Lyophilisation and concentration of chitosan polyelectrolyte complexes for delivery of DNA and siRNA

D. Veilleux1, A. Tavakoli Naeini1, M. Lavertu1, K. Biniecki2, and M. D. Buschmann1

1Ecole Polytechnique de Montreal, Montreal, QC, H3T 1J4, Canada;
2ANRis Pharmaceuticals Inc, Kirkland, QC, H9H 4R9, Canada
daniel.veilleux@polymtl.ca

ABSTRACT SUMMARY
Development of stable chitosan (CS)/DNA or siRNA nanoparticle (NP) formulations at therapeutic doses is required for clinical applications. Freeze-dried (FD) formulations containing lyoprotectants and/or a buffer were developed, and their physicochemical properties, in vitro tranfection or silencing efficiencies, and cytotoxicities were assessed. FD Formulations with 0.5% (w/V) sucrose or trehalose and 3.44mM histidine could be concentrated upon rehydration to reach high nucleic acid concentrations (> 1 mg/mL), while remaining near-isotonic. Physico-chemical properties and in vitro biological activity of these NPs were maintained post FD, while having low cytotoxicity.

INTRODUCTION
Polymer/nucleic acid NP using CS have been optimised for efficient and safe delivery of plasmid DNA and siRNA, in vitro and in vivo. [1-6] However, these polyelectrolyte complexes require preparation in dilute conditions (100 µg/mL nucleic acid) to produce small uniformly sized nanoparticles which typically display limited stability in solution due to physical or chemical degradation over long-term storage in aqueous environments [7,8]. Further development of this technology for clinical and commercial applications therefore requires stabilisation through lyophilisation. This process also offers the possibility of increasing NP concentrations in formulations by rehydrating lyophilizates in reduced volumes prior to injection. Specific compositions and methods were identified in this study to retain structural properties and biological activity of lyophylized chitosan nucleic acid nanoparticles, and to permit concentration upon rehydration to achieve high doses while remaining near-isotonic.

EXPERIMENTAL METHODS
NPs were prepared using a 10kDa CS and the plasmid eGFPLuc (DNA) or the anti-eGFP siRNA. NPs were formulated with or without 0.5% (w/V) lyoprotectant (sucrose, dextran 5kDa, or trehalose) and/or buffer (3.44mM histidine at pH 6.5). Formulations were subjected or not to FD, and rehydrated to the initial volume (Rh1X) or in lower volumes, to reach a concentration factor of 20-fold (Rh20X). NP physicochemical properties were assessed: size and polydispersity index (PDI) were measured by Dynamic Light Scattering (DLS); zeta potential, by Laser Doppler Velocimetry; and morphology, by Scanning Electron Microscopy (SEM). Formulation final osmolalities were measured by freezing point depression (FPD). In vitro transfection efficiency of CS/DNA NP was quantified using HEK293 cells, measuring the expression of eGFP and luciferase by flow cytometry and luminometry respectively; in vitro silencing of CS/siRNA NP was assessed by flow cytometry using eGFP positive H1299 cells. Formulation cytotoxicity was measured in vitro using the lactate dehydrogenase (LDH) assay, the alamarBlue assay, and the annexin V/propidium iodide apoptosis assay. All assays were performed twice (N=2) on triplicates (n=3) for each formulation.

RESULTS AND DISCUSSION
We found that both lyoprotectant and buffer were required to prevent CS/DNA or CS/siRNA NP aggregation following FD. Physicochemical properties of NPs were similar, independent of the nature of the nucleic acid used: hydrodynamic diameters ranged from 125 to 235nm, with PDI values from 0.15 to 0.25 and zeta potentials from +20 to +26mV. Optimal formulations had osmolalities between 339 and 372 mOsm after Rh20X, and their NP were mainly spherical (Figure 1D). The addition of minimal amounts of histidine at pH 6.5 increased NP stabilization upon FD at low lyoprotectant contents. This may be due to the buffering effect of histidine, which could prevent CS hydrolysis during FD while stabilizing formulation pH pre- and post- FD, but may also be the result of electrostatic interactions with NP, as suggested by their lower PDI and increased sphericity prior to FD (Figure 1C). Addition of excipients to formulations had little effect on the transfection efficiency of fresh CS/DNA NPs, with levels above 90% of fresh NPs without excipient (Figure 2A). Both lyoprotectant and buffer were required to maintain
transfection and luciferase levels of at least 50% of control after FD (Figure 2). Dextran was the least effective lyoprotectant, with luciferase expression equivalent to only 12% of control after Rh20X (Figure 2B). Similar results were obtained with CS/siRNA NP, with silencing efficiencies ranging from 60 to 75%, whether formulations were freshly prepared or FD (Rh1X or Rh20X). Excipients or FD had no impact on NP cytotoxicity, which remained below 25% loss of alamarBlue assessed metabolic activity.

CONCLUSION
The current study identified two compositions, 0.5% (w/V) sucrose or trehalose with 3.5mM histidine, that met the requirements of near-isotonicity and dose of 1 mg/mL of nucleic acid after Rh20X. NP physicochemical properties were conserved (Z-average < 250 nm; PDI ≤ 0.3; Zeta potential ≥ 20 mV) as well as in vitro biological activity (transfection and luciferase levels, or silencing efficiency, near or greater than 50% of controls), and maintained low toxicity levels. These novel biocompatible DNA and RNA delivery systems permit broad applications, increased doses, and improved storage properties. Characterization of these systems during accelerated and long term stability studies is ongoing.

REFERENCES

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