Popular Science Summary of Degree Project in Biophysical Chemistry at The Faculty of Engineering, Lund University, in collaboration with the Technical University of Denmark.

Protein Stabilization during Freeze-drying

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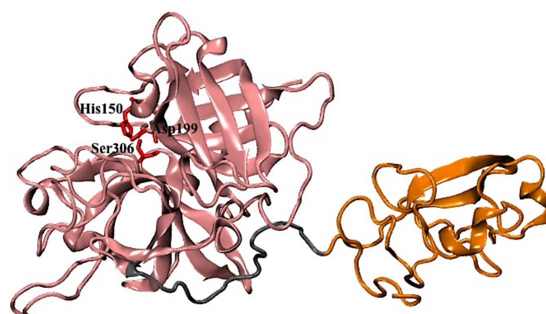
Freeze-drying is a widely used approach to improve long-term stability of protein-based biopharmaceuticals. The process involves multiple challenges and proper formulation design is a key component in product development to overcome these issues. Small molecule excipients have a vital function in protection of the protein. A thorough investigation of the relevant stabilization mechanisms is essential for optimization of screening procedures. Within these frames, computational modelling is an important tool to provide the necessary molecular level insight.

Protein-based therapeutics is a quickly growing field in the pharmaceutical industry. However, many proteins are known for low stability in solution and therefore special processing techniques are required to circumvent this problem. Freeze-drying is a method that is used frequently to produce a tolerable solid-state powder of the protein, which is resolvated before administration to the patient. The process consists of two main steps: freezing and drying of the product. These steps involve several challenges, where the protein is exposed to crucial stress factors such as low temperature and the removal of water molecules.

The correctly folded structure of a protein is essential to its function. The conditions during freeze-drying can cause the protein to unfold, which can trigger detrimental aggregation events where multiple protein units adhere to each other. Under such circumstances the pharmaceutical product will be destroyed. To prevent this from happening, suitable additives called excipients are included in the protein formulation. The excipients act in different ways to protect the protein. A main theory of stabilization relies on the concept of preferential interaction. This idea defines two separate modes for excipients to induce stability, either through favourable binding or exclusion from the protein surface. Protection is provided by shifting the energetic balance towards the folded state of the protein.

A complete understanding of the stabilizing action of excipients is utmost important in formulation design. However, this aspect is non-trivial to explore experimentally during freeze-drying. Therefore, the study presented here aimed to investigate the molecular level detail of stabilization mechanisms using the computer modelling technique of Molecular Dynamics (MD) simulation. Computational approaches are vital in biophysical chemistry to examine molecular events that are inaccessible to direct observation.

A therapeutic protein named Reteplase was used as a study model. Commercial formulations of Reteplase include either of two different excipients: arginine or tranexamic acid (TXA). A comparison of the performance of these two stabilizers could provide key insight to the features associated with an optimal selection of excipient.



The protein structure of Reteplase.

The investigation revealed that arginine is effective at a lower concentration than TXA in terms of preserving and repairing the original protein structure. The drying stage was considered the most critical step of the process, where the greatest benefit of formulation additives was observed. It was uncovered that arginine and TXA bind preferentially to exposed sensitive regions of protein in order to stabilize them against unfolding. In the specific case, ionic interactions play an important role in the binding to Reteplase.

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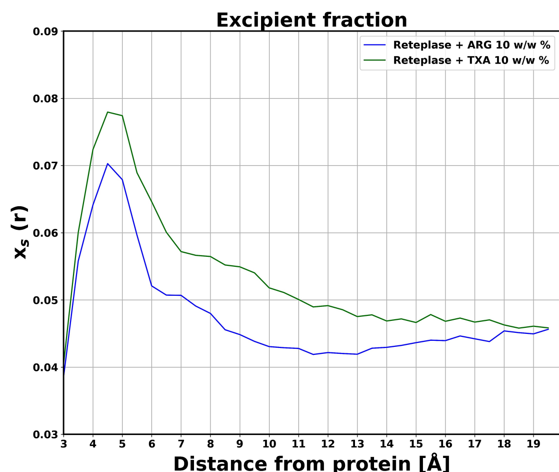

Plot displaying the fraction of excipient molecules (x_s) as a function of the distance to the protein surface. The green and blue graphs correspond to TXA and arginine respectively. The appearance of the graphs indicates that there is an accumulation of excipient molecules close to the protein. According to the theory of preferential interaction, this observation holds true for additives that stabilize a protein by means of favourable binding.

TXA displayed an overall higher strength of protein-excipient interactions. The observation could in part be explained from its smaller molecular size, which provides a good accessibility to cavities on the protein surface. The diverse chemical character of TXA is also likely to increase the number of ways that the excipient could interact with the protein. The study demonstrated that arginine has a high tendency to associate with other arginine molecules, which limits interactions with the protein. It was also found that the charge of the protein surface affects whether attraction or repulsion occurs to excipient molecules. The complexity of the protein structure was considered important to the ability of correcting damages caused by the stresses during freeze-drying.

The AMBER Molecular Dynamics suite was used for the simulations carried out in this project. Different specialized force fields were utilized for modelling of the protein and small molecules respectively. A force field defines the complete mathematical form of the energy equation that describes interactions between atoms within the simulated system. The simulation of the total freeze-drying process was divided into five parts: standard room temperature simulation, freezing, partial drying, full drying and resolvation. The protocol defining the simulation settings was inspired by similar studies. Technical details are presented in the full report. Calculations of chosen protein structural descriptors and visual observations in a graphical window were used to analyze spatial protein rearrangements during the simulations. The theory of preferential interaction was employed to investigate the mechanisms that improve protein stability in the presence of the studied excipient molecules.

Degree Project finished in 2021: *A Molecular Dynamics Simulation Study of the Protein Stability of Reteplase in Formulation with Various Excipients during Freeze-Drying Processing.*

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