reduced prevalence of disease and pests in the region.

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Resources

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[ii]www.fao.org/food-chain-crisis/how-we-work/plant-protection/fallarmyworm/en/
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[v]www.malariavaccine.org/malaria-and-vaccines/need-vaccine
[vi]https://en.wikipedia.org/wiki/Sickle_cell_disease

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Forum

Precipitation as an Enabling Technology for the Intensification of Biopharmaceutical Manufacture

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Advances in precipitation have demonstrated the capability of purifying therapeutic proteins such as antibodies from biological sources in a scalable and cost-effective manner. We discuss the latest developments in the unit operation for downstream processing applications and provide a perspective on exploring precipitation for bioprocess intensification.

Principles of Precipitation and Its Role in Bioprocessing

Precipitation has played a vital role in the industrial purification of important therapeutics, most notably blood products from plasma fractionation [1]. Whilst precipitation is widely used for the purification of low-value products, the method has gained recent interest for the downstream processing of high-value biopharmaceutical products due to higher titres challenging the efficiency of traditional chromatographic methods. Protein precipitation involves converting proteins from the soluble state to the insoluble state via destabilisation in an aqueous solution upon changes in the solution conditions. Submicron-sized particles are formed during the nucleation phase and primary growth occurs under Brownian motion. Further growth is governed via particle–particle collisions until a stable size is reached, which depends on the shear field, mixing, and the individual proteins.

In general, precipitation methods target the protein of interest, which is recovered by centrifugation or filtration and subsequently resolubilised. Whilst taking this approach, precipitation can be irreversible, which prevents dissolution of the precipitate for further processing, but it may damage the native structure and activity of the protein, which are critical to its therapeutic activity. By contrast, precipitation can also be applied to remove impurities in the precipitate whilst leaving the target molecule in solution in the native state [2]. Precipitation can be induced by a wide variety of agents including neutral salts, organic solvents, nonionic polymers, polyelectrolytes, acids, and affinity ligands

Because of the complexity of protein precipitation phenomena induced, the mechanisms responsible are not well understood; in addition, they are strongly influenced by factors such as temperature, pH, ionic strength, protein concentration, and protein surface characteristics (i.e., distribution of polar and non-polar amino acids) as well as precipitating agent. Therefore, despite

Box 1. Patents Claiming Precipitation Processes for Therapeutic Production

From 2010 to 2017, a steady growth of precipitation patents for various therapeutics has been observed with a total of 246 patent submissions (Figure I). In particular, there has been an increase in the number of patents concerning antibody and fusion protein precipitation procedures between 2014 and 2017. Many of the fusion protein precipitation-based patents recorded concern the purification of Fc-fusion-based molecules. An influx of 21 patents for mAb precipitation and 11 patents for fusion protein precipitation occurred in 2017. Given the major contribution of antibody-based products to the current biopharmaceutical market, a huge investment towards R&D in developing these processes has been made by some biopharmaceutical companies such as Novartis, Amgen, and Bristol Myers Squibb. In terms of these patents, both product and impurity precipitation for bioprocessing are described. Several enzyme and hormone patents have also been published claiming patents for insulin crystallisation, thyroglobulin precipitation, and L-asparaginase precipitation. Plasma protein fractionation patents, by contrast, have appeared to decline, largely due to the already well-established commercial process (Cohn's method and adaptations thereof) and hence less investment from R&D.

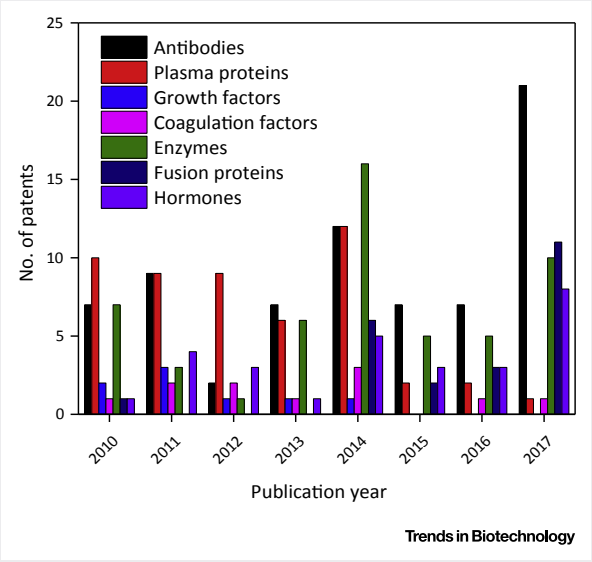

Figure I. Graphical Representation of the Number of Patent Submissions Concerning Therapeutic Purification with Precipitation.

the knowledge gained from earlier studies, developing a precipitation method for a specific macromolecule remains an empirical procedure that requires extensive characterisation.

Developments in Protein Purification with Precipitation

Bioprocesses implementing precipitation have already been established for purifying some biological products, including human serum albumin and erythropoietin [3]. Recently, many of the precipitation developments have revolved around monoclonal antibody (mAb) and Fc-fusion protein purification as an alternative to Protein A chromatography. This has led to an increased number of publications reporting precipitation strategies as well as an influx in patent submissions by some biopharmaceutical companies (Box 1). Given the complex nature of protein mixtures in cell culture fluid, this poses a challenge for precipitation processes, which are often less selective compared with Protein A chromatography for antibody capture [4]. Whilst single-step precipitation using conventional precipitants such as polyethylene glycol (PEG) [5] and ethanol [6] is capable of obtaining high antibody yields, an excess carry-over of impurities such as host cell proteins (HCPs), DNA, and aggregates is often observed. To overcome this, impurity precipitation and multistep precipitation strategies have been developed [2,6,7].

Precipitation Strategies Based on Conventional Precipitants

Calcium chloride ($CaCl_2$) and caprylic acid have been utilised as effective agents in impurity precipitation to remove DNA, HCPs, and high-molecular weight species whilst obtaining >80% mAb yields [2,8]. Caprylic acid, for example, significantly reduced HCPs from various Protein A eluate pools containing 13 different mAbs down to 38 ppm at acidic pH and up to 80 mM concentration [8]. Hammerschmidt and colleagues [4] studied a multistep process using calcium chloride flocculation and cold ethanol precipitation for mAb purification from cell culture fluid. The two-step precipitation process, involving 250 mM $CaCl_2$ (pH 8.5) at above 5 mM phosphate for DNA flocculation and 25% v/v ethanol (pH 6.5) for precipitation of the mAb product from CHO cell culture, improved DNA removal by up to 60-fold and additionally removed HCPs by up to a factor of 6 whilst obtaining >90% mAb yield with short processing times (<10 min) in a batch vessel and tubular reactor. Repeating this process for four mAbs gave purities comparable to Protein A chromatography (>95%) at the cost of lower yield (<85%) [6].

An alternative precipitation process using $CaCl_2$ for impurity precipitation in conjunction with PEG for mAb product precipitation gave mAb yields in the range of 80–95% and up to a sevenfold and 13-fold reduction in aggregate and HCP levels, respectively, depending on the mAb, PEG size, and PEG concentration [9]. A sequential application of 3% (w/v) PEG6000 (pH 4) to precipitate impurities followed by 14% (w/v) PEG6000 for mAb precipitation showed >90% mAb yield and >96% purity for batch and continuous processing [5]. A four-step

Box 2. Reactors and Scaling Considerations for Precipitation Unit Operations

Batch Stirred Tank Reactor

Precipitation processes are often performed in large stirred tanks whereby the precipitant is slowly fed to the protein solution at a constant mixing rate to avoid local concentration gradients and solution heterogeneity (Figure IA). When precipitation equilibrium or steady state is reached, the suspension is then harvested by centrifugation or filtration. This reactor configuration is often used for blood plasma fractionation [1].

Continuous Tubular Reactor

In the continuous tubular reactor (CTR) configuration, precipitant and protein solutions are fed continuously through an initial mixer (static or active) at a defined flow rate and then passed through a long tube whose length is determined by the precipitation kinetics (Figure IB) [14]. Minimal mixing occurs as the reaction proceeds through the reactor and the suspension progresses in a pluglike flow fashion. Coiling or bending of the tube minimises the use of space required for operation.

Coiled Flow Inversion Reactor

Similar to the CTR, initial mixing of precipitant and protein solution is achieved with an in-line mixer before proceeding through a series of coiled flow inversion reactors (CFIRs) [13]. Each CFIR consists of helical coils of tubing, which are bent at equidistant right angles around pipes (Figure IC). The helix configuration induces a secondary flow pattern called Dean vortices due to centrifugal forces which enhance radial mixing and a sharper residence time distribution. Right-angle bends further enhance radial mixing and provide even narrower residence time distributions to better emulate plug flow compared with the CTR configuration.

Scaling up

One approach to scale up is the Camp number (N_{Ca}), which is the product of the shear rate and aging time [14]. When $N_{Ca} > 10^5$, precipitates are said to be mechanically stable to withstand shear forces experienced in industrial separation equipment. Various reactor designs are available, which can or have the potential to accommodate large-scale precipitation processes. Whilst traditional precipitation is performed in stirred tanks, large amounts of precipitant relative to product will result in exceedingly large volumes, and mixing becomes more challenging. Scaling-up a batch process is typically based on achieving a constant power per unit volume, which requires higher energy inputs with increasing volume. To overcome this, alternative reactors – including the CTR and CFIR – have been employed for continuous precipitation and have shown similar performance to batch precipitation but with significantly higher productivity [4,13]. These reactors also have the advantage of incorporating in-line monitors to track process conditions in real-time, enabling quick responses to process changes [13]. However, precipitations that require residence times on the order of several hours may not make much sense for continuous processing as this would involve long tubing and low flow rates affecting overall productivity and hence be more suitable for batch processing.

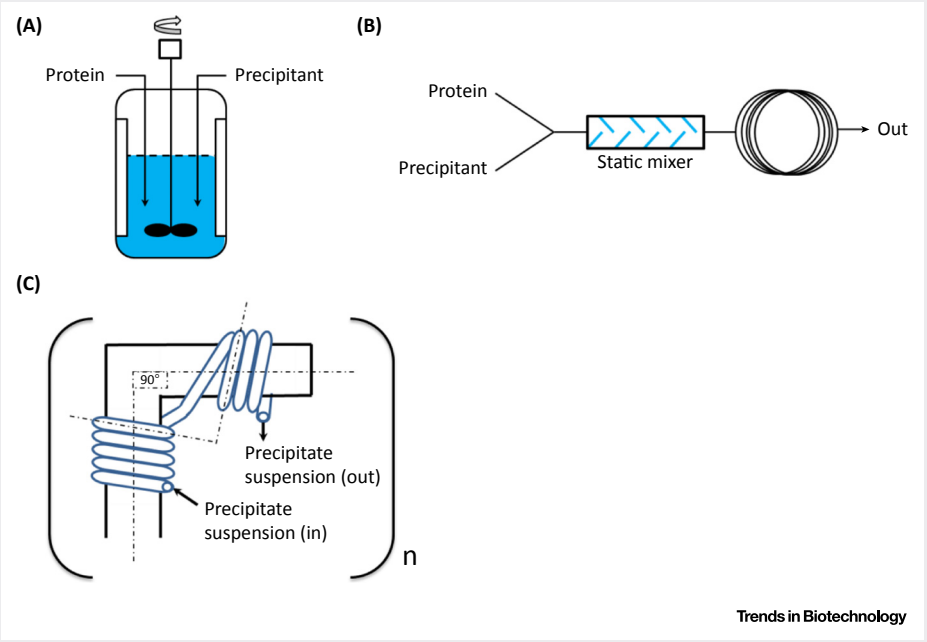

Figure I. Schematic Illustration of Different Reactors. (A) Batch stirred tank reactors, (B) continuous tubular reactors, and (C) coiled flow inversion reactors for precipitation applications.

precipitation procedure using caprylic acid (impurity precipitation), PEG (mAb product precipitation), $CaCl_2$ (impurity precipitation), and cold ethanol (mAb product precipitation) directly from cell culture fluid achieved an overall mAb yield of 70%, removal of HCPs as low as 300 ppm, and removal of DNA up to 94 ng/mL; besides, there were no detectable aggregates, all of which approach the profile of a pharmaceutical-grade product [7].

Tag- or Affinity-Based Precipitation

A novel precipitation approach is to use affinity ligands or peptide tags that can form complexes with therapeutic products and become precipitated under certain conditions [10–12]. In one such example, a pH-responsive short peptide tag derived from *Corynebacterium glutamicum* cell surface protein B (CspB) fused to proinsulins, Teriparatide and Bivalirudin, enabled a reversible and potentially cost-effective precipitation process for unstable therapeutics at nonphysiological conditions with near 100% recoveries. Similarly, the pH-responsive polyelectrolyte Eudragit S-100 fused to *Staphylococcus aureus* Protein A could precipitate 89% of mAb under pH 5.2 and 7 °C with >95% purity in a single step at a precipitant-to-mAb ratio of 6:1 [10]. Swartz and colleagues [11] used Z-domain-elastin-like polypeptide (Z-ELP) nanocages containing an IgG-binding domain to precipitate mAbs and Fc-fusion proteins with solution pH. Precipitant-to-target protein ratios of 3:1 were shown to precipitate >95% product from cell culture fluid at ambient temperature with short residence times (<30 min) and resulted in >99% removal of HCP and DNA for some mAbs depending on the pH.

In another study, Handlogten and colleagues [12] demonstrated a rapid purification procedure for the pharmaceutical antibodies trastuzumab and rituximab using a two-step process involving ammonium sulfate precipitation and affinity-based precipitation using synthetic trivalent haptens. In the first step, ammonium sulfate at 1.2 M was used for the precipitation of large impurities, and in the second step, trivalent haptens were added to the soluble fraction (1.1 haptens per antibody) to form cyclic complexes with bivalently active antibodies and immediately induce precipitation without altering the ammonium sulfate concentration. This method obtained >85% yield and >95% purity of bivalent active antibodies. Classic precipitants can be challenging to recycle, but affinity tags could be dissociated from their complexes using chromatography or filtration and reused for further purification cycles. The cost of affinity tags must however be considered to justify a tag-based precipitation for a platform process.

Concluding Remarks and Future Prospects

Given the increased demand for therapeutic production, downstream processing methods such as precipitation can offer a scalable and cost-effective alternative. Protein purification with precipitation has the flexibility of using various cheap precipitating agents for protein capture, intermediate purification, and polishing with relatively short process times, which can be adapted to batch or continuous processing. To compete with conventional chromatography, precipitation methods must undergo extensive development and optimisation, mostly in terms of impurity removal, before being considered as alternatives for an industrial process. High-throughput process development in combination with design of experiments approaches can help to understand and identify the process conditions that influence precipitation performance but remain a laborious task. Because of the large number of variables, the required process analytical technology must be identified and implemented to monitor performance and ensure process robustness in accordance with industry standards. Recent studies provide more confidence in the possibility of implementing precipitation in platform processes with precipitants such as PEG, ethanol, caprylic acid, calcium chloride, and affinity tags. However, large-scale studies are needed to determine the performance and economic viability of using these agents for inducing precipitation at commercial scale.

Applying precipitation in an industrial setting requires a number of considerations prior to formulating a scale-up strategy because performance is likely to differ with scale. Considering the short process times reported for recent antibody precipitation studies, implementing continuous reactors is appealing. Options for scaling up these reactors (Box 2) might include increasing the pipe diameter and flow rate to have the same residence time [13]. However, yields could potentially differ as a result of the differences in flow regime and mixing efficiency. Alternatively, a scale-out approach could be more desirable, which would provide identical flow patterns and yields, but at the cost of more pumps and facility space.

Over the longer term, another strategy is recycling the precipitant with tag- or affinity-based precipitation methods, but the regulatory burden associated with tag/ligand robustness or consistent impurity removal has not been addressed. Affinity-based precipitation is appealing due to its potential to provide a platform process which would be more amenable to rapid process development. Nonetheless, further development is required, and an evaluation of the costs is needed.

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