosphoramidate (lipid **2**) were synthesized as described (Mevel et al., 2008a,b).

2.1. Liposomes

Liposomes were prepared by mixing at an equimolar ratio of lipid **1** and **2** in ethanol (Perche et al., 2011). Ethanol solution was then evaporated until formation of a film. The film was hydrated for 12 h at 4 °C in 1 mL of 10 mM HEPES buffer, pH 7.4, vortexed and then the suspension was sonicated for 15 min at 20 °C at 37 kHz using an ultrasonic bath (Bioblock Scientific, Illkirch, France). Liposomes (500 μL) were dialysed (Dialysis Tubing Cellulose membrane size: 33 mm × 21 mm, MWCO 12.4 kDa, Sigma–Aldrich) at 4 °C for 6 h and then overnight against 500 mL of 10 mM HEPES buffer, pH 7.4.

2.2. Plasmid DNA

pTG11033 was a plasmid DNA of 9514 bp (kindly given by Trangène S.A., Strasbourg, France) encoding the luciferase gene of *Photinus pyralis* (Firefly luciferase, FLuc) under the cytomegalovirus (CMV) promoter. Supercoiled DNA was isolated from *E. Coli* DH5α supercompetent bacteria (Invitrogen, Cergy Pontoise, France) by alkali lysis and purification with QIAGEN Mega Kit Endotoxin free Plasmid (Qiagen, Courtaboeuf, France).

2.3. Lipopolyplexes

Polyplexes (PX) were prepared by adding dropwise PEG-HpK (15 μg in 5 μL 10 mM HEPES buffer, pH 7.4) to pDNA (5 μg in 25 μL 10 mM HEPES buffer, pH 7.4). After mixing for 4 s with vortex, the solution was kept for 30 min at 20 °C. Then, LPD100 were formed by adding liposomes (5 μL at 5.4 mM; 10 μg) to PX by up-down pipetting. The solution was kept for 15 min at 20 °C before use. For transfection with fresh LPD100, the solution was adjusted to 1 mL with serum-free medium.

2.4. Dehydration by evaporation

For dehydration, PX or LPD solution was adjusted to 500 μL with 10 mM HEPES buffer, pH 7.4. When indicated, the solution was completed with saccharose by using a 50% saccharose solution in 10 mM HEPES buffer, pH 7.4. Dehydration was carried out in 2 mL polypropylene tube during 10 h in a Speedvac (Speedvac SC110A, Savant; Refrigerated Vapor Trap RVT 400, Savant) at 35 °C under 10 mbar. Upon dehydration, LPD100 or PX were stored at −20 °C. They were reconstituted by addition of 500 μL of water and kept for 1 h at 20 °C. For transfection, reconstituted PX or LPD100 were further diluted with 500 μL serum-free medium.

2.5. DNA quantification

The quantity of pDNA in reconstituted PX and LPD100 was determined by fluorescence measurement in the presence of 12 μg/mL of ethidium bromide (Promega, Charbonnières-les-Bains, France) and 1.7 mg/mL 500 kDa dextran sulphate (Sigma) in 96 wells plates. The fluorescence intensity was measured at 620 ± 10 nm upon excitation at 531 ± 25 nm with Victor I spectrophotometer (1420 Multilabel Counter Victor, Wallac).

2.6. Size and ζ potential measurements

The size and ζ potential of PX and LPD were measured at 10 μg/mL pDNA by dynamic light scattering and electrophoretic mobility with ZetaSizer 3000 (Malvern Instruments, Orsay, France), respectively (Midoux and Monsigny, 1999). Twenty μL of LPD100 containing 10 μg pDNA were diluted with 1 mL of 10 mM HEPES buffer pH 7.4.

2.7. Cells and cell culture

Human embryonic kidney 293T7 cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in MEM medium containing 10% heat inactivated fetal bovine serum (PAA Laboratories, Les Mureaux, France), 2 mM L-glutamine (Fischer Bioblock, Illkirch, France), 100 U/mL penicillin (Fischer Bioblock), 100 U/mL streptomycin (Fischer Bioblock) and geneticin (400 μg/mL) (Brisson et al., 1999). Cells were mycoplasma-free as evidenced by MycoAlert® Mycoplasma Detection Kit (Lonza, Levallois Perret, France).

2.8. Transfection

Two days before transfection, cells were seeded in 24 well culture plates at density of 1×10^5 cells/cm^2. At the time of the transfection, the cells were 80% confluent. The cells were then incubated for 4 h at 37 °C with 0.5 mL (2.5 μg pDNA) of the transfecting solution. Then, the medium was removed, replaced by fresh complete culture medium and the cells were cultured for 44 h before measurement of the luciferase activity following a reported procedure (Midoux and Monsigny, 1999). The luciferase activity (Relative Light units, RLU) was expressed per milligram of proteins.

2.9. Cryo-transmission electronic microscopy

Samples (5 μL/10 ng) were deposited on 300-mesh holey carbon cupper grids (Ted Pella, Inc. Redding, CA). Grids were rapidly plunged into a liquid ethane bath cooled with liquid nitrogen (Leica EM CPC, Vienna, Austria) and maintained at a temperature of approximately −170 °C using a cryo-holder (Gatan, Pleasanton, CA) according to (Dubochet et al., 1988). Observations were performed under low-dose conditions using a FEI Tecnai F20 electron microscope (Eindhoven, NL) operating at 200 kV. Images were recorded with a 2 K × 2 K low scan CCD camera (Gatan).

3. Results and discussion

The transfection of HEK293T cells with a plasmid encoding luciferase was used as read-out to test the preservation of LPD100 activity upon dehydration and storage at −20 °C. Their preservation after rehydration was also evaluated by measuring their size, ζ potential and nucleic acid content. The pDNA quantification was carried out by measuring the fluorescence intensity of ethidium bromide after its intercalation into DNA. However, to measure correctly the amount of DNA contained in PX and LPD100, the fluorescence intensity was measured after dissociation of DNA complexes with a high molecular weight dextran sulphate (DS). Indeed, the fluorescence intensity of PX and LPD100 treated by DS was close (85% recovery) to that of the same amount of free pDNA whereas the fluorescence intensity of PX and LPD100 without dextran sulphate treatment was very low (11% for PX and 14% for LPD) (Fig. 1). This result indicates that this treatment is required to measure the entire DNA contained in PX and LPD100.

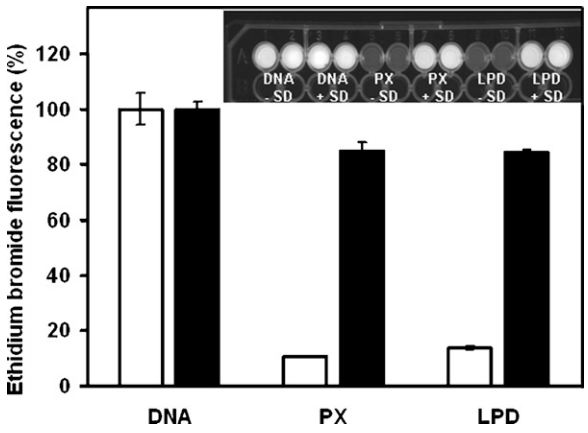

Fig. 1. Quantitative measurement of DNA in polyplexes and LPD100. The fluorescence intensity of polyplexes (PX) and LPD100 was measured in the presence of ethidium bromide without (white bars) and with (black bars) dissociation in the presence of 500 kDa dextran sulphate (DS). *Insert*: Fluorescence photography under UV light of wells containing PX or LPD100 in the presence of ethidium bromide without and with DS under.

3.1. Dehydration of lipopolyplexes

Taking into account that the freezing can cause liposomes aggregation and the freeze-thawing of liposomes can produce giant liposomes, the LPD100 dehydration was carried out by evaporation of unfrozen samples with a speedvac (Pick, 1981). The presence of saccharose has been shown allowing a better cryopreservation of LPD (Li et al., 2000; Molina et al., 2001). Thus, evaporation was performed in the presence of saccharose. Upon dehydration, the plasmid integrity has been checked by agarose electrophoresis after dissociation in the presence of DS. As shown in Fig. 2, no DNA migrated in the absence of DS indicating that the plasmid was completely associated with LPD100 upon dehydration in the presence of 10% and 20% saccharose and reconstitution. The electrophoresis upon LDP100 dissociation in the presence of DS revealed that the plasmid integrity was preserved upon dehydration in the presence of 20% saccharose. Compared to fresh LPD100, TE of reconstituted LPD100 was completely lost when LPD100 evaporation was carried out in the absence of saccharose (Fig. 3). The size of those LPD100 (370 nm) was three times bigger than that of fresh ones (130 nm) (Table 1). In contrast, when the LPD100

Table 1
Size and ζ potential of LPD100.

	Size (nm)	ζ (mV)	DNA recovery (%)[a]
Fresh LDP			
	136 ± 5.4	14 ± 2.5	
10% saccharose	144 ± 2.5	19 ± 2.6	
20% saccharose	152 ± 3	20 ± 3.3	
Dehydrated LPD			
10% saccharose	283 ± 5	15 ± 3.8	85 ± 2
20% saccharose	180 ± 31	10 ± 6.2	80 ± 3.5
LPD made with dehydrated PX			
10% saccharose	271 ± 36	31 ± 0.2	105 ± 1.5
20% saccharose	382 ± 34.5	27 ± 0.6	114 ± 1

[a]Determined by fluorescence.

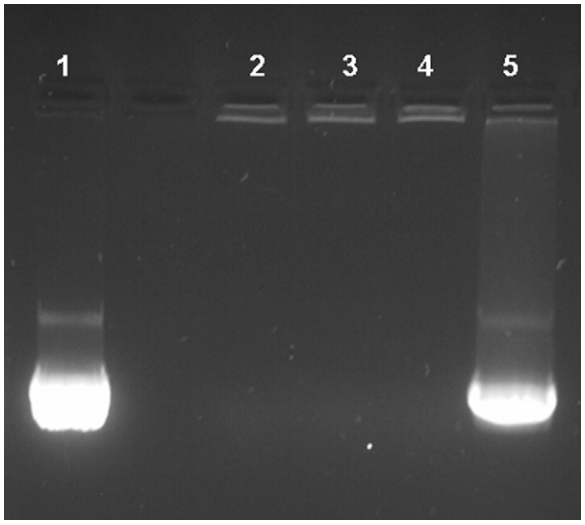

Fig. 2. Agarose gel electrophoresis shift assay of LPD100. *Lane 1*: pDNA free; lane 2 fresh LPD100; lane 3 LPD100 dehydrated in the presence of 10% saccharose; lane 4 LPD100 dehydrated in the presence of 20% saccharose and lane 5 LPD100 dehydrated in the presence of 20% saccharose and dissociated with dextran sulphate of 500 kDa. Electrophoresis was conducted under 80 V/cm through a 0.6% agarose gel containing ethidium bromide (1 μg/mL) in 95 mM Tris, 89 mM boric acid, and 2.5 mM EDTA (pH 8.6). An ultraviolet lightbox was used to visualize ethidium bromide-stained DNA in gels.

evaporation was carried out in the presence of 5% saccharose, TE of the reconstituted LPD100 was equal to that of fresh ones (Fig. 3). Moreover, when LPD100 were dehydrated in the presence of 10% and 20% saccharose, TE was much higher (2 and 6 fold higher with 10% and 20% saccharose, respectively) (Fig. 3). A similar increase of TE after dehydration of LPD and lipoplexes has been previously reported (Li et al., 2000). More importantly, the size (180 nm) and ζ potential (10 mV) of LPD100 evaporated in the presence of 20% saccharose were close to those of the fresh ones (136 nm and 14 mV) whereas 80% of DNA was recovered after reconstitution (Table 1). These common features (size and ζ potential) between fresh and reconstituted LDP100 prompted us to investigate further their morphology by electron microscopy (CryoTEM).

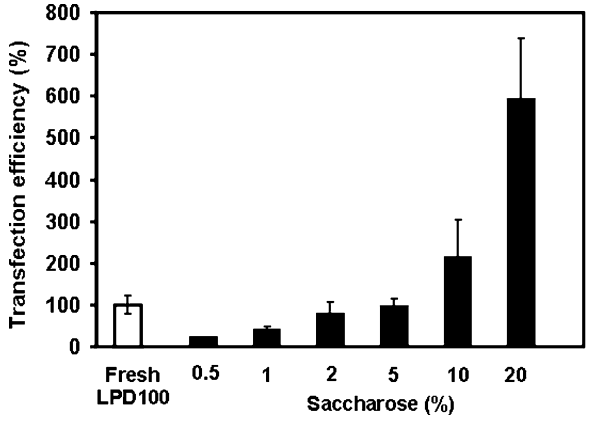

Fig. 3. Effect of evaporation on the transfection efficiency of reconstituted LPD100. 293 T7 cells were transfected with LPD100 evaporated in the absence of excipient or in the presence of saccharose. After storage for 48 h at −20 °C, water was added and LPD100 were hydrated for 1 h before use. The transfection efficiency is expressed as percent of that obtained with fresh LPD100 in the presence of the corresponding saccharose amount. The values shown are averages of three independent experiments.

3.2. Morphology of LPD100

CryoTEM observations of LPD100 with saccharose revealed the presence of globular and dense structures (Fig. 4A–C). LPD100 exhibited features resembling to typical assemblies of DNA/polymer complexes and of DNA/liposome complexes as those shown in Fig. 5. Indeed LPD100 formed intricate assemblies both composed of DNA/polymer and DNA/liposome moieties (black arrows in Fig. 4). The lamellar organization of DNA/liposome moieties appeared at the periphery of the assembly, indicating that liposomes were associated with DNA/polymer complexes with a moderate reorganization of the structure of polyplexes (Fig. 4B and C). The interactions of liposomes with DNA/polymer complexes did not lead to a complete reorganization of the DNA/polymer complexes. Interestingly, after evaporation and reconstitution, LPD100 kept a comparable size and the bipartite structure (Fig. 4D–F). Both DNA/polymer and DNA/liposome moieties remained visible, indicating that the sample treatment had less impact on the LPD100 assembly (Fig. 4E and F). This result may help to understand how the transfection efficiency is preserved after dehydration in the presence of saccharose.

LPD100 formulated in the absence of saccharose were also observed by cryoTEM (Fig. 5). While fresh LPD100 exhibited similar features than those prepared in the presence of saccharose (Fig. 6A–C), after evaporation and reconstitution, LPD100 form larger assemblies (micron-sized structure) and the multilamellar structures, typical of DNA/liposome complexes were not visible while DNA/polymer complexes was observed (Fig. 6E and F). These observations indicated that LPD100 underwent a deep structural modification during the evaporation treatment. The LPD100 assembly reorganization as well as the size increase can be a good explanation for the loss of transfection efficacy. In conclusion, the presence of saccharose does not modify the LPD100 assembly but enable the preservation of the bipartite structure made of DNA/polymer moities and of DNA/liposome moities that seems of importance for transfection efficiency.

3.3. Lipopolyplexes stability

TE of fresh LPD100 solution was not conserved more than 1 month (storage at 4 °C) since after this period of time TE was dramatically reduced (10%) when compared to fresh LPD100. Consequently, we then evaluated the stability of reconstituted LPD100 evaporated in the presence of 10% or 20% saccharose. The stability of the dehydrated powder was checked after 1 month storage at −20 °C. Rehydrated LPD100 which have been evaporated in the presence of 20% saccharose were better conserved. After one month, TE was 100% of fresh ones (Fig. 7). Comparatively, LPD100 evaporated with 10% saccharose retained only 37% of TE (Fig. 7). But their size and ζ potential were similar to that of LPD100 dehydrated in the presence of 20% saccharose (Table 1). A reduction of TE after freeze-thawing of LPD that had kept sizes of fresh ones, have been already observed (Molina et al., 2001). This suggests that the TE reduction could be due to an alteration of the LPD structure either upon dehydration or rehydration or both.

3.4. Lipopolyplexes made with dehydrated polyplexes

LPD100 were prepared with dehydrated PX and fresh liposomes in order to check that the lost of TE of dehydrated LPD100 did not come from sensitivity of PX to dehydration. It has been reported that lyophilisation of PX made with PEI or block copolymers did not impair TE (Hobel et al., 2008; Brus et al., 2004; Werth et al., 2006; Miyata et al., 2005). As expected, TE of PX was preserved after evaporation. TE of LPD100 made with PX which have been evaporated in the presence of 10% or 20% saccharose was comparable

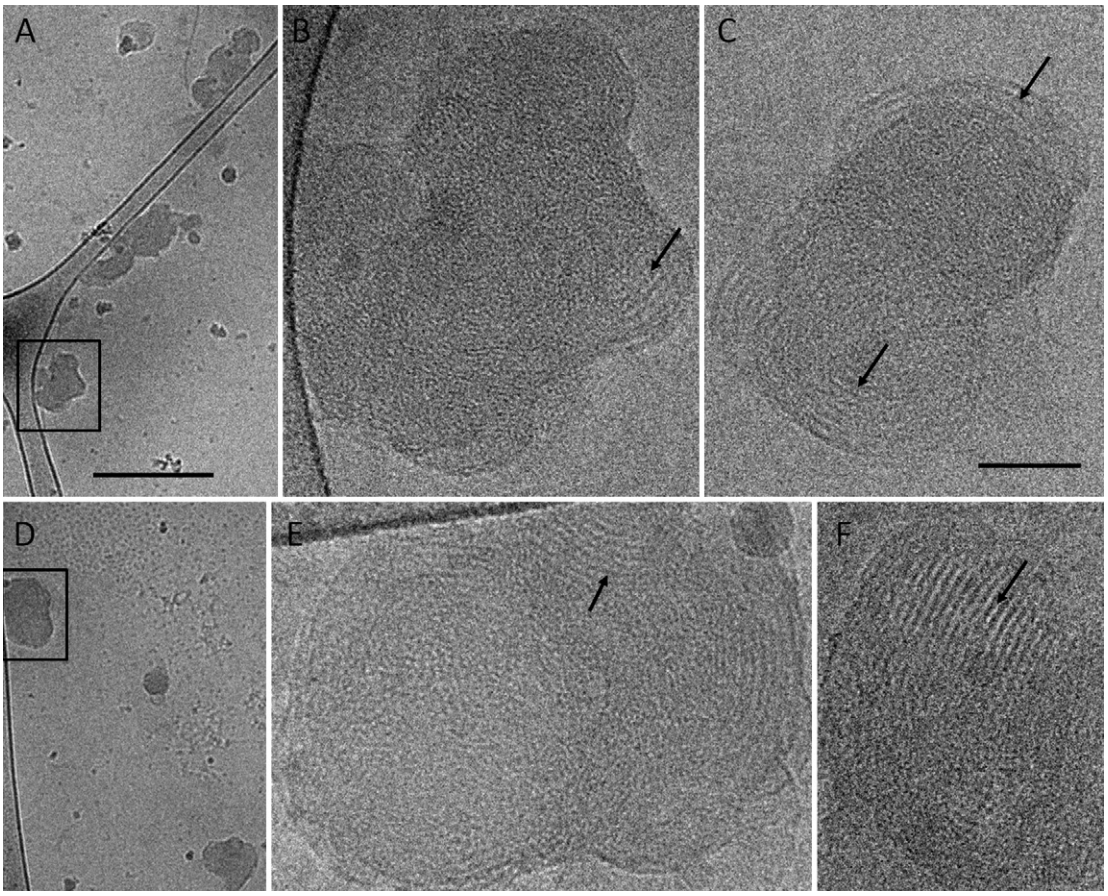

Fig. 4. CryoTEM of LPD100 in the presence of 10% saccharose. (A)–(C) Fresh LPD100 were prepared in HEPES buffer in the presence of 10% saccharose. (D)–(F) Reconstituted LPD100 after evaporation in the presence of 10% saccharose. Large views (A), (D) and enlarged areas (B), (E) marked with black squares in (A) and (D) respectively. Arrows show the typical multilamellar structures formed by the assembly of lipid moiety with DNA. *Scale bars*: 500 nm (A), (D); 50 nm (B), (C), (E), (F).

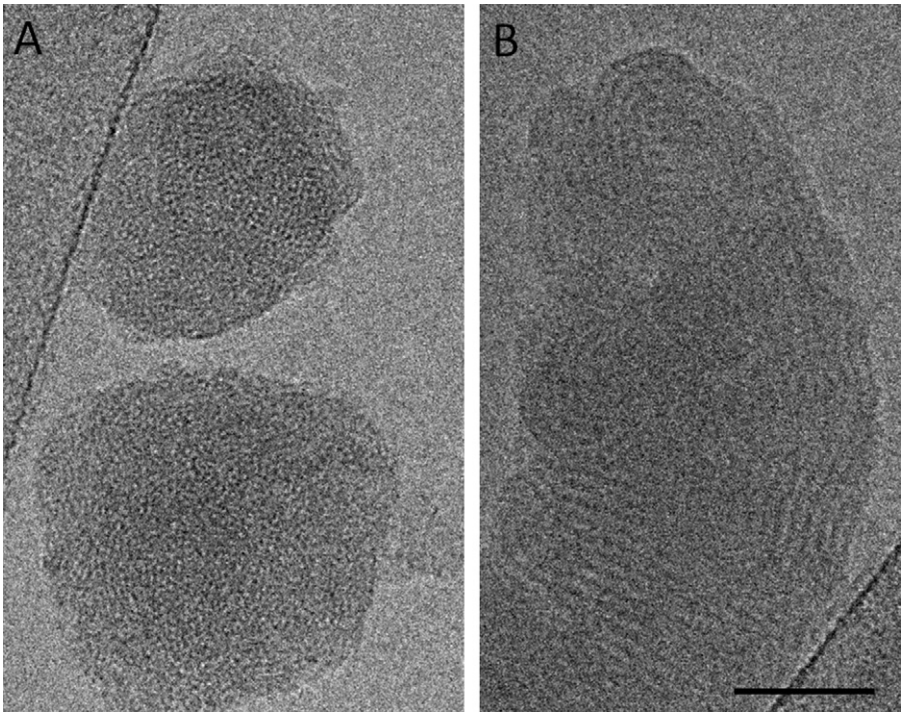

Fig. 5. CryoTEM of DNA/polymer (A) and DNA/liposome (B). Note that DNA/polymer did not form regular assembly compared to the typical 6.5 nm multilamelar structure of DNA/liposome. Scale bar 50 nm.

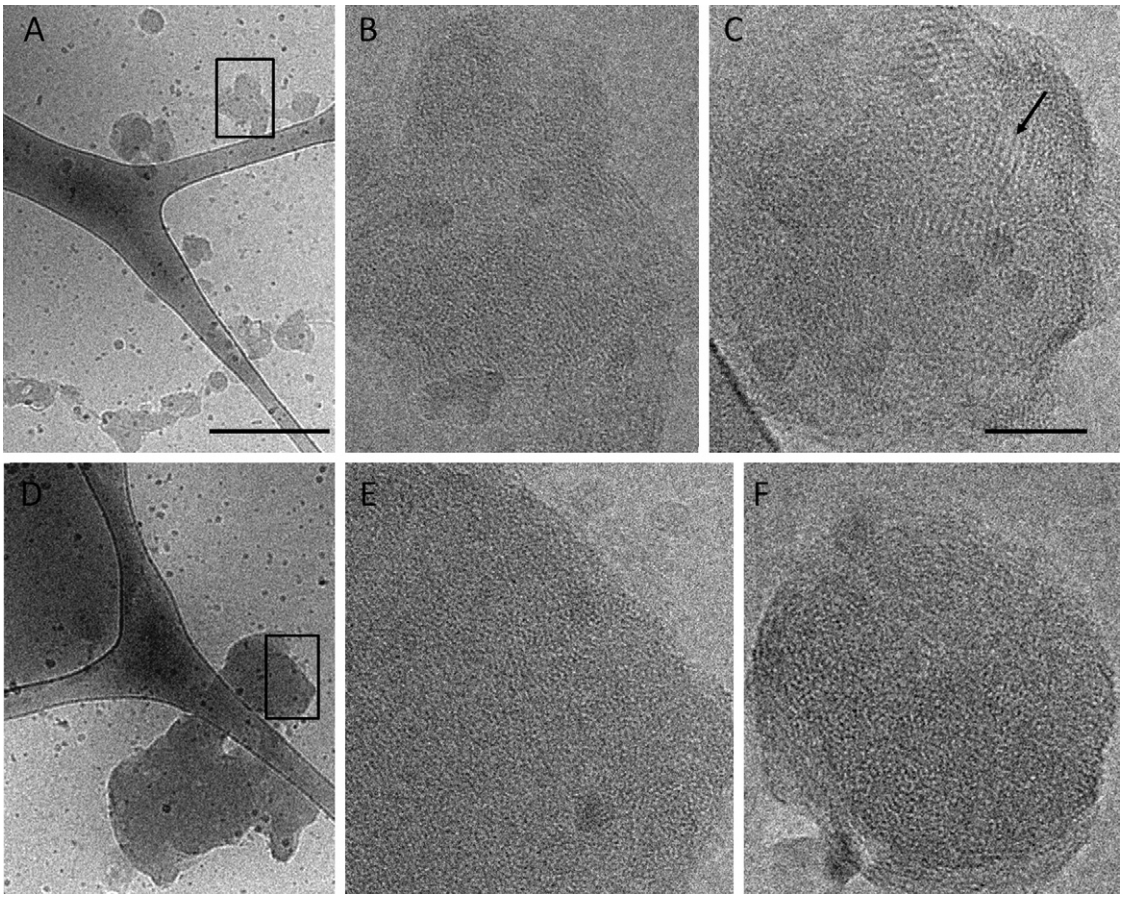

Fig. 6. CryoTEM of LPD100 in the absence of 10% saccharose. (A)–(C) Fresh LPD100 were prepared in HEPES buffer. (D)–(F) Reconstituted LPD100 after evaporation in the absence saccharose. Large views (A), (D) and enlarged areas (B), (E) marked with black squares in (A) and (D) respectively. Arrows show the typical multilamellar structures formed by the assembly of lipid moiety with DNA in fresh sample while it was rarely observed after evaporation and reconstitution. *Scale bars*: 500 nm (A), (D); 50 nm (B), (C), (E), (F).

to that of fresh ones (Fig. 8). However, the LPD100 size was 2- and 3-fold bigger than that of fresh LPD100 when PX dehydration was performed in the presence of 10% and 20% saccharose, respectively (Table 1). The saccharose concentration was probably too low to keep the size of PX unchanged. Indeed, higher sucrose/DNA ratios have been used to preserve the size of PEI polyplexes compared to that used for LPD made with protamine/DOTAP/DOPE (Molina et al., 2001). Despite their similar TE, the size of LPD100 (380 nm) made with PX which have been evaporated in presence of 20% saccharose, was two-fold bigger than that of LPD100 (180 nm) which have been evaporated with 20% saccharose. Therefore, the dehy-

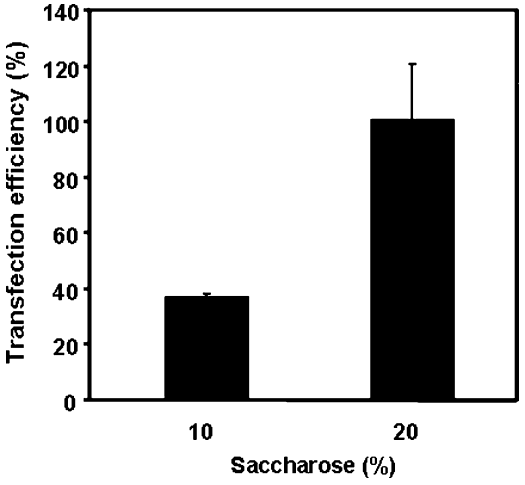

Fig. 7. Stability of dehydrated LPD100. 293 T7 cells were transfected with fresh LPD100, reconstituted LPD100 upon evaporation in the presence of 10% or 20% saccharose. Dehydrated LPD100 were kept for 30 days at −20 °C in the dark and reconstituted before the transfection. The transfection efficiency is expressed as percent of that obtained with fresh LPD100 in the presence of 10% or 20% saccharose. The values shown are averages of two independent experiments.

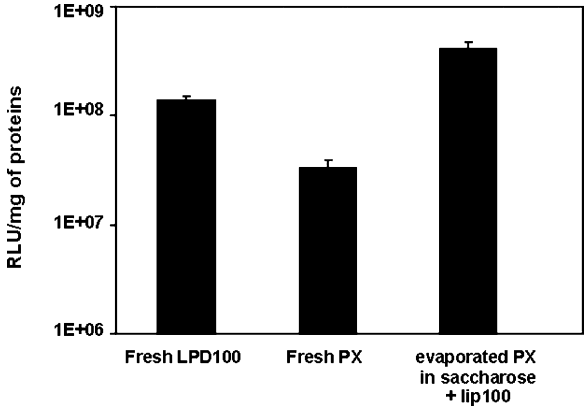

Fig. 8. Formation of LPD100 with dehydrated polyplexes. 293 T7 cells were transfected either with fresh LPD100, fresh polyplexes (PX) or LPD100 prepared with PX evaporated in the presence of 10% saccharose. Dehydrated PX was kept at −20 °C for 48 h before the preparation of LPD100 by addition of fresh liposomes 100 (lip100). The transfection efficiency is expressed in RLU/mg of proteins. The values shown are averages of two independent experiments.

dration of LPD100 allowing a reconstitution with a size less than 200 nm, is more interesting than the reconstitution of LPD100 with dehydrated PX and fresh liposomes.

4. Conclusion

Lipopolyplexes are usually assembled just before use which makes their conservation, handling and transportation risky. Here, we found that the storage of LPD100, with preservation of their physicochemical parameters, their morphology and high transfection efficiency, was obtained after dehydration by evaporation with a speedvac in the presence of 10–20% saccharose. This result will facilitate the development of LPD, especially their cross evaluation between several laboratories.

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