Supercritical carbon dioxide spray drying for the production of stable dried protein formulations

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CHAPTER 7

Summary and prospective

Supercritical carbon dioxide ($scCO_2$) processing technologies are a broad and diverse array of techniques that can be used to engineer nano- and microparticles for a variety of pharmaceutical applications, such as allowing the use of less invasive administration routes, improving drug stability, controlling the release of drug, increasing bioavailability and/or selectively targeting of particular tissues or cell types [1-3]. In each case, the $scCO_2$ can be used in different ways, for example as a solvent or anti-solvent, in order to obtain particles with the desired drug delivery profile and therapeutic efficacy [4-6]. As the functionality of the resultant nano-/microparticles is often related to the particle characteristics (such as the efficiency of the drug loading, particle size and morphology), it is necessary to provide proper particle characterization, in order to obtain crucial data for the development and optimization of the production process and the product. A review of these characterization techniques is presented in **Chapter 2**.

Supercritical carbon dioxide spray drying is a dehydration technique, which has been proposed as an alternative for freeze-drying and conventional spray-drying, to prepare dried therapeutic protein formulations and other biologics at ambient temperature, thereby avoiding thermal denaturation [7]. As the solubility of water in CO_2 is low, organic solvents, such as ethanol and acetone, were often introduced as co-solvents, to enhance the water evaporation. However, the use of co-solvents can lead to destabilization and aggregation of proteins [8]. Preliminary tests in the absence of a co-solvent resulted in up to 10%wt residual water content in the protein formulation after $scCO_2$ spray drying [9]. Yet, according to Title 21 of the Code of Federal Regulations for Food and Drugs (1990), the maximum residual moisture content in freeze-dried protein formulations should be no greater than 3%wt, in order to preserve the protein integrity during storage. Therefore, one of many challenges in $scCO_2$ spray drying is finding suitable conditions to prepare stable dried protein formulations similar to freeze-dried products, with less than 3%wt of residual water content without the need for organic solvents. In **Chapter 3**, it is shown that the residual water content in $scCO_2$ spray dried powders is related to the processing parameters. The experiments were carried out for lysozyme/trehalose formulations at 1:0, 1:4 and 1:10 weight ratios. To decrease the residual water content, the relative humidity in the high pressure CO_2 drying medium should be controlled by using a high CO_2 to solution flow rate ratio and low solution volume. Under these conditions, the resulting dried protein formulation had a residual water content of about 2.5%wt. However, the use of low solution flow rate and volume limits the production capacity of the $scCO_2$ spray drying process. In **Chapter 3** also the scalability of $scCO_2$ spray drying is examined by maintaining a

constant gas (CO_2) to liquid flow rate ratio (GLR) on a pilot-scale unit. When using the same GLR value in 4- and 10-litre drying vessels of the $scCO_2$ spray dryers, the final powdered products had comparable quality in terms of the droplet size, residual water content, protein structure and activity and particle size and morphology.

From the results obtained in **Chapter 3**, the best conditions were used to prepare $scCO_2$ spray dried formulations of other proteins. While the protein integrity for polyclonal and monoclonal antibodies, a-chymotrypsinogen A, lysozyme and a-lactalbumin was maintained after drying, the 1:10 myoglobin/trehalose formulation showed heme destabilization and myoglobin aggregation, which most likely was process induced. Parameters related to the $scCO_2$ spray drying process, such as pressure, temperature, high pressure CO_2 and spray drying, were individually studied, in order to assess their influence on heme binding destabilization and myoglobin aggregation. In the range of 65-130 bar and 25-50°C, pressure and temperature alone did not influence the myoglobin integrity. However, the results showed that myoglobin instability was induced by exposure to pressurized CO_2. Furthermore, the pH of the myoglobin formulation was found to decrease from 6.2 to about 5.0, leading to a partial loss of heme during spray drying. This study is presented in **Chapter 4**. The changes in myoglobin structure were monitored by UV-Vis spectroscopy, circular dichroism spectroscopy and size-exclusion chromatography. However, the nature of the interaction between CO_2 and myoglobin was not investigated. Consequently, it is not known whether the pressurized CO_2 only causes physical instability of the myoglobin structure, or whether there is actually a chemical reaction occurring between them. For this, the chemical modification of proteins should be observed in order to better understand the mechanisms by which the myoglobin integrity is affected by $scCO_2$, e.g., by using mass spectroscopy in combination with Fourier transform infrared spectroscopy (FTIR) spectroscopy [10].

During spray drying, atomization results in the formation of droplets and correspondingly a gas/water interface. Protein molecules can adsorb at the interface, leading to reorientation of the protein structure that results in protein destabilization and aggregation [11, 12]. However, the critical parameter study of the CO_2 spray drying process as described in **Chapter 4** was not able to directly show the influence of atomization on myoglobin instability. For this reason, a gas bubbling study was conducted, as a means to evaluate the influence of CO_2/water interface and acidification by CO_2 on myoglobin integrity. **Chapter 5** shows a series of CO_2 and N_2 gas bubbling tests on myoglobin solutions over a range of pH 4.0-7.0. CO_2 gas bubbling decreased the pH of the myoglobin solutions prepared at starting pH 4.5-7.0. However,

changes in the secondary structure of myoglobin were only found when the myoglobin solution had a final pH of 4.3, although no loss of heme was observed. In case of myoglobin solutions with a starting pH of 4.0-5.3, the effect of CO_2/water interface resulted in the formation of sub-visible myoglobin aggregates (1-100 µm diameter) with a fiber-like morphology. For the myoglobin solutions with a starting pH of 6.2 and 7.0, however, the CO_2/water interface in combination with the CO_2 acidification resulted in sub-visible aggregates with a highly irregular morphology. Moreover, the concentration of the aggregates was five times higher than what was observed for the solutions with the low starting pH. In contrast, N_2 gas bubbling did not affect the pH of the solutions, and consequently there was no change in the secondary structure and the heme binding site of myoglobin. However, N_2 bubbling also led to the formation of fiber-like myoglobin aggregates, but in this case, they were observed over the entire pH range studied. From these results, it was concluded that the gas/water interface induces the formation of aggregates, while the gas/water interface in combination with the drop in pH results in conformational changes and changes in the particle morphology.

The effects observed in the CO_2 bubbling study are not entirely comparable to those occurring at the gas/water interface formed during CO_2 spray drying, due to the different properties of gaseous and supercritical CO_2 as well as the difference in processing conditions. However, the bubbling method still suggests that heme destabilization is induced by acidification, whereas myoglobin aggregation can be caused by exposure of the protein to the CO_2/water interface and the CO_2 acidification, depending on the starting pH of the protein solutions. Moreover, this protein instability appears to be a surface-induced process, which is most likely enhanced in pressurized CO_2. Furthermore, the pH shift also suggests that the protein formulation needs a buffer agent to control the pH of the formulation upon exposure to CO_2. In this study, protein aggregation was evaluated in terms of the sub-visible particles and morphology by flow-imaging microscopy and the partial loss of monomer and protein by UV/Vis and size-exclusion chromatography. However, in order to intrinsically understand the mechanism of protein aggregation, the molecular interactions that lead to aggregation should be investigated. For example, the secondary structure of protein aggregates can be studied by FTIR [13].

In order to understand the role of the formulation in stabilizing myoglobin, a series of tests were conducted using different excipients. **Chapter 6** shows effects of sugar (trehalose), buffer agents (phosphate, citrate and histidine) and a surfactant (Tween 80) on the heme binding and aggregation of myoglobin during CO_2 spray drying. The heme was

stabilized in the myoglobin structure when trehalose was added to the myoglobin formulations. The pH of the myoglobin/trehalose formulations was maintained at pH 6.2 after CO_2 spray drying when introducing buffer salts such as 50-150 mM phosphate, 10 mM citrate and 25 mM histidine, with full recovery of the heme observed. Myoglobin aggregation was gradually reduced with an increasing concentration of phosphate buffer. Moreover, a high concentration of myoglobin exhibited self-buffering properties, leading to the stabilization of heme and a significant reduction in myoglobin aggregation. The use of the surfactant was hypothesized to act as a barrier at the interface of the high pressure CO_2 and atomized protein droplets, thereby minimizing protein adsorption at the interface that leads to protein aggregation. However, Tween 80 did not show any significant effect in reducing myoglobin aggregation. As discussed in **Chapter 5**, it is likely that the pressurized CO_2/water interface is not the same as the interface between gaseous CO_2 and water observed in the gas bubbling study, from which it may be inferred that the surfactant will not have the same effect in the supercritical CO_2 environment as it does under atmospheric conditions. Moreover, polysorbates have been shown to be unstable under certain conditions, undergoing autoxidation, side-chain cleavage, formation of short-chain acids upon exposure to changes in pH and temperature, as well as light exposure [14-16]. It is possible that one or more of these factors may influence the surfactant properties of Tween 80 during $scCO_2$ spray drying.

As shown in **Chapter 6**, it was of key importance to avoid significant acidification and exposure of the myoglobin to the hydrophobic CO_2 during $scCO_2$ spray drying, in order to stabilize the heme binding in myoglobin and to reduce myoglobin aggregation [17]. The integrity of myoglobin was preserved by adding trehalose and a suitable buffer agent. While it is anticipated that other proteins may also be destabilized during $scCO_2$ spray drying, it should not be assumed that the formulation that was used to stabilize myoglobin will be applicable in all cases. Given the diversity of proteins, the choice of the pH and the use of stabilizing agents (e.g., disaccharides, polyols, buffers and surfactants) should be tailored for each specific protein. Moreover, it is anticipated that working with a high protein concentration may allow for buffer-free protein formulations. However, the self-buffering capacity of each individual protein should be fundamentally studied, in order to understand what protein concentration is needed to maintain the pH during $scCO_2$ spray drying. The self-buffering property of proteins can be observed by following a similar experimental approach as outlined in a study by Karow et al. [18]. By acid-base titration, the buffering capacity of proteins at any working pH can be investigated in terms of the

equivalent amount of hydrogen or hydroxide ions used for changing one unit of the starting pH of a protein solution.

In general, $scCO_2$ spray drying is a promising process for preparing stable dried protein formulations for research and commercial purposes. Similar to other drying techniques, care should be taken to choose the appropriate processing conditions and formulation (**Chapter 3, 4 and 6**). Although only proteins were studied in this thesis, it is anticipated that the $scCO_2$ spray drying technique is also applicable to peptides, genetic materials (DNA and RNA) and vaccines. Moreover, $scCO_2$ spray drying could be used as a technique to engineer a broad range of different types of drug delivery systems, such as particles with core-shell structures, tunable matrix compositions, and hierarchical structures, such as microparticles decorated with nanoparticles [19].

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