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Research review paper

Renaissance of protein crystallization and precipitation in biopharmaceuticals purification

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The current chromatographic approaches used in protein purification are not keeping pace with the increasing biopharmaceutical market demand. With the upstream improvements, the bottleneck shifted towards the downstream process. New approaches rely in Anything But Chromatography methodologies and revisiting former techniques with a bioprocess perspective. Protein crystallization and precipitation methods are already implemented in the downstream process of diverse therapeutic biological macromolecules, overcoming the current chromatographic bottlenecks. Promising work is being developed in order to implement crystallization and precipitation in the purification pipeline of high value therapeutic molecules. This review focuses in the role of these two methodologies in current industrial purification processes, and highlights their potential implementation in the purification pipeline of high value therapeutic molecules, overcoming chromatographic holdups.

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1. The new trend: anything but chromatography

Recent developments in more efficient fermentation and cell culture technologies, led to an improvement in biopharmaceutical product titers from an average of 0.2 g/L in 1985 to 2.56 g/L in 2014, although monoclonal antibodies (mAbs) titers can reach 27 g/L (Perciva, 2008). This improvement in the upstream of biopharmaceuticals production led to a shift in the manufacturing bottleneck towards the downstream processing (DSP), which can weigh up to 70% of the total production costs (Broly et al., 2010). Currently, DSP face several challenges, namely the need to (i) deal with the high upstream titers and production volumes, (ii) accomplish high purities and yields with a reduced number of unit operations through process integration and intensification and (iii) comply with the trend of single-use technology DSP (Kuczewski et al., 2011; Shukla and Gottschalk, 2013).

For biopharmaceutical products with high added value, DSP design aims to achieve high quality, concentration, productivity and yield. DSP trains of biological products usually comprise four main steps:

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recovery, isolation, capture and polishing (Broly et al., 2010). The capture and polishing steps are frequently based on chromatographic operations with high costs associated with resin price (e.g. 5,000–14,000 €/L (Franzreb et al., 2014)) and operation costs (e.g. high buffer consumption of 300,000–2,000,000 L/batch). The development of new and more efficient upstream processes, leading to higher protein titers, constrains the chromatographic process of the capturing step (Gottschalk, 2006). As a result, new approaches to DSP design are being considered, due to the high costs associated. High-throughput (HTP) screenings are used in combination with statistically planned experiments (Design of Experiments, DoE) to recognize the influence of different factors, thus narrowing down the experimental conditions to be tested. In addition, Quality by Design (QbD) can also be implemented with HTP or DoE (Pathak et al., 2014; Zhang et al., 2014), combining in this way the manufacturing process with product quality, while improving process robustness and productivity.

Considering mAbs DSP, the conserved domains of mAbs led many companies to define a general purification process based on a common sequence of unit operations. The heart of the process is the protein A chromatography capture step usually followed by two polishing steps composed by ion exchange chromatography. Although stable, reliable, and reproducible, protein A chromatography is considered to be a productivity bottleneck, requiring several cycles per batch (e.g.1–8 cycles/batch mAb (Franzreb et al., 2014)), and being particularly expensive. In order to overcome the limitations demonstrated by chromatography such as high resin cost, diffusion and capacity limitations, new approaches based on Anything but Chromatography (ABC) methodologies are gaining increasing interest (Barroso et al., 2014; Dhadge et al., 2014b). Currently, new trends in non-chromatographic methodologies for DSP include membrane processes (Dong and Bruening, 2015), liquid–liquid phase extraction (Jacinto et al., 2015), magnetic separation (Batalha et al., 2010; Dhadge et al., 2014a; dos Santos et al., 2016), precipitation and crystallization (Gronemeyer et al., 2014). Non-chromatographic methods aim to reduce or even replace chromatographic operations in the purification pipeline, which can achieve high yields and purity, despite its disadvantages. On the other hand, non-chromatographic methods can cope with the high titers and volume feed produced upstream. Within the pool of non-chromatographic methods available, precipitation is being applied to the purification of some biopharmaceuticals (Hammerschmidt et al., 2015a; Sim et al., 2012a; Sommer et al., 2014), and protein crystallization is a new and challenging method that can overcome the current chromatographic challenges (Smejkal et al., 2013; Zang et al., 2011). Although crystallization and precipitation are currently being used for the purification of commercially available proteins, such as ovalbumin (Judge et al., 1995), lipase (Jacobsen et al., 1998), elastase (Lewis et al., 1956), protease I and II (Srivastava and Aronson, 1981) and lactose (Commins et al., 1980) as revised by Miranda et al., 2009, these are still not widely implemented in the industrial biopharmaceutical sector despite their potential (Gagnon, 2012).

2. Understanding protein precipitation and crystallization

Protein precipitation is based on the premise that a homogenous protein solution is only homogeneous up to a certain protein concentration from which a new phase, corresponding to the precipitate formation, starts to appear. By changing the environmental conditions, one expects to exceed the protein solubility limit, leading to the formation of the new solid phase. Protein crystallization is a form of precipitation in which the precipitate is formed in an ordered manner.

Protein precipitation is mostly used for low value products. However, due to its advantages in DSP, research has been conducted towards high value products, such as therapeutic proteins in the biopharmaceutical industry.

Protein precipitation depends on the solubility of a protein in an aqueous media, which is deeply related with the distribution of the hydrophobic and hydrophilic amino acid residues at the protein surface (Englard and Seifter, 1990). In a time where proteins were considered as colloids, the first protein precipitation experiments were carried by Antoine Fourcroy in circa 1789. Fourcroy was able to precipitate gelatin by boiling various animal by-products and albumin from egg white, blood serum and milk casein by heating, acid or alkaline precipitation (Tan and Yiap, 2009). The following studies focused on proteins that could be obtained in large quantities, namely from blood and egg white. The first studies using precipitation as a means for protein purification were developed by Edwin Joseph Cohn during World War II (1939–1945). He became famous for the separation of blood components by fractional precipitation. The Cohn fractionation process aimed at obtaining albumin to be administered to soldiers with blood loss, starting from blood plasma and taking advantage of the different solubility among the different plasma proteins (Cohn, 1941). This also showed that a protein purified by precipitation had biological activity. Since its discovery, protein precipitation has been used in proteins DSP, for purification and concentration purposes (Hinderer and Arnold, 2012; Sheth et al., 2014a).

With the development of single-crystal X-ray crystallography, the crystallization of macromolecules became one of the most common methods to determine protein structures and understand fundamental biochemical pathways or even design new drugs. However, by the time the first protein was crystallized, in the first half of the 19th century, crystallization was applied as a purification and biophysical characterization method (Giegé, 2013; Hünefeld, 1840). Nonetheless, the interest in crystallization from a purification point of view increased due to the low costs associated, the ability to cope with high volumes, and with high concentrations of the target molecule. In addition, crystallization in the DSP of biopharmaceuticals has the ability to integrate protein purification, stabilization and concentration in one single step.

Macromolecular crystallization can be performed by four different methods, namely vapor diffusion, microbatch, dialysis and free interface diffusion. In the vapor diffusion the protein sample is set to equilibrate with a reservoir solution that contains similar precipitant and buffer solution at a higher concentration. In batch crystallization, the supersaturated protein sample is mixed with the precipitants in the same mixture and then placed in a mineral oil of branched alkanes in the C20+ range allowing little to none water diffusion, in this way enabling a controlled evaporation. Crystallization by dialysis, makes use of dialysis membranes where ions can pass but not proteins or polymers, in this way slowly supersaturating the protein solution leading to crystal formation (Thomas et al., 1989). Free interface diffusion relies on carefully placing the precipitant solution on top of the protein solution in a capillary. Due to the narrow diameter of the capillary, the precipitant and protein will slowly inter-diffuse until equilibrium is reached, leading to protein supersaturation (Salemme, 1972).

Although a rational approach might be drawn the fact is that both precipitation and crystallization experiments have a rather empirical design, mainly due to the complexity of the occurring phenomena (Altan et al., 2016). Currently, there is no comprehensive theory behind the process in order to guide the experimental design. Furthermore, the wide range of macromolecules possible to crystallize enhances the complexity of the crystallization design. The guidelines for macromolecular crystallization involve a systematic search, changing individual parameters that can influence the crystallization process. Once found the set or multiple sets of parameters that yield the best precipitate/crystal outcome, an optimization can be performed to obtain the best possible crystals, which for crystallography purposes correspond to single, edge-shaped crystals.

Nonetheless, some theories have been proposed to understand protein behavior in an aqueous solution. Classical nucleation theory (CNT) aims to calculate the nucleation rate of a particular system, as a theoretical model this is an approximation (Lutsko and Durán-Olivencia, 2013; Sleutel et al., 2014). The phase diagram plots the protein concentration versus any adjustable parameter (e.g. temperature, precipitant

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