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► **To cite this version:**

Federico Perche, Olivier Lambert, Mathieu Berchel, Paul-Alain Jaffres, Chantal Pichon, et al.. Gene transfer by histidylated lipopolyplexes: A dehydration method allowing preservation of their physicochemical parameters and transfection efficiency. *International Journal of Pharmaceutics*, 2012, 423 (1), pp.144 - 150. <10.1016/j.ijpharm.2011.04.009>. <hal-02995807>

HAL Id: hal-02995807

<https://hal.science/hal-02995807v1>

Submitted on 3 Dec 2020

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

Abstract

Background: Lipid–Polycation–DNA complexes (LPD) are promising non-viral systems for nucleic acids delivery. However, LPD are ternary complexes which are prepared extemporaneously before use, a procedure that limits their storage and handling. We have examined whether LPD dehydration would be a storage option. *Results:* LPD were prepared by addition of a selected liposomal formulation on preformed PEGylated histidylated polylysine / pDNA polyplexes. LPD100 with O,O-dioleoyl-N-[3N-(N-methylimidazolium iodide)propylene] phosphoramidate : O,O-dioleoyl-N-histamine phosphoramidate liposomes. We measured the transfection efficiency (TE) of LPD upon lyophilisation or evaporation. TE up to 60% recovery was observed after LPD100 evaporation. Moreover, TE was preserved upon one month storage. When LPD100 were prepared with dehydrated DNA/polymer complexes and fresh liposomes, TE was as efficient as with fresh LPD. *Conclusion:* Evaporation rather than lyophilisation was a powerful mean for storage of dehydrated LPD100 containing lipophosphoramidates.

1. Introduction

Gene therapy aims to use nucleic acids as medicine for curing genetic deficiencies and a large variety of acquired diseases [1]. Nucleic acids include large plasmid DNA (pDNA), small DNA as antisense oligonucleotides and RNA as ribozymes, siRNA and mRNA. pDNA encodes either proteins, oligonucleotides, ribozymes or siRNA. Their electrostatic complexes with cationic polymers or lipids are proposed as non-viral delivery systems. Accordingly, the development of lipoplexes (complexes with cationic lipids) and polyplexes (complexes with cationic polymers) has drawn much attention over last fifteen years [2, 3]. A third emerging class of Lipid-Polycation-DNA complexes termed LPD or lipopolyplexes concerns ternary complexes made with liposomes, cationic peptides or polymers and nucleic acids. LPD made with 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) : cholesterol liposomes plus protamine sulfate or dioleoyl phosphatidylethanolamine (DOPE) : cholesteryl hemisuccinate liposomes plus polylysine were the first ones developed by Huang et al., [4, 5]. LPD were also used to deliver siRNA for silencing the epidermal growth factor receptor in lung cancer cells [6]. LPD were used as adjuvant for protein-based vaccines [7, 8] and carrier for mRNA-based vaccines [9, 10]. We also reported that LPD were efficient system for mRNA-based vaccine. For this purpose, mRNA encoding tumor antigen was complexed with a PEGylated histidine-rich polylysine and the resulting polyplexes were encapsulated in L-histidine-(N,N-di-n-hexadecylamine)ethylamide : cholesterol liposomes [11]. The histidine groups of these molecules promote membrane destabilization in acidic medium allowing nucleic acid delivery in the cytosol [12, 13]. LPD are ternary complexes that are usually prepared extemporaneously before their use, a procedure that limits storage of large quantities and would be difficult to handle by medical personnel for *in vivo* applications.

In the present study, we examined whether LPD dehydration would be an interesting storage option. We measured the transfection efficiency (TE) recovery of reconstituted LPD

upon either lyophilisation or evaporation. pDNA was first condensed with PEGylated histidylated polylysine and subsequently a liposomal formulation was added to form LPD. For LPD100, O,O-dioleoyl-N-(3N-(N-methylimidazolium iodide)propylene) phosphoramidate (1) : O,O-dioleoyl-N-histamine Phosphoramidate (3) liposomes were used [14, 15] (Scheme 1). The lipophosphoramidate 2 possesses an N-methylimidazolium polar head conferring a permanent positive charge for DNA condensation. The imidazole group of the neutral colipid 3 which can acquire a cationic charge when the pH of the medium drops below pH 6 was used to favour endosome destabilisation and pDNA delivery in the cytosol. We previously reported that lipoplexes formed with those liposomes exhibited efficient transfection [14, 15]. Lipophosphoramidates have a chemical structure which is bio-inspired from the natural phospholipids present in the cell membranes and most of these synthetic vectors proved to exhibit a moderate or low toxicity according to *in vitro* [16, 17] and *in vivo* [18, 19] transfection assays. The best preservation of LPD100 TE was obtained after evaporation rather than lyophilisation. Moreover, TE of evaporated LPD100 was preserved upon one month storage. When LPD100 were prepared with dehydrated pDNA/PEGylated histidylated polylysine complexes and fresh liposomes, TE was as efficient as with fresh ones.

2. Materials and Methods

2.1. Preparation of liposomes

Liposomes 100 (lip 100) were prepared by mixing at a molar ratio of 1:1, 1 mL of 5.4 mM O,O-dioleoyl-N-[3N-(N-methylimidazolium iodide)propylene] Phosphoramidate (1) in ethanol and 1 mL of 5.4 mM O,O-dioleoyl-N-histamine Phosphoramidate (2) in ethanol [14, 15]. Ethanol solution was 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 37 kHz at 20°C using a Bioblock ultrasonic bath (Bioblock Scientific, Illkirch, France).

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* DH5 α supercompetent bacteria (Invitrogen, Cergy Pontoise, France) by alkali lysis and purification with QIAGEN Mega Kit Endotoxin free Plasmid (Qiagen, Courtaboeuf, France).

2.3. Polymer

PEGylated histidylated poly-L-Lysine (PEG-HpK) was synthesized as previously described [11, 12].

2.4. Lipopolyplexes

Polyplexes (PX) were prepared at a DNA / polymer weight ratio of 1:3 (μg : μg) by adding dropwise PEG-HpK (15 μg in 5 μL 10 mM HEPES buffer, pH 7.4) to DNA (5 μg in 25 μL 10 mM HEPES buffer, pH 7.4) and mixing for 4 sec. After 30 min at 20°C, LPD were formed by adding liposomes (5 μL at 5.4 mM; 10 μg) to PX, a gentle mixing and letting the solution stand for 15 min at 20°C. For transfection assays with fresh LPD, the solution was adjusted to 1 mL with serum-free medium, made 0.15 M NaCl with a 5 M NaCl solution. For LPD dehydration, the solution was adjusted to 500 μL with 10 mM HEPES buffer, pH 7.4. When indicated, the solution was adjusted to 0.15 M NaCl with a 5 M NaCl solution and / or was diluted with a 50% saccharose solution (Sigma). For transfection assays with dehydrated LPD, the solution was reconstituted by adding 500 μL of water and 500 μL serum-free medium.

2.5. Lyophilization

PX and LPD (5 μg of plasmid DNA) at a final volume of 500 μL in polypropylene cryotubes closed with a cap pitted with holes made with a 25G \times 5/8" needle (Terumo, Guyancourt, France) were frozen progressively (-1°C per min) in isopropanol (>99.5%,

Fischer Scientific) using a Mr Frosty container (Dominique Dutscher, Brumath, France) placed in a -70°C freezer during 12h and freeze-dried at -55°C, 0.05 mbar with an Alpha 1-2 LD Plus lyophilizer (Christ, Osterode, Germany) and a vacuubrand rotary vane vacuum pump RZ 2.5 (Vacuubrand GMBH + CO KG, Wertheim, Germany) during 18h without secondary desiccation.

2.6. Evaporation

PX and LPD dehydration was carried out at a final volume of 500 µL in 1.5 mL polypropylene tube during 8h in a Speedvac (Speedvac SC110A, Savant; Refrigerated Vapor Trap RVT 400, Savant) at 35°C, 10 mbar. When indicated, PX and LPD were frozen progressively (-1°C per min) in isopropanol before evaporation.

2.7. DNA quantitation

The quantity of DNA in reconstituted PX and LPD was determined by fluorescence measurement in the presence of 12 µg/mL of ethidium bromide (Promega, Charbonnières-les-Bains, France) and 0.05 mg/mL 500 kDa dextran sulfate (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) after 30 min of agitation on an agitating plate.

2.8. Lipid quantitation

The lipid concentration in reconstituted LPD was determined by fluorescence measurement in the presence of Nile Red in 96 well plates. LPD (10 µL in 70 µL of 10 mM HEPES, pH 7.4) were mixed with 20 µL of a Nile Red solution (1 µM in methanol, Sigma). The mixture was agitated for 5 min at room temperature on an agitating plate. The fluorescence intensity was measured at 620/10 nm upon excitation at 531/25 nm with Victor I spectrofluorophotometer.

2.9. Size and ζ potential measurements

The size and ζ potential of PX and LPD were measured at 7 $\mu\text{g}/\text{mL}$ DNA by dynamic light scattering and electrophoretic mobility with ZetaSizer 3000 (Malvern Instruments, Orsay, France), respectively as previously described [12]. 20 μL of liposomes or LPD containing 10 μg DNA were diluted into 1.2 mL of 10 mM HEPES buffer pH 7.4.

2.10. 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) and 100 U/mL streptomycin (Fischer Bioblock) [20]. The cells were cultured in the presence of geneticin (400 mg/mL) to maintain selection of T7 transformants. Cells were mycoplasma-free as evidenced by bisbenzimidazole (Hoechst 33258, Molecular Probes) [21].

2.11. Transfections

Two days before transfection, cells were seeded in 24 well culture plates at density of 1.2×10^5 cells/cm². At the time of transfection cells were 80% confluent. Cells were then incubated for 4 h at 37°C with 0.5 mL (2.5 μg DNA) transfection solution. Then, the transfection medium was removed, replaced by fresh complete culture medium and cultured for 44 h before measurement of the luciferase activity.

2.12. Luciferase activity

Luciferase activity was measured using a Lumat LB9507 luminometer (Lumat LB9507, Berthold, Wildbach, Germany) and luciferase activity (Relative Light units, RLU) was expressed per milligrams of proteins according to [22]. The amount of proteins was determined by modified bicinchoninic assay and the luciferase activity was normalized to RLU per milligram extracted protein.

3. Results

3.1. Lyophilisation of lipopolyplexes

LPD were slowly frozen and lyophilized in HEPES buffer. The transfection of HEK293T cells with a plasmid encoding luciferase was used as read-out to test the preservation of LPD activity upon dehydration. As shown in figure 1, when LPD100 and LPD23 were freeze-dried without salt or any cryoprotectant, the luciferase activity obtained with reconstituted LPD was two orders of magnitude lower than that with fresh ones. When LPD23 were freeze-dried in the presence of 0.15 M NaCl, the luciferase activity decreased also strongly (Fig. 2). This was also the case with LPD100 (not shown). This strong decrease was not associated with a lower amount of DNA and lipids in reconstituted LPD. Indeed, the quantities of DNA and lipids before lyophilisation and after rehydration of freeze-dried LPD were equal (data not shown). Table 1 shows the effect of lyophilisation on LPD size. Fresh LPD100 exhibited an average size of 118 ± 37 nm in the absence of salts. At physiological salt concentration, fresh LPD100 formed aggregates of 1.7 μ m. Upon lyophilisation in the presence of salt, the size of reconstituted LPD100 was ~50% smaller than fresh ones. Sugars are often employed as excipient for liposomes cryopreservation. When 2 mg D-Mannitol were added to LPD23 solution before lyophilisation, TE of reconstituted LPD was better (15% of that obtained with fresh LPD) than when lyophilisation was carried out in the absence of D-Mannitol (Fig. 2). As checked by agarose gel electrophoresis, all pDNA did not migrate in the presence of 2 mg D-Mannitol meaning that sugar did not dissociate LPD (not shown). TE of LPD23 lyophilized in the presence of 0.15 M NaCl plus 2 mg D-Mannitol was a little bit more (20%) protected (Fig. 2). This was also the case for LPD100 (not shown). Note that under the latter conditions, the size of reconstituted LPD23 was small (168 nm) compared to that of LPD100 (Table 1). This small size could negatively affect their TE *in vitro* but it could be an advantage for transfection *in vivo*.

3.2. Evaporation of lipopolyplexes

Whilst rarely employed, evaporation with speedvac can be used for LPD dehydration. Taking into account that freezing step causes LPD aggregation, and evaporation of unfrozen LPD produces giant liposomes [23], the evaporation of unfrozen or frozen LPD100 was tested. The evaporation of unfrozen LPD100 carried out in the presence of 0.15 M NaCl did not drastically destroy their TE (Fig. 3). The luciferase activity was about half that of fresh LPD100 for a hydration time of 1h or 2h but was only of one third for 5 min or 30 min hydration time. The optimal hydration time for LPD recovery was at least 1 h. Next, we compared LPD evaporation in the presence of either salt, D-Mannitol or salt plus D-Mannitol. Evaporation of LPD100 in the presence of 0.15 M NaCl gave the best TE recovery (Fig. 4). The luciferase activity was half of that obtained with fresh LPD. For LPD23, TE recovery was 10% at the best (Fig. 4 insert). This protocol was reproducible since the TE of three different LPD100 preparations made at three different times was not statically different (Fig. 5). In contrast, TE recovery was lower when LPD were frozen before evaporation. The presence of D-Mannitol allowed reconstitution of small LPD100 (440 nm *versus* 1.7 μ m) (Table 1). Note that TE of the latter was 10% of that obtained with the fresh ones (Fig. 4). Reconstituted frozen LPD100 evaporated in the presence of salts exhibited a smaller size than fresh ones (~700 nm *versus* 1.7 μ m) (Table 1).

3.3 Lipopolyplexes stability

TE of fresh LPD was not conserved after 1 month storage at 4°C. Compared to fresh LPD100, TE were indeed 13%. We evaluated the stability of reconstituted LPD after evaporation in the presence of 0.15 M NaCl (Fig. 6 *mettre 1 mois direct*). The stability was checked after 1 month in solution at 4°C or -20°C. TE of LPD was also tested after reconstitution from powder conserved 1 month at -20°C. A good preservation of LPD100 (60% recovery) was observed after 1 month at -20°C either as solution or powder.

3.4 Lipopolyplexes made with dehydrated polyplexes

LPD were prepared with dehydrated PX (PEG-HpK polyplexes) and fresh liposomes to assume that the loss of TE of dehydrated LPD did not come from dehydration sensitivity of PX. It has been reported that lyophilisation of PX made with PEI or block copolymers did not impair TE [24-27]. As expected, TE of PEG-HpK PX was preserved after lyophilisation or evaporation (Fig. 7). TE of LPD100 made with freeze-dried PX were comparable and were only reduced by 30% to 40% compared to that of fresh LPD (Fig. 7 insert). Interestingly, TE of LPD100 reconstituted with evaporated PX was even 2-fold higher than fresh LPD100 (Fig. 8). Note that the optimal hydration time of PX was about 1 h.

4. Discussion

LPD prepared by the addition of a selected liposomal formulation on preformed polyplexes (PX) are promising non-viral nucleic acids delivery systems. Here, PX were formed between a PEGylated histidylated polylysine and pDNA and LPD were prepared by the addition of liposomes 100. We previously shown that Lipopolyplexes with 100 exhibited powerful DNA transfection [13-15]. Usually, LPD are prepared extemporaneously before their use, a procedure that limits storage of large quantities and would be difficult to handle by medical personnel. Therefore, the preparation of efficient dehydrated LPD would be an interesting option since only hydration will be carried out before *in vivo* administration. For this purpose, lyophilisation has been applied to lipopolyplexes [28-30], polyplexes [31-35], LPD [24, 31] and siRNA-liposomes complexes [32, 33]. In this last case, dehydration increased the stability of siRNA-liposomes complexes by immobilization and elimination of water which is a powerful oxidant (hydrolysis) and associated water. It has been also observed that the absence of cryoprotectant produces higher particles-sizes that reduce subsequently the transfection efficiency [36]. A rapid freezing of lipopolyplexes in liquid nitrogen before lyophilisation has been reported to preserve their activity after rehydration [34, 37, 38]. Here,

we decided to slowly freeze LPD according to [39, 40]. In addition, LPD were lyophilized in Hepes buffer to prevent acidification during water elimination [24]. But, the TE recovery of our LPD dramatically decreased after lyophilisation. Dehydration submits LPD to dehydration stress, vacuum stress, temperature stress and rehydration stress. These stresses can decrease when a cryoprotectant is added to protect both sides of the liposomal membrane. For this purpose, sugars and especially disaccharides which are naturally used by many organisms to increase resistance to dehydration, are employed as excipients for cryopreservation of liposomes [41]. In prokaryotes, protection by cryoprotectants is explained by mechanisms including water replacement hypothesis [42]. The particle isolation hypothesis has been proposed to explain stability of lipid/DNA complexes after lyophilisation in the presence of sugars [43]. These sugars included inulin [44], dextran [44], dextrose, galactose, mannose [34], sucrose [38, 45], trehalose [25, 34, 40, 46], alkylglycosides [47] and D- Mannitol [48]. Unfortunately, TE of LPD100 dramatically dropped (90% to 80%) upon lyophilisation in the presence of 2 mg D-Mannitol as well as in presence of salt plus 2 mg D-Mannitol. Higher concentration of D-Mannitol should have a better effect. But, we used this sugar concentration to prevent dilutions which have to be done after reconstitution to preserve isotonicity of solutions before administration [45]. We found that lyophilisation of PX formed with PEG-HpK polymer preserved their TE. When LPD were prepared with freeze-dried PX and fresh liposomes, 60% to 70% of TE was preserved for the two types of LPD meaning that dehydration of liposomes was the major hurdle. Nevertheless, PX dehydration should be a minimal option for storage.

Although lyophilisation is the main technique used for lipoplexes, PX and LPD dehydration, evaporation by speedvac is more seldom [49]. Speedvac is made of a centrifuge and a vacuum pump. The vacuum is inferior to that of a freeze-drier, so the vacuum stress is lower. Evaporation is carried out in a confined area at room temperature while lyophilisation

is done at -50°C. So, speedvac evaporation could be less stressing for LPD. In contrast to lyophilisation, we observed that evaporated LPD100 retained 50% TE upon reconstitution. LPD evaporation in the presence of 0.15 M NaCl without prior freezing and was found to be the optimal protocol for LPD100. Under this condition, the reconstitution of LPD is obtained just by adding water. Remarkably, LPD100 retained 60% transfection efficiency after one month at -20°C either as solution or powder.

The mixture of the two lipophoramidates (**1** and **2**) remaining miscible upon hydration is likely in favour of the high TE recovery upon LPD100 dehydration.

LPD with a small size and a good TE recovery *in vitro* could be obviously used for *in vivo* administration. Among them, ??? of salts and fresh liposomes exhibited good recovery. Despite their low TE recovery ?? could be effective *in vivo* due to their smaller size.

5. Conclusion

The storage of LPD100 with preservation of the transfection efficiency was obtained after dehydration by evaporation.

Acknowledgements

We warmly thank Dr Arabinda Chaudhuri (Indian Institute of Chemical Technology, Hyderabad, India) for the synthesis of L-histidine-(N,N-di-n-hexadecylamine)ethylamide.

Remercier Ulysse Asseline.

This work was supported by grant from Ligue Nationale contre le Cancer (Région Centre). FP was a recipient of a fellowship from Ligue Nationale contre le Cancer.

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Figure legends

Scheme 1: Lipid structures. **1:** O,O-dioleoyl-N-[3N-(N-methylimidazolium iodide)propylene] Phosphoramidate; **2:** O,O-dioleoyl-N-histamine Phosphoramidate.

Figure 1: Effect of LPD lyophilization on the transfection efficiency. 293T7 cells were transfected with either non lyophilised (fresh) or lyophilised (L) LPD23 (white bars) or LPD100 (black bars). The luciferase activity expressed as Relative Light Units (RLUs) per mg protein was measured after 48 h of culture. The values shown are averages of three independent experiments.

Figure 2: Effect of evaporation on the transfection efficiency of LPD. 293 T7 cells were transfected with unfrozen and evaporated LPD100 in the presence of 0.15 M NaCl. LPD100 were resuspended in water and allowed to hydrate for different times before the transfection. The transfection efficiency is expressed as percent of that obtained with fresh LPD100. The values shown are averages of three independent experiments.

Figure 3: Effect of NaCl and saccharose on the transfection efficiency of evaporated LPD. 293 T7 cells were transfected with LPD100 freshly prepared or evaporated either in the presence of 0.15 M NaCl; 10%, 20% or 30% saccharose. LPD were not frozen before evaporation. After evaporation, LPD were suspended in water and allowed to hydrate 1 h before the transfection. The transfection efficiency is expressed as percent of that obtained with fresh LPD100. The values shown are averages of three independent experiments.

Figure 4: Reproducibility of the transfection efficiency of LPD after evaporation. 293 T7 cells were transfected either with three different preparations of LPD evaporated in the presence of 0.15 M NaCl. The transfection efficiency is expressed in Relative Light Units per mg of protein. Statistical analysis was performed (Student's t-test): * P = 0.2, ** P = 0.3, *** P = 0.4. The values shown are averages of three independent experiments done with evaporated LPD (white bars) and black bar represents fresh LPD.

Figure 5: LPD stability. 293 T7 cells were transfected with fresh LPD 100, LPD 100 kept at 4°C or -20°C after preparation during 1 month without prior dehydration, LPD100 evaporated in the presence of 0.15 M NaCl. Evaporated LPD were reconstituted and kept for 30 days away from light at 4°C (S 4°C) or at -20°C (S -20°C). LPD were also kept in powder at -20°C for 30 days away from light and reconstituted before transfection (P -20°C). The transfection efficiency is expressed as percent of that obtained with fresh LPD100. The values shown are averages of three independent experiments.

Figure 6: Effect of PX dehydration on the transfection efficiency of PX and LPD. 293T7 cells were transfected with fresh PX, freeze-dried PX or evaporated PX. PX were lyophilized in the presence of 0.15 M NaCl. The luciferase activity expressed as Relative Light Units (RLUs) per mg protein was measured after 48 h of culture.

Figure 7: Effect of PX evaporation on the transfection efficiency of LPD. 293 T7 cells were transfected with LPD100 made with unfrozen and evaporated PX. PX were evaporated in the presence of 0.15 M NaCl. After evaporation, water was added and PX were hydrated for different times before LPD formation and transfection. The transfection efficiency is expressed as percent of that obtained with fresh LPD. The values shown are averages of three independent experiments.

Figure 8: Measure of pDNA by fluorescence either complexed or complexed and treated with 500 kDa dextran sulfate. White bars: without dextran sulfate, black bars: after addition of 10µL dextran sulfate.

Table I: LPD and PX size and ζ potential values.

LPD	Size (nm)	ζ (mV)	DNA recovery %
fresh LPD			
- NaCl	136 \pm 5.4	14.1 \pm 2.5	90 \pm 1.5
+ NaCl	1141 \pm 179	0.6 \pm 8.2	90 \pm 1.5
10% saccharose	144 \pm 2.5	19 \pm 2.6	87 \pm 2
20% saccharose	152.5 \pm 3	20.1 \pm 3.3	87.5 \pm 3
NaCl 10% saccharose	1458 \pm 15	-0.2 \pm 4.2	\pm
freeze-dried LPD			
- NaCl	370 \pm 200	11.5 \pm 2.8	41 \pm 0.6
+ NaCl	684 \pm 85	-1.9 \pm 2.1	69 \pm 7
10% saccharose	475 \pm 60	10.1 \pm 2	39 \pm 0.8
20% saccharose	702 \pm 490	10.8 \pm 1.5	35.5 \pm 1.6
NaCl 10% saccharose	1140 \pm 142	5.3 \pm 3.4	53 \pm 2
evaporated LPD			
- NaCl	\pm	\pm	6.5 \pm 1.7
+ NaCl	785 \pm 140	3.5 \pm 3.7	42.4 \pm 2
10% saccharose	283 \pm 5	15.5 \pm 3.8	24.5 \pm 0.7
20% saccharose	180 \pm 31	10.4 \pm 6.2	15.3 \pm 1.5
NaCl 10% saccharose	786 \pm 20	2.7 \pm 8.6	41.2 \pm 1.2
LPD made with dehydrated PX			
+ NaCl	658 \pm 42	\pm	\pm
PX	Size (nm)	ζ (mV)	DNA recovery %
fresh			
- NaCl	181 \pm 1	\pm	\pm
+ NaCl	1263 \pm 14	\pm	\pm
freeze-dried			
- NaCl	nd	\pm	\pm
+ NaCl	500 \pm 704	\pm	\pm
10% saccharose	\pm	\pm	\pm
20% saccharose	\pm	\pm	\pm
NaCl 10% saccharose	\pm	\pm	\pm