Summary and conclusions

Cell-based therapeutics of all varieties have been gaining interest in the pharmaceutical industry in recent years, with some of them showing very promising results. For example, CAR T-cell therapies have transformed the landscape of hematologic malignancies, especially for treatment-resistant and recurring diseases. On the side of microorganism cell-based therapies, fecal microbiota products have become increasingly interesting in preventing infections of the gastrointestinal system. However, one common factor for these therapies is that they are often very expensive. The high cost of cell-based therapies is, for a large part, the result of the intricate manufacturing processes required to produce them. One of the remaining challenges in this respect concerns the shelf life of cell-based therapies, as they are generally very unstable as a liquid formulation. Seeing that cell therapies are typically administered as a liquid, this presents an important challenge. Commonly, microorganism cell-based therapies are freeze-dried, while human cell-based therapies are frozen and stored at very low temperatures to maintain the stability of the product. However, cryogenic storage contributes to the high costs of cell-based therapies, and has several other disadvantages such as concerns of contamination. In this sense, it could be of interest to replace cryogenic storage with storage of the freeze-dried product for some of these therapies.

Freeze-drying is already commonly used in the pharmaceutical industry to stabilize labile products such as therapeutic proteins. It starts by freezing a liquid product, whereafter ice is sublimated and any remaining water is subsequently removed by desorption. The result is a product stable for a long time at relatively high temperatures (i.e., cryogenic storage is no longer needed). Considering the cost-saving potential, it is of interest to further develop the application of freeze-drying in the context of cell-based therapies. However, the process of freeze-drying itself also has flaws. Indeed, it is considered to be a slow process, with at times poor product quality (e.g., problems with batch inhomogeneity). To solve these problems, new freeze-drying technologies have been developed. One of these technologies is continuous spin freeze-drying developed by RheaVita BV. Considering the advantages of the spin freeze-drying approach, many of which are applicable specifically to cells (i.e., exceptional control of the freez-

ing phase), this thesis was largely focused on the development of this process and its application to cell-based therapies.

A general introduction in **Chapter 1** and the objectives of the thesis in **Chapter 2** were followed by the first experimental section in **Chapters 3 and 4**. These chapters focused on the development of spin freeze-drying as a technology, so that it could be applied optimally to the investigation of freeze-drying cell-based therapies in later chapters. In **Chapter 3**, the focus was on the spin freezing phase of the process. Here, a mechanistic model was developed that predicts the product temperature profile over time as spin freezing progresses. Several techniques such as uncertainty and sensitivity analyses were employed to develop and demonstrate process understanding. In **Chapter 4**, the spin freeze-drying technology was further developed by creating a control system for the entire process. Here, the spin freezing process was further improved to enable the control of the gas temperature simultaneously with gas flow rate. Additionally, a feedback control system was created for the subsequent drying process (i.e., both primary and secondary drying). Both freezing and drying control systems were combined, and the process was characterized by producing freeze-dried samples. Residual moisture content, pore structure, cake appearance and specific surface area of the freeze-dried material were investigated and related to the process settings used in the process. In the end, appropriate control of the process was demonstrated, and the system could be used for further research.

Subsequently, **Chapter 5, 6 and 7** focused on applying the developed processes in the previous chapters to freeze and freeze-dry cell-based products. In **Chapter 5**, the freeze-drying of a bacterial species with anti-inflammatory properties, *R. mucilaginosa*, was investigated. *R. mucilaginosa* has been under investigation for inflammatory diseases of the lung. One promising delivery method would be to nebulize these bacteria to transport them into the lungs where they can exert their anti-inflammatory effect. However, as with all cell-based therapeutics, bacteria are not stable for long as a liquid suspension. Therefore, it was investigated if these bacteria could be freeze-dried in order to obtain long-term stability. To this end, spin freeze-drying was compared to conventional batch-wise freeze-drying, and a formulation screen was performed to optimize long-term stability. Following formulation optimization, *R. mucilaginosa* retained its viability and anti-inflammatory activity for up to a year of room temperature storage. Impactful factors were the storage temperature, moisture content of the product and freeze-drying method.

Chapter 6 described the freeze-thawing of Jurkat T cells using spin freezing and both a DMSO-containing formulation as well as a DMSO-free formulation. In this work, the focus was to elucidate the impact of each phase of the spin freezing process in an effort to find optimal conditions for a subsequent freeze-drying process. It was found

that almost all phases of the process impact the viability of the cells post-process in different ways. The cooling rate used before the formation of ice had an impact on cell viability, likely due to osmotic effects which were formulation-specific. The degree of supercooling at the moment of ice nucleation was not found to be impactful, which we attribute to the method of controlling the product temperature during spin freezing. The rate at which ice crystals grow was expectedly impactful, as the impact of this process is already well-described in literature. Finally, the cooling rate after complete solidification of the ice also had a formulation-dependent impact, which was attributed to recrystallization effects and a risk for intracellular crystallization.

In **Chapter 7** it was attempted to freeze-dry Jurkat T cells in order to confer long-term stability. Multivariate data analysis was utilized to gauge the status of the cells, regardless of the absence of live cells. It was found that specific formulation components influence the similarity of the freeze-dried cell to a positive control. Additionally, the intracellular delivery of protectants was assessed. Although the real amount of delivered protectant is unclear, none of the investigated techniques resulted in cells that were more similar to the positive control compared to cells that did not undergo a loading treatment. Finally, spin freeze-drying was not significantly more damaging to the cells compared to conventional freeze-drying.

Finally, **Chapter 8** discussed the performed work of this thesis in the broader international context, and discussed future perspectives. The broader international context section consisted of three parts. In a first part, the relevance and efficacy of cell-based therapies are discussed to illustrate the promise of this new class of therapeutics. Secondly, the cost of cell therapies was considered, including the reasons for this high cost and ways to mitigate it. Thirdly, the promise of off-the-shelf human cell-based therapies was discussed, and the place of freeze-drying within this context was described. The future perspectives section described research initiatives relevant to this thesis and to the field of freeze-drying cell-based therapeutics in general. Relevant topics in this last part include the intracellular delivery of protectants, the importance of the reconstitution step for these products, and the difference between freeze-thawing and freeze-drying.

In summary, this thesis contains both the process development of the spin freeze-drying process, as well as its application to the investigation of freeze-drying and cryopreservation processes for cell-based products. Although there are still many challenges, this thesis represents another step towards the successful application of freeze-drying to cell-based therapies.