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Oxidative protein refolding on size exclusion chromatography: From batch single-column to multi-column counter-current continuous processing

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HIGHLIGHTS

- Mathematical model presented for its applicability for protein oxidative refolding/aggregation predictions in SMB-SEC.
- The model considers a wider loading concentration range of the model protein (lysozyme) on SEC.
- It was observed that at higher loading concentrations aggregation occurs when local protein concentration exceeds a critical concentration.
- The model was found to predict the SMB-SEC performance of solubilized protein recovery.
- No urea recovery at the product stream indicated that the refolding reaction will continue off-column to recover the native-protein product.

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ABSTRACT

Recently protein refolding on size exclusion chromatography (SEC) operated in multi-column continuous simulated moving bed (SMB) configurations (hereinafter SMB-SEC) has been investigated for future industrial applications. This is due to several advantages offered by SMB configurations particularly when process parameters are thoroughly screened and optimized. A robust mathematical model is essential for high-throughput process screening and optimization. In this work, a previously investigated single-column mathematical model was modified to extend its applicability for protein oxidative refolding/aggregation predictions in SMB-SEC. The model considers a wide loading concentration range of the model protein (lysozyme) on SEC. The potential influences of high concentrations of chaotropic reagents on kinetic and thermodynamic model parameters have been discussed based on previous experimental results and their predicted local concentrations through the SMB-SEC columns and at the product stream. It was observed that aggregation occurs when local protein concentration exceeds a critical concentration. No urea recovery at the product stream indicated that the refolding reaction will continue off-column to recover the native-protein product. Therefore, it is suggested that the developed model is tested against experimental results for total soluble protein (early intermediates and native conformations) in the presence of L-arginine additive and process performance indicators are defined based on this criterion.

1. Introduction

Despite the advances made to date for expression of protein-therapeutics using *Escherichia coli* (Huang et al., 2012), existing technologies to recover active and high-purity product still incur significant costs due to low product concentration and high buffer consumption during conventional batch dilution refolding process resulting in low volumetric productivity (Freydell et al., 2011). As

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an alternative, SEC has been widely used at lab scale (Freydell et al., 2011; Freydell et al., 2010a; Gu et al., 2001; Lanckriet and Middelberg, 2004; Park et al., 2006; Wellhoefer et al., 2014, 2013). Due to the gradual separation of denaturing and reducing agents from unfolded protein molecules and their separation from soluble aggregates, SEC allows for higher working concentrations compared to dilution refolding and results in high refolding yield and purity. However, this method in form of single-column batch processing may not enhance the process performance indicators (e.g. productivity and buffer consumption) for industrial scale production of *E. Coli*-based protein-therapeutics. On the other hand, a multi-column continuous simulated moving bed system offers several advantages compared to single-column batch operation including increased productivity per unit mass of solid phase, lower solvent consumption, and less diluted products. This configuration consists of a set of chromatographic columns connected in series and is operated in continuous mode; the inlet/outlet lines are periodically shifted synchronously in the direction of liquid-phase flow to mimic countercurrent movement between a liquid-solvent and a solid phase. Multi-column continuous simulated moving bed is a well-established process for intensified difficult separations of small molecules and fine products e.g. separation of enantiomers (Rajendran et al., 2009; Ströhlein et al., 2005). However, its application for protein refolding and separation has recently attracted the attention of researchers (Freydell et al., 2010b; Park et al., 2006; Wellhoefer et al., 2014, 2013). For example, Freydell et al. have reported a 35 times increase in productivity and 1/10th solvent consumption when a SMB-SEC was used for refolding of a model fusion protein compared to single-column processing.

Since many parameters are involved in operating a SMB-SEC (i.e. internal and external flow rates, switching time and feed concentration) systematic optimization studies are required to exploit the full potential of this system. A mathematical model is a pre-requisite for screening of process parameters and optimization. Freydell et al. used a previously investigated single-column model in order to predict refolding/aggregation of a fusion protein in a four-zone SMB-SEC configuration comprised of multiple columns connected in series. They observed considerable discrepancy between model predictions and experimental results based on native refolded protein recovery. For instance, the refolding yield was over-predicted by a factor of three. As discussed in the same work, this disagreement can be related to lower dilution factor in SMB-SEC compared to a batch single-column refolding resulting in higher local concentration of chaotropic agents (urea and DTT). And, in order to improve the SMB-SEC model- predictions in terms of native protein recovery the influence of local concentration of urea and DTT on model parameters should be considered.

The effect of lower dilution factor is twofold, as in addition to higher local concentrations of chaotropic reagents it also results in higher local protein concentration compared to a batch single-column refolding. Higher local protein concentration may additionally result in different reaction schemes. For example if for a single-column refolding experiment no aggregation was observed there would still remain the possibility of aggregation in SMB-SEC at the same protein loading concentrations.

In this work, (1) a previously experimentally verified single-column model was modified to expand its applicability for prediction of oxidative protein refolding/aggregation on SMB-SEC by considering a wide protein loading concentration range (i.e. low loading concentrations where no aggregation was observed and high loading concentrations where aggregation occurred) and the additional model parameters resulting from this modification were determined experimentally. The approach presented in this work is different from that of existing research work where only high loading concentrations are considered to model protein refolding/aggregation; (2) the sensitivity of refolding kinetics and possible complexity arising from reducing agent (DTT) carry-over have been discussed and DTT-free refolding was investigated and compared to previous studies with DTT carry-over; (3) the denaturing reagent (urea) mass transfer parameters were measured experimentally and used to predict the concentration of urea through SMB-SEC columns and at the product outlet under the current operation conditions; (4) the suitability of the developed model for process optimization was explored and an appropriate criterion for model validation was proposed; and (5) the effect of SMB-SEC operating parameters namely loading concentration and switching time on process performance indicators were predicted and the results were compared to single-column oxidative refolding of lysozyme.

2. Mathematical model and theory

2.1. Column model

The protein refolding in size exclusion column was modeled using dispersive transport in the bulk with a film linear mass transfer resistance between particle-solid and bulk-liquid phases. The formulated differential mass balances for solutes in the bulk and the solid phases are (Freydell et al., 2010a)

$$\frac{\partial C_{b,i}}{\partial t} = D_L \frac{\partial^2 C_{b,i}}{\partial x^2} - u\frac{\partial C_{b,i}}{\partial x} - Pk_{ov,i}\left(C_{eqS,i} - C_{s,i}\right) + r_{b,i} \tag{1}$$

$$\frac{\partial C_{s,i}}{\partial t} = k_{ov}\left(C_{eqS,i} - C_{s,i}\right) + r_{s,i} \tag{2}$$

where $C_{b,i}$ and $C_{s,i}$ are the concentration of solute i (unfolded, intermediates and native conformations) in bulk and solid phase respectively. Note that in Eq. (1), the total concentration of species is constant. In Eqs. (1) and (2), $t$represents time, x axial distance along the column, D_L axial dispersion coefficient, u interstitial velocity, $k_{ov,i}$ solute overall mass transfer coefficient, P phase ratio, $C_{eqS,i}$ the solid phase concentration in equilibrium with the bulk concentration. $r_{b,i}$ and $r_{s,i}$ are the net concentration change due to refolding and aggregation reactions in bulk and solid phases, which are described further in Section 2.3.

The solute solid phase concentration in equilibrium with the bulk concentration was treated as a linear equilibrium relationship with a fixed equilibrium constant (Zelic and Nesek, 2006):

$$C_{eqS,i} = K_{eq,i} C_{b,i} \tag{3}$$

where $K_{eq,i}$ is the equilibrium constant.

The boundary and initial conditions used to solve Eqs. (1) and (2) are as follows:

$$C_{b,i}\left(t, 0^-\right) = \begin{cases} C_{f,i} & 0 < t < t_{pulse} \\ 0 & t > t_{pulse} \end{cases} \tag{4a}$$

$$\frac{\partial C_{b,i}}{\partial x}(t, L_C) = 0 \tag{4b}$$

$$C_{b,i}(0, x) = 0 \tag{4c}$$

$$C_{s,i}(0, x) = 0 \tag{4d}$$

where $C_{f,i}$ is solute concentration in feed, t_{pulse} is the duration of sample injection, and L_C is the column length. The assumption that