RESEARCH ARTICLE

First comprehensive view on a magnetic separation based protein purification processes: From process development to cleaning validation of a GMP-ready magnetic separator

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Magnetic separation processes are known as integrated bioanalytical protein purification method since decades and are well described. However, use of magnetic separation processes in a regulated industrial production environment has been prevented by the lack of suitable process equipment and prejudice against the productivity of the process and its qualification for cleaning-in-place operation. With the aim of overcoming this prejudice, a comprehensive process development approach is presented, based on a GMP-compliant magnetic separator, including an optimization of the batch adsorption process, implementation into a technical-scale, and the development and validation of cleaning routines for the device. By the implementation of a two-step counter-current binding process, it was possible to raise the yields of the magnetic separation process even for very low concentrated targets in a vast surplus of competing proteins, like the hormone equine chorionic gonadotropin in serum, from 74% to over 95%. For the validation of the cleaning process, a direct surface swabbing method combined with a total organic carbon analysis was established for the determination of two model contaminants. The cleanability of the process equipment was proven for both model contaminants by reliably meeting the 10 ppm criteria.

KEYWORDS

cleaning validation, direct capturing, high-gradient magnetic separation, industrial biotechnology, process development, protein purification

1 | INTRODUCTION

Magnetic separation is known for many decades in biotechnological separation processes [1,2]. The basic principle is the selective adsorption of a molecule of interest (MOI) to magnetic particles (MP) functionalized with a target selective binding group. After adsorption, the MP can be

separated by magnetic forces in order to isolate the MOI from the feedstock. In this manner, integrated processes can be realized combining a solid-liquid separation with a capturing of the MOI [2–4]. Magnetic separation is a highly flexible technique that has been applied for various applications in biotechnology. Typically, magnetic separation is used for purification tasks like enzyme, cell [5] as well as protein purification [6–8], such as the recovery of immunoglobulins from cell supernatants or serum [9,10], recycling of immobilized enzymes [11–15], as well as cell labeling and sorting [16–18]. The use of MP is well established and routinely used in industry and is constantly opening up new fields of applications in academia

Abbreviations: CCPP, counter-current purification process; CIP, cleaning-in-place; eCG, equine chorionic gonadotropin; HGMS, high-gradient magnetic separation; LHS, liquid handling station; MOI, molecule of interest; MP, magnetic particles; SSPP, single step purification process; TOC, total organic carbon.

for analytical-scale processes [19–21]. For large-scale protein purification purposes, magnetic separation is virtually unknown in industry, although there have been most different applications and processes described in the past and interesting approaches in recent years in literature reaching from milliliter to 100 L scales [22–28]. However, the absence of magnetic separation techniques within bio-industry is mainly due to the lack of commercially available GMP-compliant high-gradient magnetic separation (HGMS) devices. This gap has now been filled with the commercialization of the first GMP-compliant HGMS device by the company Andritz GmbH at the beginning of 2017 [29]. The device allows direct capturing and purification of MOI from crude feedstocks such as cultivation broth or natural sources for example, blood or blood serum [30,31]. By the reduction of process steps, higher yields as well as time and cost savings are expected [32]. As an example, for the advantages of HGMS processes, the purification of the glycoprotein equine chorionic gonadotropin (eCG) from pregnant mare serum has been studied recently. The conventional purification method of eCG is divided into two main sections. The first section comprises multiple precipitation steps. First, with 0.5 M metaphosphoric acid followed by two additional precipitation steps with 50 and 75% v/v ethanol at 4°C. In the second section, further purification is achieved by fixed bed chromatography and gel-filtration. In addition to the expected low yield of around 50% due to high losses during precipitation and resolving, the consumption of solvents makes this process an economically and ecologically imperfect solution [33–36]. An alternative purification process via magnetic separation has been presented by Müller et al., saving 2/3 of the solvent by using magnetic anionic exchange particles after a first precipitation procedure [37,38]. The yield of this process reached up to 79%. However, due to the high conductivity of the raw material, the ionic exchange magnetic separation process could not be applied without a previous precipitation step. With the development of an affinity ligand for the specific binding of eCG and the functionalization of MP with this anti-eCG affinity ligand, it was possible to avoid all precipitation steps and purify eCG directly from untreated serum. This purification process has been implemented in a small version of an automated non-GMP-compliant ‘rotor-stator’ HGMS device [39]. Purification and concentration factor exceeded the previously presented process. The drawbacks of the affinity-based process were a lower yield of around 50% and a loss of adsorbent binding capacity of over 30% in 30 process cycles. With this poor performance data, the disadvantages of batch adsorption processes from crude feedstocks became clearly apparent. Harsh cleaning conditions as well as particle fouling due to the characteristics of the feedstock lowered the reusability of the MP. In addition, the batch adsorption process with only one equilibrium stage and low MOI concentration in the feed is responsible for the low yield.

PRACTICAL APPLICATION

Magnetic separation processes are known as integrated bioanalytical protein purification methods since decades and are well described. However, use of magnetic separation processes in a regulated production environment has been prevented by the lack of suitable process equipment and prejudice against the productivity of the process and its qualification for cleaning-in-place operation. We present a comprehensive view on a technical-scale protein purification process based on magnetic separation, including the optimization of batch-based adsorption processes as well as cleaning routines. We thus believe that this application for the first GMP-compliant magnetic separator addresses a broad range of readers. We address specialists from academia to industry where the application can be used as flexible, continues clarification, and capturing tool for protein, cell, or virus purification. We are confident that the integration in industrial processes opens new possibilities in modern biopharmaceutical production processes as well as in magnetic particle production

While there is an obvious potential for optimization of one stage batch adsorption HGMS processing, the lack of suitable large-scale GMP-compliant separation equipment and demonstrations of its cleanability represent another obstacle to a successful introduction of magnetic separation in industrial purification processes [22,40]. Cleanability – in the best case without disassembling but as automated routine in a cleaning-in-place (CIP) procedure – is an indispensable prerequisite for the industrial application of process equipment in biopharmaceutical industry and demanded by the FDA [41–44]. The basis for a successful cleaning of a process equipment is a GMP-compliant design. Besides the design of product-touching equipment parts following hygienic design regulations, the design of non-product-touching housings need to be taken into account as well [45,46]. A cleaning protocol has to be developed in order to ensure the quality of a product and avoid cross-contaminations from batch-to-batch or from different active pharmaceutical ingredients, if used as multiproduct equipment [47,48]. Furthermore, validation of the cleaning process is of great importance. Two sampling methods for the cleaning validation of surfaces are recommended. From these two, the surface swabbing test is commonly preferred over the rinse sampling test. Defined parts of the surface are swabbed with a sampling swab followed by an analysis of the resolved contaminants. Other than specific analytical techniques, such as HPLC or ELISA, also non-specific analytical methods gain importance [49,50].

Total organic carbon (TOC) analysis is simple to implement as it does not require the development of a dedicated method. Furthermore, it offers low detection limits in the range of ppb, and is less time consuming in the set-up and execution compared to more specific techniques [51–53].

For the first time, we bring the decisive elements for a biopharmaceutical protein purification process together in this study. We optimize the approach of affinity based HGMS for eCG purification directly from crude feedstock to circumvent limitations of common techniques and proof the applicability of HGMS in modern biopharmaceutical purification processes. A comprehensive concept is presented, consisting: (i) of a scale-up of a magnetic separation process for the purification of eCG from laboratory to technical-scale; (ii) the successful implementation of the process into the first commercial GMP-compliant ‘rotor-stator’ HGMS device; and (iii) a concept for the cleaning and cleaning validation of the device [29]. The process consists of a counter-current process variant which allows to increase the process yields without using larger amounts of MP. The accompanying cleaning validation of the device is conducted based on surface swab sampling tests and TOC analysis. With horse serum and hemoglobin solution, two highly concentrated model contaminants were used to validate the cleaning process. The comprehensive approach of a successful process implementation and optimization with the subsequent cleaning of the magnetic separation device has been carried out for the first time and illustrates the great potential of magnetic separation processes for a commercial protein purification in technical-scales.

2 | MATERIALS AND METHODS

2.1 | Materials

2.1.1 | Equine chorionic gonadotropin (eCG) purification process

eCG is a glycoprotein hormone consisting of two non-covalently bound subunits. The total weight of 60 kDa distributes among one α subunit with 16.96 kDa and one β subunit with 43.72 kDa. The low pI of 1.8 is caused by a carbohydrate content of 45% with 10% of sialic acid. eCG is secreted in the endometrial cups and can be detected in mare serum between day 40 and day 130 of pregnancy with highest levels within the first days. Among other applications, eCG is used to regulate the reproductive activity of cows [54]. For this study, eCG purification serves as a model process to demonstrate the optimization potential of magnetic separation processes for low concentrated targets.

Salts and chemicals for buffer preparation for the purification processes were purchased from VWR (Darmstadt, Germany) in analytical grade. Blood samples were taken from native Haflinger mares between day 50 and day 100 of preg-

nancy. Erythrocytes and fibrin were separated and the serum was stored at -20°C until further use. Prior to storage, the average eCG concentration of 20 IU/mL was confirmed via an eCG-ELISA (fzmb GmbH, Bad Langensalza, Germany) according to the manufacturer’s instruction. Each sample was measured in six dilutions. For this study, NHS activated Mag Sepharose (kindly provided by GE Healthcare, Uppsala, Sweden) was functionalized with the tailor-made anti-eCG affinity ligand developed by the Forschungszentrum für Medizintechnik und Biotechnologie (fzmb), Bad Langensalza, Germany for direct binding of eCG from crude feedstocks. The MP are based on a highly cross-linked agarose base matrix with magnetite inclusions. A mean diameter of 54 μm and a saturation magnetization of 47 Am^2/kg with a remanence of 6.6 Am^2/kg were determined. For small-scale binding and recycling studies, an automated liquid handling station (LHS) JANUSTM workstation equipped with a VarispanTM 4 fixed tip arm (Perkin Elmer, Waltham, MA, USA) as well as a magnetic separation support for 96-well flat bottom microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were used. For milliliter-scale separation tasks a MagRack 6 (GE Healthcare, Uppsala, Sweden) was applied.

‘Rotor-stator’ high-gradient magnetic separator (MES 100 RS)

For pilot-scale separation processes, the latest GMP-compliant version of a ‘rotor-stator’ high-gradient magnetic separator was used (MES 100 RS, Andritz KMPT GmbH, Vierkirchen, Germany). The heart of the separation device consists of a separation chamber surrounded by an electromagnet. The separation chamber contains a stack of densely perforated metal discs serving as matrix elements for the magnetic separation of the MP. The matrix discs are alternately mounted to the housing of the chamber and to a central rotatable shaft. The shaft can be rotated with 1500 rpm creating shear forces in the gap between the matrix elements. As spacer and sealing between the matrix discs, polyetheretherketone elements were used. Fluid streams are moved by a peristaltic pump with a maximum speed of 2.7 L/min and are controlled by two valve blocks, one below and one above the chamber. The valve blocks provide six hose nozzles for system fluid connections. The separation chamber as well as the valve blocks is designed to be self-draining. All system parts in contact with product streams were chosen according to recommendations and guidelines of the authorities. A detailed description and characterization of the separator can be found elsewhere [29,55].

2.1.2 | Cleaning validation equipment

Polyester Texwipe Alpha[®] swabs (ITW Texwipe, Kernersville, NC, USA) with a long handle were used to recover impurities from the surfaces of the separator. For resolving organic carbon related to the impurities from the swab,

ultrapure water was acidified with 6 M phosphoric acid (Carl Roth GmbH KG, Karlsruhe, Germany) to a pH of 2. For the resolving and storage, 15 mL reaction tubes (VWR international GmbH, Darmstadt, Germany) were pretreated twice with the resolving solution to reduce residual organic carbon. As model contaminants, the described horse serum as well as hemoglobin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in a concentration of 50 g/L was used. As cleaning solution, 0.5 M sodium hydroxide solution (Carl Roth GmbH KG, Karlsruhe, Germany) and COSA CIP 92 (kindly provided by Ecolab Deutschland GmbH, Monheim am Rhein, Germany) diluted with ultrapure water, according to the manufacturer's protocol, were used.

2.2 | Methods

2.2.1 | eCG process

Small-scale binding and recycling studies

Maximum binding capacity $Q_{\max}$ and dissociation constant K_D based on the Langmuir model were determined by small-scale adsorption studies on the LHS. Varying concentrations of functionalized MP (0.5–6 g/L) were equilibrated in 100 mM phosphate buffer pH 7.4 followed by the incubation with serum in a 96-well plate at a total volume of 200 μ L. After one hour of constant mixing, the MP were separated and the eCG concentration in the supernatant was analyzed via the eCG-ELISA. Equilibrium loading and concentration data were fitted using the Langmuir model. Adsorption studies were repeated in scale-up studies of 2 and 15 mL total volume with consistent particle concentrations.

Long-term recycling studies were performed by the help of the LHS. An amount of 2.5 g/L MP was incubated with 200 μ L serum for 30 min in a 96-well plate. The binding step was followed by four wash cycles with 100 mM phosphate buffer pH 7.4 and an incubation time of 2 min. For elution, the last wash solution was replaced by 50 mM ammonium acetate pH 3 and incubated for 30 min. Between each step, the MP were magnetically separated and the supernatant was completely replaced by the next solution. In total, 30 bind and elute cycles were performed. Elution fractions were neutralized with 10% ammonia and stored at 4°C until eCG concentration determination via the eCG-ELISA.

Small-scale purification process

Small-scale eCG purification processes were performed in two different modes. For the single step purification process (SSPP), 4 g/L functionalized MP were incubated with serum in two scales. In the first scale, the MP were twice equilibrated with 2 mL of 100 mM phosphate buffer pH 7.4 before incubation with serum for 1 h and constant mixing at a total volume of 2 mL. After separation of the MP and removal of the supernatant eCG, loaded MP were washed four times with 2 mL equilibration buffer. Elution was performed with 2 mL

FIGURE 1 Schematic illustration of a counter-current batch adsorption process. The feedstock is pumped countercurrently to the magnetic particles. Fresh feedstock (S_0) is contacted with pre-loaded magnetic particles (M_1) and subsequently used in a second binding process as once used feedstock (S_1) with un-loaded magnetic particles (M_0). The magnetic particles are washed in between the binding steps and washed as well as eluted after the second binding step

of 50 mM ammonium acetate solution pH 3 for 1 h. The eCG concentration of all fractions was determined via the eCG-ELISA. A scale-up of the SSPP was performed with a total volume of 10 mL. To reach higher eCG concentrations in the eluates, the elution volume for this study was reduced to 2 mL.

The counter-current purification process (CCPP) (Figure 1) was performed in the same scales and with the same particle concentration as the SSPP. Unused MP (M_0) were incubated with serum that was already once in contact with MP (S_1). This 'preloaded' MP (M_1) were washed two times and subsequently incubated with fresh serum (S_0). After the second incubation step, the MP (M_2) were washed and eluted as described for the SSPP. The once used serum (S_1) from the second binding step in the first process cycle was incubated again with unused MP (M_0). In summary the MP and the serum are used in a two-stage counter-current process where the serum is incubated once with 'preloaded' MP and once with 'unloaded' MP. The MP were eluted after the second incubation and subsequently equilibrated for the next CCPP. In total, four counter-current cycles were performed. All fractions were collected and their eCG concentrations were determined.

Technical-scale purification process

The technical-scale eCG purification from pregnant mare serum was executed as CCPP. The amount of functionalized Mag Sepharose available for this process was approximately 9 g. This amount corresponds to 70 mL of sedimented chromatographic resin. A particle concentration of 3 g/L was used. The incubation of the MP with the serum was conducted for 30 min in an external vessel with constant mixing. After the binding phase, the suspension was pumped from the bottom valve block to the top. The MP were separated by the magnetized matrix elements and the once used serum was collected. Three wash steps with 1.5 L of wash solution each were executed. In each step, the solution being present in the separation chamber was displaced by new wash solution. The MP were resuspended by switching off the magnetic field and

rotating the inner shaft with the connected matrix elements at 1500 rpm for 2 min. Afterwards, the MP were recaptured before replacing the process liquor. For all process steps, the same buffer compositions as used for the small-scale experiments were applied. A closer description of the system control of the rotor-stator separator can be found elsewhere [55]. After the wash steps, MP were eluted with 1 L of elution buffer for 30 min by resuspending the MP in the separation chamber. Finally, the MP were equilibrated three times with 1.5 L of equilibration solution, in order to prepare them for the next binding step. The eluted MP were recovered from the system with the once used serum and incubated again for 30 min before the pre-loaded MP were separated and washed again. The twice used serum was disposed and the pre-loaded MP were recovered from the system with a batch of fresh serum. The fully loaded MP were washed, eluted, and equilibrated as described, while the once used serum was used for the next particle recovery and binding step. In total, four counter-current cycles were performed.

2.2.2 | CIP procedure and cleaning validation

The cleaning process of a device used in biopharmaceutical production has to be considered early in the planning and construction phase. The equipment design is a significant factor for development of the cleaning process, and decides if a CIP is possible. Automated cleaning protocols reduce downtimes for dis- and reassembling of process equipment, prevent human error, and deliver consistent cleaning results. Therefore, it should always be aimed for a CIP. A CIP always includes a recorded standard operation procedure with details of the process steps to be carried out and the cleaning agents to be used. Furthermore, a validation and control of the process has to be established. The cleaning is achieved mainly by chemical action and can be supported by physical action if possible. Critical cleaning parameters that have to be considered are action time of the cleaning solution, temperature, and concentration. For the study presented here, we have adhered to the specifications of the supplier for the industrial detergent. A typical cleaning process consists of a pre-water wash, a hot alkali step supported by surfactants, and a final rinse with water for injection.

Total organic carbon analytic

The organic carbon content of the cleaning validation samples was determined by a Sievers M9 Total Organic Carbon Analyzer (GE Analytical Instruments, Manchester, UK). The TOC of the sample was oxidized and passed a CO₂ permeable membrane for detection by a conductometry. The detection range was specified by the manufacturer from 0.03 ppb to 50 ppm with a precision of $\pm 2\%$. The calibration was linear in a range from carbon free samples up to 1.5 ppm carbon.

Method validation

A linear relation between the contaminants to be detected and the amount of TOC detected by the analytical method is required for a successful validation of the analytical methods. For the calibration of the TOC amount of the contaminants, varying concentrations of serum and hemoglobin were added to 12 mL of ultrapure water acidified with phosphoric acid. Additionally, two sample swab heads were added and the sample tube was vortexed at maximum speed for 30 sec.

Extraction efficiency from sampling swabs

To determine the usability of the selected sample swabs, the amount of TOC that can be resolved from the sample swabs was determined. An amount of 200 μ L varying concentrations of contaminants were applied to both sides of one dry sample swab by a pipette, and subsequently incubated with an additional sample swab head in the resolving solution as described.

Recovery efficiency from separator surfaces

To verify the accuracy of the test method, the amount of TOC that can be recovered from the surfaces of the separation chamber was quantified. The surfaces of the chamber include metal matrix elements as well as polyetheretherketone sealings and the metal hose nozzles. Known concentrations of contaminants were applied to the surface of pre-cleaned matrix elements and evenly spread over one half of the element with the aid of a pipette. The contaminated elements were dried at 25°C for 1 h. One sample swab head was wetted in the resolving solution and the contaminated surface was swabbed in horizontal lines to ensure a total surface coverage. After the initial pass, the swab was turned and the surface was swabbed with the fresh side of the swab in an orthogonal direction to the previous lines. After the second pass, the head of the swab was transferred to the sample tube from which it was wetted and the procedure was repeated with a fresh dry sample swab. Both heads were incubated as described. The sample tubes were stored at -20°C until TOC analysis.

2.2.3 | Cleaning validation of the separation device

To validate the CIP procedure for the separation device, the separation chamber was contaminated with two model impurities, followed by an automated cleaning routine with two different cleaning agents and a surface swabbing test with TOC analysis. The separation chamber was flushed with the impurities from the lowest valve connection at the bottom valve block to the highest at the top one. After the separation chamber was completely filled with solution, the central shaft with the connected matrix elements was rotated at 1500 rpm for 30 sec. The system was completely drained after contamination and three times flushed with 2 L of water. Between each new water batch, the central shaft was rotated

FIGURE 2 (A) eCG eluate concentrations in relation to the concentration determined in the eluate of the first cycle for 30 reuses of the particles. (B) Corrected and original concentration data from eCG binding studies to anti-eCG functionalized Mag Sepharose fitted with the Langmuir model. A maximum binding capacity of 16.9 IU/mL and a dissociation constant of 0.5 IU/mL were determined

with 1500 rpm for 1 min. After the last water flush, the system was drained. The cleaning protocols with the cleaning agents, 0.5 M sodium hydroxide and COSA CIP 92, were carried out as follows. The separation chamber was flushed with 5 L of cleaning agent tempered to 70°C from the bottom to the top. The batch of cleaning agent was pumped in a loop through the system with a pump speed of 1.5 L/min from bottom to top, while the central shaft was rotated with 1500 rpm for 10 min. This procedure was repeated three times with alternating the pump direction. After draining the last batch of cleaning agent, the separation chamber was flushed three times with 2 L of ultrapure water. The water batches were looped and mixed as described for the cleaning agents for 1 min. After the cleaning procedure, the separation chamber was drained and perfused with compressed air for drying. Subsequently, the separation chamber was disassembled and swab samples were taken from the inside of the chamber cover and the hose nozzle of the cover, as described for the method validation. Furthermore, samples were taken from the head of the central shaft, matrix elements at the top, in the middle, and at the bottom of the matrix stack as well as from the hose nozzle at the bottom of the separation chamber.

3 | RESULTS AND DISCUSSION

3.1 | Small-scale binding and recycling studies

The long-term stability and reusability of Mag Sepharose particles functionalized with a custom-made anti eCG-affinity ligand were determined in small-scale bind and elute studies with the help of an automated LHS. During 30 reuses, comparable yields could be measured, indicating the stability of the

affinity ligand against the applied low pH elution conditions, and that the magnetic particles aren't prone to fouling, despite their porous structure (Figure 2A). The number of cycles the MP can be reused without a significant decrease in binding capacity is a crucial economic factor for the overall process. The prices for MP as well as for the development and production of a tailor-made affinity ligand are high and determine the overall costs of the separation process [22]. In order to determine application related binding isotherm parameters, adsorption studies for eCG from horse serum onto functionalized MP were performed. Consistent results of the 200 μ L as well as 2 mL and 15 mL scales were observed; the observed Langmuir isotherm shape with a steep slope at the beginning and saturation of binding capacity in case of higher equilibrium concentrations was expected. Besides, an unexpected feature in the isotherm shape shows. Even at high concentrations of adsorption particles applied, a fraction of around 20% of the original eCG activity remains in solution (Figure 2B). Because the phenomenon occurs in different test series independent of used batch volume, we don't believe in an experimental error but that the reason can be found in different variants of the protein eCG. Due to the high degree of glycosylation of this protein, such variants are likely [56–58]. It seems that not all of them bound with the same strength to the used affinity ligand. It appears that eCG variants with low affinity do not bind to the functionalized particles in a batch adsorption process due to the low number of separation stages. In column separation processes, with a higher number of equilibrium stages it might be possible to capture these variants. For this reason, in all further calculations, two variants of eCG are considered with 78% of a binding variant and 22% of a non-binding variant. The percentage of the non-binding fraction is calculated by dividing the value of the intersection of

the original isotherm (see Figure 2B) with the x-axis by the start concentration of eCG used.

The fraction of 22% non-binding eCG variant is subtracted from all ELISA results of binding tests with serum, resulting in a corrected Langmuir isotherm which is shown in Figure 2B. Of course, economic optimization of the process would require the screening for new affinity ligand having the potential to bind all eCG variant, however, for the purpose of our investigations regarding process optimization and cleaning validation, the available sorbents are as well suitable. The corrected Langmuir isotherm results in a maximum binding capacity of 16.9 IU/mg and a dissociation constant K_d of 0.5 IU/mL by fitting the concentration data to the Langmuir model. Mass balances from single bind and elute cycles provided information about the predicted performance of the single step purification process (SSPP) operation mode of direct eCG purification from serum. Serum with an eCG concentration of 15.3 IU/mL corresponding to approx. 12 IU/mL of binding eCG was used for an SSPP with 4 g/L adsorbents, leading to a concentration of 2.7 IU/mL of binding eCG in the supernatant after binding. Three wash steps were performed showing low eCG concentrations followed by one elution step with a concentration of 10 IU/mL in the eluate. From the resulting mass balance, it can be concluded that three wash steps and one elution step are sufficient to wash off weakly bound product and recover all bound eCG. The overall yield in the elution fraction of the SSPP operation mode reached 83% with a closed mass balance. From the numbers, it becomes clear that the major loss of eCG is unbound product in the supernatant. This is due to the batch mode character of the purification process, where only one equilibrium stage is applied, corresponding with the known tradeoff between the product amount left in the supernatant and the loading capacity reached. To tackle the disadvantages of low MOI concentrations in combination with batch adsorption processes, a CCPP was established in order to process maximum batch size with minimum use of MP and high yields. To predict the expected yield in dependence of the used MP concentration of the CCPP, the mass balance and the determined isotherm parameters are used to calculate the predicted concentrations and particle loadings. From the results of the simulations of the CCPP, it can be concluded that a two-stage process with a MP concentration of 3 mg/mL should deliver very good yields of more than 95%.

CCPP cycles with 3 mg/mL MP concentration were executed in total volumes of 2 and 10 mL. One initial single bind and elute cycle to start the process and to produce, a one-time treated serum and four counter-current cycles were performed (Figure 3). During these cycles, the yield increased constantly, starting with a value of 74.6% in case of the initial binding and elute step to over 95.8% yield in the full CCPP cycles three and four. Taking into account all five cycles, an overall yield of 91.4% could be reached. The overall process yield

FIGURE 3 Yield of the eCG elution fractions of the four pilot-scale CCPP cycles is plotted in black. eCG elution yields of small-scale CCPP cycles are plotted in grey

will converge to the yield of the last cycles if performing more counter-current cycles and therefore approaches the value of the theoretically calculated optimal yield.

3.2 | Pilot-scale process studies

The process sequence of small-scale eCG purification was up-scaled to a pilot-scale process including one initial SSPP and three full CCPP cycles. In Figure 3, the resulting eCG yields of the elution fractions are plotted. Although the scale of eCG purification was increased by a factor of around 1000 and manual particle handling was replaced by a fully automated particle management within the developed magnetic separator, the results are almost similar. The yield of the initial SSPP cycle with only one bind and elute step was rather low (47%), while applying full CCPP cycles, the yield quickly increased and yields above 95% in the second and third pilot scale CCPP cycle were achieved. Approaching elution yields close to 100%, it also shows that the CCPP process achieved steady state operation and that elution conditions are sufficient for practically complete elution in the separator too. In conclusion, applying a two-step CCPP operation, it was possible to increase the batch size which can be treated per cycle by reducing the required adsorbent concentration while simultaneously increasing the yield of the process. At the same time, the CCPP operation also has some drawbacks, namely longer cycle times and increased washing buffer consumption. However, short binding times due to batch adsorption, and fast processing due to high fluid velocities in the magnetic separation process implicate that even a two or three step CCPP operation using magnetic adsorbents requires less process time than conventional column-based chromatography while achieving a substantial gain in product yield.

FIGURE 4 (A) Determination and validation of the accuracy of the chosen analytic for hemoglobin (inverted triangles) and serum (dots); y-axis: hemoglobin and serum amount applied to control, x-axis: measured TOC after dissolving procedure. (B) Recovery efficiency of organic carbon from the matrix elements for hemoglobin (inverted triangles) and serum (dots); y-axis: TOC amount applied to matrix elements, x-axis: measured TOC after swabbing and dissolving procedure

3.3 | CIP procedure development and cleaning validation

The two commonly used sampling techniques for cleaning validation are rinse and surface swab sampling, whereby the surface swab technique is preferred by the FDA and therefore used as sampling method for the cleaning validation of the magnetic separator. The TOC analytic was chosen due to its variable applicability for different contaminants and high sensitivity. The TOC method is a non-specific analysis taking into account all organic contaminants. Therefore, it does not allow conclusions to be drawn regarding the type of carbon source, including the active pharmaceutical ingredients. In return, this method is more flexible and does not require any complex method development as it would be required for a HPLC method for instance.

3.3.1 | Method validation

A linear relationship between the contaminant amounts applied to sample swabs and the measured TOC amount after conducting the resolving procedure is a prerequisite for the use of the chosen analytical method. For both contaminants, serum as well as hemoglobin solution, linear relationships were observed with proportional factors of 9.6 mg/mg for serum and 2.75 mg/mg for hemoglobin (Figure 4A). Average recovery rates of 90% for hemoglobin and 84% for serum confirmed the chosen resolving procedure of the sample swabs, including mixing duration and resolving solution. In addition, the recovery of hemoglobin and serum from matrix elements

which were spiked with contaminant amounts increasing from 0.01 to 2 mg showed a linear correlation over the tested range (Figure 4B).

3.3.2 | Cleaning of the magnetic separator

The separation device was rinsed with two kinds of contaminants, horse serum as well as a 50 mg/mL hemoglobin solution. Afterwards, an automated CIP protocol was conducted for each contaminant with two main cleaning agents. An industrial commercially available cleaner (COSA CIP 92) and 0.5 M sodium hydroxide solution were used. After the main cleaning step, the system was flushed with ultra-pure water until neutral pH was reached. Surface swab samples were taken at different places of the separation chamber and the validated TOC analytic was carried out. The TOC amounts recovered from selected parts of the separation chamber were converted to amounts of hemoglobin, respectively serum, and multiplied with the total number of parts in the separation chamber as well as a recovery and safety factor of 0.73. The result is given as absolute values in milligram and additionally as TOC concentrations which theoretically could be found in the following smallest production batch (Table 1). The safety factor results from the lowest recovery factor found in the method validation. The measured TOC concentrations were taken as given, without taking into account that parts of the TOC signal might be remains of the cleaning agent. This explains the slightly higher total mass of hemoglobin and serum found after the cleaning process using COSA CIP 92 compared to sodium hydroxide. COSA

TABLE 1 Combined mass of hemoglobin or horse serum calculated from the recovered TOC values and the mean recovery values found after the cleaning process with COSA CIP 92 or 0.5 M sodium hydroxide on the equipment surface as well as the maximum TOC concentration for the smallest following batch

Contaminant Cleaning agent	Hemoglobin				Horse serum			
	COSA CIP		NaOH		COSA CIP		NaOH	
	mass [mg]	TOC [ppb]	mass [mg]	TOC [ppb]	mass [mg]	TOC [ppb]	mass [mg]	TOC [ppb]
Matrix elements	1.07	432	0.14	55.3	0.79	82.4	0.45	46.9
Sealing	0.83	336.6	0.17	66.9	0.7	72.9	0.77	80.3
Others	0.07	26.9	0.01	5.2	0.06	6.26	0.03	3.13
Total	2.0	795.5	0.32	127.4	1.59	161.6	1.27	130.3

CIP 92 contains 10–20% fatty alcohol ethoxylates, soap in a concentration of 5–10% and 3–5% alkylamine ethoxylates as additional carbon sources. The final washing procedure with ultra-pure water might lead to an incomplete removal of surfactants of the cleaning agent. The maximum amount of contaminants being carried to a following batch sum up to 2 mg for hemoglobin and COSA CIP 92, which is the highest amount of contaminants found in this study and therefore the worst case scenario. The smallest possible following batch size is given by the volume of the separation chamber with 1 L. Therefore, the maximum concentration of contaminants is 2 mg/L corresponding to 2 ppm. From this, it is clear that the 10 ppm criterion is reached for all cases.

4 | CONCLUDING REMARKS

In this work, counter-current operation as well as cleaning validation of a rotor-stator magnetic separator for protein purification is presented. As a model process, direct capture of the hormone eCG from unclarified serum was chosen, due to the low MOI concentration in combination with a strong cleaning challenge resulting from contact of the equipment with whole serum. The main focus of the eCG purification process development was the optimization of the process yield. Superior purification performance using an eCG specific mAb ligand has been shown before and sufficiently discussed. In order to overcome the disadvantages of batch adsorption processes in case of low concentrated targets, we present a CCPP strategy. This process, performed at pilot-scale, enabled an increase of process yields from about 80 to almost 100% without changes in MP concentration or a decrease in batch volume. Furthermore, in comparison to commonly used eCG purification strategies, the use of solvents for precipitation process steps could be completely avoided. The process has been successfully scaled-up from laboratory-scale to pilot-scale with the use of the presented GMP-compliant ‘rotor-stator’ HGMS device. Finally, an automated CIP procedure for this separation device was developed and validated successfully. The system was cleaned from horse serum as well as concentrated hemoglobin solution with a commercially available cleaning

agent or sodium hydroxide solution in an automated process. For the validation of the cleaning process, surface swab test and TOC analytics were established and validated. Linear correlations for both contaminants and good recovery rates from the swabs could be realized. To the best of our knowledge, this work presents for the first time a complete magnetic separation process development, starting from small scale batch binding tests, over process scale-up and implementation of a fully automated GMP-ready magnetic separator to the cleaning validation of the equipment. Therefore, it can be seen as a first example of the implementation of a magnetic separation process into biopharmaceutical protein purification considering not only the process development and scale-up to a technical scale but also the implementation of an adequate cleaning and cleaning validation.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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