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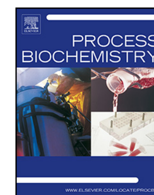

Real-time monitoring of protein precipitation in a tubular reactor for continuous bioprocessing

Monika Zelger [b], Siqi Pan [b], Alois Jungbauer [a,b], Rainer Hahn [a,b,*]

[a] Department of Biotechnology, University of Natural Resources and Life Sciences Vienna, Muthgasse 18, 1190 Vienna, Austria
[b] Austrian Centre of Industrial Biotechnology, Muthgasse 18, 1190 Vienna, Austria

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ABSTRACT

Real time monitoring is critical to apply continuous bioprocesses successfully. This requires the integration of flow cells into continuous reactors. Potential continuous steps include protein precipitation, used in plasma purification, antibody enrichment and virus inactivation. Consequently we developed real-time monitoring of protein precipitation in a tubular reactor by pH, conductivity, turbidity and image processing. Feasibility studies were then performed for each monitoring method. Since integrated flow cells can affect time to reach steady state due to its inherent residence time distribution (RTD) property, we first determined flow behavior in flow cell is best described by laminar flow in pipes model, in order to find acceptable levels of volumetric flowrates that is not impractically high. Separately, we found pH highly important for protein precipitation while conductivity has high sensitivity to changes in buffer content. Turbidity signals are lumped values of floc properties while image processing can separately measure flocs by area, size and count. Also, the time and behavior to reach steady state amid changing precipitating conditions was investigated, to select for correct collection times to ensure high product quality. Lastly, we report the technical considerations in reactor design, construction and operation.
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1. Introduction

The benefits of continuous processing of biopharmaceuticals include universal platform, steady state, closed systems, easier scale up, minimized hold times, continuous flow, high volumetric productivity, flexible capacity, simplified operation and is compatible with disposable technology [1–3]. Examples include refolding [4,5], precipitation [6,7] and chromatography [8–10]. Economically, continuous processing can reduce cost of goods in different process scenarios [11]. Furthermore, continuous processing for biosimilars becomes economically favorable as the biopharmaceutical sector matures [12].

Among the bioprocessing steps, protein precipitation is simple and versatile. It can enrich proteins [13], remove contaminants [14], and allow selective precipitation of proteins [15]. Economically, it is an alternative to expensive chromatographic resins [16], where precipitation could replace the initial chromatography steps (eg. ion exchange for capture). Furthermore, the mixing of protein solution to precipitant to desired conditions in precipitation enables easy transfer from batch to continuous reactors [6,7,17].

However, industrial application of continuous processes can only be realized when quality by design (QbD) requirements are met [18,19]. Particularly, continuous bioprocessing in downstream require better process analytical technology (PAT), with reliable control and hardware than is currently available [12]. This requires real time monitoring of process, where critical process parameters can be automatically adjusted in response to disturbances to ensure that the quality attributes consistently conform to the established acceptance criteria, required for industry to adopt continuous process [20].

Consequently, as an extension to previous work [5,6], we integrated several inline monitoring probes and used image processing for monitoring the precipitation step in a tubular reactor. Feasibility studies were then performed for each monitoring method. Specifically, we investigated 1) flow behavior of processing liquid caused by integrated flowcells, 2) characterize the different monitoring methods based on pH, conductivity, turbidity and image processing, 3) time to reach steady state amid changes in process conditions and 4) relationship between process conditions (pH, protein concentration) to precipitate properties via turbidity measurements and image processing. Additionally, we report the technical considerations for future work to better satisfy QbD and PAT paradigm.

* Corresponding author at: Department of Biotechnology, University of Natural Resources and Life Sciences Vienna, Muthgasse 18, 1190 Vienna, Austria.
E-mail address: rainer.hahn@boku.ac.at (R. Hahn).

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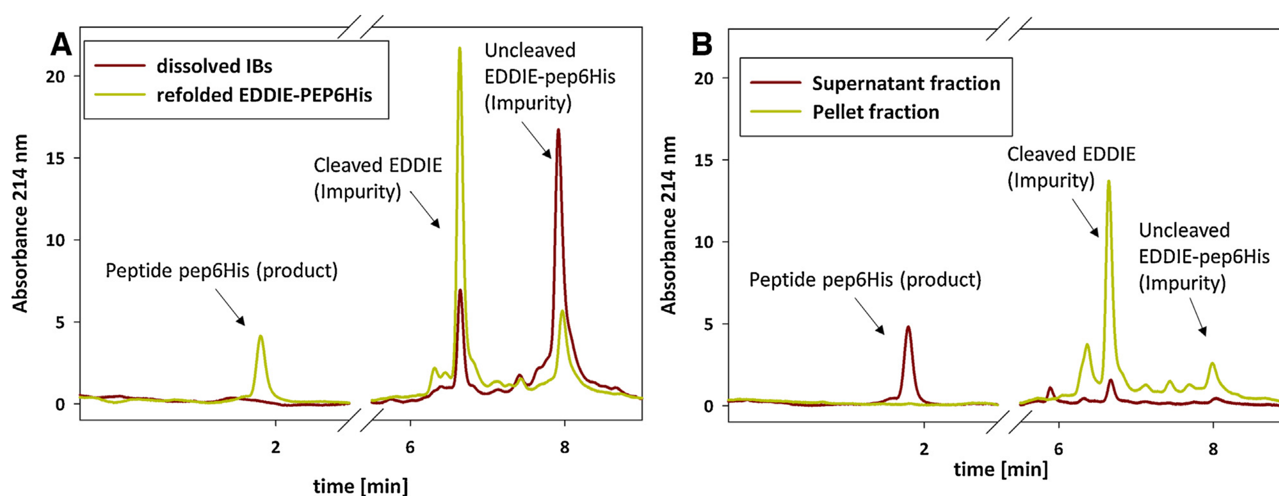

Fig. 1. Representative chromatograms; A) for dissolved IBs before refolding and refolded EDDIE-pep6His. B) Targeted peptide (pep6His) is purified and remains soluble in supernatant after acid precipitation at pH 4.4.

In current study, precipitation was performed in a small scale tubular reactor. The model protein used to perform precipitation was the refolded autoproteolytic fusion protein EDDIE-pep6His, which was overexpressed as inclusion bodies (IB) in *Escherichia coli* [21]. Under denaturing conditions of the dissolved IBs, EDDIE remains fused to its peptide partner, pep6His (a short 16 amino artificial peptide consisting of 10 randomly chosen amino acids and a C-terminal polyhistidine tag) [21]. However under refolding conditions, EDDIE releases its fused target pep6His (Fig. 1A). After refolding, the cleaved EDDIE and remaining uncleaved EDDIE-pep6His was precipitated out by lowering processing solution to below pH 5.0, while pep6His product remains soluble in supernatant (Fig. 1B). In this study, the precipitation step was performed in a tubular reactor and monitored in real time.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Merck (Darmstadt, Germany) and Sigma (Steinheim, Germany) respectively, if not indicated otherwise.

2.2. Recombinant protein expression and IBs isolation

The recombinant protein EDDIE-pep6His were overexpressed in *E. coli* BL 21 with a pET30a plasmid (Novagen, Madison, WI, USA) containing the corresponding coding gene [21]. *E. coli* fed-batch cultivation was performed with a semi-synthetic medium on a 5-L scale according to Kaar et al., and isolation of inclusion bodies (IBs) was performed with an APV 2000 lab homogenizer (Invensys, Albertslund, Denmark) [22].

2.3. Batch dissolution and refolding of EDDIE-pep6His

Refolded material of EDDIE-pep6His for precipitation in tubular reactor was prepared by dissolving 1 part IBs to 4 parts dissolution buffer containing 10 M urea, 50 mM Tris and 100 mM monothioglycerol (MTG) at pH 7.3. To measure protein concentration, dissolved samples were filtered and measured on a Cary 50 UV–vis Spectrophotometer using the theoretical extinction coefficient 1.098 (mg/mL protein) cm^{-1} at 280 nm. Subsequently, overnight refolding was initiated by mixing 1 part dissolved protein to 6 parts refolding buffer containing 1 M Tris, 0.25 M sucrose (Acros Organics), 2 mM EDTA, 20 mM MTG (pH 7.3).

2.4. Residence time distribution (RTD) analysis of flowcell

A tubular reactor was set up with one IPC-4 peristaltic pump (ISMATEC, Wertheim, Germany), short hose of Tygon tube at 3.2 mm inner diameter (ISMATEC, Wertheim, Germany), respective flowcells shown in Fig. 2 (Swagelok W/TWO TUB (Hamilton) and self-designed turbidity flowcell (Bilek and Schüll GmbH, Vienna, Austria) and a fraction collector (Frac-920, GE Healthcare) (Fig. 3). To evaluate the residence time and the effective volume of the flowcell positive step input experiments at two different flow rates, 1 mL/min and 5 mL/min were performed. A distinct amount of Vitamin B12 dissolved in 1x PBS buffer served as positive step input. The whole set up was filled with 1x PBS buffer before the Vitamin B12 input was started. The solution was pumped through the flowcell at the respective flow rate and collected in fractions (a 0.6 mL and 0.5 mL) after exiting the flowcell by mean of a fractionizing collector. Absorbance at 550 nm of each fraction was measured by NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) and concentration calculated using a self-established calibration curve. Concentrations were normalized by the initial concentration of the step input and plotted against time in order to obtain the cumulative distribution function $F(t)$. Subsequently, the best fitting model for the $F(t)$ curve was investigated using excel solver and table curve 2D (Systat Software).

2.5. Construction and operating parameters of tubular reactor

Using data from RTD analysis, acid precipitation experiments were carried out in the tubular reactor at a total flow rate of 5.00 mL/min. Tubular reactor was set up using IPC 4 and IPC-N peristaltic pumps (ISMATEC, Wertheim, Germany), GXM mixer (Stamixco, Winterthur, Switzerland), Tygon tubes of 3.2 mm inner diameter (ISMATEC, Wertheim, Germany), Swagelok W/TWO TUB flowcell equipped with pH and conductivity sensor (Hamilton, Bonaduz, Switzerland) and fractionizing collector (Frac-920, GE Healthcare). Conductivity and pH in the flowcell were recorded by Hamilton Device Manager (HDM) software every 5 s. Conductivity sensor was calibrated offline and temperature compensation factor was set at 2%/°C.

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