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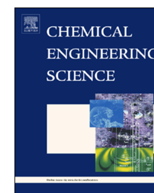

Design of industrial crystallization of interferon gamma: Phase diagrams and solubility curves

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HIGHLIGHTS

- Kind of precipitant highly influences the economics of crystallization processes.
- Phase diagrams are the key factor to design robust crystallization processes for industrial applications.
- Homogeneous nucleation is the driving force of the crystallization of rhINF-γ.
- Formation of uniform crystals takes place by Ostwald ripening instead of growth out of the liquid phase.

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ABSTRACT

Phase diagrams and solubility curves for engineering of crystallization for interferon gamma have been developed by small scale precipitation and crystallization experiments. A scale up was carried out to proof the accuracy and the suitability of the created phase diagram with sodium sulfate for bulk crystallization. To provide nucleation and a high yield the operating point was chosen at a protein concentration of 6 mg/mL and a sodium sulfate concentration of 25% [w/v]. Crystallization was completed within 5 h with a yield of $>95\%$. The double logarithmic plot of crystallized fraction versus time yielded in an overall kinetic crystallization constant of $5.79 \times 10^{-2}\ \text{min}^{-1}$. Regular cubic crystals can be observed which contradicts the low Avrami coefficient. The Avrami coefficient of 1 is interpreted that nucleation takes place throughout the entire crystallization process and larger crystals are formed by Ostwald ripening instead of growth out of the liquid phase. The established process with its conditions is suited for industrial protein crystallization.

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1. Introduction

Phase diagrams and solubility curves are the basis for engineering of crystallization processes. Batch crystallization has the potential to become an efficient recovery and concentration process in manufacturing of biopharmaceuticals (Przybycien et al., 2004). Development of crystallization conditions for a protein suitable for bioprocessing is still difficult and requires knowledge about the thermodynamic properties of the protein solution. Crystals should be formed in a reasonable time less than 24 h, relatively pure and with a size, shape and mechanical strength that they can be easily separated from the mother liquid. This is different to the search of crystallization conditions for structural biology purposes. The high demand in crystallization conditions suited for bioprocessing may have discouraged a lot of engineers to apply batch crystallization as a recovery process. For the structural biology applications these criteria except crystal size and purity are not important. High throughput techniques (HTP-techniques) for crystallization became a powerful tool for finding optimal conditions (Joachimiak, 2009). Such techniques provide crystallization conditions, but do not provide phase diagrams, which are important to optimize and engineer crystallization conditions.

Crystals for structure analysis differ from those for bulk crystallization. For structure analysis large single crystals without grid irregularities and high packing quality, which diffract highly, are required, but no phase diagrams and induction kinetics. All kinds of chemicals and additives can be used for generating crystals and

Abbreviations: rhINF-γ, recombinant human Interferon gamma; PEG 4000, Polyethylene glycol 4000; HCP, host cell proteins; HTP-techniques, high throughput techniques; UV_{280}, ultraviolet light with a wavelength of 280 nm

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optimizing their structure (Mueller et al., 2007; Peters et al., 2005). Excipient, costs, yield and scalability are not relevant and growth kinetics could take months. A method published for successful crystallization of rhINF-γ for structure analysis lasts one week and additionally a harmful additive, benzamidine, a serin proteinase inhibitor, had to be used (Landar et al., 2000). For bulk crystallization the objectives of the process are different. Precipitants must be inexpensive, the compounds should be of pharmaceutical grade, the growth kinetics should be fast, within several hours, the crystallization yield must be high and the protein must not lose its potency after crystals are redissolved (Jacobsen et al., 1997). Crystal grid irregularities and a large single crystal shape are not as important as purity and scalability of the process (Peters et al., 2005). For that reason the crystallization time, solubility and precipitation range of the target protein is important, when implementing it into a purification process, e.g. as a purification or formulation step. But complete phase diagrams for prediction of crystallization are rarely available. A general strategy to implement protein crystallization into a purification process was proposed as follows (Lee et al., 2000; Schmidt et al., 2004): (a) screening for appropriated crystallization conditions with vapor diffusion techniques and pure protein (purity $> 95\%$), (b) optimization of the conditions (addition of additives, exchange of buffers, etc.), (c) generating a phase diagram of protein and precipitant concentration with microbatch method, (d) amplifying of the phase diagram with other parameters like temperature and pH, (e) fitting of the phase diagram range with or without the help of seeding for contaminated protein solutions and (f) finally scale up to pilot or large scale process. The number of such implemented processes is rare (Przybycien, 1998). Only a few reports have been published so far about bulk protein crystallization (Hekmat et al., 2007). Some examples for that or crystallization of protein from impure solutions are the crystallization of lipase (Jacobsen et al., 1998), lysozyme (Lorber et al., 1993; Judge et al., 1998), ovalbumin (Judge et al., 1995), L-methionine γ-lyase (Takakura et al., 2006), insulin (Schlichtkrull, 1956) and an aprotinin variant (Peters et al., 2005). We present a systematic approach to establish a crystallization step of a biopharmaceutical relevant protein, recombinant Interferon gamma (rhINF-γ) as a purification step. The human interferon gamma is a cytokine produced by activated T-cells and natural killer cells. The active form is a 45 kDa heterodimer, which contains a 20 kDa and a 25 kDa glycopeptide. It exhibits pleiotropic biological activities (Dijkmans and Billiau, 1988) and is particularly used in clinics for treatment of visceral leichmaniasis and chronic granulomatous.

In order to evaluate precipitants to be adequate for preparative batch crystallization solubility curves of rhINF-γ were determined to find the point in time, when the solubility reach the equilibrium and how this can affect the establishment of the phase diagram and further the protein crystallization. The obtained phase diagrams with different precipitants will be compared and discussed. Based on the generated phase diagrams, a favorable operating point for batch crystallization was selected and the crystallization was performed as 150 mL batch crystallization.

2. Materials and methods

2.1. rhINF-γ

rhINF-γ was produced in *Escherichia coli* (not glycosylated) and obtained from the pilot plant at Boehringer Ingelheim RCV GmbH & Co KG, Vienna, Austria.

2.2. Chemicals

Acetic acid, ammonium acetate, ammonium phosphate dibasic, hydrochloric acid, Polyethylenglycol 4000, sodium acetate tri-hydrate, sodium chloride, sodium hydroxide and sodium sulfate were purchased by Merck KGaA, Darmstadt, Germany. Ammonium sulfate was obtained from Mallinckrodt Baker Inc., New Jersey, USA.

2.3. Generating of protein stock solutions by ultra-and diafiltration

Ultra- and diafiltration of rhINF-γ was performed using Amicon Ultra-15 Centrifugal Filter Units with Ultracel-3 membranes from Millipore GmbH, Vienna, Austria. Ultrafiltration was performed by centrifuging the filter units several times at 5000 rcf for 10 min (Centrifuge 5810R from Eppendorf AG, Hamburg, Germany) until a protein concentration of approximately 40 mg/mL was achieved. Diafiltration was performed by adding the same volume of diafiltration buffer (0.1 M ammonium acetate pH: 6.0), followed by a twofold concentration. This procedure was repeated seven times.

2.4. Buffer preparation

All buffers were prepared with water deionised and purified using an ultra-pure water system (SG Water, Barsbüttel, Germany) or a Millipore Milli-Q system (Millipore Corp., Bedford, MA, USA). The pH of these buffers was adjusted with hydrochloric acid, sodium hydroxide or acetic acid.

2.5. Determination of protein concentration

Protein concentration was determined by UV absorption at a wavelength of 280 nm using a Genios Pro TM UV plate reader from Tecan Crailsheim, Germany. An extinction coefficient $\varepsilon_{0.1\%}$ of 0.75 for rhINF-γ was used for calculation of the protein concentration.

2.6. Determination of solubility curves

A constant volume of rhINF-γ stock solution with a concentration of 40 g/L was mixed in 1.5 mL tubes (Eppendorf tube, Eppendorf, Hamburg, Germany) with different volumes of the adequate precipitant stock solution (40% [w/v] PEG 4000, 30% [w/v] sodium chloride, 30% [w/v] di-ammonium hydrogen phosphate, 50% ammonium sulfate, 37.5% [w/v] sodium sulfate) resulting in different salt and protein concentrations. After mixing, the solutions were incubated for 1–4 days using an end over end mixer (Stuart SB 3 Blood Tube Rotator Mixer, Bibby Scientific Limited, Stone, Staffordshire, UK) with 20 rpm at room temperature. Formed precipitates were separated by centrifugation of the samples at 16,110g at 25 °C for 30 min (Centrifuge 5415R, Eppendorf AG, Hamburg, Germany). The supernatant was removed and protein concentration measured by UV_{280}. The initial and obtained protein concentrations were plotted against the belonging salt concentrations to get the solubility curves.

2.7. Determination of phase diagrams

The protein stock solution was mixed with different amounts of the precipitant stock solution and distilled water respectively in 1.5 mL tubes (Eppendorf tube, Eppendorf, Hamburg, Germany) to get the desired protein and precipitant concentrations. 1 mL batches with different protein and precipitant concentrations were generated as initial conditions. The batches were incubated at room temperature with gently mixing (Assistent 348 Roller, Karl Hecht GmbH & Co KG, Sondheim, Germany) for 20 h, in case of using di-ammonium hydrogen phosphate as precipitant for 4 days. After incubation samples are investigated for crystals, precipitates or other structures by light microscopy (Labovert FS microscope, Leitz, IL, USA). The batches are centrifuged (20 min; 13,400 rpm; MiniSpin centrifuge, Eppendorf AG, Hamburg, Germany), filtrated (0.22 μm) and measured

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