Research Article

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Simulation of mono-PEGylated lysozyme separation in heparin affinity chromatography using a general rate model

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Abstract

BACKGROUND: The bioavailability of therapeutic proteins is improved through PEGylation. This chemical modification involves the production of isomers with different numbers and sites of attached PEG chains, which are difficult to separate efficiently. Their purification with chromatography requires an understanding of the operation and the evaluation of different operational conditions. The General Rate Model (GRM) was applied for modelling the linear salt gradient elution of mono-PEGylated and native lysozyme in Heparin Affinity Chromatography (HAC) considering mass balance equations for proteins in the bulk-fluid phase, in the particle phase and the kinetic adsorption.

RESULTS: The model was able to simulate the individual proteins and the separation of these in a PEGylation reaction using as proof-of-concept a mono-PEGylated and native lysozyme mixture under changes of operational parameters such as the gradient length (5, 13, 25 column volumes) and flow (0.8 and 1.2 mL min^{-1}) with a relative error in retention times of less than 6% and correlation coefficients greater than 0.78.

CONCLUSION: Simulation of the elution curves of PEGylated lysozyme in HAC was performed in this work and the diverse information generated by the model is explained through the physicochemical protein properties. This simulation represents a tool for optimization, prediction and future scale-up of PEGylated proteins purification, which would reduce the investment in time and resources to test several operating conditions.

Supporting information may be found in the online version of this article.

Keywords: simulation; mono-PEGylated lysozyme; heparin affinity chromatography (HAC); general rate model (GRM); PEGylation

NOTATION

Bi_i	Biot number for mass transfer for component I, $k_i R_p/\varepsilon_p D_{pi}$
Co_i	Maximum concentration of protein i, equal to initial feed concentration of the component
C^∞	Maximum capacity of the column
$C_{A,N+1}$	Initial dimensionless concentration of the modulator in the mobile phase
c_{bi}	Dimensionless concentration of component i in the bulk-fluid phase
$C_{fi(\tau)}$	Feed concentration of component i
c_{pi}	Dimensionless concentration of component i adsorbed to the resin
d	Inner diameter of the column
Da_i^a	Damköler number for adsorption of component i, $Lk_{ai}Co_i/v$
Da_i^d	Damköler number for desorption of component i, L_{kdi}/v
D_{bi}	Axial dispersion coefficient of component i
d_{mi}	Solute molecular diameter
d_p	Pore diameter of the particles
D_{mi}	Molecular diffusivity
D_{pi}	Effective diffusivity of component i
F	Flux of the mobile phase
L	Column length
k_{ai}	Adsorption rate constant for component i
k_{di}	Desorption rate constant for component i
k_i	Mass transfer coefficient of component i

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N	Number of proteins in the sample. Modulator corresponds to component N+1
Nr	Radial dimension, interior orthogonal collocation points
Nz	Axial dimension, finite element discretization points
Pe_{Li}	Peclet number for mass transfer component i, vL/D_{bi}
r	Dimensionless radial coordinate
R_p	Radius of the adsorbent particle
Re	Reynolds number, $2R_p v\rho/\mu$
Rh	Viscosity radii
v	Interstitial velocity, $4F/\tau d^2\varepsilon_b$
z	Dimensionless axial coordinate
α_i, β_i	Experimental parameters for the exponential elution relationship
ε_b	Bed void volume fraction
ε_p	Adsorbent particle porosity
τ	Dimensionless time
τ_{tor}	Tortuosity
ηi	Dimensionless parameter for component i, $\varepsilon_{pi}D_{pi}L/Rp^2v$
μ	Viscosity of the mobile phase,
ξ_i	Dimensionless parameter for component i, $3Bi_i$ η_i $(1-\varepsilon_b)/\varepsilon_b$
ρ	Density of the mobile phase

INTRODUCTION

PEGylated proteins are therapeutics with improved biodistribution, physical and chemical properties caused by the addition of polyethylene glycol (PEG) to the protein such as good solubility, resistance to proteolysis, retarded kidney elimination, and non-toxicity.[1,2]

Lysozyme is a model enzyme which interacts with large substrates, and it has demonstrated bactericidal activity with numerous applications as a food preservative, antibiotic and pharmacological agent;[3,4] it is for this reason that the PEGylation of this protein and its use are being widely studied.[5,6] The lysozyme PEGylation reaction contains reactive PEG, unmodified proteins and PEG-modified proteins or conjugates; of the latter only the mono-PEGylated conjugates have the appropriate characteristics and efficacy for their beneficial action. Despite the fact that diverse chromatographic modes in a packed bed column have been tested in one-single step purification, such as size exclusion (SEC), ion exchange (IEX), reverse phase (RPC) and hydrophobic interaction (HIC), the yield and purity are usually low.[7] Recently, it has been demonstrated that heparin affinity chromatography (HAC) is able to separate the products of the lysozyme PEGylation reaction with high yield and purity.[8] However, purification of mono-PEGylated lysozyme and any other PEGylated proteins at pilot production level demands knowledge about the mechanism governing the separation so as to understand, optimize, control, predict and scale-up the chromatographic operation. Simulation and modelling of chromatographic processes is a tool to reach these aims,[9] in addition to selecting strategic directions in the design, thus saving time and resources.[10] Until now, simulation of chromatographic separation of polymer grafted proteins has not been studied and it can help to overcome or make efficient the current challenges and difficulties in the post-production of PEGylated proteins. Since the original physical and chemical properties of the PEG-modified proteins are changed by the PEGylation,[11] it is interesting to evaluate if the separation of PEGylated proteins can be predicted through the simulation. In this way, many trials in the chromatographic purification of PEGylated proteins may reduce costs in the optimization of expensive PEG-modified proteins.

The theoretical relationships in a process such as chromatography are described in a mathematical model, a set of expressions; then these equations are solved under specific conditions.[12] Among the most used models to represent and simulate the behavior of adsorptive chromatography with proteins is the general rate model (GRM).[10,13] However, in affinity chromatography, examples of the application of the GRM are few. These include the scale-up of the separation of a bovine serum albumin (BSA) and hen egg white lysozyme solution on a Cibacron Blue F-3GA column,[14] the salt gradient elution of bovine serum albumin (BSA) and rabbit hemoglobin (Hb) from Blue Sepharose CL-6B, and the pH gradient elution of a three-mouse antibody mixture (Ig G1, Ig G2a and Ig G2b) from protein A.[15]

The objective of this research was to model and simulate the elution curves of mono-PEGylated lysozyme and native lysozyme in HAC with a linear salt gradient elution applying the theory of the GRM. Also the simulation efficiency with changes in operational parameters (flow and gradient length) was evaluated.

THEORY

General rate model

The general rate model (GRM) is a mathematical model used for studying chromatographic phenomena using rate expressions which represent the mass transfer of the components (protein and modulator or salt) in the system. The GRM considers in the mathematical formulation the adsorbent properties, process conditions and different mass transfer processes.[13,16] The model is integrated by three sets of differential equations (Equation (1) to (3) in Supplementary material), two of them describing the mass balance of the components in the bulk-fluid phase and in the particle phase inside the bed,[17] and the third representing the adsorption mechanism of the proteins to the adsorbent.

In the formulation of the GRM, the assumptions considered are: isothermal chromatography, spherical and uniform diameter of adsorbent particles, negligible radial dispersion in the column and no convective flow inside the macropores. There is an instantaneous equilibrium between macropore surfaces and the stagnant fluid inside the particles; diffusional and mass transfer parameters are constant and independent from the mixing effects of the components involved; the column was pre-equilibrated. Before the sample load the system does not contain protein; symmetric distribution of the compounds inside the adsorbent; and the column outlet protein dispersion flux is null.[13,17]

Mass balance of the protein in the bulk-fluid phase takes into account diffusion, convection, accumulation and interfacial flux from bulk-fluid to particle (Equation (S1) in Supplementary material). For the salt component, interfacial mass transfer is not considered. The mass balance of the particle inside the pore involves accumulation in the micropore, accumulation in the macropore, and radial diffusion inside the porous particle (Equation (S2) in Supplementary material). Finally, the adsorption of the protein is modeled as a second-order kinetic binding reaction (Equation (S3) in Supplementary material) and initial and boundary conditions are given by Equations (S4) to (S10) in Supplementary material.

The affinity of the proteins for the ligand and the modulator concentration in the mobile phase were described with a linear

relationship proposed by Melander *et al.*[18] and validated by Sandoval *et al.*[15] in affinity chromatography:

$$\log_{10} b_i = \alpha_i - \beta_i C_{b,N+1} \quad (1)$$

with b_i being a parameter in the Langmuir isotherm, in Equation (2), that considers equal saturation capacities (C^{∞}) for all the components:

$$c_{pi}^{*} = \frac{a_i c_{pi}}{1 + \sum_{j=1}^{N} b_j C_{0j} c_{pj}} \quad (2)$$

a_i and b_i are related to Damkhöler numbers of adsorption and desorption through Equation (3)

$$b_i C_{oi} = \frac{Da_i^a}{Da_i^d}, \quad a_i = C^{\infty} b_i = c_i^{\infty} \frac{Da_i^a}{Da_i^d} \quad (3)$$

MATERIALS AND METHODS

Materials

The resin Heparin Sepharose 6 Fast Flow (Cat. No. 17099801) was purchased from GE Healthcare (Uppsala, Sweden). Lysozyme from chicken egg white (Cat. No. 10837059001) was acquired from Sigma Aldrich. Methoxy-PEG-propionaldehyde (Cat No. A3001-10) with a nominal molecular weight of 20 kDa was obtained from Jen Kem Technologies (TX, USA). Tris buffer grade (Cat. No. TR-16514) came from Winkler LTDA (Santiago, Chile). Sodium chloride (Cat. No. 106404) came from Merck Millipore (MA, USA). The mono-PEGylated lysozyme standard was prepared and purified as indicated by Mayolo-Deloisa *et al.*[19]

Chromatographic experiments

Resin was packed into a 5/5 HR column (5 cm length, 0.5 cm diameter, Pharmacia Biotech) and chromatographic experiments were performed in an Äkta Purifier 10 System (GE Healthcare, Uppsala, Sweden) equipped with a 200 μL injection loop using a linear salt gradient of 1 mol L^{-1} of sodium chloride as was pointed out by Mejía-Manzano *et al.*[8] As mobile phases A and B, 20 mmol L^{-1} Tris-HCl pH 7.5 and 20 Mm Tris-HCl pH 7.5 containing 1 mol L^{-1} NaCl were used, respectively. The detection of native and mono-PEGylated lysozyme was done at 215 nm.

Individual standards of the proteins were injected separately to obtain the elution curves at a flow of 0.8 mL min^{-1} and 13 column volumes (CVs). To represent the lysozyme PEGylation reaction, mixtures containing mono-PEGylated and native lysozyme in mass ratio (4:1)[8] were prepared at a total protein load of 1 mg mL^{-1}. These mixtures were analyzed in the Äkta Purifier 10 System at different combinations of flow and gradient length as shown in Table 1.

Software and numerical methods for simulation

The formulated rate model was translated to algorithms programmed in Matlab® R2014a software (The Mathworks, Natick, MA, USA) based on programming guides established by Sandoval *et al.*[15] and Gu *et al.*[17] The bulk-fluid and the particle phase expressions were discretized in space with finite elements (with 5 quadratic elements) and orthogonal collocation methods to obtain an ordinary differential equations (ODEs) system.[15] This ODEs system was solved with the Matlab *ode15s* routine. All simulations were carried out on a laptop computer with Windows 8.1 operating system.

Table 1. Operating conditions used for simulating elution of mono-PEG and native lysozyme mixture in HAC

Tested condition	Flow (mL min^{-1})	Gradient length (CVs)
A	0.8	13
B	1.2	13
C	1.2	5
D	0.8	25
E	0.8	5
F	1.2	25

Table 2. Physical parameters used in chromatographic simulations

Physical parameter	Value
Bed void volume fraction, ε_b	0.2[a]
Column capacity, C ∞(M)	Lys nat 0.660[b]; Lys mon 0.622[b]
Column length, L(cm)	5[c]
Column volume, V (mL)	1
Density of the mobile phase, ρ (g cm^{-3})	0.99823[d]
Inner diameter of the column, d (cm)	0.5[c]
Macroporous particle diameter, d_{porous} (nm)	300[e]
Molecular weight, MW (kDa)	Lysnat 14.7[f]; Lysmon 34.7[g]
Particle porosity, ε_p	Lysnat 0.75[h]
Particle radius, R_p (cm)	0.0090[i]
Tortuosity, τ_{tor}	2[j]
Viscosity of the mobile phase, μ (g cm^{-1} s^{-1})	0.010015[d]

[a] [20, 21],
[b] [8],
[c] Column dimensions (Pharmacia Biotech),
[d] [22],
[e] [23],
[f] Sigma Aldrich, lysozyme (Cat. No. 10837059001),
[g] Calculated,
[h] [21],
[i] [20],
[j] [14]

Parameter definition and estimation of kinetic parameters for individual protein standards

Parameters are classified in physical, operational, dimensionless mass transfer parameters and adsorption kinetic parameters. The first refers to the physical characteristics of the adsorbent, sample, phases and column (Table 2), and were found and established according to Orellana *et al.*,[20] Hahn,[21] Geankoplis[22] and Hage and Cazes.[23] Since for this particular chromatography the reference in the values of tortuosity (τ_{tor}) and bed void volume fraction (ε_b) was absent, it was decided to perform preliminary simulations with individual standards and select their adequate value in the approximation of the kinetic parameters. The values tested for τ_{tor} were 2, 4 and 6 and for ε_b were 0.2, 0.3 and 0.4, taking as positive criteria the absence of an initial peak and how long took the simulation. Also interior orthogonal collocation points (Nr) with values of 2, 4 and 8 were evaluated.

The dimensionless mass transfer parameters and related variables (Re, Pe_{Li}, Rh, D_{pi}, d_{mi}, D_{mi}, k_i, Bi_i) were determined through

Table 3. Kinetic parameters estimated and used to simulate chromatographic profiles of individual and protein PEGylation mixtures in Heparin Affinity Chromatography

Protein	α	β	Da^d	Error (%)	Corr*
Native lysozyme	3.849	16.956	1.765	1.62	0.995
Mono-PEGylated lysozyme	2.626	42.881	0.558	0.87	0.997

α, β and Da^d are dimensionless kinetic parameters.

*Correlation coefficient between simulated and experimental absorbance data.

the same equations as in Sandoval *et al.*[15] and Orellana *et al.*[20] (Equation (S11) to (S20) in Supplementary material).

Adsorption kinetic parameters (α, β, Da^d) were estimated applying Equation (3) for each protein from the experimental curves of the pure proteins at a flow of 0.8 mL min^{-1} and a gradient length of 13 CVs, starting off with approximated values and after obtaining the precise values through the algorithm 'fminsearch' of Matlab with termination tolerance on $1x10^{-4}$.

Operational parameters are those which can be modified during each run such as flow, protein concentration and gradient length.

Simulation of PEGylation mixture separation at different operational conditions

Once kinetic parameters were determined for mono-PEGylated and native lysozyme, protein mixtures at different conditions were simulated.

Statistical analysis

To evaluate the simulation effectiveness, two criteria were considered: the relative error between simulated and experimental retention times and the correlation of the simulated and experimental data. Relative error in retention times was calculated using the following equation:

$$Error\ (\%,) = \left| 1 - \frac{t_{sim}}{t_{\exp}}, \right| \cdot 100 \qquad (4)$$

where t_{sim} is simulated retention time and t_{exp} is the average of experimental retention time. Correlation (Corr) was estimated by comparing point by point the simulated absorbance with the experimental absorbance.

RESULTS AND DISCUSSION

In the present work, PEGylated lysozyme separation in Heparin Chromatography from native and mono-PEGylated lysozyme were simulated applying the general rate model (GRM) theory and resolving the derived ODEs system through a numerical method on Matlab software.

Parameter definition and estimation for individual protein standards

As has been described in methods, preliminary tests with different τ_{tor}, ε_b and Nr were done to select the best values for the simulation and kinetic parameter estimation. Therefore, an adequate combination in this pre-selection was $\tau_{tor} = 2$, $\varepsilon_b = 0.2$ and Nr = 4.

The estimated kinetic parameters for individual standards of proteins (native and mono-PEGylated lysozyme) are shown in Table 3. From this table it can be seen that each kinetic parameter is different for both proteins. Constant α encompasses all the characteristic system parameters (electrostatic and hydrophobic interactions), β is only a descriptor parameter of the electrostatic interactions,[18] and Da^d is a dimensionless term which describes the relationship between the dissociation velocity of the ligand and protein and the mass transfer rate.[24] In this study, we observed that β is higher for mono-PEGylated than for native lysozyme, as it was expected that retention of the proteins in this heparin support is inverse to the magnitude of β parameter. Despite the fact that β is a function of diverse properties such as protein charge (number, distribution and size), salt counter-ion and charge of the stationary phase,[25] it is obvious that the change in β is attributed to the decrease in number and size of charges in mono-PEGylated conjugates with respect to native proteins, since that elution was performed under the same elution conditions and with the same heparin adsorbent. As described by some authors,[11,26] there is a charge-shielding effect due to the PEGylation, and in ion exchange chromatography PEGylated proteins are weakly retained. The difference observed in the β parameter can also be explained by a change in the isolectric points (pI), Some studies,[5,27,28] have pointed out that mono-PEGylated lysozyme may be modified in other 5 lysine residues alternatively to the N-terminal residue (position 1), however, the most abundant isomers of mono-PEGylated lysozyme are at position 1 and lysine 33, which has a calculated pI between 11.07 and 11.12 in comparison with 11.28 of the native lysozyme,[28] so a slight decrease in pI occurred, although a specific correlation is difficult to establish at this moment, more analysis including calculated pI of other PEGylated proteins would need to be done. The Damköler number for desorption (Da^d) was greater for the native than for the modified lysozyme. For the Damköhler desorption numbers (Da^d), the values were intermediate without indicating apparently some prevalence of desorption on diffusion rate. From the Da^d, the respective Damköhler adsorption numbers (Da^a) were estimated (9.18×10^{-18} for native and 3.02×10^{-45} for mono-PEGylated) and the adsorption rate is the rate-limiting mass transfer step ($Da^a < 1$).[24] The respective Da^a for each protein shows that the adsorption of the PEG-conjugate shape occurs faster than in the native lysozyme. In general, to compare our estimated kinetic parameters using the Melander relationship with those published earlier is difficult since affinity supports and proteins are different.

The dimensionless number at flow 0.8 mL min^{-1}, such as Re, showed that the flow through the packed heparin bed is laminar ($Re \ll 100$). Pe were greater than 280, thus indicating that the mass transfer process in both proteins is controlled by convection rather than diffusion. The estimated Bi numbers for the studied proteins were greater than 100; therefore, the external film mass transfer is negligible in the pore diffusion,[10] predominating the intraparticle diffusion rate. In this last case, although diffusion has little influence in the separation, the calculated molecular diffusivity (Dm) showed that mono-PEGylated lysozyme (1.12×10^{-6} cm^2 s^{-1}) diffuses slower than the unmodified lysozyme (8.40×10^{-7} cm^2 s^{-1}). Estimated diffusivity for native lysozyme was in agreement with the experimental coefficient measured by Brune and King[29] for this same protein in water. This reinforces the possible use of the mono-PEGylated lysozyme coefficient in future calculations. The diffusivity correlates inversely (if the Stokes–Einstein equation is considered and the proteins are treated as rigid spheres[30]) with estimated protein viscosity radii (Rh) using the Fee and Van

Table 4. Retention times and relative error of simulated and experimental peaks for individual and protein PEGylation mixture at different operational conditions using Heparin Affinity Chromatography

Mixtures of proteins (conditions)	Native lysozyme			Mono-PEGylated lysozyme			
	t_{sim}	t_{exp}	Error (%)	t_{sim}	t_{exp}	Error (%)	Corr*
A	7.86	7.90	0.51	5.76	5.68	1.41	0.991
B	5.11	5.33	4.13	3.77	3.82	1.31	0.920
C	4.00	4.29	6.76	3.48	3.53	1.42	0.853
D	10.31	9.72	6.07	6.41	6.30	1.75	0.798
E	6.10	6.32	3.48	5.33	5.15	3.50	0.943
F	6.63	6.42	3.27	4.21	4.25	0.94	0.784

*Correlation coefficient between simulated and experimental absorbance data.

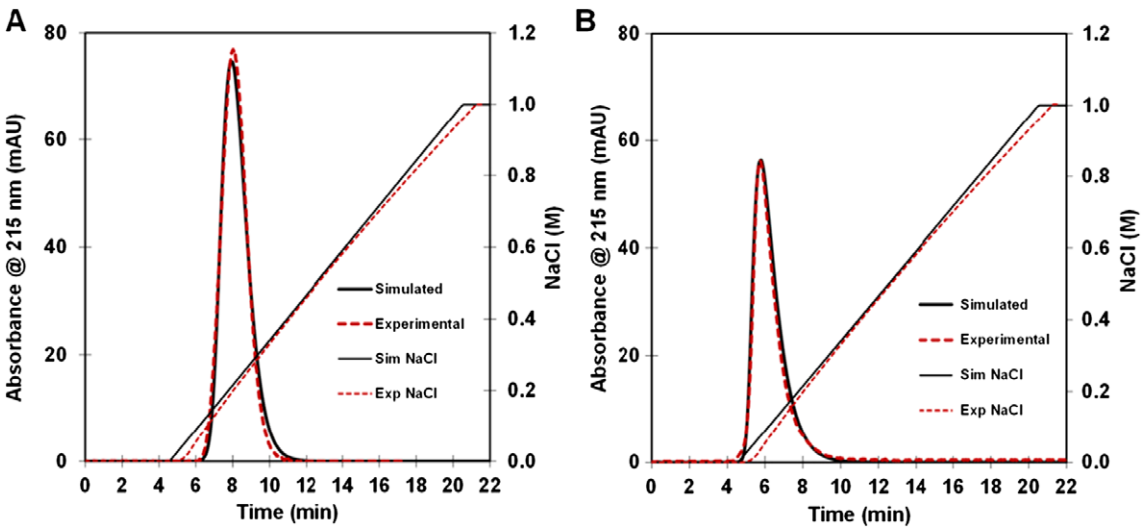

Figure 1. Experimental (dotted line) and simulated (continuous line) profiles in Heparin Affinity Chromatography of individual standards and salt concentration: native (**A**) and mono-PEGylated lysozyme (**B**) at flow 0.8 mL min^{-1} and gradient length 13 CVs**.**

Alstine proposed model,[31] 20.087 Å for native and 50.31 Å for mono-PEGylated. Hence it can be concluded that the viscosity radii in the modified protein is approximately 2.5 times greater than that for unmodified lysozyme, and as a consequence it diffuses faster. So, the separation may be slightly driven by these diffusivity differences in addition to other main processes such as convection and adsorption rate.

The experimental curves for native and mono-PEGylated lysozyme are shown in Fig. 1(A) and 1(B). The peak of the native lysozyme is symmetric while the PEGylated one is asymmetric presenting peak tailing, which has been associated with low desorption reaction rate when some fraction of the molecules bound to the ligand are dissociated slowly.[17] The peak tailing observed for the mono-PEGylated conjugates makes it more difficult to simulate; however, simulated and experimental absorbance data at 215 nm of both proteins had correlation coefficients (Corr) higher than 0.990. Regarding the retention times, standards showed a low relative error (below 2%). Therefore, individual standards were successfully simulated at 0.8 mL min^{-1} and at a linear elution gradient of 13 CVs.

Simulation of PEGylation mixture at different operational conditions of flow and gradient length

The comparison in retention times between the simulation and experimental profiles for mixtures at each operational condition from Table 1 is indicated in Table 4, while their respective chromatographic profiles are shown in Fig. 2.

The protein mixture tested at condition A (flow at 0.8 mL min^{-1} and 13 CVs, Fig. 2(A)) represents lysozyme PEGylation separation at the optimal conditions for the purification of mono-PEGylated lysozyme using a linear salt gradient found by our group in a previous study.[8] The relative error for the retention time of native lysozyme in mixture A (0.51%) was lower than that in the individual standard (1.62%), while for the mono-PEGylated lysozyme the error increased slightly (0.87 vs 1.41%). The correlation between the simulation and the experimental curve in this mixture was also good (0.991), only the peak of the simulated unmodified protein was slightly smaller than in the experimental mixture. Gradient of NaCl or modulator was also well simulated. The error in retention times of the native lysozyme at the other conditions increased relative to that observed in the individual standard. The same behavior was observed for the modified protein.

The biggest relative errors in the simulation of the mixtures were for the native lysozyme, up to 7%, while the PEG-protein errors did not exceed 4%. The error of retention times of native lysozyme in mixtures was the highest (above 6%, Fig. 2(C)–(D)) when the mixture was simulated at extreme and opposite tested operational conditions: a high flow (1.2 mL min^{-1}) with a short gradient length (5 CVs) as the mixture C or a low flow (0.8 mL min^{-1}) with a large gradient length (25 CVs) as the mixture D. The error

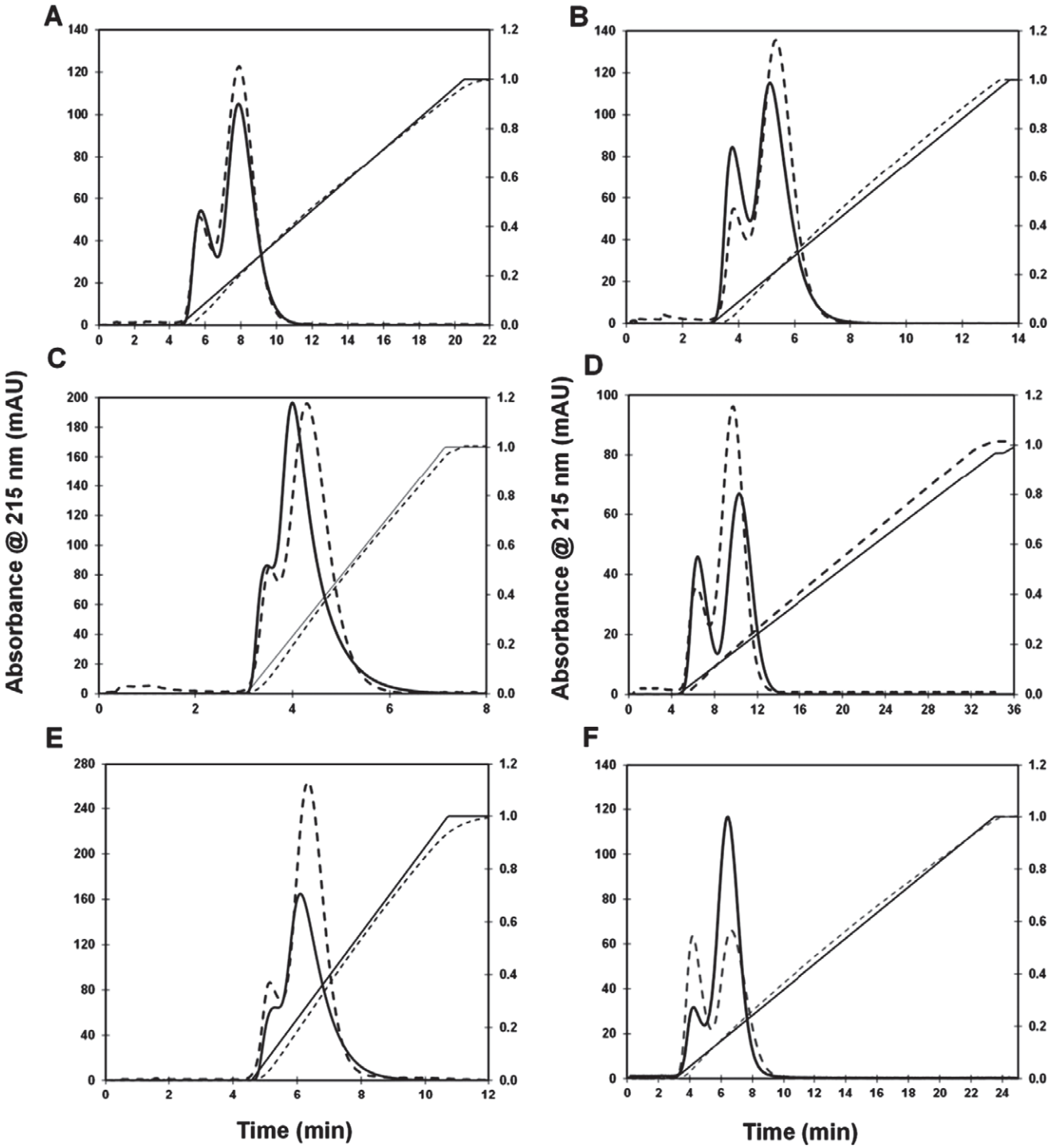

Figure 2. Experimental (dotted line) and simulated (continuous line) profiles in Heparin Affinity Chromatography of protein PEGylation mixture (native and mono-PEGylated lysozyme): (**A**) 0.8 mL min^{-1} and 13 CVs, (**B**) 1.2 mL min^{-1} and 13 CVs, (**C**) 1.2 mL min^{-1} and 5 CVs, (**D**) 0.8 mL min^{-1} and 25 CVs, (**E**) 0.8 mL min^{-1} and 5 CVs, (**F**) 1.2 mL min^{-1} and 25 CVs.

for mono-PEGylated lysozyme was kept below 2% in almost all mixtures except for case E (Fig. 2(E)), when a gradient length of 5 and a flow at 0.8 mL min^{-1} were used. In summary, mono-PEGylated lysozyme is better simulated than unmodified protein in mixtures. These results show a slightly bigger difference in the modelling at operational conditions (flow and gradient) with respect to the simulation in the separation of conalbumin, α-lactalbumin and BSA with the anionic Q-Sepharose Fast Flow, in which errors ranked from 0 to 4.6%;[20] but our relative errors are lower than those obtained in the separation of BSA and hemoglobin with Blue Sepharose (values between 1.78 and 17.62%).[15]

The correlation indicates the overlapping of the curves and indirectly the amount of the predicted protein. In mixtures, the less accurate correlation for the simulations was for mixtures D and F (0.798 and 0.784, respectively). These had in common the same gradient length of 25 CVs but a different flow; also, the relative

error for native lysozyme was somewhat higher. This may be due to small changes of the kinetic parameters at different conditions as seen by Orellana *et al.,*[20] which would require their re-adjustment at each tested condition.

The average time in the simulation of the mixtures takes between 40 and 100 s, which is a very short time compared with performing a chromatogram (6 min) at the fastest conditions (flow at 1.2 mL min^{-1} and gradient length of 5 CVs), without considering the time involved in the preparation of samples or equipment. This suggests that simulation saves time and experimental costs in the determination of adequate operational conditions.

It is important to point out that in the mentioned simulations, despite the fact that native lysozyme is a well known protein, the mono-PEGylated conjugate has not been completely studied and characterized, and its properties are unknown with precision (molecular weight, viscosity radii, diffusivity), nevertheless, the results allowed validation of the properties used or calculated .

In our study, di-PEGylated lysozyme was not included in the PEGylation protein mixture because in our previous work[8] this protein was shown not to be retained in the heparin support at dynamic conditions and it was also shown that a mixture of mono-PEGylated lysozyme and native lysozyme in a 4:1 ratio represents the separation observed in a lysozyme PEGylation reaction. Presently, model predictions based on pure proteins provide a good approximation to the real separation.[32]

The modelling of elution curves of PEGylated proteins or any other kind of polymer grafted-protein has not been researched; hence the results shown here establish a reference for future simulation of polymer-protein conjugates, particularly PEGylated proteins. In the same way, mass transfer data obtained in the simulation offers a guide for future scale-up procedures.[12]

As applications of the present work, we suggest the simulation of other PEG–protein conjugates: di-PEGylated, tri-PEGylated or poly-PEGylated isomers varying the size of the linked mPEG-chains in other random PEGylation mixtures. Actually, simulation of lysozyme PEGylation separation in other types of adsorption chromatographies such as ion exchange, hydrophobic or reverse phase and their comparison (from the technical and economical viewpoints) will provide information about the most robust technique in its purification. Also, the simulation of step gradient methods may increase its range of performance. As mentioned above, scale-up of Heparin Chromatography with lysozyme PEGylation reaction at pilot scale design process is a future recommended application of the simulation when conditions as the flow and gradient length are varied.

CONCLUSIONS

The purification of a suitable PEGylated protein conjugate from a reaction mixture with high yield and purity continues to be a challenge**.** Therefore, the purification of mono-PEGylated lysozyme in HAC is limited by both the understanding of the operation itself and the great number of conditions to test; thus, its simulation and modelling is a strategy to deal with these hurdles**.** In this work the separation of a lysozyme PEGylation mixture, representing the PEGylation reaction, was simulated under different operational conditions (flow and gradient length) using the GRM approach. Retention times for both proteins in mixtures were predicted with relative errors less than 6%, indicating that unmodified lysozyme was slightly more difficult to simulate in extreme and opposite conditions of flow and gradient length. Correlation between simulated and experimental data was the lowest when a large gradient was used; however, the rate model was able to simulate the elution curves of the separation between mono-PEGylated and native lysozyme in HAC. The processes that controlled separation were the adsorption/desorption rate, convection and pore diffusion.

In the future, scale-up to pilot plant purification through HAC may be done taking as a basis the information collected by this simulation; furthermore, the application of the GRM to chromatography of PEGylated proteins may be extended to other modes for optimizing each process individually**.**

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Supporting Information

Supporting information may be found in the online version of this article.

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