



Continuous separation of protein loaded nanoparticles by simulated moving bed chromatography

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ABSTRACT

For scale up and efficient production of protein loaded nanoparticles continuous separation by size exclusion chromatography in simulated moving bed (SMB) mode helps do reduce unbound protein concentration and increase yields for perfectly covered particles. Silica nanoparticles were loaded with an excess of beta casein or bovine serum albumin (BSA) and the loaded particles purified by size exclusion chromatography using Sephacryl300 as stationary phase in a four zone SMB. We determined our working points for the SMB from batch separations and the triangle theory described by Mazzotti et al. with an SMB setup of one Sephacryl300 26/70 mm column per zone with switch times of 5 min for BSA and 7 min for beta casein. In the case of BSA the Raffinate contained loaded nanoparticles of 63% purity with 98% recovery and the extract was essentially particle free (95% purity). We showed that the low purity of the Raffinate was only due to BSA multimers present in the used protein solution. In the case of beta casein where no multimers are present we achieved 89% purity and 90% recovery of loaded nanoparticles in the Raffinate and an extract free of particles (92% purity). Using a tangential flow filtration unit with 5 kDa cutoff membrane we proved that the extract can be concentrated for recycling of protein and buffer. The calculated space–time–yield for loaded nanoparticles was 0.25 g of loaded nanoparticles per hour and liter of used resin. This proves that the presented process is suitable for large scale production for industrial purposes.

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1. Introduction

Large scale separation of protein loaded nanoparticles is a pending problem. Simulated moving bed (SMB) chromatography using size exclusion chromatography is a way to separate the loaded particles from residual unbound protein in solution. SMB offers a way to scale up to large scale. Moreover, at the time more sophisticated nanoparticles with different characteristics are produced with open applications to different fields ranging from agriculture such as herbicide [1,2] to medicine such as vaccines, cancer treatment and drug delivery [3–9]. At the moment the separation of protein loaded nanoparticles is mostly done by ultracentrifugation [10] if done at all [9]. Some papers reported counter current separation of different kinds or sizes of nanoparticles, but didn't account for special needs of protein-nanoparticle separation and are dependent on

specific interactions of the nanoparticle and the chromatography material [11]. However, for applications where targeting is necessary because of side effects of unbound protein, efficient separation is mandatory. Ultracentrifugation has some serious downsides, it can only be operated in batch-mode, and therefore suffers from low productivity and high buffer consumption, and it is only applicable to relatively dense particles. SEC can be used for separation of small particles from other solutes and can be operated also in a continuous mode to reduce buffer consumption and can be operated to achieve higher productivities [12].

We present a method in which only the active compound is retained by the column, and the covered nanoparticle is excluded from all volumes within the chromatographic material. Used this way, this method is applicable to a wide variety of combinations of active compounds and nanoparticles. For productivity and to be able to recycle the unbound active compound and buffer, we used a 4 zone SMB chromatography as described by Mazzotti [12] with one SEC column per zone and showed separation of nanoparticle and protein with productivities suitable for large scale production. For this proof of concept we used 70 nm sized nanoparticles because this size roughly corresponds to virus sizes (or for that matter virus

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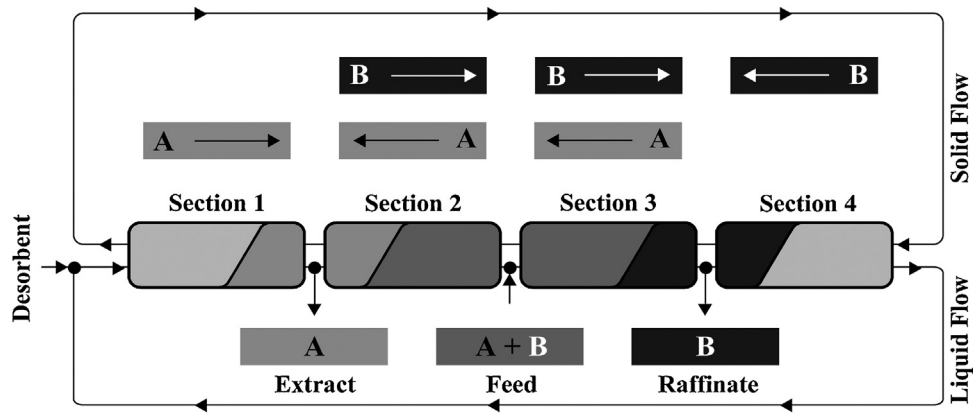


Fig. 1. Scheme representation of a four zone true moving bed.

like particles), which are commonly between 50 and 150 nm big. We think that especially the application as drug delivery vehicle and/or as vaccine are interesting routes which can benefit from special uptake mechanisms that have evolved to specifically deal with viruses efficiently.

1.1. Theory

Optimal operation conditions for an SMB system can be hard to determine empirically because of many adjustable and interconnected parameters. Different methods have been proposed to estimate suitable conditions for SMB. Ruthven and Ching [13] proposed a method later refined by Guiochon [14] which assumes linear isotherms but has to apply a safety margin on the modelled parameters to account for inaccuracy. An alternative process to find suitable parameters using equilibrium constants was presented by Storti and Mazzotti [15] and was termed triangle theory. For this model they also assumed linear adsorption isotherms, but also made the assumption that axial dispersion and mass transfer resistance are negligible. Later on, the triangle theory was refined by Mazotti et al. [12] and we based our parameters on the equation in this paper. The triangle theory works by defining a ratio for each of the four sections in the SMB, assigning different tasks to each section (Fig. 1). Zone 1 regenerates the chromatographic resin and section 4 regenerates the solvent, where section 2 and 3 are separating 2 species in a binary mixture.

According to Mazotti *et al.* we can formulate restrictions for each of these sections if we know the equilibrium constants of the species involved. To find this restriction one has to calculate the m_i values for each section, given by the following equation:

$$m_i = \frac{Q_i t^s - V\varepsilon}{V(q - \varepsilon)} \quad (1)$$

Q_i is the volumetric flow, t^s is the switch time, V is the column volume and ε is the column porosity. Mazotti et al. showed that for complete separation these m_i values have to fulfill certain restrictions. To find this constrains one has to experimentally determine the Henry constants of the two components, preferably in the concentration range which is intended to be used in the SMB system. The first section m_1 has to completely regenerate the column resin; likewise the fourth section has to completely regenerate the eluent. Therefore the following conditions can be formulated (2).

$$m_1 \geq h_1 \quad (2)$$

$$m_4 \leq h_2 \quad (3)$$

where h_1 is the Henry coefficient of the more retained species and h_2 is the Henry coefficient of the less retained species. Sections 2

and 3 are more critical in regards to parameter settings, as in these two sections the separation takes place. Again, we assume linear adsorption isotherms for all species involved, which lead us to the following set of restrictions for m_2 (4) and m_3 (5).

$$h_2 < m_2 \leq h_1 \quad (4)$$

$$h_2 \leq m_3 \leq h_1 \quad (5)$$

To find optimal parameters Mazotti et al. suggested to fix the values of m_1 and m_4 and to explore the m_2/m_3 plane to find optimal parameters, reducing the complex problem of interconnected parameters to a 2D plot. A representation of the restrictions found by Eqs. (2)–(5) is the triangle (hence the name triangle theory) build up by the m_2/m_3 plane (Fig. 2).

The zone of complete separation (triangle build up by points XYZ) is where all restrictions of Eqs. (2)–(5) are met, and the optimal production point in terms of productivity would be point X. However, this point is highly unstable as small variations in feed concentration or other parameters shift the actual working condition into Zone E or R leading to incomplete separation and loss of purity and productivity. Moreover, the triangle is only valid for strictly linear isotherms, which is rarely the case in any application. In real

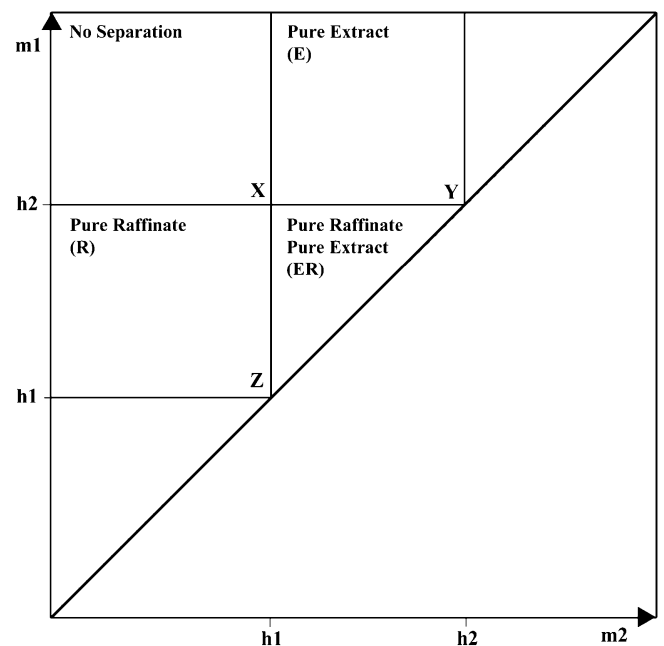


Fig. 2. m_2/m_3 plane under the assumption of linear isotherms.

systems the zone of complete separation is distorted [15] which makes operation on this point X impossible. In this work we concentrated on designing a stable system for a proof of concept and therefore chose conservative values in the middle of the triangle. The productivity of the system is decreased by approaching the line of ZY because of the reduction of the feed flow rate, but this approach also diminishes the risk of failure due to non-linear isotherms in the system and due to flow fluctuations or imprecise determination of the Henry constants. For future work or real industrial applications a thorough investigation of the adsorption isotherms and the m_2/m_3 is recommended for maximal productivity.

2. Material and methods

Chemicals without explicitly stated manufacturer were purchased from Sigma Aldrich (St Louis, USA).

2.1. Nanoparticles

Silica nanoparticles with amidine surface modification in the size of 30 nm, 70 nm, 200 nm and 1000 nm were purchased from Kisker (Steinfurt, Germany). The size and monodispersity was confirmed by TEM and DLS.

2.2. Determination of adsorption isotherm

To determine the adsorption isotherm of model proteins on nanoparticles, different concentrations of model protein were mixed with nanoparticles to obtain different protein concentrations combined with a fixed particle concentration of 2.5 mg/mL for 30 and 70 nm particles and 5.0 mg/mL for 200 and 1000 nm particles. The samples were incubated for 12 h to reach equilibrium. The concentration of model protein was determined by analytical SEC analysis as described below. No further sample preparation was necessary.

2.3. Analytical SEC

A Superdex 200 prep grade 10/200 GL size exclusion column (GE Healthcare, Piscaway, USA) was connected to an Agilent 1100 Series (Agilent Technologies, Santa Clara, USA) and equilibrated with SEC low salt running buffer (50 mM HEPES, pH 7.0) at 1.0 mL/min. 100 μ L of sample was injected and absorbance was monitored at 280 nm. The concentration of protein was determined by comparison to a calibration curve prepared from a standard solution of the same protein. The amount of nanoparticle was only determined relatively to the feed.

2.4. Preparative SEC

A Superdex 200 prep grade 10/200 GL size exclusion column (GE Healthcare) or a self-packed Sephacryl300 10/200 column (GE Healthcare) was connected to an ÄKTA-explorer system (GE Healthcare) and equilibrated with SEC low salt running buffer (50 mM HEPES, pH 7.0) at 1.0 mL/min. 200 μ L or 1000 μ L of sample was injected, the absorbance was monitored at 280 nm and the peaks were collected.

2.5. Simulated moving bed chromatography

The Semba System (Semba Biosciences, Madison, USA) was connected to 4 Sephacryl300 26/70 size exclusion columns (GE Healthcare) used in a 4 zone SMB mode with 1 column per zone. The flow rates used were determined through the triangle-theory model and were different for different proteins. For BSA the flow rates were: feed: 0.57 mL/min, extract: 1.70 mL/min,

Raffinate: 2.58 mL/min, recycle: 1.45 mL/min, switch time: 5 min. For beta-casein the flow rates were: feed: 0.61 mL/min, extract: 1.33 mL/min, Raffinate: 1.41 mL/min, recycle: 1.55 mL/min, switch time: 7 min. The absorbance was monitored at 280 nm for the Raffinate. Fractions were collected for the extract and Raffinate for each switch and investigated by analytical SEC for protein concentration and nanoparticle content.

2.6. Tangential flow filtration

The extract of one complete SEC-SMB run was collected and transferred to a Labscale TFF System (Millipore, Billerica, MA, USA) equipped with a Pellicon XL 50 Ultracel-5 PLCC Cassette with cut-off 5 kDa (Millipore). The system was operated at pressures of 10 psi transmembrane pressure for 80 min. Samples of 1 mL were collected from the Permeate and from the circulating flow of concentrated extract every 10 min and analyzed by analytical SEC for nanoparticle content and protein concentration.

3. Results and discussion

3.1. Binding of proteins to nanoparticles

To select model nanoparticles, two important factors have to be considered. The particle has to bind enough protein to be detectable by our analytical methods, and the size should be as close as possible to the size of a virus (roughly 70–150 nm). Additionally, the process parameters to ensure completely covered nanoparticles have to be found. The saturation range of protein loaded nanoparticles can be found by adsorption isotherms. To find optimal conditions for perfectly loaded nanoparticles for different sized nanoparticles, silica nanoparticles with amide functionalization in the size of 30 nm, 70 nm, 200 nm and 1000 nm were studied together with two model proteins: BSA and beta-casein (Fig. 3). The surface modification of these particles allows proteins to not only adsorb to the negatively charged silica surface, but provide additional positively charged binding opportunities. For both proteins the adsorbed amount of protein decreased with increasing particle size because of changed surface to volume ratio. This data show that our intended model of 70 nm particles adsorbs enough protein (8.14 ng/mm² for BSA and 3.11 ng/mm² of Beta Casein) to be detectable for our analytical system and is therefore a suitable model system.

Some strange behavior can be detected if we normalize the maximum amount of adsorbed protein (given by the Langmuir fit) with the available surface area (Table 1). The adsorbed amount per surface area is reasonable stable for BSA but beta-casein adsorption shows a different behavior. The amount of adsorbed beta-casein per surface area changes with different sized nanoparticles, which was unexpected. Probably the changing amount of adsorbed beta-casein per surface area as function of particle size may indicate multilayer adsorption or other complex adsorption mechanisms.

Table 1

Maximum amount of bound protein estimated by a Langmuir fit or adsorption isotherms per surface area on differently sized nanoparticles for BSA and beta-casein.

Nanoparticle size (nm)	Max. adsorbed BSA [ng/mm ²]	Max. adsorbed beta-casein [ng/mm ²]
30	7.32	2.65
70	8.14	3.11
200	6.96	5.17
1000	7.40	7.00

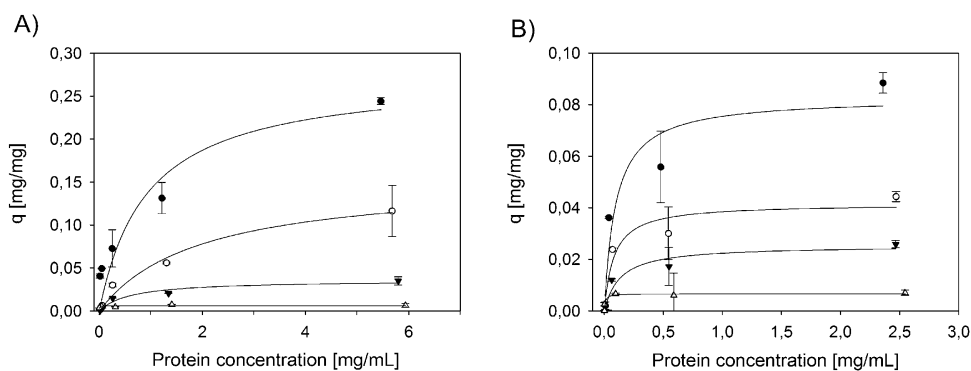


Fig. 3. Adsorption isotherms curves of BSA (A) and beta casein (B) on differently sized nanoparticles: (●) 30 nm, (○) 70 nm, (▼) 200 nm, (△) 1000 nm sized nanoparticles. The solid line represents a Langmuir fit of the data.

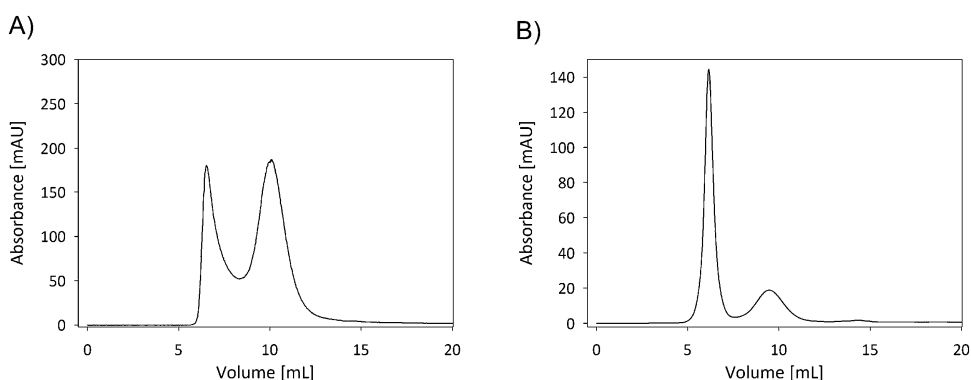


Fig. 4. UV280 trace of SEC chromatography of 70 nm silica nanoparticle mixed with (A) BSA and (B) beta casein.

3.2. SEC batch

The operating parameters of our SMB system were estimated from batch experiments. We used the triangle theory described [12] and for this purpose Henry constants and distribution coefficients are required.

70 nm silica nanoparticles loaded with BSA or beta-casein were separated from free protein (Fig. 4) by Sephacryl300 batch experiment. Protein loaded nanoparticles passed through the column and were eluted in the void fraction, but the protein was retained. The differences in the peak height of eluted nanoparticles loaded with different proteins are explained by the different molar extinction coefficients of the protein as well as the different amount of adsorbed protein. We did not achieve base line separation for BSA and protein loaded nanoparticles, but SMB does not require base line separation to yield pure fractions of a binary mixture [12].

From these chromatograms we deduced the peak moments of the substances and the distribution coefficients. Henry constants were estimated from the distribution coefficients and directly yield the constraint for m_1 and m_4 according to Eqs. (2) and (3). The missing constraints were calculated according to the triangle theory and Table 2 shows the process parameters we selected as well

Table 2

Restrictions for m values calculated by the triangle theory and selected m values for separation of 70 nm silica nanoparticles and BSA or beta-casein.

	BSA restrictions	BSA selected values	Beta-casein restrictions	Beta-casein selected values
m_1	$0.39 \leq m_1$	0.50	$0.41 \leq m_1$	0.50
m_2	$0 < m_2 \leq 0.39$	0.13	$0 < m_2 \leq 0.41$	0.09
m_3	$0 \leq m_3 \leq 0.39$	0.26	$0 \leq m_3 \leq 0.41$	0.28
m_4	$m_4 \leq 0.01$	-0.31	$m_4 \leq 0.00$	-0.15

as constraints for these parameters calculated from batch experiment data. We selected these values by an educated guess to be in the middle of the restrictions predicted by the triangle theory. The selection of the values was very conservative, being not too close to any restriction in case of non-ideal behavior of the system, to ensure a functional process and could be further optimized for an industrial process.

3.3. SMB results

Fig. 5 shows the time trace of protein concentration and particle concentration in the extract and Raffinate of the SMB separation of 70 nm protein loaded nanoparticles and free bovine serum albumin. It can be seen that the system is stable after approximately 12 switches (or 3 complete cycles). We achieved a good separation of protein and particle as the extract fraction is almost free of particles; however the Raffinate is getting contaminated with BSA multimers after 9 switches (or roughly 2 cycles). This incomplete separation can be explained by the setup of the system which was to separate BSA monomers and particles. We assumed the separation of a binary mixture when setting up the parameters according to the triangle theory, but in fact the BSA multimers add an additional third species to the system, which is not covered by the theory we used. The BSA multimer peak is between these two species and is therefore found in both fractions, extract and Raffinate. Table 3 shows the corresponding composition of feed, extract and Raffinate based on UV adsorption at 280 nm as well as the recoveries for particles and proteins. The extract is sufficiently pure of particles, but because of BSA-multimer problem the Raffinate purity is insufficient.

In comparison to BSA as model protein, beta-casein does not build up any multimers, therefore we expected no such problem

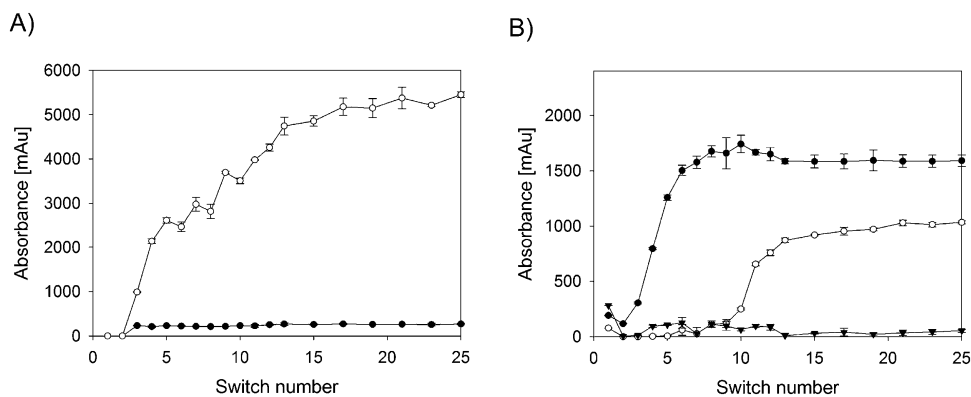


Fig. 5. Timetrace of the (A) extract during SMB-SEC separation of 70 nm silica nanoparticles and BSA: (●) particle and (○) protein concentration and (B) Raffinate during SMB-SEC separation of 70 nm silica nanoparticles and BSA: (●) particle, (▼) protein monomer and (○) protein dimer concentration.

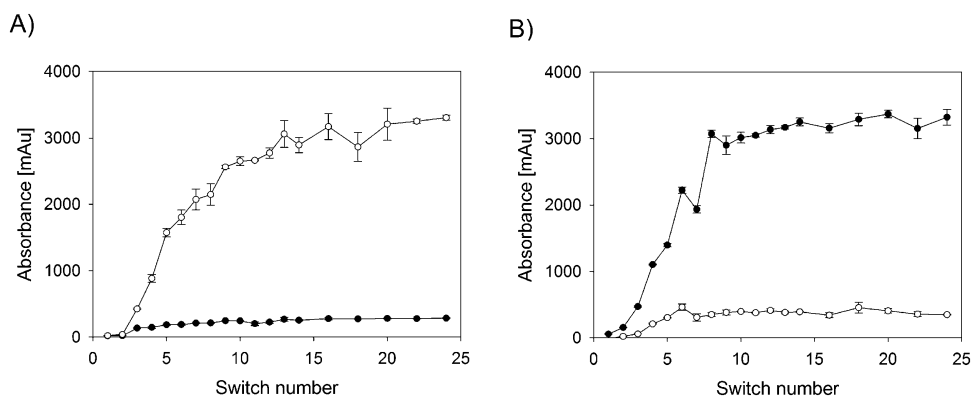


Fig. 6. Timetrace of the (A) extract and (B) Raffinate during SMB-SEC separation of 70 nm silica nanoparticles and beta-casein: (●) particle and (○) protein concentration.

Table 3

Composition of feed, extract and Raffinate for SMB-SEC separation of 70 nm silica nanoparticles and BSA as well as recoveries.

	Feed (%)	Extract (%)	Raffinate (%)	Recovery (%)
Particles	16	5	63	98
BSA monomer	63	76	1	100
BSA multimer	21	19	36	

as seen for BSA. Fig. 6 shows the time traces of separation of 70 nm silica nanoparticles and beta-casein, and as expected the system is also stable after roughly 3 complete cycles with good separation of protein and particles. This system fulfils the assumption of a binary mixture, resulting in almost pure fractions of proteins and nanoparticles in the extract and Raffinate, respectively. Table 4 shows the corresponding purities and recoveries, we achieved good recovery and good purity for protein and particles around 90% and the calculated productivity was 0.25 g/L/h of purified protein loaded nanoparticle per volume of column resin, which makes this process suitable for industrial production. Chromatography and especially SMB offers great scalability advantages over Ultracentrifugation, being only restricted by the available column and pump sizes and not dependent on particle density or size. The scale up itself would be straight forward as only flow rates and column diameters have

Table 4

Composition of feed, extract and Raffinate for SMB-SEC separation of 70 nm silica nanoparticles and beta-casein as well as recoveries.

	Feed (%)	Extract (%)	Raffinate (%)	Recovery (%)
Particles	57	8	89	90
Beta-casein	43	92	11	96

to be adjusted to achieve the same residence time in small and large scale.

To further reduce costs and buffer consumption, this system was tested in combination with tangential flow filtration (TFF) to reuse buffer and to concentrate the protein fraction. Reusing the buffer greatly decreases buffer consumption and concentrating the protein fraction ensures that protein is not wasted during the production of protein loaded nanoparticles, which is especially interesting when the active compound is very expensive.

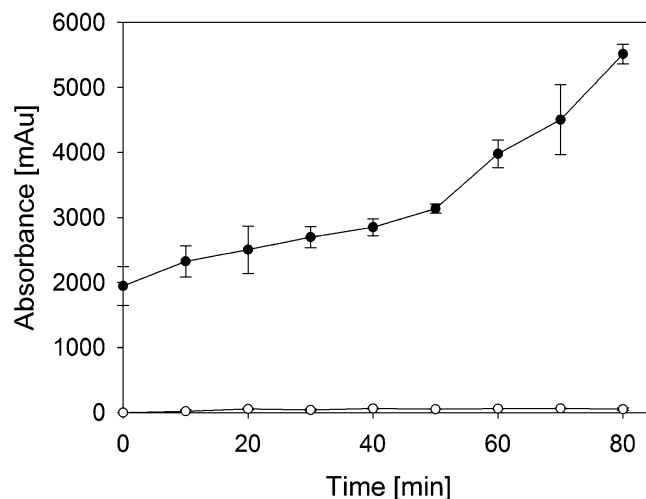


Fig. 7. Timetrace of (●) Retentate protein concentration and (○) Permeate protein concentration of TFF concentration of SEC-SMB extract.

To prove the applicability of TFF for concentration of protein and reuse of buffer we collected the extract of the SMB–SEC separation of 70 nm silica nanoparticles and beta-casein and concentrated it over the course of 80 min (Fig. 7). We achieved a total concentration factor of 2.8, which allows this solution to be used again for loading of nanoparticles, reaching a yield of 96%. We also proved that the resulting buffer is free of protein and ready to be used in the SMB system again (Fig. 7).

4. Conclusion

We were able to prove the applicability of SEC–SMB to separate perfectly protein-covered nanoparticles from proteins resulting in high yield (>90%) and purity (>90%). The productivity achieved for the system (0.25 g/L/h of protein loaded nanoparticle) is suitable for an industrial process and can surely be further optimized as no optimization was done in this work. Cost of goods can be held low due to the demonstrated recycling of protein and buffer using TFF. The features of high purity, high recovery, and low costs due to a continuous process using recycling makes this methods highly suitable to fulfill the need of protein/nanoparticle separation not only in lab scale, but also in production scale. The generic setup of SEC chromatography allows for separation of a variety of active compound–nanoparticle combinations, even allowing to use the same parameter setup when purifying different nanoparticles, but the same protein, as the retention time for different nanoparticles is the same as long as they are unable to diffuse into the chromatographic resin. The easy scalability of the chromatographic system, as well as the TFF system by increasing either column diameter

or membrane area additionally adds to the industrial value of this process.

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