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Stability of Cryo-Concentrated Complexes

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Introduction

Nucleic acid-based molecules (NA) have a great potential as therapeutics. However, their clinical use is limited due to their lack of natural mechanisms enabling them to pass biological barriers like cell membranes, blood-brain barrier, endothelium *etc*. This is mainly because of the high molecular weight and negative charge of NA. What is more, these NA-s are generally rapidly degraded by extracellular and intracellular enzymes [1]. Therefore, there is a great need for efficient and non-toxic delivery vectors which are able to cross these barriers and carry macromolecule-based therapeutics with them to the intracellular targets.

Cell-penetrating peptides (CPP) are relatively short (ranging from approximately 5 to 30 residues) amino acid sequences which are able to permeate cell membranes and deliver covalently or non-covalently bound cargo, such as NA (Figure 1) [2]. Although covalent bonding strategy allows number of cargo molecules per CPP to be controlled resulting in a defined structure, non-covalent formulations have their advantages, especially in case of NA, due to simpler preparation and versatility. In case of plasmids (pDNA), which are circular NA molecules, the non-covalent attachment is more preferred as it does not involve complicated modifications of the plasmid. However, as non-covalent bonding strategy is mainly based on electrostatic interactions, the formulated nanoparticles may have heterogeneous size-distribution and low stability [3,4]. Therefore, they are rapidly cleared from the general blood circulation and achieve high tissue accumulation levels only in case of certain organs when assessed in mammalian organism. Since physical parameters such as size and size distribution influence biodistribution and toxicity of nanoparticles [5], it is important to prepare stable, homogeneous and well-defined nanoparticles for *in vivo* application.

Fig. 1. Spontaneous non-covalent formulation of nanoparticles based on electrostatic interactions between positively charged CPP and negatively charged cargo.

Results and Discussion

Recently, a new formulation approach for CPPs called cryo-concentration was introduced by our research group [6]. Cryo-concentration is based on nanoparticle formulation in diluted solution following their reconstitution in a concentrated, *in vivo* compatible solution after freeze-drying. In more detail, the process involves formulation of CPP-cargo complexes in a 10x diluted concentration compared to the final injection concentration. After reconstitution in water, the obtained cryo-concentrated complexes (CCC) are ready for use in *in vivo* application (Figure 2).

Cryo-concentration enables preparing stable and homogeneous nanoparticles between CPP NF55 and pDNA which do not aggregate after reconstitution, have excellent stability against enzymatic degradation and show significantly higher bioactivity *in vivo* [6]. Additionally, these ready-made

Fig. 2. Process of preparing cryo-concentrating complexes (CCC) from formulation to in vivo application.

CCC-s are convenient to use as they can be used immediately after taking up into solution without further mixing, further incubation processes, nor do they require any additional reagents aside from Milli-Q® grade water.

However, the shelf-life of these nanoparticles is currently unknown. Therefore, the stability of these nanoparticles was tested by storing them at 3 different conditions for 31 weeks: -20 ºC, +4 ºC and room temperature (RT). Freshly prepared CCC was used as a control throughout the experiments. It was observed that while CCC stored at room temperature began to show decrease of transfection efficacy after 4 weeks, efficacy of CCC stored at -20 ºC and +4 ºC remained at the same level with freshly prepared CCC throughout the whole tested time period (Figure 3). Therefore, the CCC approach allows the preservation and use of CPP-NA complexes over extended time period compared to previously used freshly prepared CPP-NA complexes.

As a conclusion, cryo-concentration is a simple and versatile tool to achieve stable and homogeneous nanoparticles with higher bioactivity *in vivo*. They can be conveniently used as there is no need for additional procedures after reconstitution of the nanoparticles. What is more, as the efficacy of CCC stored at +4 ºC or below remained at the same level with freshly prepared complexes throughout the 31 week-period, the shelf-life of CCC is at least 6 months.

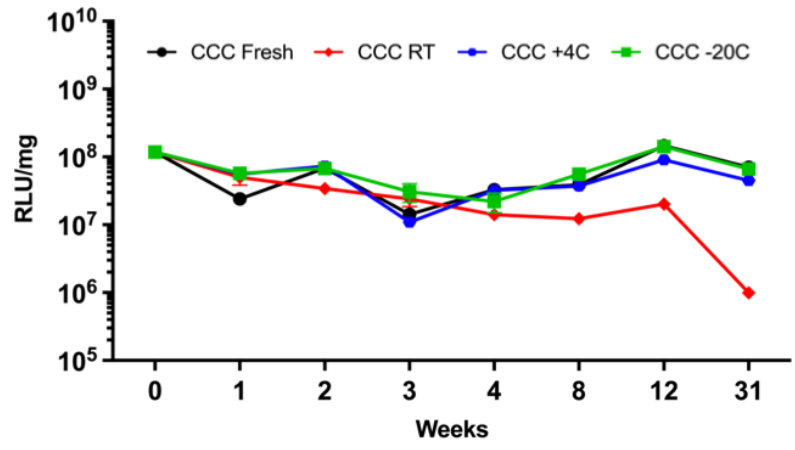

Fig. 3. Stability of cryo-concentrated complexes (CCC) stored at different conditions. Experimental conditions: CHO K1 cells, 10K cells per well, 96 wp, full media, pFluc dose 0.1 g per well. Complexes were formulated between NF55 and pDNA at charge-ratio 3. Luminescence was measured 24 h post-transfection and normalized to protein content.

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