Investigations on Polyplex Stability During the Freezing Step of Lyophilization Using Controlled Ice Nucleation—The Importance of Residence Time in the Low-Viscosity Fluid State

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ABSTRACT: The aim of the study was to comprehensively investigate the influence of the freezing step during lyophilization on the stability of gene-delivery particles in order to better understand particle stabilization during freezing. Particle size of plasmid/linear polyethylenimine (LPEI) polyplexes at two DNA concentrations and at increasing sucrose–DNA ratios was investigated separately as a function of freezing procedure, ice-nucleation temperature, residence time of the particles in a partially frozen state, or incomplete freezing. Using a numerical model, the increase in sucrose concentration and system viscosity and corresponding bimolecular reaction rates were theoretically estimated. Freezing with a temperature-hold step after ice nucleation negatively influenced particle stability. Ice-nucleation temperature had an impact only at low DNA concentrations. Particle stability was highly reduced during the early part of freezing ($<-3^{\circ}C$), especially at low shelf-ramp rates. In this phase, bimolecular reaction rates increase greatly at still low system viscosity. Below a critical temperature ($\leq\sim-18^{\circ}C$) and at high system viscosity, no further particle aggregation occurred. In conclusion, the initial sample viscosity rather than the unfrozen volume and the residence time of particles in the low-viscosity state are the predominant factors in particle stabilization, which likely apply to aggregation in any system. © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:929–946, 2013

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INTRODUCTION

Nucleic-acid-based therapeutics are a promising new class of biopharmaceuticals.[1] The most common non-viral carriers for the nucleic acids are lipoplexes and polyplexes.[1] These nanoparticulate carriers are based on cationic lipids or cationic polymers that form condensed complexes with the negatively charged nucleic acids.[1] One general limitation in the long-term stability of nanoparticles, specifically nonviral vectors, is their tendency to aggregate in aqueous solutions, which is known to correlate with a loss in transfection efficiency of nucleic acid-based systems.[2] Consequently, lyophilization has proven to be an attractive way for the production of long-term stable, transfection efficient gene-delivery formulations. Although lyophilization is generally considered a very gentle drying process, it involves two potential stresses: freezing and drying.[3] These stresses typically cause damage of macromolecules and nanoparticles, unless appropriate stabilizers are added to the formulation.[4]

In a previous study, we found that the aggregation of plasmid/linear polyethylenimine (LPEI) polyplexes occurs predominantly during the freezing and not during the drying step of the lyophilization process.[5] Similar observations were also reported for lipid/DNA complexes.[6–8] In general, selected excipients, especially disaccharides like sucrose, are very efficient stabilizers when present above a critical mass ratio of stabilizer to gene-delivery particle.[7,9–11]

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During freezing, there are two main stress factors that could impact the stability of gene-delivery particles: ice formation and cryoconcentration.[12–14] Ice formation can threaten particle stability via their exposure to ice–liquid interfaces or because of mechanical damage by growing ice crystals.[14] Cryoconcentration leads to both solute- and particle-rich liquid phase and might facilitate particle aggregation because of an increase in ionic strength, pH shifts, or simply particle crowding.[4,7,14]

Several mechanisms addressing how cryoprotectants may stabilize nonviral gene-delivery particles during freezing have been discussed.[2,15] The "preferential exclusion hypothesis," which was established to explain protein stabilization, states that solutes are preferentially excluded from a protein surface leading to the formation of a stabilizing solvent layer.[2,15,16] However, it is doubtful whether the "preferential exclusion hypothesis" can be adapted to nonviral vectors.[15] According to the "glass formation or vitrification hypothesis," nonviral vectors are entrapped in the amorphous glassy matrix that is formed when the sample is cooled below the glass-transition temperature of the maximally freeze-concentrated system (T_g').[2,15] The high viscosity of the glassy matrix immobilizes gene-delivery particles and, thus, prevents particle aggregation.[2,15] However, the "vitrification hypothesis" alone is not able to explain the requirement of a critical mass ratio of stabilizer to gene-delivery particle.[15]

In a study on the freeze–thaw stability of lipid/DNA complexes, Allison et al.[7] demonstrated that glass formation is not obligatory as sugars like glucose are also able to preserve particle size at temperatures above T_g' . Consequently, the "particle isolation hypothesis" was proposed, which is based on the fact that the separation of individual particles in the unfrozen fraction prevents particle aggregation during freezing.[7] At higher stabilizer–particle mass ratios, the volume of the unfrozen fraction increases, leading to greater dilution and fewer particle–particle contacts.[7] Moreover, if the system has very high viscosity, diffusion is too slow to form new particle–particle contacts on the relevant time scale. In a further study, Armstrong and Anchordoquy[17] showed that particle aggregation can result from the formation of ice, as well as from prolonged incubation in the frozen state above, but not below a critical temperature well above T_g' at which particles are already sufficiently immobilized in the freeze concentrate.[17]

Until now, no detailed study on the influence of different freezing parameters, such as ice-nucleation temperature or freezing rate, on the stability of nonviral gene-delivery particles is available. These parameters impact the concentration and temperature history and therefore the residence time in a concentrated but relatively fluid system and thus, might also highly impact particle stability. Studies of the impact of freezing variations on the stability of nonviral gene-delivery particles are limited to several reports of fast freezing in a dry ice/alcohol bath, in liquid nitrogen, or in a -70°C freezer resulting in better preservation of lipoplexes compared with slow freezing in a -20°C freezer or at -2.5°C/min to -40°C.[6,18]

However, these fast freezing methods are difficult to control and are not practical on large scale. Instead, shelf-ramp freezing, where the filled vials are placed on the shelves of the freeze-drier and the shelf temperature is decreased linearly with time, is the most commonly used freezing method.[13] During shelf-ramp freezing, ice nucleation occurs stochastically, resulting in vial-to-vial or batch-to-batch heterogeneity and scale-up issues.[13] Therefore, different methods based on ultrasound, electro-freezing, vacuum-induced surface freezing, an ice fog, or depressurization have been developed to control nucleation at the desired nucleation temperature.[13] Depressurization is a rather newly established method that has gained substantial interest.[19,20] Using this method, Bursac et al.[19] reported larger pores, lower specific surface area (SSA), and improved lactate dehydrogenase recovery for samples frozen by depressurization at $\sim -3^{\circ}$C with an $\sim$15-min hold step after nucleation, as compared with samples produced by shelf-ramp freezing.

The objective of this study was to systematically investigate the influence of different freezing procedures on the stability of plasmid/LPEI polyplexes, in order to get an extended understanding of critical freezing parameters and the underlying stabilization mechanisms during freezing of nonviral gene-delivery particles. The polyplexes were formulated at different plasmid DNA concentrations and at increasing sucrose–polyplex mass ratios. In the first step, the influence of the "standard" depressurization method, which refers to the use of a 15-min hold period after nucleation, in comparison with conventional shelf-ramp freezing on polyplex particle size was investigated. Second, the influence of the ice-nucleation temperature and the residence time of the particles above T_g' during freezing on polyplex particle size were studied individually with the aid of the depressurization method to control nucleation. Furthermore, the increase in sucrose concentration and sample viscosity during freezing was theoretically calculated and correlated with bimolecular reactions rates. These theoretical results were then compared with the experimental data. The identification of the critical parameters in freezing and a better understanding of the stabilization mechanisms involved during freezing should help to design less stressful lyophilization processes and lyophilized products with improved quality.