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Stirred batch crystallization of a therapeutic antibody fragment

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ABSTRACT

Technical-scale crystallization of therapeutic proteins may not only allow for a significant cost-reduction in downstream processing, but also enable new applications, e.g., the use of crystal suspensions for subcutaneous drug delivery. In this work, the crystallization of the antigen-binding fragment FabC225 was studied. First, vapor diffusion crystallization conditions from the literature were transferred to 10 μL-scale microbatch experiments. A phase diagram was developed in order to identify the crystallization window. The conditions obtained from the microbatch experiments were subsequently transferred to parallelized 5 mL-scale stirred-tank crystallizers. This scalable and reproducible agitated crystallization system allowed for an optimization of the crystallization process based on quantitative measurements. The optimized crystallization process resulted in an excellent yield of 99% in less than 2 h by increasing the concentration of the crystallization agent ammonium sulfate during the process. The successful scalability of the Fab fragment crystallization process to 100 mL-scale crystallizers based on geometric similarity was demonstrated. A favorable crystal size distribution was obtained. Furthermore, a wash step was introduced in order to remove unfavorable low-molecular substances from the crystals.

1. Introduction

Over the past years, production processes of therapeutic proteins have been optimized with regard to higher protein concentrations in order to satisfy the increasing demand. The downstream processing, however, still relies heavily on costly, difficult to scale-up technologies, i.e., preparative chromatography. Consequently, the purification of higher amounts of protein per production batch becomes increasingly challenging (Low et al., 2007; Shukla and Thömmes, 2010). For therapeutic proteins, the chromatography steps are usually complemented with filtration steps for virus reduction, concentration, and formulation (Kelley, 2007). In addition to the drawbacks of existing downstream processing technologies, current protein formulations, i.e., primarily aqueous solutions or amorphous precipitated lyophilisates, often exhibit low product activity and low product stability (Manning et al., 2010). The high dosages required for some therapeutic proteins call for highly concentrated protein formulations, which, in case of aqueous solutions, lead to an increased viscosity and increased protein aggregation propensity (Shukla et al., 2007). The stabilization of protein formulations using additives or excipients often results in a reduction of the product activity (Andya et al., 2003). Furthermore, these substances may be unfavorable for pharmaceutical applications.

Current research on alternative purification technologies, e.g., precipitation, aqueous two-phase extraction, membrane-based techniques, or application of magnetic adsorbent particles, mainly focuses on the remedy of the limitations of downstream processing (Franzreb et al., 2006; Thömmes and Etzel, 2007; Saxena et al., 2009; Asenjo and Andrews, 2012). In contrast to this, protein crystallization can serve as an attractive, alternative technology for protein formulation as well. This is due to the fact that crystalline proteins have an increased product activity and are significantly more stable compared with state-of-the-art formulations (Shenoy et al., 2001; Elkordy et al., 2002, 2004). Additionally, protein molecules are tightly packed in the crystals, thus allowing for higher concentrated protein formulations with lower viscosities than comparable aqueous solutions, e.g., for the subcutaneous delivery of therapeutic proteins (Yang et al., 2003; Basu et al., 2004; Pechenov et al., 2004).

Only few studies on systematic approaches to the technical-scale crystallization of therapeutic proteins can be found in the literature apart from the most prominent example, the crystallization of the polypeptide insulin (Schlichtkrull, 1957; Harrison et al., 2003). Giffard et al. (2011) demonstrated the initial capture and purification of the therapeutic enzyme urate oxidase by batch crystallization from fermentation broth with a purity of 87%. However, no details on the reaction volumes were given and the crystallization system was not clearly defined. Yields ranging

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from 51% to 100% were observed after a rather extensive process duration of 1 month. Trilisky et al. (2011) reported a study on the crystallization of 20 monoclonal antibodies and 2 Fc fusion proteins in vapor diffusion, batch, and evaporation crystallization experiments with volumes up to 350 μL. While diffraction-quality crystals were obtained for 4 antibodies, the other antibodies only exhibited liquid–liquid phase separation. No detailed information on the crystallization kinetics and on the crystal yield was given. However, it was stated that a yield >90% may be achievable within a few hours. Zang et al. (2011) crystallized the monoclonal full-length antibody mAb04c in μL-scale vapor diffusion and microbatch experiments. Furthermore, mAb04c was crystallized from impure solutions spiked with protein contaminants, DNA or bacteriophages, and from clarified cell culture supernatant with final purities above 90%. However, an unsatisfactory low yield of approximately 30% was observed. Other work focused on finding an interpretation of protein phase behavior by analyzing the second osmotic virial coefficient B_{22} which determines protein–protein interactions. Such a study was performed, e.g., using the monoclonal antibody IDEC-152 (Lewus et al., 2011). However, it was found that the rigorous prediction of protein crystallization conditions based on the second osmotic virial coefficient B_{22} is not yet feasible (George et al., 1997; Lewus et al., 2011; Liu et al., 2011). Up to now, only limited information is available on the large-scale crystallization of therapeutic proteins in stirred tanks. The most prominent example is the crystallization of the recombinant therapeutic protein Apo2L/TRAIL within 8.5 h with anticipated volumes up to 800 L (Matthews and Bean, 2006; Thömmes and Etzel, 2007). On the other hand, the results from crystallization studies in stirred tanks using technical proteins may also apply for therapeutic proteins. Hekmat et al. (2007) evaluated the transfer of vapor diffusion crystallization conditions of lysozyme into scalable stirred tank crystallizers with a volume of 4 mL. Schmidt et al. (2005) was able to separate two isoforms of an undisclosed protein by crystallization in stirred tanks with volumes up to 100 mL. Variations of the stirrer speed had little effect on the crystallization kinetics. However, larger crystals were obtained at lower shear forces, i.e., a lower mean volumetric power input by the stirrer. L-Methionine γ-lyase was purified from pretreated crude enzyme solution in a 100 L stirred tank (Takakura et al., 2006). This three-step crystallization process had a yield of 87% within approximately 3 days. Other examples are the 1 L-scale crystallization of ovalbumin (Judge et al., 1995) or the crystallization of fungal lipases with volumes up to 500 mL (Jacobsen et al., 1998; Lee et al., 2000).

While the feasibility of large-scale protein crystallization was generally demonstrated in the aforementioned literature, only few of the studies regarding the crystallization of therapeutic proteins were performed in well-defined, scalable stirred vessels. In addition, rather long process durations and/or low yields were observed in most cases. Hence, further work is required in order to establish the technical-scale crystallization of therapeutic proteins as an alternative to the state-of-the-art downstream processing techniques. The most interesting class of therapeutic proteins, i.e., full-length monoclonal antibodies, is often difficult to crystallize. This is most probably due to their large size and structural flexibility. Therapeutic antigen-binding antibody fragments (Fabs), however, are easier to crystallize. Consequently, the successful μL-scale crystallization of a triple-digit number of human Fabs is reported in the RCSB Protein Data Bank. Based on this data, the successful development of an efficient, scalable crystallization process for therapeutic Fabs is expected to be feasible.

The present work has the following aims: (a) to transfer known vapor diffusion crystallization conditions of the antigen-binding fragment of the antibody C225 (FabC225) from literature to microbatch experiments, (b) to subsequently transfer the microbatch crystallization conditions to 5 mL-scale stirred-tank batch crystallizers, (c) to perform scale-up of the crystallization process to a 100 mL stirred tank, (d) to evaluate the obtained crystal morphologies and crystal size distributions, and (e) to investigate the removability of unwanted substances like salts or other crystallization agents from the protein crystals by a wash step.

2. Materials and methods

C225 full-length antibody in aqueous solution (5 g L^{-1}) and Fractogel EMD SO_3^- (M) chromatography medium were kindly provided by Merck KGaA (Darmstadt, Germany). The buffer of the C225-solution was exchanged to double-distilled H_2O by dialysis and diafiltration prior to papain digestion. The FabC225 was freshly generated from the C225-solution by papain digestion. Depending on the amount of FabC225 necessary for the respective crystallization experiments, volumes of 100 mL, 2 L, and 20 L were used for the digestion. The digestion solution contained 1 g L^{-1} C225, 1 mM EDTA, 40 mM L-cysteine, and 40 mM sodium phosphate buffer. The pH was adjusted to 7.0 using sodium hydroxide. The reaction was started by addition of papain to a final concentration of 2.5 mg L^{-1}. The mixture was incubated in a stirred-tank reactor at 37 °C for 1 h. The reaction was stopped by adjusting the pH to 5.0 using acetic acid. The digest was stored at 4 °C until purification. FabC225 was separated from the digestion buffer via cation exchange chromatography using Fractogel EMD SO_3^- (M). The chromatography column was equilibrated with 40 mM sodium acetate buffer at pH 5.0. The papain digestion mixture was diluted 2.5-fold to reduce the ionic strength (final volumes: 250 mL, 5 L, and 50 L, respectively) and subsequently loaded onto the column. The fractions were eluted using a stepwise increase of NaCl concentrations. The fraction containing FabC225 was eluted using 150 mM NaCl in 40 mM sodium acetate buffer at pH 5.0. The fraction containing the Fc fragment and the papain was eluted using 300 mM NaCl in 40 mM sodium acetate buffer at pH 5.0. The purity of FabC225 was verified by SDS-PAGE analysis and size exclusion chromatography.

10 μL-scale microbatch crystallization was performed in 72-well Terasaki plates (Greiner, Frickenhausen, Germany). The pH was adjusted to 6.25 using 40 mM sodium citrate buffer in all experiments. Separate protein and crystallization agent stock solutions were prepared for each crystallization condition. The drops were prepared by gently mixing the protein and crystallization agent stock solutions in the wells. Triplicate measurements were performed for all crystallization conditions. The plates were sealed with 8 mL paraffin oil and stored at 20 °C in a refrigerating incubator (Binder, Germany). After 4 days, microphotographs of all wells were taken in order to categorize the crystallization conditions by their qualitative outcome (crystallization, precipitation, and clear drops).

Two geometrically similar unbaffled stirred tanks were used in order to study the agitated batch crystallization of FabC225 on the 5 mL-scale and the 100 mL-scale (Fig. 1).

Pitched-blade impellers were used for gentle mixing of the crystal slurry. This type of impeller is known to exert low shear forces on the fluid (Mirro and Voll, 2009). The impellers of both stirred tanks were operated in the scoping mode (upward flow near the stirrer axis). The stirrer speed was set within a range of 100–300 min^{-1}. The custom-built crystallizers had a fill-height to inner diameter ratio of 1.0 and an agitator diameter to tank inner diameter ratio of 0.5. Ports in the vessel head allowed for sampling and feeding of crystallization agent solutions. Standard round-bottom centrifuge tubes made of glass were used as vessels. The vessels were immersed in a refrigerating bath for temperature control. The crystallization was started by mixing protein and crystallization agent stock solutions in the stirred tanks. The progress of the crystallization process was monitored by sampling at variable