Preparation of Concentrated Chitosan/DNA Nanoparticle Formulations by Lyophilization for Gene Delivery at Clinically Relevant Dosages

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ABSTRACT

Chitosan/DNA polyplexes have been optimized for efficient and safe in vitro and in vivo gene delivery. Clinical application of this technology requires the development of formulations with higher concentrations to reach therapeutic dosages. Polyplexes were prepared using chitosan and EGFPLuc plasmids. Freeze-thawing and freeze-drying studies were performed to identify and optimize lyoprotectant and buffer contents in formulations. Freeze-dried samples were rehydrated in reduced volumes to increase their final DNA dose. Nanoparticle physicochemical properties were analyzed, and their transfection efficiency and cytotoxicity were measured in human embryonic kidney 293 cells. Data showed that 3.5 mM histidine buffer (pH 6.5) combined with one of 0.5% wt/vol sucrose, dextran 5 kDa, or trehalose was required to prevent polyplex aggregation during freeze-drying. Optimal formulations could be concentrated 20-fold, to a clinically desired ~1 mg of DNA/mL, while maintaining near physiological pH and tonicity. Polyplexes were predominantly spherical, with diameters below 200 nm, polydispersity indexes below 0.32, and zeta potentials above +19 mV. Rehydrated formulations had transfection efficiencies no less than 65% of fresh polyplexes without excipients and had no effect on viability and metabolic activity of human embryonic kidney 293 cells. These concentrated formulations represent an important step toward clinical use of chitosan-based gene delivery systems.

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further stabilizing them through hydrogen bonding with their polar groups upon water removal (water replacement hypothesis).\textsuperscript{10,12} Disaccharides, oligosaccharides, polysaccharides, and polymers have all been used as lyoprotectants to stabilize compositions during FD.\textsuperscript{12-17} Freeze-thaw studies are an efficient approach to rapidly screen for potential lyoprotectants while optimizing formulations for lyophilization.\textsuperscript{18,19} Buffers may also be used to stabilize pH in the cryoconcentrated phase, preventing nanoparticle acid hydrolysis or modification of their surface charge.\textsuperscript{20} Ideal buffers should have near-neutral pH values for clinical use, yet be slightly acidic to prevent CS/DNA polyplex aggregation upon rehydration and maintain their transfection efficiency.\textsuperscript{21,22} Phosphate, succinate, or tartrate salts must however be avoided, as they are prone to crystallization or precipitation at low temperatures, causing pH shifts of up to 4 U during lyophilization.\textsuperscript{20,23-26} L-histidine has interesting properties for lyophilization, with a pK\textsubscript{A} of 6.1 and negligible crystallization upon FD at pH between 5.5 and 6.5.\textsuperscript{27}

Attainable concentration factors are generally limited by the amounts of excipients required to preserve nanoparticle integrity during lyophilization and the need for near-isotonicity in the reconstituted formulations (e.g., 10% sucrose).\textsuperscript{28,29} Previously, investigation of lyophilization of CS/siRNA polyplexes by Andersen et al.\textsuperscript{28} suggested that 5%-10% sucrose was necessary to preserve the reconstituted formulations (e.g., 10% sucrose).\textsuperscript{30,31} Preparations for lyophilization are prone to crystallization or precipitation at low temperatures, causing pH shifts of up to 4 U during lyophilization.\textsuperscript{20,23-26} l-histidine has interesting properties for lyophilization, with a pK\textsubscript{A} of 6.1 and negligible crystallization upon FD at pH between 5.5 and 6.5.\textsuperscript{27}

The aim of this study was to develop bio-compatible, near-neutral (pH), CS/DNA polyplex compositions that could be lyophilized and rehydrated to concentrate DNA, while preserving nanoparticle physicochemical properties and biological activity, and retaining isotonicity. Therefore, preliminary freeze-thaw and FD studies were sequentially performed to respectively identify minimal concentrations of lyoprotectants, including mannitol, sucrose, dextran 5 kDa, or trehalose, along with histidine buffer required to prevent CS/DNA polyplex aggregation following lyophilization. Optimized compositions were freeze-dried and rehydrated to higher concentrations, and polyplex hydrodynamic radius, polydispersity, zeta potential (ZP), morphology, and transfection efficiency were assessed. The influence of CS and lyophilized CS/DNA polyplexes on viability and metabolic activity of human embryonic kidney 293 (HEK 293) was measured and compared to the gold standard among nonviral gene delivery vectors, branched polyethyleneimine (BPEI), and BPEI/DNA polyplexes, which are known for their toxicity in vitro.\textsuperscript{32,33} Although lyophilization is also used to increase shelf life of therapeutic agents, the long-term storage stability of these formulations will be assessed in future studies.

**Material and Methods**

**Materials**

CS was from Marinard. Deuterium oxide (Cat #151882), deuterium chloride 35% wt in deuterium oxide (Cat #543047), sodium hydroxide (Cat #S5881), sodium nitrite (Cat #431605), 1 N hydrochloric acid solution (Cat #318949), glacial acetic acid (Cat #338826), sodium azide (Cat #S2002), sodium acetate trihydrate (Cat #57670), 1 N sodium hydroxide solution (Cat #319511), mannitol (Cat #M1902), sucrose (Cat #S5389), trehalose dihydrate (Cat #T0167), l-histidine (Cat #H6034), sodium chloride (Cat #S6191), 2-(N-morpholino)ethanesulfonic acid (MES) hydrate (Cat #M2933), sodium bicarbonate (Cat #S5761), triton X-100 (Cat #T8532), and BPEI (M\textsubscript{w} = 25 kDa, Cat #408727) were from Sigma-Aldrich. Dextran 5 kDa (Cat #5510 0005 8006) was from Pharmacosmos. Serum vials (Cat #223683) and aluminum seals (Cat #22418201) were from VWR. Butyl stoppers (Cat #73828-13) were from Kimble Chase. Optitran BA-S 83 membrane (Cat #10439394) was from Whatmann. HEK 293 cells were from ATCC (ATCC #CRL 1573). Dulbecco's Modified Eagle Medium high glucose (DMEM HG, Cat #12800-017), fetal bovine serum (FBS, Cat #26140), lipofectamine (Cat #11668-019), trypsin-ethylenediaminetetraacetic acid (EDTA) (Cat #25200-056), DH5a competent cells (Cat #18263-012), and AlamarBlue Cell Viability Reagent (Cat #DAL0125) were from LifeTechnologies. Bright-Glo Luciferase Assay System (Cat #E2620) and Glo Lysis Buffer (Cat #E2661) were from Promega. BCA Protein Assay Kit (Cat #23227) was from Pierce Biotechnology. Plasmid EGFP-Luc (Cat #6169-1) and LDH Cytotoxicity Detection Kit (Cat #D30117) were from Clontech Laboratories. The EndoFree Plasmid Mega Kit (Cat #12391) was from Qiagen. Complete EDTA-free Protease Inhibitor Cocktail (Cat #11873580001) was from Roche Life Science.

**Preparation of CS/DNA Nanoparticle Formulations**

Commercial CS was heterogeneously deacetylated using concentrated sodium hydroxide and was depolymerized using nitrous acid, as previously described.\textsuperscript{34} A degree of deacetylation of 92%-93.7% was measured by \textsuperscript{1}{H} NMR, according to Lavertu et al.,\textsuperscript{36} and a number-average molecular mass (M\textsubscript{n}) of 7.5-11.3 kDa (polydispersity index [PDI] 1.23-1.52) was determined by gel permeation chromatography multiance light scattering, as described in Nguyen et al.\textsuperscript{37} Dry CS was dissolved in 28 mM HCl overnight at room temperature (RT) to a final stock solution concentration of 5 mg/mL. The stock solution was sterile filtered, then diluted to 271 μg/ml with Milli-Q water; and/or sterile mannitol, sucrose, dextran, or trehalose; and/or histidine (pH 6.5). The plasmid EGFP pluck stock solution was prepared and characterized by UV spectrophotometry, as described in Lavertu et al.\textsuperscript{38} The plasmid DNA solution was diluted to 100 μg/mL with Milli-Q water and/or excipients, as described for CS. All polyplexes were formed at CS amine to DNA phosphate ratio of 5 (N/P = 5), by mixing equal volumes of CS and DNA, pipetting CS up and down approximately 10 times immediately following addition to DNA. Samples were left to stabilize at RT for 30 min prior to use.

**Freeze-Thawing, FD, and Rehydration of Nanoparticle Suspensions**

A total of 400 μL samples were transferred to cryovials and then were frozen to −80°C overnight, using a Nalgene Mr. Frosty freezing container to maintain a cooling rate of −1°C. Samples were thawed 30 min at RT prior to use. Sample volumes of 400 μL or 2 mL were freeze-dried in 2 or 10 mL serum vials, respectively, with 13 or 20 mm butyl lyophilization stoppers. Sample trays were covered with a Whatmann Optitran BA-S 83 membrane to prevent dust or bacterial contamination. FD was carried out in a Laboratory Series Freeze-Dryer PC/PLC (Millrock Technology, Kingston, NY), using the following cycle: ramped freezing from RT to −40°C in 1 h, then maintaining isothermal at −40°C for 2 h; primary drying for 48 h at −40°C, at 100 mTorr; and secondary drying at 100 mTorr, increasing temperature to 30°C at a rate of 0.1°C/min, and then maintaining isothermal at 30°C for 6 h. Samples were backfilled with argon, stoppered, crimped, and stored at 4°C until reconstitution, 30 min prior to use. The FD temperature was fixed at −40°C based on glass transition temperatures of maximally cryoconcentrated solutions (T\textsubscript{g}) of lyoprotectants (sucrose, dextran, and trehalose), histidine (pH 6.5), and different lyoprotectant--histidine mixtures, supplied or not with CS or CS/DNA polyplexes. T\textsubscript{g} was measured using modulated dynamic scanning calorimetry. Since
the presence of CS or polynplexes had negligible effect on Tg at working concentrations, the Fox equation for multicomponent systems could be used to predict formulations Tg’ using excipient contents only.40 All formulations had Tg’ greater than or equal to −33°C, depending on the lyoprotectant used (data not shown).

All FD cakes were white and showed no collapse, although minimal retraction was seen along the edges of the vials. Samples were reconstituted to their initial concentration (Rh1X) using 400 µL or 2 mL Milli-Q water, or to 20 times their initial concentration (Rh20X) using 20 µL or 100 µL Milli-Q water. All formulations rehydrated instantaneously at Rh1X, and rehydrated in less than a minute at Rh20X. For Rh20X samples, samples were diluted back to 1X with Milli-Q water just prior to use, 30 min post reconstitution. For toxicity studies, parts of the Rh20X samples were diluted back to 10X (Dil.10X), instead of 1X (Dil.1X). Preliminary dynamic light scattering (DLS) analyses revealed that the dilution of Rh20X samples back to their initial concentration prior to analysis had no impact on nanoparticle properties (data not shown). This method was therefore implemented to circumvent possible complications associated with analysis of concentrated formulations, namely interactions between polynplexes and increased viscosities due to higher excipient, free CS, and nanoparticle content.

**Formulation pH and Osmolality**

A total of 100 µL samples (n = 3) were used to assess formulation pH using a Accumet Model 20 pH meter and a Ross glass combination microelectrode (Thermo Fisher Scientific, Waltham, MA). A total of 250 µL samples (n = 3) were used to measure osmolalities using an Advanced Model 3250 Single-Sample Osmometer (Advanced Instruments, Norwood, MA). Rh20X samples were not diluted to 1X for these analyses, but multiple samples were combined when necessary.

**Nanoparticle Size and Surface Charge Analysis**

Particle hydrodynamic diameter (Z-average and intensity mean diameter) and PDI were measured by DLS with a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK), using disposable microcuvettes. Each sample was analyzed 2 consecutive times at 25°C, where each analysis consisted of 12–20 successive readings (10 s photon counts/reading) averaged, as optimized by the instrument. Particle ZP was measured by laser Doppler velocimetry with the Zetasizer Nano ZS, using disposable capillary cells. Each sample was diluted to a volume of 800 µL and a final NaCl concentration of 10 mM, and was analyzed 3 consecutive times at 25°C. Nanoparticle ZPs were calculated from their electrophoretic mobilities using the Smoluchowski equation.41 Diluent viscosity and refractive index were adjusted according to excipient content.

**Nanoparticle Scanning Electron Microscopy Imaging**

Small volumes of sample were pulverized on polished silicon wafers using a gas spray method, and then were sputter coated with gold. Observations were performed using the high vacuum mode of a Quanta FEG 200 environmental scanning electron microscopy (SEM; FEI, Hillsboro, OR): accelerating voltage 20 kV, spot size 3, and working distance ~5 mm. Six to 10 images were taken per sample, measuring the length of 18–45 particles on each image.

**In Vitro Transfection**

Previous work showed that CS/DNA (N/P = 5) polynplexes have optimal transfection efficiency in HEK 293 cells in slightly acidic medium (pH 6.5), in the presence of 10% FBS.22 Under these conditions, polynplexes are positively charged, given their pK∞p of ~7,21 allowing for high cell uptake. Consequently, HEK 293 cells were cultured in DMEM HG supplemented with 1.85 g/L of sodium bicarbonate and 10% FBS, at 37°C in 5% CO2. Cells were maintained and subcultured according to ATCC recommendations, without any antibiotics. The 24-well culture plates were seeded with 70,000 cells in 500 µL of culture medium, and incubated 24 h to reach ~50% confluency for transfection. DMEM HG with 5 mM MES, 10 mM sodium bicarbonate, and 10% FBS, subsequently referred to as transfection medium, was equilibrated overnight at 37°C, 5% CO2 and pH was adjusted to 6.5 with 1 N sterile HCl prior to transfection. Culture medium was removed from cells and replaced with 450 µL of transfection medium and 50 µL of CS/DNA formulation, for a final DNA concentration of 2.5 µg/well. Cells were incubated 24 h with polynplexes at 37°C, in 5% CO2, and then the medium was removed and cells were replenished with 500 µL of culture medium. After an additional 24-h incubation period, cells were observed under a fluorescence microscope (Zeiss Axiovert TV 100; GmbH Germany) to monitor any morphological changes, and percentage of transfection and transgene expression levels were assessed by flow cytometry and luminometry respectively. Lipofectamine was used as positive control, as described in Lavertu et al.,1 and naked DNA was used as a negative control.

**Flow Cytometric Determination of Percentage of Transfection**

Cells were trypsinized (trypsin 0.25% EDTA) and transferred to 5–10 mL flow cytometry tubes. For each sample, enhanced green fluorescence protein (EGFP) expression was measured for 20,000 events using a MoFlo XDP cytometer (Beckman Coulter, Mississauga, ON) equipped with a 488-nm argon laser for excitation (model ENTCCII-621; Coherent, Santa Clara, CA) and a 510/20-nm (FL1) band pass filter, with photomultiplier tube voltages of 500, to detect fluorescence. Forward and side scatter were used to establish a collection gate to exclude dead cells and debris. The Summit software (V4.3.02, Build 2451) was used to determine the EGFP positive events by a standard gating technique: a control sample (cells only) was displayed on an FL1 Log versus Counts graph, and the gate drawn such that control cells were excluded. The percentage of positive events was the number of events within the gate, divided by the total number of events.

**Luminometric Determination of Luciferase Expression**

Transfected cells were washed with 100 µL phosphate buffered saline (PBS), then lysed with 100 µL of Glo Lysis Buffer. Cell lysate aliquots of 25 µL were transferred to 96-well white luminescence plates and were supplemented with an equal amount of Bright-Glo substrate prior to measurement on an Infinite M200 microplate reader (Tecan, Manndorf, Switzerland). Cell lysate aliquots of 25 µL were used to determine the protein content using BCA Protein Assay kit. The luminescence relative light units were normalized to the protein content of each sample.

**Cytotoxicity Assays**

Cells were transfected as previously described, but with the following modifications: 10,000 cells per well were seeded in 96-well flat bottom culture plate 24 h prior to transfection, then culture medium was replaced with 90 µL of equilibrated transfection medium and 10 µL of sample. BPEI/DNA polynplexes (N/P = 5) and free BPEI were used as toxic controls, and were prepared as described in Richard et al.21 Excipients only, untreated cells, and blanks were used as controls to normalize the cytotoxicity levels measured. Cytotoxicity was assessed 24 h post transfection.
Cell viability, or integrity of the cell membrane, was determined with the lactate dehydrogenase (LDH) assay. A total of 100 µL of prewarmed PBS was added to wells, samples were centrifuged 10 min at 250 g, and 80 µL of supernatants (conditioned media) were transferred to a 96-well plate. The remaining supernatant was discarded and cell pellets were resuspended in 100 µL of 1% Triton-X 100 in 1X Complete Protease Inhibitors and 100 µL of transfection medium. After incubation for 30 min at 37°C, the samples were centrifuged 10 min at 250 g, and 80 µL of supernatants (cell lysates) were transferred to a 96-well plate. Conditioned media and cell lysates were supplemented with 100 µL of LDH reaction mixture and were incubated 30 min at RT on the orbital shaker. Absorbance was then measured at 492 nm, and was subtracted with absorbance measured at the reference wavelength of 690 nm. Viability is expressed as follows:

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\text{Viability (\%) = } 100 - \frac{Abs_{med}}{Abs_{med} + Abs_{lys}}
\]  

where \(Abs_{med}\) is the absorbance of conditioned media and \(Abs_{lys}\) is the absorbance of cell lysates. Less than 10% LDH release, therefore viability above 90%, was regarded as nontoxic in our experiments.42,43

Metabolic activity was assessed by adding 10 µL of AlamarBlue reagent to each sample well and incubating an additional 4 h. Then, 80 µL of the sample was transferred from each well to a 96-well plate, absorbance was measured at 570 nm, using 600 nm as reference wavelength, and data were analyzed using the general method described by the manufacturer.

**Statistical Analysis**

Unless otherwise indicated, data were expressed as mean value ± standard deviation (\(n = 3-6\)). Statistically significant differences between groups were assessed using paired or unpaired, 2-tailed, Student t-tests. Statistically significant cytotoxicity of different groups was assessed using one-sample t-tests, with a threshold of 90%. Both tests were considered statistically significant at \(p < 0.05\).

**Results and Discussion**

**Optimization of Lyoprotectant Concentration**

Freeze-thaw studies were carried out to identify potential lyoprotectants and their minimal concentration required to prevent particle aggregation. CS/DNA nanoparticles were formed, diluted to 25 µg of DNA/mL in 0–3% wt/vol lyoprotectant, freeze-thawed once, and then analyzed in DLS (Fig. 1).

Nanoparticles were severely aggregated following freeze-thawing in the absence of lyoprotectant, whereas addition of various concentrations of lyoprotectant prevented aggregation. Mannitol had poor cryoprotective potential compared to the other 3 agents tested: at least 1% wt/vol was required to prevent large size increases post freeze-thawing, and higher PDIs were seen for all concentrations of mannitol versus other lyoprotectants. Possible crystallization of mannitol could explain this inefficiency in preventing particle aggregation.44 Sucrose and trehalose, which form amorphous phases upon freezing, preserved both nanoparticle Z-average (80-100 nm) and PDI (0.19-0.24) upon freeze-thawing in concentrations of at least 0.5% wt/vol. Dextran was large enough to scatter light in DLS, leading to underestimation of Z-average and overestimation of PDI in concentrations higher than 0.5% wt/vol so that intensity mean distributions were used to determine particle size when dextran was present. As previously published for PEI/DNA polyplexes, 0.5% wt/vol dextran was sufficient to prevent nanoparticle size increases or aggregation.33

**Optimization of Buffer Concentration**

Typically, freeze-dried compositions have buffer concentrations of 10-100 mM, to ensure buffering while minimizing pain upon injection. Rather than subjecting samples to a freeze-thaw cycle, as previously done for the optimization of lyoprotectant concentrations, optimization of buffer concentration in the formulations was done by subjecting samples to a FD and rehydration cycle. CS/DNA nanoparticles were formed with 0.5% wt/vol lyoprotectant and decreasing concentrations of histidine buffer (pH 6.5), using a final DNA concentration of 25 µg/mL. Given the pKₐ of the imidazole ring on histidine (pKₐ = 6.1), at pH 6.5 about 72% of histidine can buffer the formulation upon acidification through protonation of their imidazole ring, whereas the remaining 28% of histidine molecules are already protonated. The maximal histidine concentration used was set at 14 mM, to have approximately 10 mM of histidine available to buffer the formulation upon acidification during lyophilization. Histidine molar concentration was then gradually lowered to 3.5 mM, a molarity exceeding that of the ionizable groups of CS and DNA, the active ingredients, while being low enough to allow for significant concentration upon rehydration without exceeding 100 mM. These samples were freeze-dried, rehydrated to initial volume (Rh1X), and their Z-average and PDI were analyzed (Fig. 2).

The absence of histidine buffer led to severe aggregation of all lyophilized samples, independent of the lyoprotectant, with Z-average and PDI values above 1,900 nm and 0.9, respectively (Fig. 2). This could be due to polyplex insolubility after rehydration, the hydrochloric acid used to solubilize CS having evaporated during the FD cycle, or to hydrolysis of CS in the acidic cryocoagulated phase. The former is supported by data showing that the average pH of formulations without histidine increases from 5.8 ± 0.2, prior to FD, to 7.0 ± 0.2 after Rh1X, compared to no detectable pH change in the presence of histidine. None of the samples containing histidine and sucrose or trehalose were aggregated after Rh1X. Polyplexes in 3.5 mM histidine had significantly higher PDIs than those in 7 or 14 mM histidine following Rh1X, yet PDIs were below 0.25 for all histidine concentrations. Lower PDIs in the presence of histidine were also reported for PEI/DNA nanoparticles11 and might be due to nanoparticle stabilization through cation–π interactions between amines groups of polycations and aromatic imidazole rings of histidine molecules.33 These cation–π interactions would be greater than cation–water interaction, and further explain the stabilizing effect of histidine on nanoparticles upon FD through a mechanism analogous to the water replacement hypothesis. In the presence of dextran, decreasing histidine concentration from 14 to 3.5 mM led to significantly smaller Z-averages after Rh1X (not indicated in Fig. 2), whereas significant reductions in PDIs were seen upon decreasing histidine from 14 to 7 or 3.5 mM. Dextran having reduced conformational flexibility compared to sucrose or trehalose, its stabilizing hydrogen bond interactions with nanoparticles may be hindered by histidine in high concentrations.13,45 A balance between dextran hydrogen bonding and histidine cation–π interactions could be required to stabilize CS/DNA polyplexes during FD. About 3.5 mM histidine was sufficient to buffer all formulations, their average pH varying from 6.42 ± 0.05, prior to FD, to 6.50 ± 0.06, after Rh1X, thus ensuring solubilization of polyplexes upon rehydration.

**Optimization of Nanoparticle Concentration Upon Rehydration**

To maximize final nanoparticle concentration in samples, and possibly reach the DNA concentrations of at least 1 mg/mL typically employed in clinical trials,33 CS and DNA were diluted with the
excipients prior to polyplex formation, resulting in formulations with 50 μg of DNA/mL prior to FD, which was twice that previously used. Nanoparticle physicochemical properties were measured prior to FD, and after Rh1X and Rh20X (Fig. 3), and their sizes and morphologies were assessed by SEM (Fig. 4).

Diluting CS and DNA with excipients prior to polyplex formation had no significant impact on fresh polyplex Z-averages, but their PDIs were significantly lower than without excipients (Ctl). All formulations showed significant increases in particle size following FD (Rh1X and Rh20X; Fig. 3a), as compared to freshly prepared samples, whereas PDIs were significantly higher for polyplexes FD (Rh1X and Rh20X) in 0.5% Dex-His (3.5) and polyplexes Rh20X in 0.5% Tre-His (3.5). However, this approach allowed to double the initial DNA concentration prior to FD, from 25 to 50 μg/mL, while maintaining excipient concentrations constant. This increase in nanoparticle concentration allowed reaching a final DNA dose of 1 mg/mL following Rh20X, which is a suggested dose typically required for clinical trials.33 Rh20X formulations containing sucrose or trehalose yielded particle sizes below 200 nm, with PDIs below 0.25 (Fig. 3a), and would therefore be suitable for in vivo parenchymal cell transfection.46 As previously discussed, histidine interaction with polyplexes led to significantly lower PDIs prior to FD. SEM imaging revealed that polyplexes were more spherical in these samples due to the presence of histidine (not shown). Formulations containing excipients (lyoprotectant and histidine) had ZP varying between +19 and +21 mV, whether samples were freshly prepared, Rh1X or Rh20X; ZP was independent of the type of lyoprotectant or FD (Fig. 3b). These ZP values were significantly lower than that of fresh CS/DNA polyplexes without excipients (+32 mV) due to the buffering effect of histidine at pH 6.5, which reduced charge density on CS.4 The final average pH of Rh20X formulations was 6.48 ± 0.02. As anticipated, osmolalities of Rh20X formulations containing sucrose or trehalose were nearly isotonic, at 372 and 339 mOsm, respectively, whereas all other formulations were hypotonic (<200 mOsm).

Nanoparticles observed in SEM were mostly spherical and rod-like, whether freshly prepared or FD and rehydrated in the presence of excipients (Fig. 4). FD in the absence of excipients led to severe aggregation (Fig. 4a). Mean particle sizes measured on SEM images for fresh polyplexes were significantly different between all 3
lyoprotectants, whereas, of all the FD formulations, significant differences were only seen between Rh20X samples containing sucrose and dextran. Polyplex sizes measured ranged within the following limits: fresh nanoparticles 118-131 nm, Rh1X particles 104-108 nm, and Rh20X nanoparticles 130-142 nm. Polyplexes observed were smaller than those measured in DLS, which was expected since SEM yields number-weighted average sizes and DLS yields intensity-weighted average sizes.\(^47\)

**In Vitro Transfection**

Transfection and luciferase expression levels (Fig. 5) were expressed as a percentage of the values obtained for nanoparticles freshly prepared in the absence of excipients (Ctl), which transfected 53% of cells and had luciferase expression levels of \(6.9 \times 10^7\) relative light units/min/mg of proteins. In the absence of excipients, FD nanoparticle transfection efficiencies and luciferase expression levels were no more than 20% and 15% of Ctl, respectively. In the presence of excipients, FD formulations had important variability, whether fresh or FD, suggesting this lyoprotectant may affect cell metabolism in vitro. In terms of gene expression, sucrose seemed a better lyoprotectant than trehalose, with mean luciferase levels higher than 100% following FD, compared to trehalose-containing formulations, which had mean luciferase levels as low as 75% of Ctl after Rh20X. It is however important to mention that only Rh20X samples containing sucrose had luciferase expression levels significantly lower than the fresh samples of the same formulation.

It is currently unclear why transfection efficiency is lower post FD, with lowest transfection levels following Rh20X. Given all formulations yielded particles ranging from 100-200 nm, it is unlikely to be related to an increase in particle size. Transfection efficiency was lower following Rh1X, suggesting that the formulations would not fully protect polyplexes from the physical or chemical stresses associated with the FD and rehydration cycle. Rehydration of samples at 20-fold the initial concentration led to further decrease in transfection efficiency. At higher CS/DNA polyplex, free CS, and

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**Figure 2.** Optimization of histidine concentration for lyophilization of CS/DNA polyplexes. Polyplexes were formulated in 0.5% wt/vol sucrose, dextran, or trehalose, and 0-14 mM histidine at pH 6.5, at a final DNA concentration of 25 \(\mu\)g/mL. Samples were FD, rehydrated to initial volume (Rh1X), and their Z-average diameter and PDI were compared to those of fresh polyplexes without excipients (no lyo fresh). A total of 3.5 mM histidine was sufficient to prevent severe aggregation following Rh1X. Z-average (w) and/or PDI (x) of FD polyplexes were significantly different (\(p < 0.05\)) from fresh polyplexes without excipients (no lyo fresh). Significant differences (\(p < 0.05\)) in PDI (y) between polyplexes FD with concentrations of histidine varying from 14 to 3.5 mM. Mean ± standard deviation ( \(n = 4\)).

**Figure 3.** Optimization of nanoparticle concentration by FD and rehydration to lower volumes. Polyplexes were formulated with 0.5% wt/vol sucrose, dextran, or trehalose, and 3.5 mM histidine at pH 6.5, namely 0.5% Suc-His(3.5), 0.5% Dex-His(3.5), and 0.5% Tre-His(3.5), at a DNA concentration of 50 \(\mu\)g/mL. Formulations were freeze-dried, rehydrated to initial (Rh1X) or to 20-fold the initial concentration (Rh20X), and their physicochemical properties were compared to those of fresh polyplexes without excipient (Ctl). Formulations could be concentrated 20-fold without particle aggregation. PDI (w) and/or ZP (x) of fresh polyplexes and FD polyplexes in excipients were significantly different (\(p < 0.05\)) from fresh NPs in the same excipients. Mean ± standard deviation (\(n = 6\)).
histidine concentrations, formulation colloidal stability may be affected, leading to irreversible modifications of the nanoparticles. A hypothesis that would require further investigation is that the FD process itself, or rehydration to higher concentrations (Rh20X), would lead to a decrease in free CS in the formulations, the polycations accumulating at the surface of the polyplexes. This would provide an explanation for the increases in particle size observed after FD, and could potentially be responsible for the decrease in transfection efficiency, as previous work showed free CS is required to successfully transfect HEK 293 cells in vitro.48

In Vitro Cytotoxicity

Although both sucrose and trehalose-containing formulations have great potential for in vivo applications, given their near-isotonicity and their high transfection efficiency following Rh20X, trehalose was the preferred lyoprotectant retained for in vitro toxicity assays due to its superior properties with regard to stability in FD form, namely its higher glass transition temperature.49 Fresh, Rh1X, or Rh20X CS/DNA nanoparticles formulated in 0.5% wt/vol trehalose and 3.5 mM histidine (pH 6.5) were prepared. Of the Rh20X samples, part were diluted back to 1X prior to addition to cells (Rh20X-Dil.1X), and part were diluted back to 10X (Rh20X-Dil.10X), therefore subjecting cells to a polyplex concentration 10 times higher than those incubated with fresh, Rh1X, or Rh20X-Dil.1X samples. CS/DNA polyplex formulations tested had no significant toxic effect on HEK 293 cells in vitro, with viability and metabolic activity levels at least 90% that of nontransfected samples incubated with corresponding excipient concentrations (Fig. 6a). These results are in accordance with our previously published AlamarBlue data for CS/DNA polyplexes freshly prepared without excipients.22 Increasing CS/DNA formulation 10-fold had no significant impact on cell viability. Free CS cytotoxicity was also assessed since it represents the major form of CS upon CS/DNA formation at N/P ratio of 5 or above.21,50 As previously reported,51 low Mn CS showed no significant in vitro toxicity at the concentrations tested (14 and 68 μg/mL).

Two BPEI/DNA nanoparticle (N/P = 5) formulations were tested on cells: the former was prepared at same DNA concentration as fresh, Rh1X, or Rh20X-Dil.1X CS/DNA polyplexes, the latter at 10 times that concentration, therefore the same DNA concentration as Rh20X-Dil.10X CS/DNA polyplexes. The low concentration BPEI/DNA formulation (3.3 μg of BPEI and 5 μg of DNA/mL of media) showed no significant cell toxicity, whereas the more concentrated BPEI/DNA

Figure 4. SEM images of CS/DNA polyplexes formulated without or with excipients, after FD and rehydration to 20-fold the initial concentration (Rh20X). Particles are severely aggregated in the absence of excipients (a), whereas their morphology was more spherical and their sizes were maintained in the presence of both 0.5% wt/vol sucrose (0.5%Suc), dextran (0.5%Dex), or trehalose (0.5%Tre), and 3.5 mM histidine at pH 6.5 (His (3.5)); (b) 0.5% Suc-His(3.5)-Rh20X, 142 ± 45 nm (n = 326); (c) 0.5% Dex-His(3.5)-Rh20X, 130 ± 41 nm (n = 149); and (d) 0.5% Tre-His(3.5)-Rh20X, 135 ± 39 nm (n = 160).
formulation (33 μg of BPEI and 50 μg of DNA/mL of media) showed metabolic activity significantly lower than 90%. Previous data reported higher in vitro cytotoxicities for PEI/DNA polyplexes, for a similar PEI dose as the highest shown in Figure 6a.37 These polyplexes were however prepared at N/P ratio of 23, representing much higher doses of free PEI in the samples, thus partly explaining their higher cytotoxicities.37,52 More importantly, cells incubated with the same CS/DNA polyplex concentration (50 μg of DNA/mL of media) had significantly higher metabolic activity despite a CS concentration 4.25-fold higher than that of BPEI in the formulations. Diluted-free BPEI (25 kDa) at 3.3 μg/mL was nontoxic to HEK 293 cells, whereas concentrations of 33 and 65 μg/mL of media led to significant toxicity, with cell viabilities of 68% and metabolic activities of 31%. Both cell viability and metabolic activity of free CS at 68 μg/mL were significantly higher than those of free BPEI at 65 or even 33 μg/mL. Overall, CS (8.3 kDa, 92.5% degree of deacetylation) was less cytotoxic than BPEI (25 kDa), whether bound to DNA or not.

Conclusions

This study identified formulations that preserve CS/DNA polyplex physicochemical properties and biological activity after lyophilization using minimal amounts of both lyoprotectant and buffer. Nanoparticles lyophilized in 0.5% wt/vol sucrose, dextran, or trehalose, and 3.5 mM histidine at pH 6.5. Formulations were freshly prepared, or freeze-dried and rehydrated to initial (Rh1X) or to 20-fold the initial concentration (Rh20X). Results are expressed as a percentage of the values obtained for fresh polyplexes without excipient. Lyoprotectant and buffer were necessary to preserve transfection efficiencies and luciferase expression levels after FD. Transfection efficiencies were similar between formulations; luciferase expression was highest with sucrose and most variable with free BPEI (25 kDa) at 3.3 μg/mL, significantly higher with sucrose and trehalose-containing formulations. Luciferase expression (formulations; luciferase expression was highest with sucrose and most variable with free BPEI (25 kDa) at 3.3 μg/mL was nontoxic to HEK 293 cells, whereas concentrations of 33 and 65 μg/mL of media led to significant toxicity, with cell viabilities of 68% and metabolic activities of 31%. Both cell viability and metabolic activity of free CS at 68 μg/mL were significantly higher than those of free BPEI at 65 or even 33 μg/mL. Overall, CS (8.3 kDa, 92.5% degree of deacetylation) was less cytotoxic than BPEI (25 kDa), whether bound to DNA or not.

analysis of the stability profiles of these formulations over short- and long-term storage, as well as evaluation of their in vivo efficiency, will advance these CS/DNA gene delivery systems toward clinical use.
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