Characterization and Validation of a Novel GMP-Compliant Magnetic Separator – Process Development and Optimization for Protein Recovery

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Für meine Familie

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Abstract

The current biopharmaceutical market is dominated by monoclonal antibody drugs. To keep up with the steadily growing market demand for this kind of biopharmaceuticals platform purification processes have been developed and intensively optimized. Established biopharmaceutical companies made large investments in large but quite inflexible stainless steel production sides. The emergence of more flexible and less capital binding single-use techniques allowed also smaller companies and a growing number of contract manufacturers to gain market shares. An increasing competition on the market leads to a constant search for cost, time and process optimization opportunities. In addition, the growing market of individualized medicine created a demand for more flexible and faster purification processes. One approach to save cost and rise the productivity of a process is to combine unit operations and create integrated process operations. The biggest impact of such a process is in early stages of the purification procedure. Against this background, process operations combining the harvest and capturing/purification of the molecule of interest (MOI) are of great interest. Typical examples for the combination of solid-liquid separation - during harvest - and an isolation of the MOI - during capturing and purification - are aqueous two phase systems (ATPS) or expanded bed adsorption (EBA) processes. Whereas ATPS requires immense development efforts and demands further purification processes to disparage introduced process contaminates, EBA is prone to fouling processes and is strongly limited in the maximum flow velocity which leads to limited process turnovers. Magnetic separation, as another representative of integrated purification tools, has the potential to satisfy the needs of the industry for a cost saving, fast, and flexible purification method. The combination of functionalized magnetic particles with high-gradient magnetic separation (HGMS) devices allows a capturing of the MOI direct from the cultivation broth in combination with an isolation and purification. The development effort of magnetic separation processes is low compared to ATPS and EBA operations. Ligands for the adsorption of MOIs can be used from common column chromatography processes and the variety of magnetic particles on the market is enormous. Furthermore, magnetic separation processes are well described for small-scale analytical methods. Besides the small development efforts, the process itself offers fast binding kinetics, due to batch adsorption processes, and nearly no limitations in flow velocity. The wide choice of particles and ligands make magnetic separation processes flexible and fast

to develop which saves costs. Maybe even more important are time and cost savings during production as well as a rise in productivity due to the reduction of process unit operations. However, the lack of suitable GMP-compliant HGMS devices prevented the application of magnetic separation processes in industrial-scale protein purification processes until now. Traditional designs of the separation matrix as well as insufficient sealing concepts could not meet the rigorous requirements of regulatory authorities for the pharmaceutical drug production. Essential for the introduction of a new device in a GMP regulated environment is besides the functionality also the proof of the cleanability. A complete cleaning and cleaning validation is required to prevent contaminations of the product and ensure the patients safety. The lack of GMP-compliant HGMS equipment has been tackled within a cooperation project of the company Andritz GmbH and the Karlsruhe Institute of Technology in the course of this doctoral thesis. Based on the idea of a 'rotor-stator' matrix design, which was developed and patented by Franzreb et al. over ten years ago, the first GMP-compliant high-gradient magnetic separator was developed and commercially launched at the beginning of 2017. The developed separation device is presented in section 4.3.1. The main novelty is the completely reengineered sealing concept which allows effective cleaning and sterilization in place. Polyetheretherketone (PEEK) elements serve as sealing as well as spacers between the matrix elements providing a closed procedural space without any dead spaces. Furthermore, the chamber as well as valve blocks are designed to enable a self-draining of the device. In addition, high surface finishes prevent deposits and simplify the cleaning of the device. Two kinds of commercially available magnetic particles, Mag Prep magnetic particles with a mean diameter of 100 nm to 200 nm and M-PVA magnetic particles with a mean diameter of 1 µm to 3 µm, were used to access key performance data. Filter capacities of more than 270 g of Mag Prep magnetic particles until a 1 % breakthrough and even 430 g of M-PVA magnetic particles per liter of separation chamber volume could be observed. The separation capacity decreased only slightly when more viscous feedstock was used, leaving the main parameter influencing the separation capacity with the particle properties and not with the fluid phase. In addition, high particle recovery rates of over 99 % and process related performance figures for the process optimization could be determined. Based on this we conclude that the system is able to process more than 200 L crude feedstock per day and capture more than 1.6 kg target compounds.

This process performance data had to be validated by the implementation of an industrial relevant protein purification process. The purification of a monoclonal antibody from Chinese Hamster Ovary cell culture was chosen to compare the magnetic separation process and the new device with a bench mark process from industry. Five consecutive purification

cycles were performed with constant yields of over 85 %, purities over 95 % and host cell protein reduction levels of more than 2.5 log. Process yields over 85 % can easily compete with the highly optimized platform processes whereas purities are expected to be slightly higher than 95 % in column based processes. The purity issue can be tackled with the further optimization of the wash protocol and choice of wash solutions. The main advantages were the enormous saving of time and process equipment for clarification procedures and column chromatography. By combining harvest and purification operations 25 % of the overall process cost which are attributable with harvest operations can be saved. The comparison with column based processes showed a three times higher productivity for the magnetic separation process. In addition, the separation device could be used as an *in situ* product removal tool due to stable cell viabilities during the magnetic separation process.

The main disadvantage of conventional magnetic separation processes is based in the batch mode binding process of the MOI to the magnetic particles. Only one equilibrium stage is used which results in low MOI loadings on the magnetic particles especially if the binding affinity is only moderate. High loadings are desired to process large batch volumes and use the magnetic particles as economically as possible. In order to approach these conditions, a cross-flow binding process is presented in section 4. Here, process magnetic particles and feedstock are moved counter currently in a two-stage batch binding process. It was possible to rise the yields from 74 % for a single stage batch adsorption process to nearly 100 % for the cross-flow process.

Aside from the process development and optimization, the development of cleaning strategies and the validation of the cleaning process is essential for the production under GMP guidelines. Therefore, a cleaning in place procedure was developed for two model contaminants. For this model process hemoglobin solution and horse serum were used as contaminants and COSA CIP 92, an industrial cleaning agent based on sodium hydroxide and pure sodium hydroxide solution, were used for the cleaning of the system. The cleaning procedure delivered promising results leaving only a total of less than 2 mg of contaminants in the whole system. For the validation of the process a surface swabbing test with a connected total organic carbon (TOC) analytics were established and validated. The correlation between TOC content and the mass of the contaminants showed linear relationships over a wide range of concentrations. Furthermore, high recovery rates of the contaminants from the swabs could be achieved which enabled in combination the validation of the cleaning process.

The combination of characterization, process development and optimization as well as a successful cleaning validation of the first GMP-compliant HGMS device is a comprehensive approach to describe the new system and lays the foundation for a successful integration of magnetic separation processes in industrial biopharmaceutical purification processes.

Zusammenfassung

Monoklonale Antikörperpräparate dominieren heutzutage den biopharmazeutischen Markt. Um mit dem stetig wachsenden Bedarf Schritt halten zu können wurden über die Jahre standardisierte Aufreinigungsverfahren entwickelt und verfeinert. Etablierte Konzerne haben durch immense Investitionen große, unflexible Produktionseinrichtungen geschaffen. Das Aufkommen von flexibleren und weniger kostenintensiven Einmalprodukten und Techniken ermöglichte es auch kleineren Unternehmen und einer steigenden Zahl von Auftragsproduzenten in den Markt einzusteigen. Die dadurch steigende Konkurrenz führt zu einem enormen Kostendruck und somit zu einer stetigen Optimierung von Prozesszeiten, Kosten und Ausbeuten. Zusätzlich führt der wachsende Markt für personalisierte Arzneimittel zu einem verstärkten Bedarf an flexiblen und schnellen Aufreinigungstechniken. Ein Ansatz um die Prozesskosten zu senken und die Produktivität zu steigern ist die Kombination bzw. Zusammenfassung einzelner Prozessschritte zu integrierten Prozessen. Den größten Nutzen hat diese Kombination von Prozessschritten zu Beginn des Aufreinigungsprozesses. Aufgrund dessen sind Verfahren von Interesse, welche die Zellernte mit der Isolation beziehungsweise der ersten Aufreinigung des Zielproduktes vereinen. Typische Beispiele für die Kombination von Fest-Flüssigtrennung, eingesetzt zur Zellernte, und der Isolation des Zielproduktes sind wässrige Zwei-Phasen-Systeme (ATPS) oder Expanded-Bed-Adsorption (EBA). Der Nachteil von ATPS-Systemen besteht in der aufwendigen Entwicklung geeigneter Systeme und der Einbringung prozessbedingter Verunreinigungen, die zur Ausbildung der Phasen von Nöten sind und anschließend durch zusätzliche Prozessschritte entfernt werden müssen. EBA hingegen ist anfällig für Alterungsprozesse des Mediums. Außerdem wird der Durchsatz des Prozesses stark durch die maximale Flussrate limitiert um ein Austragen des Mediums zu vermeiden. Als weiterer Vertreter integrierter Aufreinigungsmethoden hat die Magnetseparation das Potential die industrielle Nachfrage nach einem flexiblen, kostensparenden und schnellen Prozess zu befriedigen. Die Kombination aus funktionalisierten Magnetpartikeln mit hoch-gradienten Magnetseparation ermöglicht eine spezifische Aufreinigung des Zielproduktes direkt aus der Fermentationsbrühe. Der nötige Entwicklungsaufwand ist verglichen mit ATPS und EBA gering, da eine Vielzahl an funktionelle Gruppen bzw. Molekülen aus der Säulenchromatographie bekannt und umfassend beschrieben sind. Magnetische Partikel und Prozesse sind zusätzlich als analytische Technik weit verbreitet und somit ist eine

große Auswahl von Partikeln kommerziell erhältlich. Die Vorteile der Magnetseparation liegen neben dem geringeren Entwicklungsaufwand in den schnellen Prozesszeiten aufgrund der erwarteten kurzen Bindezeiten. Zusätzlich besteht nahezu keine Limitierung in den Flussraten. Die Auswahl an Magnetpartikeln und funktionellen Gruppen macht dieses Verfahren flexibel einsetzbar und ermöglicht eine schnelle und kostengünstige Prozessentwicklung. Des Weiteren können während der Produktion Zeit und somit auch Kosten eingespart werden, bei gleichzeitiger Steigerung der Produktivität durch Verringerung der Anzahl von Prozessschritten. Trotz all dieser Vorteile konnte sich die Magnetseparation bisher nicht als Aufreinigungsmethode im industriellen Umfeld durchsetzen. Dies ist vor allem auf das Fehlen geeigneter GMP-konformer Magnetseparatoren zurückzuführen. Herkömmliche Konstruktionen der Trennmatrix sowie unzureichende Dichtungskonzepte entsprachen nicht den strengen Anforderungen der Zulassungsbehörden für die pharmazeutische Arzneimittelherstellung. Wesentlich für die Einführung eines neuen Geräts in einer GMP geregelten Umgebung ist neben der Funktionalität auch der Nachweis der Reinigungsfähigkeit die für Magnetseparatoren meist nicht gegeben war. Der Nachweis der Reinigbarkeit und die Validierung dieses Prozesses ist erforderlich um die Sicherheit und Gesundheit der Patienten zu gewährleisten. Im Rahmen des Kooperationsprojektes der Andritz GmbH und des Karlsruher Instituts für Technologie sollte der Mangel an einem industriell einsetzbaren Magnetseparator behoben werden. Basierend auf einer ‚Rotor-Stator' Separationsmatrix, welche von Franzreb et al. bereits vor mehr als zehn Jahren entwickelt und patentiert wurde, konnte der erste GMP-konforme Magnetseparator entwickelt werden, welcher seit Anfang 2017 kommerziell erhältlich ist. Der entwickelte Separator wird in Abschnitt 4.3.1 vorgestellt. Die wichtigste Neuerung ist das komplett überarbeitete Dichtungskonzept, welches eine effektive Reinigung und Sterilisation ohne Zerlegen des Apparates ermöglicht. PEEK-Elemente dienen sowohl als Abdichtung als auch als Abstandhalter zwischen den Matrixelementen und bieten so einen leicht zu reinigenden, geschlossenen Verfahrensraum ohne Toträume. Die Kammer sowie die Ventilblöcke sind so ausgelegt, dass eine Selbstentleerung des Gerätes möglich ist. Darüber hinaus verhindern hohe Oberflächengüten das Anlagern von Verunreinigungen und vereinfachen die Reinigung des Gerätes. Zur Bestimmung der Systemeigenschaften wurden zwei unterschiedliche kommerziell erhältliche Magnetpartikel verwendet. Zum einen Mag Prep Partikel, welche einen mittleren Durchmesser von 100 nm bis 200 nm aufweisen. Zum anderen wurden M-PVA Partikel verwendet. Diese besitzen laut Hersteller einen mittleren Durchmesser von 1 µm bis 3 µm. Es konnten maximale Filterkapazitäten von 270 g Mag Prep Partikeln bis zu einem 1 %igen Durchbruch erreicht werden. Die Filterkapazität für M-PVA Partikel lag mit 430 g pro Liter

Kammervolumen sogar deutlich höher. Die Verwendung viskoser Flüssigphasen führte zu etwas geringeren Filterkapazitäten, wobei der Haupt Einflussparameter auf die Separationseffizienz auf die Eigenschaften der Partikel zurückzuführen ist und nicht bei der Flüssigphase liegt. Hohe Partikelrückgewinnungsraten von über 99 % in Kombination mit prozessrelevanten Daten zu den Partikelbindkapazitäten ermöglichte es die Produktivität des Systems rechnerisch zu ermittelt und zu optimieren. Es zeigte sich, dass mehr als 200 L Fermentationsbrühe pro Tag prozessiert und dabei mehr als 1,6 kg Zielprodukt aufgereinigt werden können.

Diese Produktivität sollte durch die Implementierung eines industriell relevanten Proteinreinigungsprozesses validiert werden. Zu diesem Zweck wurde ein Aufreinigungsprozess eines monoklonalen Antikörpers aus einer Zellkultur von Chinesischen Hamster Ovarien für den Magnetseparator entwickelt, um das neue Gerät mit einem Benchmark-Prozess aus der Industrie vergleichen zu können. Es konnten fünf Aufreinigungszyklen mit konstanten Ausbeuten von über 85 %, Reinheiten von über 95 % und einer Reduktion des Wirtszellproteins von mehr als 2,5 log-Stufen hintereinander durchgeführt werden. Die erreichten Prozessausbeuten von über 85 % können mit den intensiv optimierten Plattformprozessen mithalten, während die Reinheiten von 95 % bei säulenbasierten Prozessen etwas höher liegen dürften. Zur Verbesserung der Reinheit kann eine weitere Optimierung des Waschprotokolls und die Wahl optimierter Waschlösungen erwogen werden. Der Hauptvorteil des Magnetseparationsprozesses liegt allerdings in der enormen Zeit- und Materialersparnis durch die Kombination aus Produkternte und chromatographischen Prozessschritten. Durch die Kombination von Zellernte und Reinigung des Zielproduktes können bis zu 25 % der Gesamtprozesskosten eingespart werden, die durch Operationen in der Fest-Flüssig Trennung verursacht werden. Der Vergleich mit säulenbasierten Verfahren ergab zudem eine dreimal höhere Produktivität für den Magnetseparationsprozess. Darüber hinaus kann dieser Prozess aufgrund stabiler Lebendzellzahlen während des gesamten magnetischen Trennprozesses auch als *in-situ* Trennmethode eingesetzt werden.

Der Hauptnachteil magnetischer Trennverfahren liegt in der Batchcharakteristik des Bindeschrittes des Zielmoleküls an die magnetischen Partikel. Bei diesem Prozess wird nur eine Gleichgewichtseinstellung erreicht, was zu einer geringen Beladung der Partikel mit Zielmolekül, insbesondere bei suboptimaler Bindungsaffinität, führt. Allerdings sind hohe Beladungen erwünscht. Zum einen können große so Volumina verarbeiten werden und zum anderen ist eine wirtschaftliche Nutzung der Partikel nur bei hohen Beladungen gegeben. Auf Grund dessen wurde ein Gegenstrom-Bindeprozess entwickelt, der in Abschnitt 2.6.2

vorgestellt wird. Bei diesem Verfahren werden magnetische Partikel und Fermentationsbrühe in einem zweistufigen Batch-Bindungsprozess im Gegenstrom bewegt. So war es möglich die Ausbeute von 74 % für einen einstufigen Prozess auf fast 100 % für den Gegenstromprozess zu steigern.

Wie bereits angesprochen ist neben der Prozessentwicklung und Optimierung die Entwicklung von Reinigungsstrategien und dessen Validierung essentiell für eine Produktion unter GMP Richtlinien. Daher wurde für zwei Modellverunreinigungen ein CIP-Verfahren (Cleaning in Place) entwickelt. Für diesen Modellprozess wurden Hämoglobinlösung und Pferdeserum als Kontaminanten verwendet. Als Reiniger kamen COSA CIP 92, ein industrielles Reinigungsmittel auf der Basis von Natriumhydroxid und reine 0,5 M Natronlauge zum Einsatz. Das Reinigungsverfahren lieferte vielversprechende Ergebnisse, da insgesamt weniger als 2 mg Verunreinigungen im gesamten System verblieben. Zur Validierung des Prozesses wurde ein Oberflächenwischtest mit nachgeschalteter Analyse des gesamten organischen Kohlenstoffes (TOC) validiert und verwendet. Die Korrelation zwischen dem TOC-Gehalt und der Konzentration der Kontaminanten zeigte lineare Zusammenhänge über einen weiten Konzentrationsbereich, zudem konnten hohe Rücklöseraten der Kontaminanten aus den verwendeten Tupfern erzielt werden, was die Validierung des Reinigungsprozesses ermöglichte.

Die Kombination aus Charakterisierung, Prozessentwicklung und Optimierung sowie einer erfolgreichen Reinigungsvalidierung des ersten GMP konformen hochgradienten Magnetseparators ist ein umfassender Ansatz zur Charakterisierung und Inbetriebnahme des neuen Systems und legt den Grundstein für eine erfolgreiche Implementierung von Magnetseparationsprozessen in industriellen biopharmazeutischen Proteinaufreinigungsprozessen.

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1 Introduction

Magnetic separation processes as well as the concept of Biotechnology have been known for decades. The early uses of magnetic separation processes date back to the late 18th century. The first patent in the field of magnetic separation processes has been filed by William Fullarton describing a process to separate iron minerals in process streams in 1792 [1,2]. With the development of new separation devices, the separation tasks became more complex. Until 1909 already 300 patents have been filed in the field of magnetic separation but it was the development of high-gradient magnetic separation (HGMS) devices in the 1950s that expanded the range of applications [3]. Faster and more complex separation tasks were now possible [4,5]. High magnetic fields in combination with a magnetizable matrix such as steel wool placed in the field producing enormous field gradients enable HGMS devices to separate also weakly magnetic, or very small magnetic particles from feed streams [6]. This opened the application, for example, for waste water treatments but later on, during the 1980s, also in the fast growing field of biotechnology [5,7–10]. The term of biotechnology was firstly used by Erky in 1919 [11]. Modern biotechnology is based on the developments in the 1950s with the discovery of the DNA as genetic material, the production of the first antibody in 1975 as well as on the production of first recombinant insulin in 1978 [12–14]. Henceforth the biotechnology market has grown constantly. Today biopharmaceutical products are one of the main products in pharmaceutical industry with market shares of over 200 billion dollar and growth rates of 15 % [15]. Among the products, such as proteins, viruses or virus like particles, monoclonal antibodies (mAbs) are the blockbusters dominating the market with shares of 50 billion dollars [16,17]. mAbs are produced with highly optimized platform processes in large scales [18]. The process typically consists of two parts. First part is the production of the mAb by Chinese Hamster Ovary (CHO) in a defined cell culture process which is called ‘up-stream process’ (USP). For secreted products such as mAbs the cultivation is followed by a solid-liquid separation to isolate the product in the supernatant called ‘harvest’. The harvest operations are usually assigned to the USP and represent also the first purification step. The second part is the purification of the mAb by several orthogonal purification methods before it can be applied

as pharmaceutical drug substance. These process steps can be summarized under the term 'down-stream process' (DSP)[19]. Each applied process step of the DSP increases the purity and concentration of the mAb but also add costs and reduce the overall yield of the process [20]. A possibility to meet the industry's desire for cost reduction and to rise the productivity are integrated purification techniques such as expanded bed adsorption (EBA), aqueous two phase systems (ATPS) or HGMS [21]. These techniques combine classical purification steps in DSP such as clarification, concentration and purification. The boundaries between USP and DSP blur due to the integration of typical harvest process steps in the expanded bed adsorption or magnetic separation process.

The development of magnetic separation processes in biopharmaceutical purification processes and applications are addressed in section 2.3. However, due to the lack of suitable GMP-compliant magnetic separation devices this technique has not found an application in GMP regulated industrial DSP processes yet. The guidelines under which a device has to be designed to be suitable for biopharmaceutical production and the validation process are outlined in section 2.7 and section 2.8, respectively. Based on the conclusions from these sections this work focusses on the characterization and validation of the first GMP-compliant high-gradient magnetic separator in section 4 and in the following sections on the implementation of an integrated mAb purification process followed by the development and validation of cleaning routines.

1.1 Research Proposal

This work is based on several doctoral theses performed under the guidance of Prof. Franzreb. Hoffmann [22], Meyer [23] as well as Ebner [24] worked on various topics of protein purification using different HGMS systems. The development of a new matrix design for a HGMS device by Franzreb et al., which was patented over a decade ago laid the foundation for the construction of a new type of HGMS devices [25]. The findings associated with this new design were published by Müller [26], who developed and used this separator design for protein purification studies from crude feedstocks. The work showed the superiority of the 'rotor-stator' matrix design when it comes to washing and recovery of magnetic particles. However, the lack of GMP-compliant separation equipment prevented the use of magnetic separation processes in biopharmaceutical industry. On the basis of this, the aims of this thesis are

- Development of a GMP-compliant 'rotor-stator' high-gradient magnetic separator in cooperation with Andritz GmbH as industrial partner
- Commissioning, characterisation and validation of the developed separation device
- Development of protein purification processes via magnetic separation in small-scale studies, scale-up to technique-scale and implementation in the new device
- Development and validation of cleaning strategies for the new separator.

2 Fundamentals

2.1 Protein Purification Processes

A protein purification process typically consist of several unit operation with the aim to increase product concentration and purity while decreasing the process volume [27]. The determining factors in process development are therefore recovery of the product, robustness, scalability and efficiency of the process as well as availability of raw materials [28,29]. Four main process steps can be defined in a DSP: recovery followed by purification, polishing and formulation. Each process step consists of several unit operations (Figure 1).

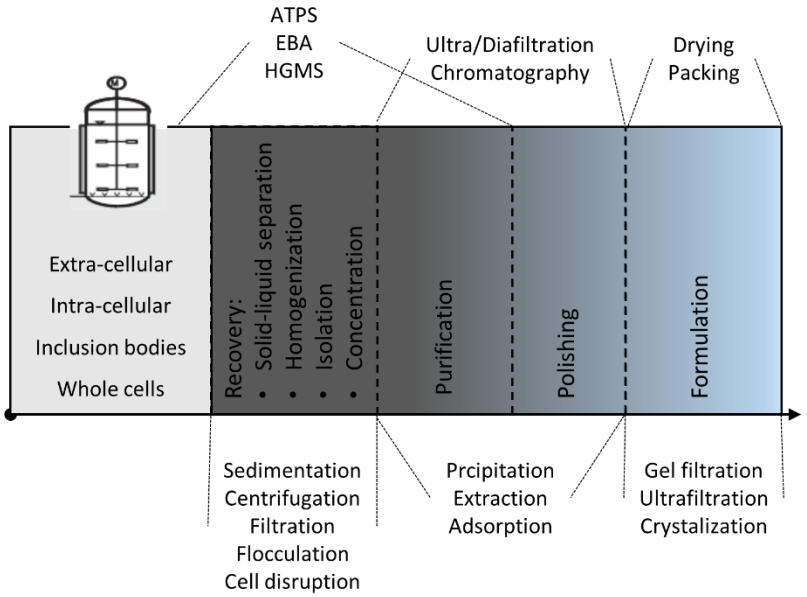


Figure 1: Schematic overview of the stages during a protein purification process. In dark grey: main process steps process during a protein purification process with separation principles below the box and process operations above the box. The use of the purification techniques depends on the expression system (light grey) during the USP. Adapted from Hubbuch et al.[30].

The boundary between the fermentation or USP and the DSP is usually set by the recovery of the product from the expression system, although this border is not totally fixed and more fluent especially by the rise of integrated purification techniques. Process operations for the recovery of a product depend strongly on the expression system used in USP. For

intracellular products a cell concentration is followed by cell disruption to release the product. From there on the process is comparable with products directly secreted in the supernatant [28]. Typical unit operations for the recovery of a product from solid components of the expression system are centrifugation followed by depth filtration [30]. Main challenges for the development of a centrifugation step are the small size and density differences of product and impurities and thus similar settling velocities. Since low solid contents are required for further unit operations multiple centrifugation steps might be required. After depth filtration, to remove last solid remains, the main purification task is performed by solid bed chromatography, which is also termed the 'workhorse' of DSP [31]. The separation principle is based on a reversible interaction of the molecule of interest (MOI) to a surface leading to the adsorption of the molecule. The mechanism of interaction is influenced by the biological function and the chemical structure of the MOI-surface combination [32]. Interactions typically used for target separation are for example hydrophobic, electrostatic, Van-der-Waals interactions, hydrogen bounds or a combination of these. Figure 2 provides an overview of the most commonly used chromatography methods. Purification is followed by the product polishing. Process or product related impurities like charge variants or product aggregates are removed by further chromatography steps during the polishing. Finally, the product is formulated in the final dosage form or in storage conditions. Techniques for this process step strongly depend on the dosage form.

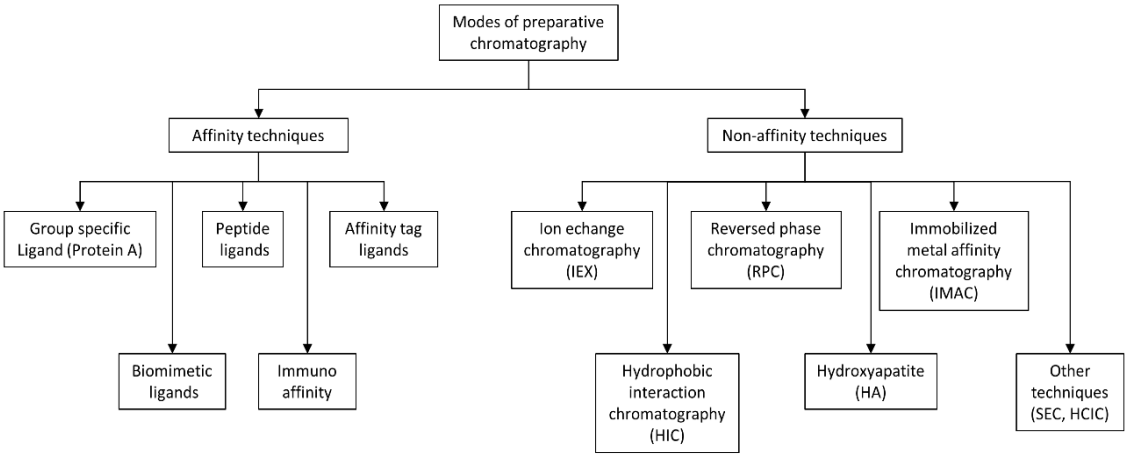


Figure 2: Overview of classical protein purification methodes using column chromatography [28].

In the following the purification process for mAbs is described in more detail because of its relevance for this work and especially for industry. Within the biopharmaceutical market, which has market shares above 200 billion dollars and a growth rate beyond 15%, mAbs are dominating as blockbusters and will do so for the next years with more than 50 candidates currently in late-stage development [15,29,33–35]. The DSP of mAbs was subject of far-reaching optimization and standardization processes [34]. Today platform processes are established and used for purification which differ only in the polishing steps of the process (Figure 3). The solid-liquid separation by centrifugation and depth filtration is followed by protein A column affinity-chromatography for product recovery, concentration and purification. With this specific, highly selective process step main impurities such as culture media components, host cell proteins (HCPs) or DNA are removed and the product is concentrated. The interaction of the affinity ligand and the mAb is reversible by lowering the pH, followed by a low pH hold for virus inactivation. Further process steps are virus filtration and as polishing steps chromatographic separation technologies. The number of added process steps is dependent on the properties of the system. Whereas cation exchange chromatography (CEX) is mainly used in bind and elute mode, hydrophobic interaction (HIC) and anion exchange chromatography (AEC) are used in flow-through mode for further reduction of DNA, HCPs, leached protein A ligands and endotoxins [18,19,36,37].

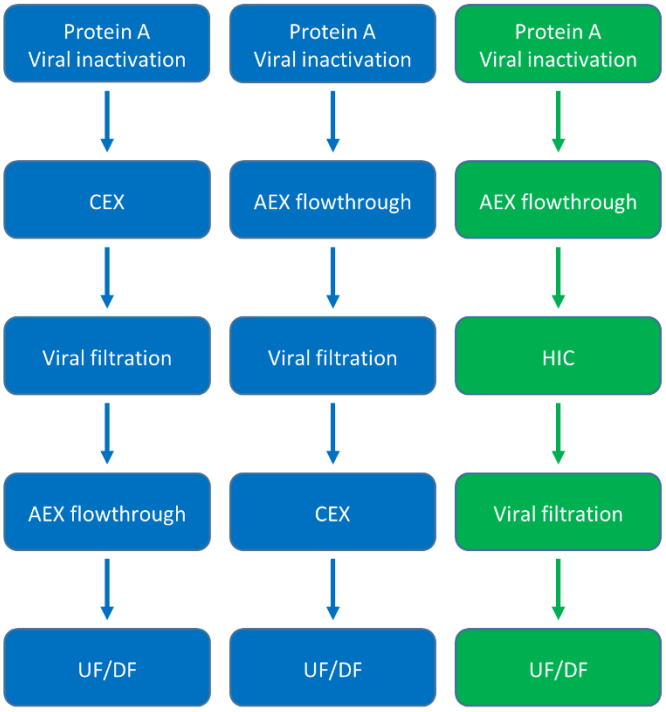


Figure 3: Examples for mAb purification platform processes. Blue boxes depict Genentech platform processes and green boxes Biogen platform process [29].

2.2 Integrated Purification Processes

The constant growing market of biopharmaceutical products and constant developments in fermentation creates a demand of new protein purification strategies in DSP. Established companies have tried to tackle the bottleneck in DSP by heavily investing in large production capacities of stainless steel plants. Standardization and platform processes have been established using structural similarities, which has improved productivity significantly [34]. The introduction of disposables and ready to use products as well as more flexible equipment have opened the biopharmaceutical production market also for smaller companies as well as for a growing number of contract manufacturers [38]. However, the cost for DSP remain with 50 – 80 % of the overall production cost high [30,39,40]. The cost contributions is shown in Figure 4. Therefore, it is of interest to combine the processes causing the highest cost in order to reduce overall cost.

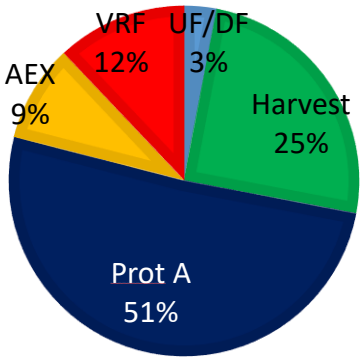


Figure 4: Cost for goods during a large-scale mAb platform purification process broken down to process unit operations [18].

Aside from the economical factor of high costs, the use of multi-stage purification processes result in low process yields. Seven to nine process steps are often necessary for the purification of antibodies [41]. Each step will add cost and product loss. Even if each individual process step reaches yields of 90 % the overall process yield will be 43 % for eight process steps (Figure 5). An obvious approach is the reduction of process steps. The combination of process steps can be realized most effectively at the beginning of the DSP [30,42]. Different methods are known to combine the solid-liquid separation with the first product recovery and concentration step. Techniques such as ATPS, EBA or HGMS unite the

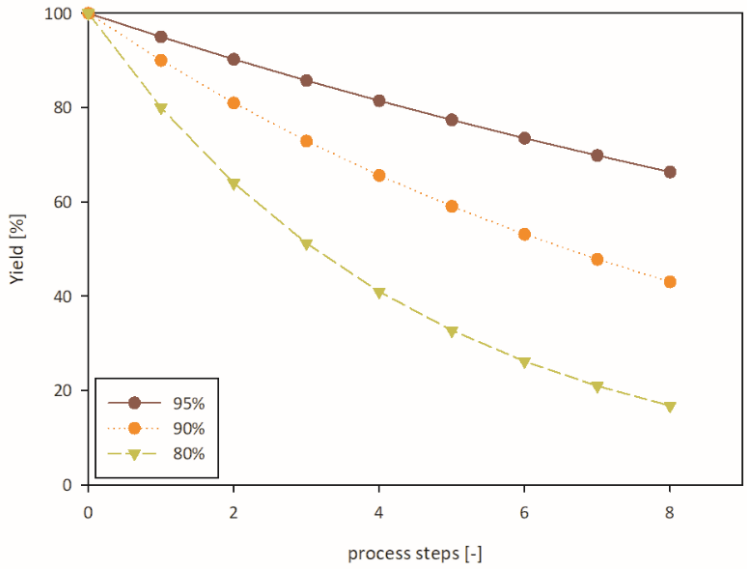


Figure 5: Process yield in dependence of the number of process steps needed to reach the desired purity [169]

ability to isolate the target molecule from a complex cultivation feed stream. A closer description of ATPS and EBA can be found in the following chapters. The principle of HGMS and the use of HGMS in downstream processing can be found in chapter 2.3. Rosa et al. [31] demand from these purification processes to be robust, reliable, easy to scale-up and of cause to be capable to remove process related impurities as prerequisite to be introduced as industrial process. Any processes meeting these requirements are extremely interesting because they offer a way to increase overall yield, lower requirements for capital investments and consumables, and most importantly, reduce process time [21,43].

2.2.1 Expanded Bed Adsorption (EBA)

From the described integrated purification unit operations in biopharmaceutical DSP EBA is closest related to common column chromatography. A bed of chromatographic media is expanded by an upwards directed fluid stream and this way allows to capture target biomolecules from complex particulate-containing feedstocks. For elution of the target molecules the flow direction is changed to execute the elution process step in a packed bed. By this method high concentration factors as known from regular column chromatography can be achieved [44]. Significant difference in physical properties of feedstock debris and chromatographic media are required for applying EBA. It is a challenge to ensure a stable

fluidized bed without risking a loss of chromatographic media from the column when feed streams containing for example cell or cell debris [45]. Furthermore, the generation of a stable expanded bed is prerequisite for economic use of EBA. It will minimize axial dispersion which leads to back mixing and reduces the theoretical stages and therefore, recovery rates [43,46,47]. Responsible for the creation of a stable fluidized bed is the combination of a size and density distribution of the particles and the flow velocity of the process. The use of small particles will lead to slow fluid velocities to avoid a discharge of the particles and therefore to long process times. Large particles require high flow rates for the fluidization. In this case short contact times limit the protein adsorption due to restricted diffusion into the chromatographic media. Hjorth describes the optimal size range of the media from 50 µm to 400 µm with a density between 1.1 and 1.3 g/mL to operate an EBA process economically [46]. A good overview of chromatographic media for EBA applications is given by Hubbuch et al. [43]. First applications using EBA in protein purification were described in the 1990s [48]. Since then EBA has shown its applicability for various products and feed streams and even entered industrial processes [49–51]. However, the small operation window of flow velocities, particle size and density make a successful scalability of EBA processes difficult. To ensure a homogeneous flow into the column in order to avoid channeling and back mixing is one of the biggest challenges in up-scaling. Furthermore, fouling of the chromatographic media, due to its porous structure, leads to a shift in density distribution of the particle bulk and reduction of binding capacity. This in combination with the high sensibility to process changes and the restrictions in process velocity prevent wide application of this technique [52].

2.2.2 Aqueous Two Phase System (ATPS)

An ATPS is a particle-free integrated purification method and therefore not susceptible to media fouling. The purification principle is based on the separation of target molecules, impurities, and solid components from the complex feedstock in different liquid phases [53]. The phase separation of the system with a high water content is achieved by mixing two solved hydrophilic polymers or a polymer- chaotropic salt combination. Above a critical concentration, temperature and ionic strength two phases form. The target separation is based on a complex interplay of the characteristics of the ATPS and the MOI [54]. Proteins are more likely to accumulate in the polymer phase, which is hydrophobic and less polar, whereas cells can be found at the phase boundary. The accumulation can be enhanced by

changing the characteristics of the ATPS, multistep processes or functionalization of the polymers [55]. Due to cost for functionalized polymers, recycling of ATPS components might be necessary for an economical operation. ATPS are known for more than 50 years in bio separation processes and many applications have been described from DNA purification, cell separation to antibody or enzyme purification. A collection of purification tasks and used ATPSs are described by Glyk et al. or Rosa et al. [31,56]. Drawbacks are elaborate investigations of suitable ATPS. Due to the poor understanding of the separation mechanism the development of new systems is mainly based on empirical analysis. Furthermore, the introduction of process impurities for phase separation such as poly-ethylene glycol (PEG) and high salt concentrations require further purification steps [21,31,57].

2.2.3 *'In Situ'* Product Removal

A further approach for combining processes is to integrate purification techniques directly into fermentation processes. '*In situ'* product removal (ISPR) is mainly used for low molecular weight products. Recently, due to the strong demands for new purification methods, ISPR is also described for high molecular weight products [21]. The idea is to integrate solid-liquid separation process operations directly into the fermentation. After the separation of the MOI the cell suspension is reintegrated into the process and fermentation is constantly continued. Aside from product removal ISPR offers further advantages. Product related impurities, inhibitors as well as toxic substances preventing an optimal fermentation process or damaging the product can be removed as well [58,59]. Techniques such as perfusion stirred-tanks or hollow-fibre bioreactors offer the possibility for constant product removal while proceeding with fermentation in order to create a continuous process [35,60]. These techniques are not selective to a MOI. In contrast to this, magnetic separation offers a selective product removal as well as an easy integration into the fermentation process equipment due to its batch binding character and can therefore be described not only as an integrated process but also as an ISPR process [59].

2.3 Magnetic Separation in Biotechnology

As mentioned, magnetic separation is known as integrated purification method as well as ISPR tool for a target orientated separation of molecules from fermentation processes. Beyond this, magnetic separation is applied in a wide variety in the field of biotechnology. The roots of the industrial use of magnetic separation are far older than the biotechnology industry. Magnetic separation processes have been known since the 1850s in the mining industry for the removal of strongly magnetic particles from feed streams such as iron ore magnetite. With the development of HGMS devices new applications like the removal of weakly or small magnetic particles in waste water treatment, chemical process recycling or the recovery of nonferrous low grade ores have been developed [3,4,61–63]. In biotechnology industry magnetic separation has been firstly used by Dunnill and Lilly in the 1970s for the immobilization of enzymes and later on as bio-affinity adsorbents material for chromatographic bind and elute processes [8]. Today magnetic separation is mainly applied on a lab-scale basis for analytical purposes. Typical applications are DNA purification, cell sorting or labelling as well as enzyme immobilization [64–71]. The principle of HGMS processes is as simple as powerful. Small functionalized target-selective magnetic particles bind the MOI directly from crude unclarified cultivation broth [72]. Known and well described molecules from common chromatographic methods can be applied as functional ligands [73]. A simple stirring tank arrangement, for the incubation, prevent the system to get blocked by solids as it is known from common column based chromatography. The use of small and mainly non-porous magnetic particles minimizes the risk of fouling by pore blocking. Furthermore, the large surface areas provide high binding capacities and the non-porous character prevents limitations in binding kinetics caused by slow diffusion coefficients [73]. Magnetic particles are easy to separate after incubation via magnetic forces. This fast but gentle separation technique is not limited by flow velocities as seen for EBA or dependent on highly complex empiric process development as for ATPS [52]. After the recovery of the MOI, impurities are washed out of the system and the purified product is available [74]. Examples for the successful application of HGMS processes for processing a wide variety of molecules have been developed over the years. Comprehensive lists have been presented by Franzreb et al. [73], Safarik et al. [74] as well as Borlido et al. [75]. Even several studies have been published presenting approaches for the purification of industrially relevant products in relevant scales up to 100 L [76–79]. As a continuation of the list of Franzreb et al. an overview of magnetic separation processes used for protein

purification in scales larger than 20 mL feed solution or gram amounts of particles starting from 2006 is given in Table 1. Besides the list of protein purification processes via magnetic separation in millilitre-scale numerous studies dealing with this topic during the same time period in small-scales have been published [80–91]. This small selection of publications demonstrates the great relevance of magnetic separation in analytical scale. The by fare shorter list of publications concerning magnetic separation in larger scale illustrates the low impact of HGMS processes in academia and for these reasons it is not surprising that HGMS processes have not yet found an application in biopharmaceutical industry. This is mainly due to a lack of investments and research in this field caused by the dominance of column based purification processes. Therefore, suitable GMP-compliant separation equipment has to be developed in adequate scales and the prices of functional magnetic particles have to drop. Currently particle prices and production scales are oriented towards the comparatively small demand in bioanalytics leading to disproportional high prices [27,73,92,93].

Table 1: Protein purification studies using magnetic separation processes in scales larger than 20 mL or gramm amounts of magnetic particles from the year 2006 up to today

System	Adsorbent/ Ligand	c_0	c_p	Feed	Yield	Purity	Reference
Protease from B. licheniformis fermentation	M-PGA/ bacitracin	-	1.8 g/L	2.25 L	50 %	-	Käppler 2009 [59]
Gonadotropin (eCG) from horse serum	M-PVA/ DEAP	39-57 IU/mL	4 g/L	20 mL	79 %	1300 IU/mg	Müller 2011 [94]
Gonadotropin (eCG) from horse serum	M-PVA/ affinity	40 IU/mL	2 g/L	1 L	50 %	488 IU/mL	Müller 2011 [95]
Lactoferrine/Lactoperoxidase from crude whey	M-PVA/ pAAc		2.5 g/L	2 L	49/58 %	-	Brown 2013 [96]
Gonadotripin (eCG) from horse serum	M-PVA/ DEAP	30-43 IU/mL	4.5 g/L	4.6 L	80 %	-	Müller 2014 [97]
His-GFP from E.coli	M-Silica/ EDTA	8.5 g/L	22.3 g/L	1 L	93 %	96 %	Fraga García 2015 [79]

2.3.1 Theory of a Magnetic Separation Process

Permanent magnetic materials are characterized by the phenomena that due to the movements of unpaired electrons in the orbitals of the atoms and coupling and rectification of the resulting microscopic moments throughout the Weiss' domains a macroscopic magnetic moment results.

In contrast to permanent magnetic materials a magnetic field with the strength H [A/m] around a conductor is caused by a current flow (I) in the conductor. If the resulting magnetic field around the conductor is superimposed by an external orthogonal magnetic field a power F is acting perpendicular on the conductor. The response of the material described as the magnetic induction or flux density B [Tesla].

$$B = \frac{F}{I * L} \qquad 2.1$$

In the case of a current-carrying coil the field strength around the conductor can be represented by equation 2.2:

$$H = I * \frac{n}{L} \qquad 2.2$$

L describes the length of the conductor, whereas n is the number of windings. The direction of H equals the direction of the flux density.

The linear relation between the flux density and the field strength can be described by equation 2.3 [98]:

$$B = \mu_0 * (H + M) \qquad 2.3$$

μ_0 is the permeability in free space. Generally, the permeability depends on the medium and is often a non-linear function of H [99]. The magnetization M of a material is defined by this equation.

$$M = \kappa * H \qquad 2.4$$

For all materials, exept ferro- and ferrimagnetic substances the magnetization is proportional to the field causing it. The proportionality factor, the susceptibility κ, is defined by.

$$\kappa = \mu_r - 1 \qquad 2.5$$

With equations 2. and 2. the magnetic induction of a material in an external field can be simplified to.

$$B = \mu_0 * \mu_r * H \qquad 2.6$$

The relative permeability μ_r is a material constant. Depending on the atomic structure as well as temperature all materials will show a magnetic behaviour which is more or less pronounced [98]. Materials can be classified according the value of constant μ_r. Paramagnetic materials have $\mu_r > 1$ while diamagnetic materials have $\mu_r < 1$. Paramagnetic materials increase an external magnetic field while diamagnetic materials weaken the surrounding field [100]. For ferro- and ferrimagnetic substances the susceptibility is not proportional to the external field but reaches a maximum and decreases for higher fields. Thus μ_r is generally a non-linear function of H. The linear relationship for dia- and paramagnetic substances can be seen if the magnetization is plotted versus the magnetic field strength (Figure 6A). In contrast to this the magnetization curve of a ferromagnetic material, never exposed to a magnetic field bevor, will show a strong dependency of the magnetization in correlation to the field strength. Increasing fields will eventually lead to a saturation effect where the material is completely magnetized reaching its saturation magnetization M_s. After removing the external field these substances will show a permanent

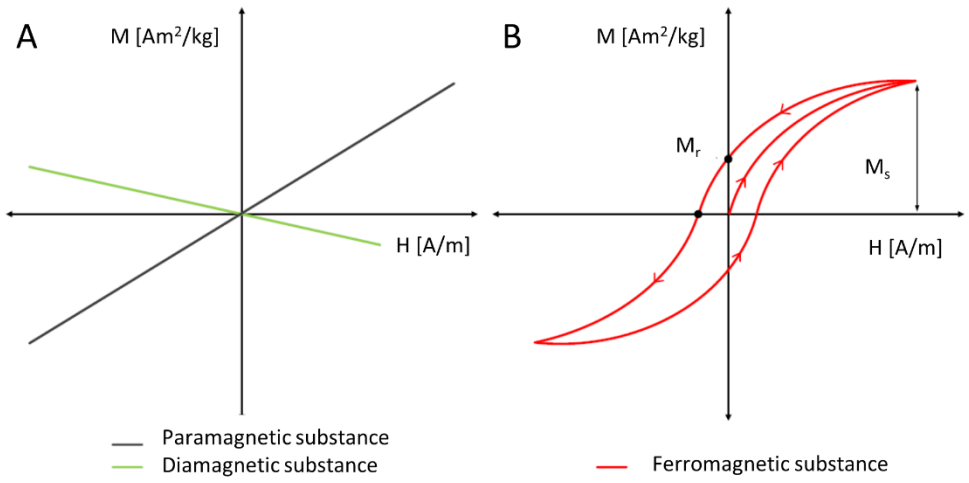


Figure 6: (A) Magnetization curve of a diamagnetic substance (green line) and magnetization curve of a paramagnetic substance (black line). (B) Magnetization curve, and hysteresis loop of a ferromagnetic substance [100][170].

magnetization known as remanent magnetization M_r (Figure 6B). A special case of paramagnetic behaviour can be seen for iron oxide crystals smaller than about 20 nm,

described as superparamagnetic behaviour. For these particles saturation can be observed for magnetization but no remanence.

The magnetic separation of a superparamagnetic particle in an external magnetic field for example is a physical separation based on an interplay between different forces [62,92]. On one side the magnetic force F_m acts on a magnetic particle depending on its magnetization, the volume V and the gradient of the magnetic field H [99,101].

$$F_m = \mu_0 * V * M * \nabla H \qquad 2.7$$

On the other side the magnetic fore has to dominate system related competing forces such as gravitational, fluid drag and diffusional forces. Additionally interparticular forces such as electrostatic forces may influence the separation by forming agglomerates which change the particle size or the separation behaviour by trapping different substances [7,62,101].

2.3.2 High-Gradient Magnetic Separation

If the liquid phase and the type of magnetic particles used are fixed, the efficiency of separation depends solely on the strength of the magnetic field and its gradient. To achieve high field gradients HGMS has been developed [4,100]. The basic principle of HGMS can be described as deep filtration. A filtration matrix consisting of ferromagnetic stainless steel plates, steel wool or a stack of wire meshes is introduced in the separation chamber and an external magnetic field is applied. The wires de-homogenize the external field, concentrate

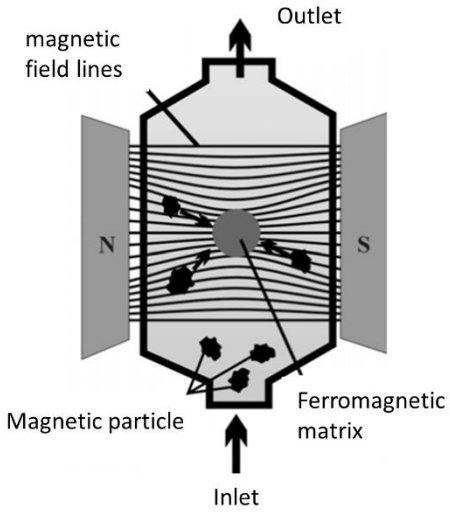


Figure 7: Schematic illustration of the behaviour of the magnetic field lines in a single wire HGMS device [24].

the magnetic field and thus create strong field gradients in the surrounding of the matrix elements, dragging magnetic particles to distinct regions of the matrix [28,102,103]. For a single wire the basic principle is illustrated in Figure 7. For the orientation of matrix elements, fluid flow direction and magnetic field three arrangements can be described

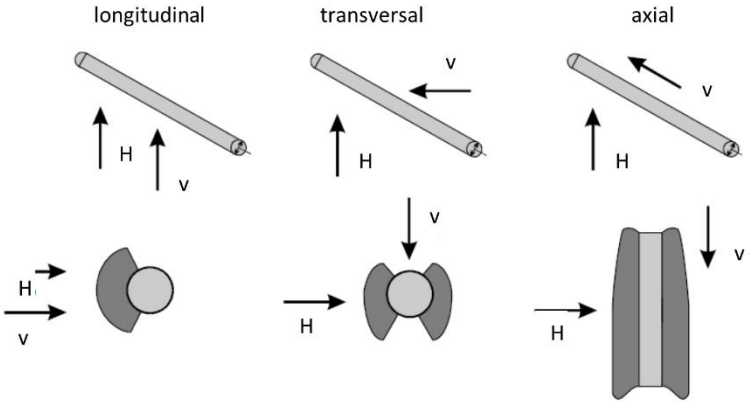


Figure 8: Geometrical arrangements of separation matrix, fluid flow and magnetic field and magnetic particle recovery areas for each case.

(Figure 8). For the later described HGMS device the longitudinal configuration is crucial. In this case the fluid flow *v* and the magnetic field *H* are parallel and perpendicular to the separation matrix. The induced magnetic pols are located at the side facing the fluid flow as well at its backside. Particle separation will mainly occur at the front side of the matrix. Furthermore, transversal and longitudinal orientations are possible. Flow behaviour, magnetic field orientation and particle recovery areas can be seen in Figure 8. To describe the simplified separation efficiency for a magnetic particle onto a single wire HGMS device the ratio between magnetic velocity (v_m) and the applied fluid velocity (v_0) can be applied. With the assumption that the Stockes equation is valid the magnetic velocity in the immediate vicinity of the wire can be considered as the maximum particle velocity caused by the magnetic field.

$$v_r = \frac{v_m}{v_0} = \frac{2\mu_0(\kappa_p - \kappa_f)M_w H b^2}{9\eta a v_0} \qquad 2.8$$

Where μ_0, M, H are described in 2.3.1, κ_p and κ_f are the susceptibility of the particle and the fluid. The fluid is also described by its viscosity η. Particle and wire size are given by its radius b and a. Executing a more detailed study on particle trajectories and capture in high gradient magnetic separation it can be derived, that for $v_r < 1$ a fuzzy breakthrough

behaviour can be expected and therefore an inefficient separation. In contrast, $\nu_r \gg 1$ results in sharp breakthrough and loading fronts [104,105].

The first HGMS devices were constructed in the early 1970's by Kolm for waste water treatment [106]. Since the first processes based on magnetic separation have been proposed for biotechnological applications a variety of designs have been published, however, none of them suitable for biopharmaceutical industry. The principle of applying HGMS processes for selective binding of bio-products is also known as high-gradient magnetic fishing (HGMF), emphasizing the target orientated separation (fishing) of MOI from crude process feeds [77].

2.4 Magnetic Separation Devices

Most magnetic separation devices in the field of biotechnology are small-scale and developed to solve a specific separation problem. Simple constructions using permanent magnets are mostly sufficient for small-scale applications. Using permanent magnets for these tasks has the advantage of low purchase price as well as no following cost due to power consumption and no need of a magnet cooling. Disadvantages are the fact that permanent magnets can't be switched off as well as the limited separation volume, particle size and flow in this case. These devices are used mainly in scales up to 50 mL and for simple batch separations in lab-scale. A large number of devices is available for different applications [3]. For more complex and larger separation task, HGMS devices are used. These devices consist typically of a container filled with a ferromagnetic matrix providing high field gradients in an external magnetic field and large surface areas for the separation of magnetic particles. Prerequisite for the processing of solid as well as magnetic particles containing feed streams is a loose patch of matrix elements allowing non-magnetic components to pass the chamber unhindered and preventing clogging up [3]. The matrix usually consists of a filamentary construction, steel wool, layers of rolled or flat wire meshes or plate stacks. The matrix designs are manifold and mainly customized to the specific separation task but have generally showed good separation performances with over 90 % of captured magnetic particles [107–109]. The selection of the matrix determines the separation and recovery performance of magnetic particles as well as the cleaning of the system. Thin wires create high field gradients as well as large surface areas but the mechanical stability and cleanability must also be considered [4,62,73]. As for small-scale applications the magnetic field can be provided by a construction of permanent magnets. For larger separation volumes electro magnets have been preferred. Besides the magnetic fields, the simple on-off characteristics of electro magnets is an important factor. In return the higher investment as well as running cost due to a cooling system of the magnet have to be accepted [98]. A number of requirements for the successful construction of a HGMS device have been published[103]. Setchell gives a more general description of factors that have to be considered. Matrix size, space and magnetic field have to be adapted to the flow velocity and viscosity of the system. Furthermore, automatization, energy consumption and scalability are crucial factors for a successful device development. Franzreb et al. [73] have elaborated on key performance figures that a HGMS device should deliver in more detail. Magnetic fields of at least 0.3 T and separation capacities of 100 kg magnetic particles / m^3

of chamber should be achieved. High particle separation efficiencies of over 99.9 % and resuspension efficiencies over 98 % are required for the successful application of a magnetic separation device. Moreover, parts directly linked to the separation task and also supporting equipment have to be considered. As an example, pumps shall provide the following features: capable to work with high contents of solids and easy to clean while still providing sufficient pump rates. A comprehensive overview of HGMS processes and HGMS devices is provided by Franzreb et al. [73]. All these devices have in common that they have never been intended to be used in industrial-scale processes of the biopharmaceutical industry. Due to strict regulation of the authorities for the production of pharmaceuticals, equipment must conform to a GMP-compliant process. None of the presented devices has met those requirements. The greatest potential for developments of a GMP-compliant device lies in the field of matrix optimization. Whereas good separation results have been achieved, the recovery of the particles and a cleaning friendly design have been paid less attention [110–112]. Wire meshes as well as steel wool matrix designs offer a multitude of places with low fluid velocities and hiding places for particles or contaminants. Besides the cleaning problems and the associated batch to batch contamination, particle losses are a critical economic factor due to high prices on the market [73,74,92]. Different approaches have been presented how to enhance the recovery and resuspension of magnetic particles. The simplest approach is a high flow rate and a circulation of the process fluid through the separation chamber. Further approaches are mechanical shaking of the separation chamber, which is certainly limited in consideration of an upscaling, and the combination of special surface coatings on the matrix with ultra-sonic devices. Such a combination showed promising results but is limited in upscaling due to the range of the ultra-sonic devices and the power input to the system [102,111,113,114]. To be able to provide a robust up-scale, the design of the matrix structure has been reconsidered by Franzreb et al.. A new approach has been presented with the ‘rotor-stator’ matrix concept [25]. The matrix consists of an alternating stack of densely perforated metal discs (Figure 9B). Every second disc is connected to an inner rotating shaft, whereas the other discs are mounted to the housing of the separation chamber. By rotating the inner shaft, shear forces can be introduced in the gap between the discs making the resuspension of particles and the cleaning of the system possible (Figure 9A). Several HGMS devices based on the ‘rotor-stator’ principle have been built in various setups and scales. A broad range of applications

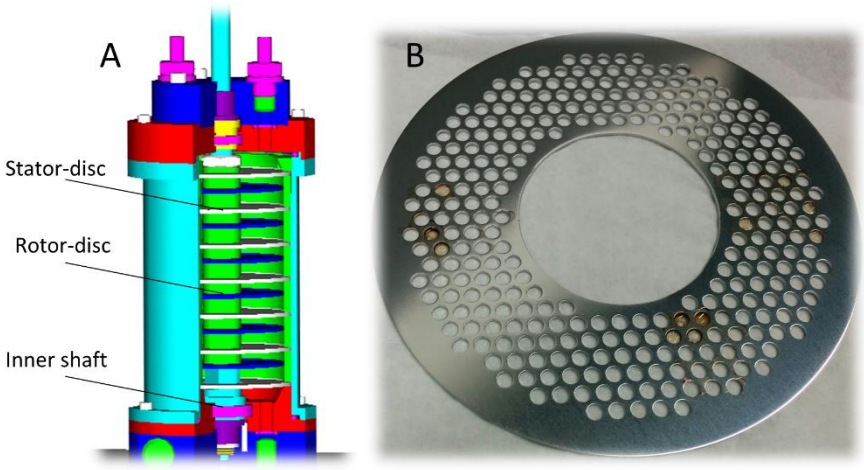


Figure 9: (A) Schematic example of the separation chamber of a 'rotor-stator' HGMS device. Stator matrix elements (yellow) are mounted to the housing of the chamber, rotor elements (blue) are connected to the inner rotation shaft. (B) Matrix element for the separation of the magnetic particels, densily perforated with holes to allow solid contaminants to pass unhindered through the chamber.

have proven the reliability of the system design in numerous purification processes and by different groups [72,77,79,95–97,115]. Simulations of the matrix design even led to an optimization of the whole structure of the matrix elements [116]. However, a GMP-compliant and suitable for technical-scale separation processes version of a 'rotor-stator' has not been developed until the collaboration of Andritz GmbH and KIT. The consortium launched the first GMP-compliant 'rotor-stator' HGMS device at the beginning of 2017. The characterization, validation, cleaning and process development for the separation device is the major part of this work and will be addressed in chapters three to five.

2.5 Magnetic Particles

The limited choice of HGMS devices on the market face a vast range of commercially available magnetic particles in addition to numerous publications presenting custom-made magnetic particles. The choice of the optimal magnetic particle is crucial for a successful process development. An overview of the commercially available particles has been presented by Franzreb et al. [28], Kudr et al [117] as well as Olsvik et al. [117] and Berensmeier [118]. To select the optimal magnetic particle for the individual task from these inexhaustible variety of magnetic particles Franzreb et al. [28] have presented a guideline with key figures. Therefore, magnetic particles should be superparamagnetic to be easily redispersed after separation. This requires that they contain iron oxide crystals smaller than 20 nm. They should be not smaller than 500 nm in order to be easily separated with a magnetization saturation of at least 35 Am^2/kg [73]. Beyond that the particles should be non-porous to prevent particle fouling if they get in contact with solutions containing crude solids. While the main advantage of porous particles is the high specific surface area available for functionalization. In this work only commercial available magnetic particles

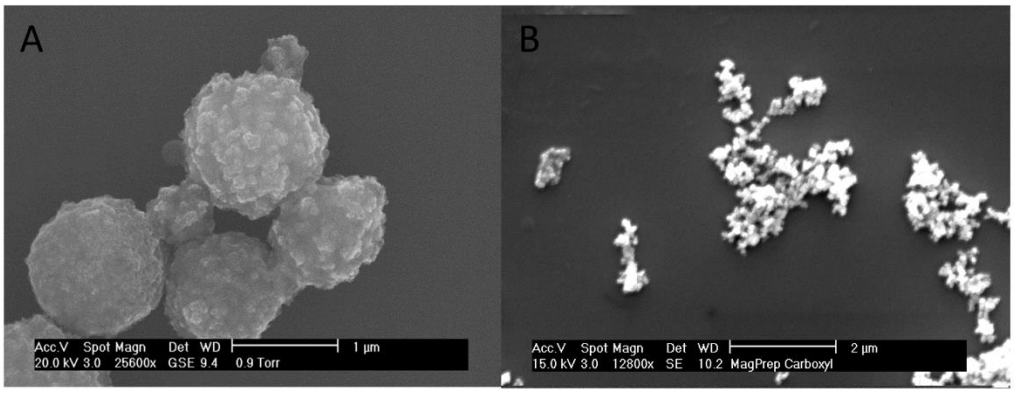


Figure 10: Scanning electron micrographs of (A) M-PVA chemagen magnetic particles, image taken in an enviamental scanning electron mocroscope mode in a magnification of 25600 and (B) Mag Prep magnetic particels image taken in a secondary electron microscope mode in a magnification of 12800

have been used whereas porous as well as non-porous particles have been applied. For the characterization of the new separation device polymer-coated magnetic particles (M-PVA magnetic particles, PerkinElmer chemagen Technologie GmbH, Baesweiler, Germany) as well as MagPrep Silica (Merck Milllipore, Darmstadt, Germany) were applied. MagPrep particles consist of monocrystalline magnetite with a thin silica coating. The mean diameter specified by the manufacturer is 100-200 nm. These magnetic particles show a saturation

magnetization of 77.3 Am^2/kg with a remanence of 25.6 Am^2/kg (Figure 11C). The high remanence indicates that a superparamagnetic behaviour is not given for these particles. Due to their 'magnetic memory' these particles will tend to agglomerate as well as stick to metal surfaces after a magnetic field is removed. Due to the silica coating of the particles they are mainly used for DNA or RNA purification without further functionalization. MagPrep particles were successfully applied in protein purification processes, for example with protein A ligands in antibody purification processes with up to 100 L scales [76]. Chemagen M-PVA magnetic particles consist of magnetite crystals encapsulated in a polymer matrix of cross-linked polyvinyl alcohol which makes them hydrophilic. Two sizes of M-PVA particles are available. Particles with a particle size range of 0.5-1 µm and such with a size range of 1-3 µm. The later one were used for the presented work. A magnetic saturation of 30 Am^2/kg with a remanence of 15 mAm^2/kg which showed that these particles are practically superparamagnetic (Figure 10A). Chemagen M-PVA particles are available with a wide variety of functional groups for further ligand attachments. M-PVA particles have been used with all kinds of functionalization for various purification tasks

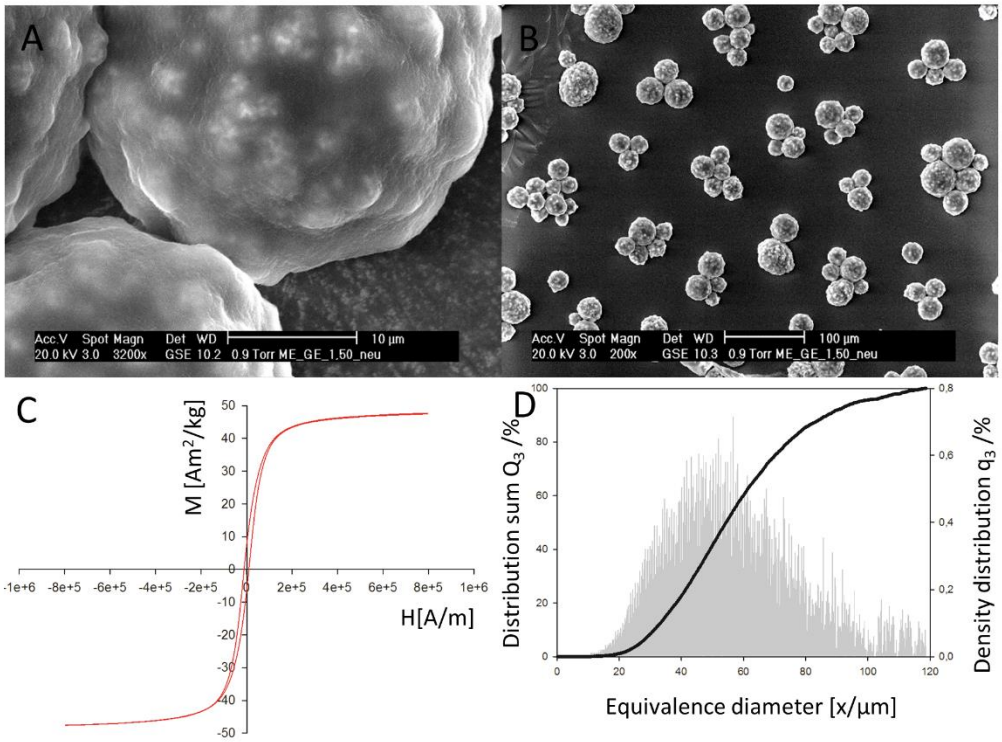


Figure 11: (A,B) Enviromental scannin electron micrographs of Mag Sepharose magnetic particels in magnifications of 3200 fold and 200 fold magnetization, respectively. (C) Hysterese loop of Mag Sepaharose showing a saturation magnetization of 47 Am^2/kg and a remanence of 6.6 Am^2/kg. (D) Distribution sum and density distribution of Mag Sepahrose.

[94,96,119,120]. Mag Sepharose magnetic particles (GE Healthcare, Uppsala, Sweden) are a representative of porous magnetic particles. They consist of a highly cross-linked agarose matrix with magnetite inclusions. The mean diameter was determined as 54 µm and they are the largest particles used for this work. They exhibit a para-magnetic behaviour with a saturation magnetization of 47 Am^2/kg and a remanence of 6.6 Am^2/kg (Figure 11C). Despite their size, the particles are still easy to suspend after separation. The porous structure of the Mag Sepharose typically prolongs binding times caused by diffusion processes and makes these particles more prone to fouling effects. Adequate washing protocols have to be developed in order to avoid capacity losses due to pore blocking. On the other hand, the porous structure results in a large specific surface, leading to high binding capacities.

2.6 Magnetic Protein Purification Process and Process Optimization

In the following section a typical protein purification process using an automated HGMS device is described. Magnetic separation processes in protein purification are determined by batch processes. In a first step the functionalised magnetic particles are mixed with the unclarified feedstock containing the MOI. The feedstock can either be from a natural source like whole blood or blood serum for instance or from a cell cultivation [121]. Typical cultivation feedstocks are CHO systems where the MOI typically will be expressed and has to be purified from the supernatant whereas in *E.coli* cultivations the product is typically intracellular and a cell lysis has to be performed before the purification [88,122]. The mixing of the magnetic particles and the feedstock is called binding step. It is performed in an external stirring vessel to ensure an adequate mixing and to process larger volumes than the separation chamber of the HGMS device is able to hold. The binding process is characterized by a batch adsorption process. During this process one equilibrium state is reached between protein adsorbed to the functionalised particles and the supernatant [109]. After the equilibrium is reached no further changes of the MOI concentration in the supernatant will occur. The binding can be described among others by the Langmuir adsorption model and fitted to the Langmuir isotherm equation $q^* = \frac{c^* * q_{max}}{k_D + c^*}$. The amount of MOI adsorbed onto the particles after the equilibrium is reached is described by q^*. q_{max} is the maximal loading of the particles and c^* the concentration of the MOI in the supernatant in the equilibrium. k_D is the equilibrium parameter and a direct measure for the stability of the binding. Smaller k_D values represent higher binding affinities. The duration of the binding step is determined by the kinetic of the binding process which is dependent on the ligand on the particle and the MOI as well as the diffusion processes. For the optimization of process times it is recommended to perform small-scale binding studies. Following the binding process, the particle-feedstock suspension is pumped through the separation device while a sufficient magnetic field is supplied for the magnetization of the separation matrix. The magnetic particles will be separated at the matrix while the feedstock is displaced by a washing solution. To ensure adequate washing results, which means to reduce the level of contaminants such as cells, cell debris as well as HCP, DNA or loosely bound MOI, the magnetic field is removed. Moreover, an optimal mixing of the magnetic

particles and the wash solution is required. After every wash step the magnetic particles are recaptured at the matrix before replacing the fluid phase. Generally, several washing steps are required to reduce the level of impurities by several orders of magnitude, since this process is a dilution wash process. Appling a wash solution with low buffer capacities during the last washing step can support the following elution. To desorb the MOI from the magnetic particles the reversible bound between the molecule and the ligand has to be dissolved by a change in the condition of the fluid phase. The elution process step is executed in the same way then the washing steps. Several elution steps might be necessary to elute all MOI from the particles. The smallest possible elution volume and therefore the highest concentration of the MOI is limited by the size of the separation chamber. After elution the magnetic particles have to be cleaned from remaining product as well as hard to remove contaminants. Typically sodium hydroxide solutions or acids with pH values around two are applied [48,123]. Finally, the particles are suspended in a solution for storage of the particles and then recovered from the HGMS device for further use. The overall process time is mainly determined by the times for fluid exchanges and therefore by the pump speed. The pump speed is only limited by the separation performance. However, magnetic separation processes are by far faster than column based processes, due to fast binding kinetics. Together with the high pump speeds applicable, they are especially attractive for multicycle arrangements in process scale-up [28]. A comparison of the productivity of a column based process with a magnetic separation process can be found in section 5.6.7.

2.6.1 Batch Adsorption Process and its Evaluation

As described earlier, the magnetic separation purification process is based on batch adsorption processes. The selection of magnetic particles and the functionalization of the particles take a key position in the process. The estimation of yield and productivity of the process, under selected boundary conditions, can serve as a tool for the selection of suitable magnetic particles, type of functionalization, as well as process conditions. The binding process can be described by a mass balance (equation 2.9) and the Langmuir adsorption isotherm mentioned above.

$$c_0 * V_{batch} - c^* * V_{batch} = q^* * m_p \qquad 2.9$$

Whereas c_0, c^* and q^* are known from the Langmuir model, V_{batch} describes the volume of the batch process and m_p the amount of magnetic particles which is dependent on the specific

filter capacity σ and the separation chamber volume V_{sep}. The volume of the batch can also be described in dependence of the capacity ratio CR.

$$V_{batch} = \frac{\sigma * V_{sep} * q_{max}}{CR * c_0} \qquad 2.10$$

CR represents the ratio between the theoretical maximum amount of target molecules which can be bound by the used mass of magnetic particles and the amount of target molecules provided in the actual batch volume. Either the batch volume determines the amount of magnetic particles that has to be used, or more likely, the amount of magnetic particles that can be separated and the CR required for the aimed yield determine the operable batch volume. With the expression for the mass balance, the isotherm and CR a dimensionless expression for $\frac{c^*}{c_0}$ can be found which results in a yield estimation.

$$\frac{c^*}{c_0} = \frac{1}{2} * \left[1 - \frac{k_D}{c_0} - CR + \sqrt{4 * \frac{k_D}{c_0} + \left(CR - 1 + \frac{k_D}{c_0}\right)^2}\right] \qquad 2.11$$

and

$$Y = 1 - \frac{c^*}{c_0} \qquad 2.12$$

As described by Franzreb et al. [124] two terms influence the yield. With increasing CR the yield will increase. To reach yields higher than 90 % it can be necessary to use larger amounts of particles than expected if the simple adoption CR = 1 is assumed. The ratio $\frac{k_D}{c_0}$ between the Langmuir parameter k_D and the initial MOI concentration in the batch c_0 describes in which section of the isotherm the binding will take place. If $c_0 >> k_D$ ($\frac{k_D}{c_0} << 1$) the loading of the magnetic adsorbents approaches q_{max} and the required capacity ratio is close to its optimal value of CR = 1, if $c_0 < k_D$ ($\frac{k_D}{c_0} > 1$) the loading of the magnetic adsorbents is less than half of q_{max} and capacity ratios CR > 2 will be required to reach acceptable yields. High values of $\frac{k_D}{c_0}$ are either caused by low concentrations of the MOI in the feedstock or a low binding affinity of the ligand and the MOI, which should lead to a review of the choice of ligand.

Besides the yield the productivity P of the process is crucial.

$$P = \frac{m_{MOI}}{t_{cycle} * V_{sep}} \qquad 2.23$$

The productivity of a process is described by the amount of MOI (m_{MOI}) produced in one cycle in relation to the time needed for the process t_{cycle} and the volume of the device used. The time t_{cycle} of the process includes the loading time as well as the operation time of the separator for the purification steps. The operation time consist of the times for washing, elution, cleaning of the particles, equilibration and recovery. While the loading time of the magnetic particle suspension into the separator is linearly dependent on its initial batch volume, the time needed for the remaining process steps is constant [125]. Furthermore, the process times are limited either by the maximum pump speed or the separation behaviour of the magnetic particles. Small particles or high viscous solutions could demand lower pump speeds to allow the magnetic particles to separate at the matrix of the device. The following case study shall illustrate the influence of the choice of system parameters. First of all, optimal process performance results are expected if the separation device is used close to the maximum filter capacity, which is defined by the 1 % breakthrough point. To reach optimal purities along with process yield and productivity optimal washing and elution conditions have to be maintained. It was shown that a maximum loading of 80 % of the maximal filter capacity still provides good washing results. The maximum loading strongly depends on the properties of the used magnetic particles (section 4.2). The first two variables of the process simulation are therefore fixed by the choice of magnetic particles (m_p) and the scale of the separation device (V_{sep}). For the case study process data from a mAb purification using Mag Sepharose functionalized with a protein A ligand and system data from the later presented GMP-compliant HGMS device, MES 100 RS is applied (Table 2). The optimum process parameters are then determined by computing productivity, purity and yield for varying *CR*. The value of *CR* where the product of productivity and yield reaches is maximum value is the optimal process *CR*. The choice of the described product is based on the here presented case study. Due to the high affinity of the protein A affinity ligand-antibody binding complex, competing binding and therefore low purities do not appear to be significant. This is a derivation from the process evaluation described by Franzreb at al. [124] which is described above.

Table 2: Process and system data for the case study that is kept constand in all cases.

	value	abbreviation
Volume of magnetic particles [ml]	480	m_p
MOI start concentration [g/L]	2.3	c_0
Maximum binding on particles [g/mL]	0.087	q_{max}
Volume of the separator [L]	1	V_{sep}
Process time without loading [min]	40	t_{cycle}-t_{load}
Pump speed [L/min]	2.7	

Three cases are presented whereby the values from table 2 were kept constant and the k_D value was varied from 0.01 g/L to 1 g/L causing different values for an optimal process CR and differing process yields. The third case elucidated the importance of $\frac{k_D}{c_0}$. The k_D value of 1 g/L was applied while demanding a process yield of at least 90 %. This leads to an inevitable adjustment of CR. As described the separation time of the process scales linear with the batch volume. The remaining process time including the time for load, wash, elution and recovery operation steps is assumed to be 40 min. The fixed value for k_D in case 1 (0.01 g/L) leads to a $\frac{k_D}{c_0}$ of 0.00435. The optimal process *CR* for the highest value of *P***Y* is 1.1 leading to a V_{batch} of 16.5 L and a purified amount of MOI of 36.7 g representing a yield of 96.8 % with an overall process productivity of 0.8 g/min*L (Table 3, Figure 12. For low k_D values which corresponds with high affinities between der functionalisation on the magnetic particles and the MOI, *CR* values close to 1 are expected. For higher k_D values such as k_D = 1 and $\frac{k_D}{c_0}$ = 0.434 as presented in case 2 the ratio between magnetic particles and start batch has to be adapted in order to reach acceptable yields. As optimal *CR* 2.2 was determined which leads to a V_{batch} of 8.25 L. However, yield will be rather low with 76.7 % (Table 3, Figure 13). To achieve yields over 90 % the *CR* has to be adjusted to five. Consequently, V_{batch} drops to 3.6 L and the overall process productivity will be just 0.18 g/min*L (Table 3, Figure 13). This illustrates the strong dependency of the batch purification process on the optimal choice of the magnetic particles and their functionalization.

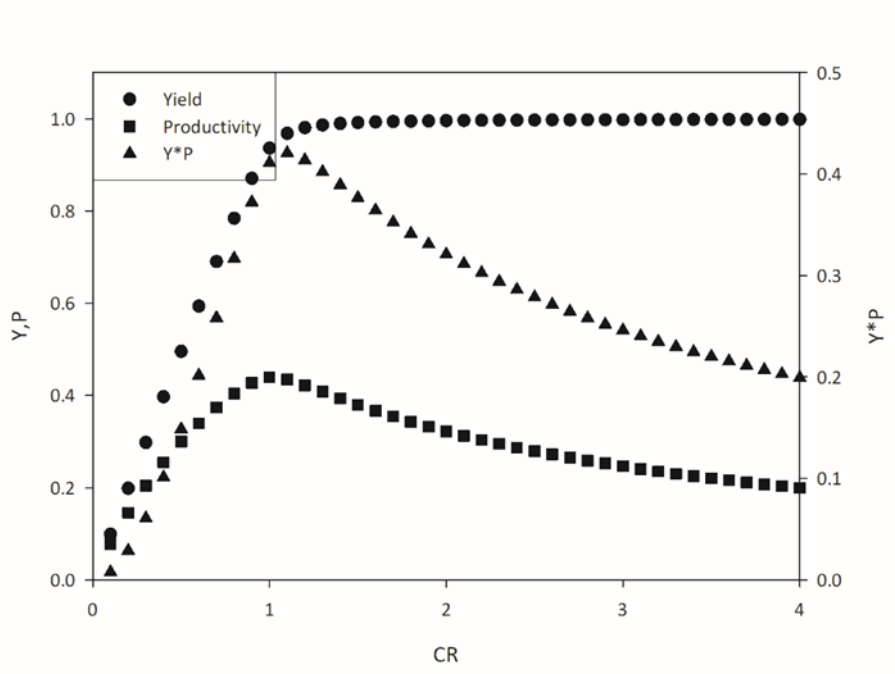


Figure 12: Yield and productivity and well as the product of yield and productivity as function of the CR for the first case (k_D=0.01 g/L). The peak value for the product of yield and productivity indicates the optimum process CR

Table 3: Process and system data for case 1: k_D of 0.01 g/L is assumed leading to the parameters presented below for an optimal CR of 1.1.; case 2: k_D of 1 g/L is assumed leading to the parameters presented below for an optimal CR of 2.2.; case 3: k_D of 1 g/L is assumed leading to the parameters presented below for a CR of 5 due to the demanded yield of over 90 %.

	case 1	case 2	case 3	abbreviation
Capacity Ratio	1.1	2.2	5	CR
Equilibrium parameter [g/L]	0.01	1	1	k_D
Equilibriums concentration [g/L]	0.073	0.535	0.22	c*
Volume of the processed batch [L]	16.50	8.25	3.63	V_{batch}
Protein produced per cycle [g]	36.75	14.56	7.55	m_{prot}
Total cycle time [min]	46.11	43.06	41.34	t_{cycle}
Productivity [g/(min*L)]	0.797	0.34	0.18	P
Yield [%]	96.80	76.7	90.4	Y

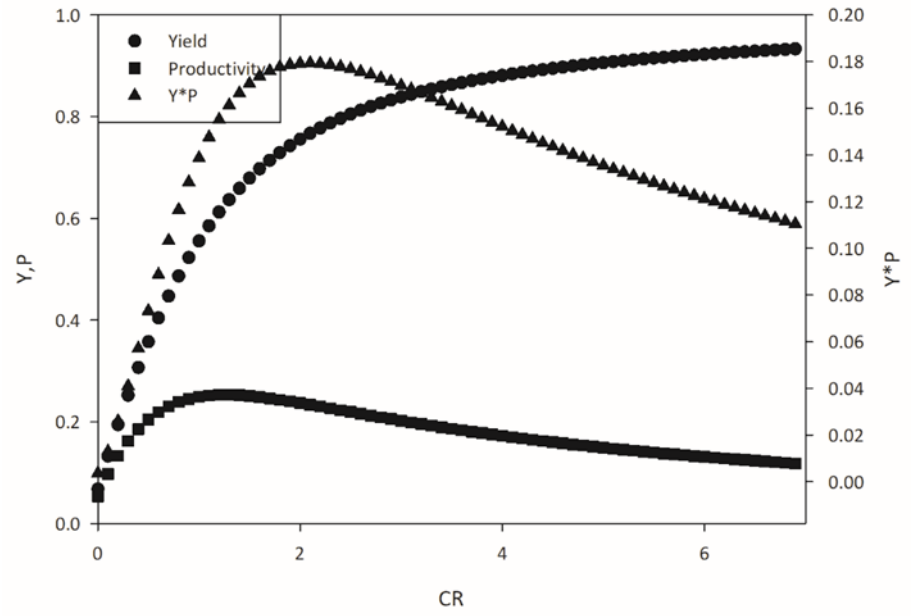


Figure 13: Yield and productivity and well as the product of yield and productivity in as function of the CR for the second case (k_D=1 g/L). The peak value for the product of yield and productivity indicates the optimum process CR of 2.2 with a low process yield. Due to the expected yield >90 % the process has to be adapted. A CR of 5 achieves the expected yield at the expense of a lower productivity

2.6.2 Process Optimization by a multi-stage counter-current Process

To take advantage of the fast binding kinetics and the short process times of the magnetic separation purification process, a multi-stage counter-current arrangement can be applied. As described one equilibrium stage is reached between the MOI adsorbed to the particles and MOI in the supernatant during the batch adsorption processes. The amount of MOI bound on the particles is dependent on the number of binding sides as well as on the concentration of MOI in the supernatant. Due to the ad- and desorption processes some part of the MOI will always remain in the supernatant. In order to bind the remaining MOI from the feedstock and raise the yield of the process further binding steps are required. In a batch adsorption processes this can be achieved using several reaction chambers R connected in a counter-current way [52]. Figure 14 illustrates a two-stage binding process schematically. Adsorbing particles and feedstock containing the MOI are moved in a counter-current flow,

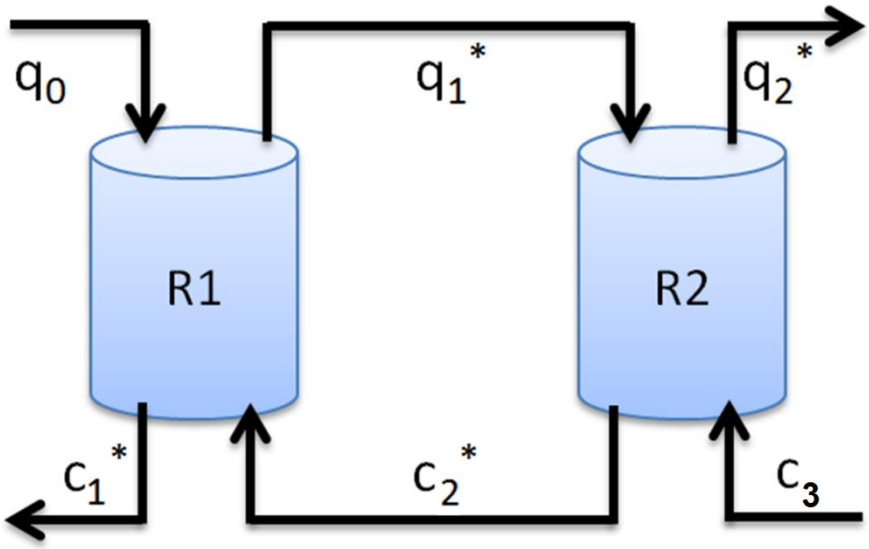


Figure 14: Scematic demonstration of a counter-current process to raise the yield of a batch adsorption process by increasing the number of binding steps

whereas both are contacted twice with each other. In reaction chamber R_1 the fresh and unloaded particles q_0 are in contacted with previously used process liquor and the unbound MOI from reaction chamber R_2. The fresh particles are able to bind a further part of the MOI from the supernatant and an equilibrium is reached. Any unbound MOI is leaving the reaction chamber R_1 with a concentration c_1^*. The preloaded particles are transferred from chamber R_1 to chamber R_2 with a loading q_1. They are incubated with fresh process liquor with a MOI concentration c_3. Do to the higher concentration of MOI a further binding on the particles takes place and second equilibrium concentration is reached in the reaction

chamber R_2. The concentration of the MOI will be c_2^* after the equilibrium is reached which is the initial concentration for R_1. The twice loaded particles are eluted in the following and can be reused for the next process. Every reaction chamber R_i is described by its mass balance and an adsorption isotherm.

$$m * q_{i-1} + L * c_{i+1} = m * q_i + L * c_i \qquad 2.34$$

$$q_i = \frac{q_{max} * c_i}{k_D + c_i} \qquad 2.45$$

In these equations index *i* describes an arbitrary reaction chamber. The isotherm equation describes the loading q of the particles with the mass m of the MOI in dependency of the concentration c of the MOI in the volume L.

As a case study the yield for the binding of equine chorionic gonadotropin (eCG) to Mag Sepharose functionalized with eCG-affinity ligands was numerically solved. A start concentration of the solution of 20 IU/mL was assumed. Furthermore, calculations used an initial particle loading of 0 IU/mL, a k_D of 1 IU/mL and a maximum binding of 12 IU/mL. As expected, the yield of the process increase with higher particle concentrations and the number of process steps (Table 4). It becomes clear that high yields in a single binding step are only possible if a very high concentration of magnetic particles is used, corresponding to low productivities and purities. Practically, at least two binding steps are preferred, which facilitate already yields over 98 % with a particle concentration of 2 g/L. Any further increase in binding steps or particle concentration enables only a small increase in yield and is therefore economically not worth considering.

Table 4: Expected process yield in percent in dependence of the number of cross-flow steps performed and particle concentration

Particle concentration [g/L]	1 step	2 steps	3 steps	4 steps
0.5	28.05	28.57	28.57	28.57
1	54.1	57.05	57.14	57.15
1.5	75	84.87	85.68	85.71
2	86.88	98.86	99.95	100
2.5	92.06	99.66	99.99	100
3	94.47	99.83	99.99	100
3.5	95.81	99.89	99.99	100
4	96.64	99.92	100	100

2.7 GMP in Process Equipment Development

Good Manufacturing Practise along with a 'Quality Risk Management' is part of a 'Pharmaceutical Quality System' which is meant to achieve quality and reliability in medical manufacturing processes. The Quality System includes all aspects influencing the product and is design to avoid any risk for the customer due to inadequate safety, quality or efficacy. Resulting from this, GMP or cGMP as it is called by the FDA, is just a small part of the overall quality system concerning the investigation and manufacturing of pilot-products, the technology transfers until the manufacturing of market relevant products as well as the quality control. The aim is the evaluation of the manufacturing and the protection of product and patient [126,127]. Eleven key components are defined by the European Commission for Health and Consumers Directorate-General as basic requirements:

1. All manufacturing processes are clearly defined, systematically reviewed in the light of experience and shown to be capable of consistently manufacturing medicinal products of the required quality and complying with their specifications;
2. Critical steps of manufacturing processes and significant changes to the process are validated;
3. All necessary facilities for GMP are provided including: Appropriately qualified and trained personnel; Adequate premises and space; Suitable equipment and services; Correct materials, containers and labels; Approved procedures and instructions, in accordance with the Pharmaceutical Quality System; Suitable storage and transport;
4. Instructions and procedures are written in an instructional form in clear and unambiguous language, specifically applicable to the facilities provided;
5. Procedures are carried out correctly and operators are trained to do so;
6. Records are made, manually and/or by recording instruments, during manufacture which demonstrate that all the steps required by the defined procedures and instructions were in fact taken and that the quantity and quality of the product was as expected.
7. Any significant deviations are fully recorded, investigated with the objective of determining the root cause and appropriate corrective and preventive action implemented;
8. Records of manufacture including distribution which enable the complete history of a batch to be traced are retained in a comprehensible and accessible form;

9. The distribution of the products minimises any risk to their quality and takes account of Good Distribution Practice;
10. A system is available to recall any batch of product, from sale or supply;
11. Complaints about products are examined, the causes of quality defects investigated and appropriate measures taken in respect of the defective products and to prevent reoccurrence [128].

This compilation illustrates that GMP guidelines covers significantly more than the adequate design of the process equipment. Only point three mentioned the importance of 'suitable equipment' without further explanations. Therefore, more specific guidelines are published by the European Commission dealing specifically with the premises, equipment and the manufacturing of biological active substances as well as medicinal products for human use [129,130]. These guidelines provide lists of requirements for the design and the process development under GMP guidelines. Besides that, some examples for the practical implementation of these requirements are given. Suggested measures are the use of cleaning and sterilisation in place systems (CIP, SIP), an effective design of the draining system, the use of single-use components and closed single purpose systems to avoid cross-contaminations. Superordinate part of all GMP development and production processes is a comprehensive and validated documentation system [131]. A good overview of the complexity of the quality assurance in biopharmaceutical production and the components of GMP in the EU as well as the USA are given by Müller et al. [126].

Based on the developed GMP-compliant HGMS device the following section gives an introduction on equipment requirements which are mandatory for pharmaceutical DSPs. As described above, GMP guidelines cover the entire process of development and production including also the equipment design as a small part but the guidelines give not direct design criteria. Guidelines for the design of GMP-compliant process equipment can be found for example compiled specially for bioprocess equipment by the American Society of Mechanical Engineering (ASME), by the 3-A Sanitary Standards, Inc or the European Hygienic Engineering and Design Group (EHEDG). The last two institutions mainly deal with hygienic standards for the food industry. All these institutions have in common, that the provided reference books are costly.

The overall development and construction of a new device for biopharmaceutical production is determined by the striving for optimal cleaning and sterilization results. All decisions on materials, construction or design elements are subordinated to the

cleanability. The specifications and suggestions supported by the ASME [132] and the EHEDG [133] have been taken into account in the design of the here used new magnetic separator as follows. As the overall goal was to design a clean device with a minimum of bioburden a CIP system has been installed. This requires that all surfaces, whether they are in contact with the product or not has to be cleanable. Surfaces which come in contact with product have to be for example free of any kind of imperfections, such as cracks. Furthermore, they have to be temperature, pressure as well as corrosion stable. It is crucial to ensure that all surfaces are resistant to chemical cleaning agents without any sign of corrosion and can withstand temperatures of 130 °C during the sterilization process. Therefore, preferred materials are 316 steel, stainless steel or higher alloy due to their homogenous, inert, non-absorbing, non-toxic and insoluble properties. The separation matrix of the new device as well as the head and the bottom part of the separation chamber is constructed from stainless steel taking further design criteria into account. Rank among these are the avoidance of horizontal areas, connection angels of 135 ° or less as well as radii of at least 3.2 mm to avoid low flow velocity areas, dead spaces to assure optimal draining and cleaning properties. The surface finish of at least 0.8 µm Ra and electro polished finish have been realized. O-ring sealing should be avoided in contact with the product to minimize dead spaces. Therefore, custom made PEEK sealing elements were constructed for sealing and spacing of the matrix elements. Polymers with product contact have to meet the same criteria as metal surfaces. Beside adsorption of product components and temperature resistance, leaching properties are of particular interest. When Polymers and steel are used together different expansion coefficients during the sterilization process has to be considered. In the here used separation device, this has been solved by a spring loading of the separation chamber with a pre-set tension to ensure tightness of the system at all temperatures. Further parts of the separator which are in contact with the product are for example the pump and the valve blocks. A hose pump is used which is a simple solution to avoid the contact of moving parts or seals with the product. The required tubing is a single-use product and therefore this solution is in accordance with GMP. The design of the valve blocks is also driven by the drainability and the avoidance of dead spaces. For this reason, diaphragm valves in a block construction are preferred. The separation device has two such valve blocks. One below and one on top of the separation chamber. All electrically powered components of a device have to be sufficiently sealed to prevent short circuits and in case of motors, leakage of lubricants. This was ensured by the spatial separation of production and system control area within the housing of the separation device. In addition,

also the housing of the device needs to have an easy-to-clean design, including a appropriate surface finish and corrosion-resistant material. Finally, production devices are design to be applied in a clean room. Due to the cost of clean rooms the footprint of the device should be as small as possible and if possible production relevant parts should be separated from the control unit to save space and shift the control unit outside of the clean room. This has not been realized with the MES 100 RS yet. However, the housing of the system consists of two parts, the control cabinet and the production side. Due to this, a separation seems to be easy to realize. In summary, it becomes clear that all parts of the design and construction are guided by the optimization of the cleaning of the system, which is the basis for the successful development of a cleaning protocol and the subsequent validation of the cleaning process.

2.8 Equipment Cleaning in Biopharmaceutical Production

The development of cleaning strategies has to start with the idea of a new production device. The equipment design has to combine the application or operation with the simplest possible cleanability and maintainability [134]. Regulatory bodies, like the FDA, expect cleaning procedures to be established and validated since 1963. Since 1978 a cleaning procedure is also mandatory according to GMP guidelines [135]. In general, the development of a cleaning strategy consists of two main parts. The development and validation of the cleaning procedure itself and the development and validation of a suitable analytics [136]. The main task is to secure costumer safety by ensuring the product quality. The key component of a successful cleaning process development and validation is the documentation of the process. Written standard operation procedures are mandatory. They have to include all information concerning the use of the process equipment, all process fluids, responsibilities, cleaning and process methods and of cause cleaning criteria as well as acceptance criteria. Key questions like: What, Who, When, How, Where have to be addressed [134,137,138]. The documentation is needed to ensure the efficiency, effectiveness as well as consistency and reproducibility of the cleaning process. Cross-contaminations of product batches as well as microbiological contamination but also contaminations of residual cleaning agents have to be avoided [139,140]. For the successful implementation of a cleaning process a comprehensive process understanding as well as the definition of the objective is essential. The nature of the impurities in combination with surfaces, cleaning agents and toxicity analysis make every process unique and therefore cleaning processes have to be adapted to every new task after performing a risk assessment [127].

In general, the cleaning process can be carried out in three ways. A manual cleaning of the equipment includes normally a disassembling followed by a manual scrubbing of all parts with the cleaning agent. This process is susceptible to deviations due to human labour. In addition, the contact with cleaning agents includes risks for the health and safety of the workers. The second option is a disassembling and automated cleaning of the device. This is supposed to deliver consistent cleaning results but is still dependent on human labour as well as time for the disassembling process. These methods can be summarized under the term of ‘cleaning out of place’ (COP). Preferred by the regulatory body is the third option. ‘Cleaning in place’ (CIP) implies the automated cleaning of the equipment without any

constructional changes. This process variant eliminates human error and improves safety for the workers. CIP processes deliver consistent cleaning results and usually reduce cleaning times and therefore process downtimes and cost [27,141].

2.8.1 Cleaning in Place (CIP) Process

The cleaning process can be described as the physical removal of soilds, organic debris and particulates from the surface of the production device. Sanitization, which is not addressed here, would be the removal or elimination of vegetative bacteria cells [142]. As mentioned, most important for the successful development of a cleaning protocol is the knowledge about the kind of contaminants and their properties as well as the interactions to the surface of the device. The parameters related to the removal of impurities in a CIP process are described by Holst [143] as TACT, time, action, concentration and temperature of the process. The fluid flow rates and regime in the device or tubing can be taken as action. Turbulent flows, high fluid velocities and a total coverage of the surface are needed for a successful surface cleaning. The flow rates and coverage can be achieved in large equipment parts with spray balls if the device is not flooded completely. For common a combination of alkaline, acid and neutral cleaner with oxidizing or tensidic surfactants are used [140,141]. Based on this a cleaning protocol consist of three essential steps. A prerinse with water for injection (WFI) to remove free material, such as solids from the system. The prerinse is followed typically by an alkaline wash. Sodium hydroxide is used in concentrations between 0.1 and 1 % with a temperature of 70-80 °C and contact times of 10 to 30 min. Alkaline washes are used to remove organic materials such as proteins, fats, or fatty acids. These cleaners can be boosted by the use of surfactants for better surface coverage and to increase the solubility of organic material. A further option is the addition of sodium hypochlorite as an oxidizer to increase the cleaning performance. The cleaning process is finalized by a WFI wash to remove all cleaning agents from the system. In some cases, it may be necessary to add an acid wash with e.g. nitric acid to the process. Acid cleaner are used to remove inorganic impurities such as iron oxide films ore milk stone [27,141]. The composition of the cleaning agents is not only dependent on the impurities. In most cases the concentration and the chemical configuration is determined by the surface materials of the device. High acid or salt concentrations might lead to corrosion of metal surface; oxidizer might influence the lifetime of polymer sealing. In addition, analytical techniques and sampling methods

have to be established and validated to prove the efficiency of the cleaning process and detect the absence of all cleaning agents after the cleaning process.

2.8.2 Validation of a Cleaning Process

The validation of the cleaning process is the documented proof of the efficiency of the developed process. This includes the validation of the cleaning process as well as the validation of the analytics used during the process. Like for the cleaning process knowledge about the process and the impurities is crucial during the validation. Analytical techniques can be divided into direct and non-specific techniques. Direct techniques allow a direct quantification of a specific contaminant but are time consuming in the development and execution as well as limited in information value if mixtures of contaminants are present. Typical examples of direct techniques are high performance liquid chromatography, protein assays, specific ELISA or DNA assays [136]. Non-specific techniques, such as the determination of the total organic carbon (TOC), offer low detection limits in the range of ppb and a 'worst case' scenario. All organic carbon will be detected from all sources such like cleaning agents, process contaminants or sampling aids and therefore deliver the highest possible contamination value [27,144]. Also fast and easy implemented methods like UV, pH or conductivity measurements provide information on the efficiency of the final water wash for example [137].

The samples to be analyzed can be taken as rinse sample or direct surface swab sample. For the rinse sample the device is flooded after the cleaning process with an adequate solvent in order to solve all remaining impurities. Drawbacks are the low expected concentrations and the problem finding a solvent which is capable to solve the remaining impurities after the cleaning process. This solvent might be of a better use during the cleaning process. The sampling technique preferred by the regulatory bodies for the validation of a cleaning process is the direct surface swabbing. The dried surface is wiped off with a moist swab to physically remove remaining impurities and dissolve them for subsequent TOC analysis. Each sampling procedure must be validated per se. Therefore, the expected contaminants have to show a linear correlation in the TOC measurements over a broad range of concentrations. Furthermore, the dissolvability is to be determined from the swab. Values above 80% recovery should be aimed for [140]. Finally, the recovery of the impurities from the surfaces has to be a linearly correlated to the TOC values. Sampling technology in particular plays an important role and must always be carried out in the same way [145].

After a successful validation of the cleaning process critical to clean areas of the process equipment have to be determined for later sampling. This can be done by a standard riboflavin test. The riboflavin test is a fluorescence test where the device is totally wetted with a riboflavin solution and cleaned afterwards. Critical areas can be determined due to the visible fluorescence of riboflavin if stimulated at a wavelength of 365 nm [146]. For the evaluation of the cleaning process, acceptance criteria for the maximum allowable carry-over (MACO) of an active ingredient (AI) into the next process batch must be defined by a risk analysis. Early in a development process, a general limit like one half of the lethal dose for an animal (LD_{50}) or the 10 ppm criteria can be used. The 10 ppm criteria means, that 10 ppm of an AI is permitted in the smallest possible subsequent batch. A more precise criterion would be 0.001 of the minimum daily dose that may be found in the next product. This, however, requires advanced knowledge of the product and can be applied in later development stages [138,139].

Only the combination of GMP-compliant design, development of adequate cleaning processes and a comprehensive documentation and validation of the cleaning process leads to a successful process development which is essential for modern biopharmaceutical production processes and especially necessary if the process equipment is challenged with crude cultivation feedstocks as the developed magnetic separation device in this work.

3 Overview of Publications

This section provides an overview on the publications this thesis is based on. The publications are ordered chronologically as well as logically. The main topics of commissioning, characterization and validation of the first GMP-compliant HGMS device, the prove of applicability by implementation of an integrated mAb purification strategy and the optimization of a batch based process as well as the development and validation of cleaning routines for the device are addressed.

The manuscript '*Magnetic Separation on a New Level: Characterization and Performance Prediction of a cGMP Compliant 'Rotor-Stator' High-Gradient Magnetic Separator*' introduces the design of the GMP-compliant high-gradient magnetic separator 'MES 100 RS'. Typical parameters characterizing the performance of the separator have been investigated, applying two commercially available types of magnetic particles. Beyond this, process prediction calculations were performed to demonstrate the potential of the device in down-stream protein purification processes.

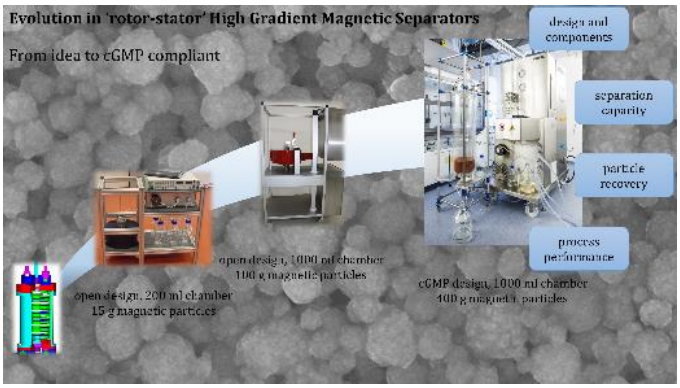
The second manuscript, '*One-step Integrated Clarification and Purification of a Monoclonal Antibody Using Protein A Mag Sepharose Beads and a cGMP-compliant High-gradient Magnetic Separator*', focuses on the process implementation of the separation device. A mAb purification process was chosen in order to compare the magnetic separation process with widely spread and fully optimized platform processes used in biopharmaceutical industry.

In the third manuscript, *'First Comprehensive View on a Magnetic Separation based Protein Purification Processes: from Process development to Cleaning Validation of a GMP-ready Magnetic Separator'*, the previous disadvantages of magnetic separation processes, which prevented the industrial use, were addressed. Besides the optimization of batch adsorption processes as used in magnetic separation, the cleaning of the MES 100 RS was addressed. The development of cleaning strategies and the validation of the cleaning process are key elements of this manuscript and prerequisite for the manufacturing of biopharmaceuticals.

Magnetic Separation on a New Level: Characterization and Performance Prediction of a cGMP Compliant 'Rotor-Stator' High-Gradient Magnetic Separator

Moritz Ebeler, Florian Pilgram, Kai Wolz, Gunnar Grim, Matthias Franzreb

Biotechnology Journal DOI 10.1002/biot.201700448

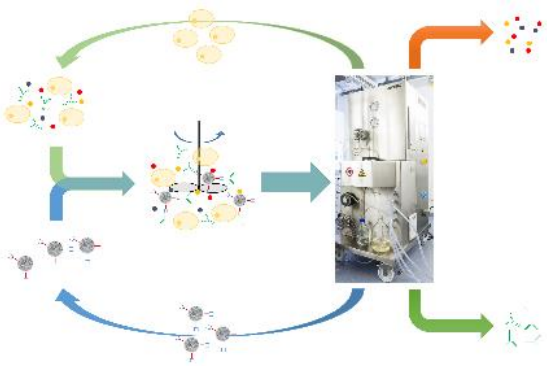


This work presents the first GMP-compliant high-gradient magnetic separator for the use in biopharmaceutical protein purification processes. The separator is based on the 'rotor-stator' matrix design, which is well described and used for various applications since the invention of the design almost ten years ago by Franzreb et al.. In order to meet GMP guidelines the design of the separator was completely reengineered. The main innovation is the sealing concept of the separation chamber. This allows working in a closed chamber which is in combination with the surface finish and the draining concept a prerequisite for the successful cleaning of the device. For the characterization of the device, specific separation performance data of two commercially available types of magnetic particles is introduced. Separation capacities of up to 400 g magnetic particles per litre volume of the separation chamber could be achieved. Beyond that, a full recovery and only negligible losses during ten resuspension and capturing cycles could be determined. Based on the characterization data a prediction of protein purification process performance is presented.

One-step Integrated Clarification and Purification of a Monoclonal Antibody Using Protein A Mag Sepharose Beads and a cGMP-compliant High-gradient Magnetic Separator

Moritz Ebeler, Ola Lind, Nils Norrman, Ronnie Palmgren, Matthias Franzreb

New Biotechnology DOI 10.1016/j.nbt.2018.02.007



Based on the development of the previously described new GMP-compliant magnetic separator a mAb purification process has been developed and conducted with this device. The process combines the typical process operations of solid-liquid separation and purification of the mAb in one unit operation and serves as alternative for the widely used platform processes for antibody purification. Protein A functionalized Mag Sepharose particles were used to selectively bind a mAb directly from the cell culture. Five consecutive process cycles have been performed showing stable yields over 85 % with purities over 95 %. Furthermore, an HCP reduction of 2.5 log-scales has been determined and stable cell viability throughout the entire process was given. A comparison with the commonly used column based process revealed the clear productivity advantages of the magnetic separation process due to short process times resulting from fast mAb binding kinetics and high pump rates.

First Comprehensive View on a Magnetic Separation based Protein Purification Processes: from Process development to Cleaning Validation of a GMP-ready Magnetic Separator

Moritz Ebeler, Florian Pilgram, Thomas Wellhöfer, Katrin Frankenfeld, Matthias Franzreb

Based on the successful process implementation of a mAb purification process, the field of applications of the novel GMP-compliant magnetic separator has been expanded. A magnetic separation based purification process of eCG from horse serum has been developed and optimized in small-scale and transferred to technical-scale. The yield around 80 % of the conventional magnetic separation bind and elute process could be increased by the introduction of a counter-currant process variant to nearly 100 %. The increase in yield has been achieved without changes in particle concentration or process batch sizes.

Furthermore, the cleaning in place of the separator has been shown for two model contaminants. Beside the development of cleaning routines, the set-up and validation of the accompanying analytic was of particular importance.

This publication presents for the first time a complete picture of a GMP-ready magnetic separation process in biopharmaceutical protein purification concerning the process device, process development and optimization as well as the cleaning and cleaning validation of the process equipment.

4 Magnetic Separation on a New Level: Characterization and Performance Prediction of a cGMP Compliant 'Rotor-Stator' High-Gradient Magnetic Separator

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4.1 Abstract

The growing market of biopharmaceuticals and the constant developments in upstream fermentation have generated a strong demand for new downstream purification methods. Magnetic separation in combination with functional magnetic particles has been known for many years as a promising candidate for a direct capturing tool in protein purification but the lack of suitable GMP-compliant purification equipment has prevented the launch of this technology in large scale bioprocessing. To tackle this bottle-neck, the principle of a 'rotor-stator' high-gradient magnetic separator was fully redesigned to meet the rigorous requirements of modern cGMP biotechnology purification processes. In order to fulfill regulatory requirements, the separation chamber was reengineered to allow effective cleaning and sterilization in place while maintaining excellent separation capacities and efficiencies. Two kinds of commercially available magnetic particles were used to validate key performance data and determine system related parameters in order to calculate process performance figures for process optimization of the new magnetic separation device. With separation capacities of over 400 g of magnetic particles per liter of separation chamber volume and separation efficiencies as well as recovery rates over 99 %, the system is able to process more than 200 L crude feedstock per day and capture more than 1.6 kg target compounds.

Keywords: direct capturing; downstream processing; GMP; high-gradient magnetic separation; industrial biotechnology

Abbreviations: GMP, good manufacturing practice; **HGMS,** high-gradient magnetic separation; **MP,** magnetic particles; **CIP,** cleanable in place; **SIP,** sterilizable in place; **PEEK,** polyether ether ketone; **EHEDG**, European Hygienic Engineering and Design Group

4.2 Introduction

The large-scale purification of biopharmaceuticals from of crude bioprocess feedstock and natural sources by conventional chromatography is often limited. Competing protein concentrations of sometimes over 50 g/L in serum or fermentation broth homogenates combined with high concentrations of particulate contaminants in the feedstock require elaborate multistep filtration and purification procedures. As a consequence, low yield, long processing times and high costs can be expected [18,60]. Alternatives are direct or integrated capturing methods. High-gradient magnetic separation (HGMS) is an elegant method combining classical purification process steps of solid-liquid separation, capturing and concentration in one-unit operation [73,92,147]. HGMS in combination with target-selective magnetic particles (MP) enables protein purification directly from unclarified feedstocks [72]. Using small non porous MP prevents fouling due to pore blocking and provides large surface areas, resulting in high binding capacities and fast kinetics [73]. MP are easy to handle in a batch stirring tank arrangement for the binding step and fast to separate through magnetic force [124]. Magnetic separation has been known for many years in industries such as mining or water recycling [61,63,148]. HGMS systems were designed to recover micron-sized and weakly MP from dry and liquid feed streams. Fixed ferromagnetic matrices, such as filamentary rods, steel wool, thin wire meshes, plates, or pebble beds offer large surface areas while generating high field gradients when placed in a magnetic background field, making them suitable for the different separation tasks in the mentioned industries [4,73,148]. However, in the pharmaceutical industry, magnetic separation has not yet been used for purification processes on a commercial, industrial scale [73,92,93]. Magnetic separation of biomolecules is mainly employed on a lab scale for cell sorting, DNA purification and analytical applications [66,74,149]. Nevertheless, there are several studies dealing with the purification of commercially interesting biomolecules via magnetic separation on various scales [76–79]. For this purpose, custom-made prototype magnetic separators have been developed with a wide variety of approaches of shape, sizes and magnetic background field but still following the classical fixed matrix design. Rolled or staged wire meshes or filamentary rod constructions are favoured as a defined matrix structure [107–109]. The use of these matrix designs results in good separation performances with over 90 % of MP separation from the feed-streams, but the resuspension efficiency needed in order to wash and recover the MP from the system for further use has been mostly ignored. However, the complete recovery of the MP from the separator is

essential for economic reasons due to the high MP costs and also to avoid batch to batch contaminations [73,74,92]. Only a few approaches for enhanced MP recovery have been described, ranging from high flow rates by circulation of the process fluids over mechanical shaking of the system up to special coatings of the matrix in combination with ultra-sonic systems [111,113,114]. These approaches have been used in ml scale chamber volumes and face major difficulties in scale-up. To overcome disadvantages of the filamentary matrix structures such as irregular shapes and junction points that offer MP and impurities areas of low flow speeds, the matrix design itself has to be optimized. This was done with the development of the 'rotor-stator' matrix design [25]. The concept of the 'rotor-stator' magnetic separator is now known in various setups and scales and has proven its broad range of application possibilities in numerous purification processes in different groups [72,77,79,115]. However, these processes have still not been developed past laboratory scale and are still suffering from the lack of suitable large scale GMP-conforming magnetic separation equipment. In this work, we present the first GMP-compliant high-gradient magnetic separator suitable for industrial use. The separator follows the concept of the established 'rotor-stator' high-gradient magnetic separator developed in our group previously [25]. Furthermore, we prove the applicability with performance data on separation efficiency as well as separation capacity for two commercially available MP. Additionally, we give an introduction to the process economics of the magnetic separator on the basis of dimensionless key figures describing the purification process in order to show the great potential of the new magnetic separator in a production environment.

4.3 Materials and Methods

4.3.1 'Rotor-Stator' High-Gradient Magnetic Separator

Previous HGMS systems typically used filamentary wire meshes as a separation matrix in order to strengthen and concentrate the magnetic field as well as enlarge the separation area for MP. The drawback of this kind of matrix is the lack of cleanability as well as the problems associated with recovery of MP from the system. To overcome this difficulties, the 'rotor-stator' matrix design was developed. The matrix consists of a stack of metal discs (Figure 15C). The discs are densely perforated with holes in order to pump the process solutions through. The metal ligaments between the holes serve as the separation matrix. These areas get highly magnetized to strongly attract MP in the magnetic field of the external electro magnet. The alternating connection of discs to a central rotatable shaft and to the housing of the separation chamber allows a fast rotation of every second disc, while the opposing discs form a stator which stands still (Figure 15B). This arrangement means that high shear forces for particle detachment, resuspension and mixing can be generated in the gap between the rotor and stator discs when required during washing and elution steps. However, existing models of 'rotor-stator' HGMS systems are not designed to meet GMP guidelines. An open matrix arrangement without a sealing concept to the inner shaft nor the surrounding chamber as well as the O-ring sealing at all chamber openings and metal-metal contacts create dead volumes and therefore pose high risk areas for the cleaning or sterilization of the device. Furthermore, the separation chamber, hose connections and valves are not designed to be self-draining. This effect is additionally reinforced by the surface and weld quality of the matrix and separation chamber. Finally, the housing of the separation device is not meant to be cleaned. In order to meet demanding requirements of industrial GMP-complaint equipment and develop a device which is cleanable (CIP) and sterilizable (SIP) in place, the design of the existing system was fully reengineered in a close cooperation between the company Andritz KMPT GmbH and the Karlsruhe Institute of Technology. This newly developed fully capsulated version of a 'rotor-stator' magnetic separator (Figure 15A) meets common hygienic design standards such as 3-A Sanitary Standards or EHEDG and is suitable for applications in clean rooms up to class 7. All surfaces are cleanable and there is no hazard associated with leaching of components into the products. Surface finishes of at least 0.8 µm Ra with minimum radii of 3 mm and

electro polished matrix elements are additionally realized [133]. Metal-metal contacts are avoided and the system is designed to be fully self-draining as well as CIP and SIP compatible. The separator includes of a two-way hose pump with a maximum capacity of 160 dm^3/h. The fluid streams are controlled by two CIP and SIP enabled multi-port diaphragm valve blocks (GEMÜ, Ingelfingen-Criesbach, Germany) with six connections on the top and bottom of the system which are EHEDG certified. In this system, a switchable electro magnet generates the magnetic field for the separation of MP. The magnet consists

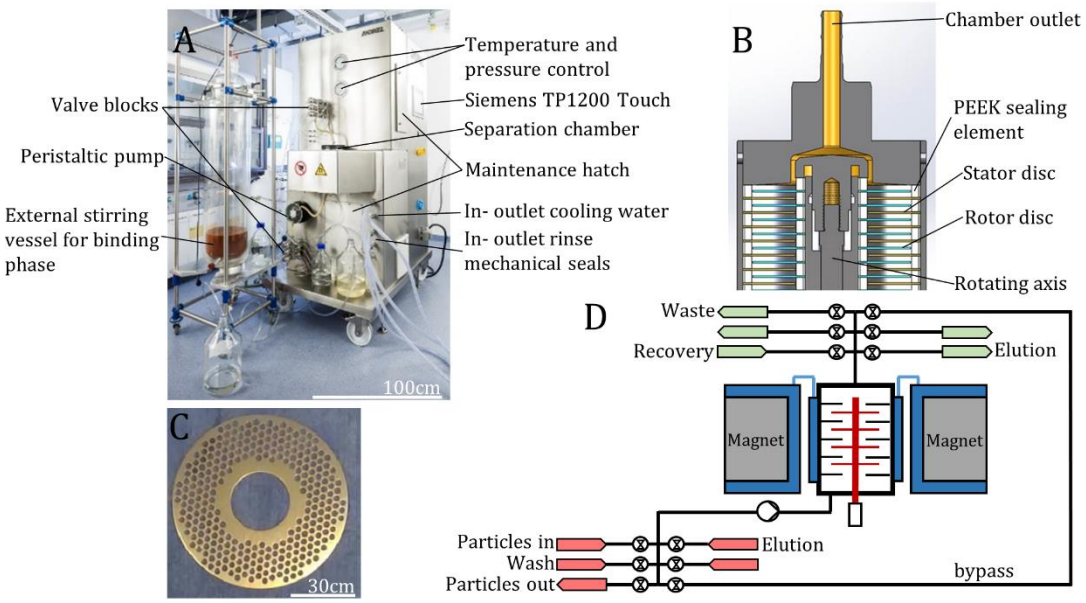

Figure 15: (A) Annotated photograph of the 'rotor-stator' high-gradient magnetic separator MES 100 RS, including external stirring vessel for batch adsorption; (B) CAD drawing of the upper part of the separation chamber; (C) perforated separation matrix rotor disc; (D) schematic drawing of the separator including top and bottom valve block with indications of connected feed-streams and flow path

of an aluminium winding coil generating a field strength of at least 0.25 Tesla at a power input of 3.6 kW. The impact of the matrix on the magnetic field inside the chamber was simulated by Multiphysics Modeling Software (COMSOL, Goettingen, Germany). The simulation of the field strength shows a magnetic induction up to 0.6 Tesla with the separation matrix installed and a macroscopically homogenous magnetic field over the whole length of the separation chamber. A water cooling jacket comprising the top and bottom lid as well as the inner bore of the magnet controls the temperature which is monitored by a Pt-100 element at the outer winding. The separation chamber has an inner volume of approximately one liter and contains the alternating stack magnetizable filter discs illustrated in Figure 15B. The discs are made from magnetizable stainless steel with an electro polished surface finish. The rotatable discs can be moved at 1500 rpm. Discs are

sealed to each other in order to provide a defined and closed process chamber. For this purpose, custom made PEEK elements were developed. The sealing is designed to avoid any dead spaces and reduce bypass flow around the discs. The inlet and outlet of the chamber allow a plug flow geometry as well as an optimal emptying of the chamber. All parts of the separator which are in contact with product are constructed to be sterilizable with superheated steam. To compensate for the extension of the disc stack and PEEK sealing due to temperatures of up to 125 °C during the sterilization the process chamber is equipped with pre-loaded springs at the top and bottom. In order to avoid O-ring sealing and the associated cleaning problems, a double acting mechanical seal is used as shaft sealing. The mechanical seal consists of non-magnetizable stainless steel to avoid any collection of MP; furthermore, the seal is sterilizable from both sides. Important system parameters such as temperature and pressure of the sealing liquid as well as core temperature of the magnet are monitored and displayed during the entire separation process. The maintenance requirements of the device are minimized by its design. Tubing can be easily connected via hose nozzles and due to the avoidance of grinding by using mechanical seals the system does not have to be disassembled frequently in order to replace wearing parts. To access the separation matrix, the separation chamber consisting of matrix discs, PEEK sealing, spring packs and a housing can be easily removed from the bore of the magnet in one piece. The system can be fully controlled and programmed from a human interface type Siemens TP1200 Touch integrated into the housing of the separator. The software is based on a Simatic S7 PLC interface programmed to be highly modular. The operator is flexible in the process design and execution. It is possible to choose from different operating modes such as a manual mode where the operator controls all functions in real time or a fully automated mode where the system runs purification protocols independently.

4.3.2 Magnetic Particles

Two commercially available MP were used to test the performance of the developed HGMS system: Chemagen M-PVA MP from PerkinElmer (Waltham, Massachusetts, United States) and MagPrep Silica MP from Merck Millipore (Darmstadt, Germany). The M-PVA particles consist of nano-sized magnetite particles surrounded by a matrix of cross-linked polyvinyl alcohol. According to the manufacturer, the mean diameter ranges from 1-3 µm. The saturation magnetization, determined with an alternating gradient magnetometer (Micromag 2900 Princeton Measurements) amounts to 29.6 ± 0.4 Am^2/kg with a remanence

of 14.8 mA^2/kg. MagPrep particles consist of monocrystalline magnetite with a thin silica coating. The mean diameter specified by the manufacturer is 100-200 nm. These particles have a saturation magnetization of 77.6 ± 3 Am^2/kg and a remanence of 25.6 Am^2/kg. The particle concentration of collected particle suspensions was determined gravimetrically by dry mass [79]. For concentrations lower than 0.2 g/L the concentration was determined by absorbance which is linearly dependent on concentration at a wavelength of 860 nm. In this case the samples were transferred to a microtiter plate and measured with a grid of 21 measuring points in triplicates using a plate reader (EnSpire Multimode, PerkinElmer).

4.3.3 Operating Procedure

All operation protocols were programmed and executed via the integrated control panel. Feedstocks containing MP were stirred constantly to ensure a homogenous particle distribution. In order to test the maximum filter capacity of the magnetic separator, MP suspensions with a concentration of 22 g/L for M-PVA MP and 20 g/L for MagPrep MP were prepared with PBS (137 mM NaCl, 2.7 mM KCl and 12 mM Phosphate, pH 7.4) as liquid phase. A 35 % (w/w) sucrose solution was used to increase the viscosity of the feed solution. For separation capacity tests, an MP suspension was pumped at 2.2 dm^3/min through the separator from the bottom valve block to the top (Figure 15D) while the electro magnet was switched on. Samples were drawn every 15 s and the particle concentration was determined. In order to test MP loss after resuspending and recapturing of MP, 90 g of M-PVA MP were loaded into the system. To perform a recirculation of particles as it occurs during buffer change or washing of the MP the system was flushed with two liters of the new buffer system. In the next step, the MP were resuspended by switching off the electro magnet and rotating of the central shaft with the connected discs at 1500 rpm for 30 s. For recapturing MP after suspension, the magnet was switched on again and MP were pumped through the system in a loop from the top to bottom valve block at 1.4 dm^3/min for 30 s in order to collect all MP at the separation matrix. Samples were drawn from the effluent of every buffer change of ten consecutive process cycles. To recover MP from the chamber, the electro magnet was switched off and the particles were suspended by rotating the central shaft. Different recovery protocols were tested (data not shown). The lowest recovery volume, which represents the ideal case, was achieved by flushing the system from top to bottom with recovery buffer at 100 % pump speed and a rotator speed of 150 rpm. The first

two fractions contained two liters; for all following fractions the volume was reduced to one liter of buffer.

4.4 Results

4.4.1 Separation Performance

In this study, the performance of the new HGMS device was tested in regards to separation performance, recyclability and recovery of MP. For successful process development, crucial particle dependent parameters were investigated. Separation capacity of the filter matrix was determined by breakthrough experiments at a constant flow rate of 90 dm^3/h. For M-PVA MP, the average filter capacity until a 1 % breakthrough of the feed concentration was 430 g of MP. Running the system at 90 dm^3/h and 22 g/L feed particle concentration led to a separation duration of 8 min and 54 s. During this time, an average separation efficiency of 99.91 % was achieved. Separation of M-PVA MP from a feed solution with higher viscosity led to the slightly lower separation capacity of 395 g and less sharp breakthrough behaviour, resulting in a lower separation efficiency of 99.82 %. For the smaller MagPrep MP breakthrough was observed after separation of 270 g MP, within 6 min and 8 s. The average separation efficiency for this beads was 99.98 %, indicating a very sharp breakthrough behaviour (Figure 16A). The combined particle loss of the system during ten

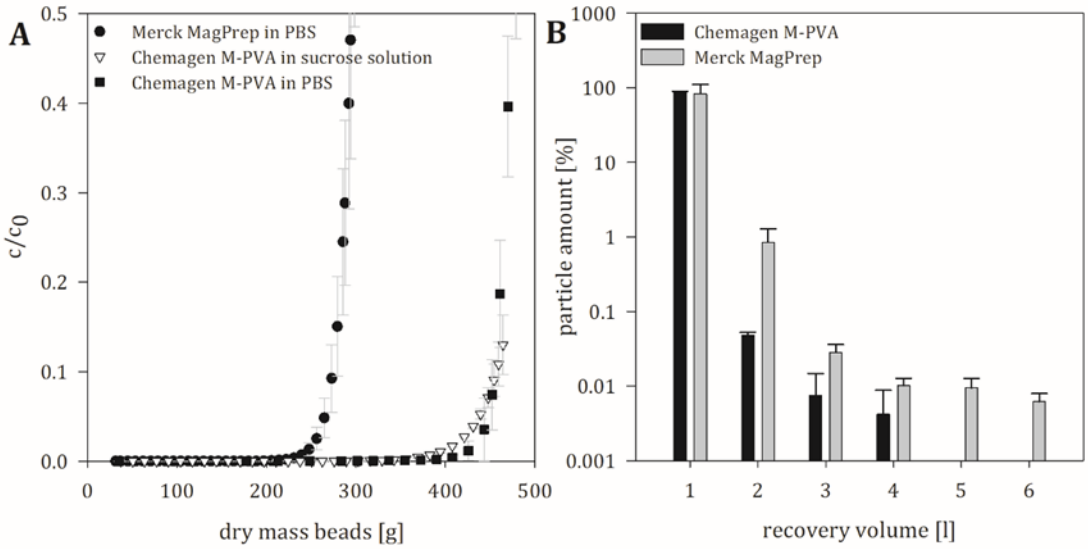

Figure 16: (A) Breakthrough curves for two kinds of commercial MP. Dots: Merck MagPrep MP; Inverse triangle: Chemagen M-PVA MP in 37 % (w/w) sucrose solution; Squares: Chemagen M-PVA MP. (B) Recovery volumes for two kinds of commercial MP. Y-axis: Fraction of MP in per cent of the total loaded amount. X-axis: Number of applied 1 L batches of recovery solution. In black bars, chemagen M-PVA MP and in grey Merck MagPrep MP.

suspension and recapturing steps, representing a complete protein purification operation

including all washing, elution and regeneration steps, accounted for 0.3 % of the total loaded particle mass for M-PVA and 0.2 % for MagPrep MP. Finally, results from particle recovery experiments for the two kinds of MP are displayed in Figure 16B. Three recovery steps were needed to recover 99.6 % of the loaded M-PVA MP. Nearly all particles were found in the first two liters of the first step. No MP were detected in the drain of the fifth and subsequent recovery steps or in the separation chamber after dissembling. However, six recovery steps were required to recover 99.8 % of the MagPrep MP.

4.4.2 Process Performance

As a model system and in order to predict the performance of the new magnetic separation device data obtained by the use of commercial M-PVA MP (#IDA2_0118071) with covalently-bound iminodiacetic acid groups charged with Cu^{2+}-ions were used to simulate the large scale purification of his-tagged green fluorescent protein (his-GFP). In lab-scale experiments, the particles showed a maximum binding for his-GFP directly from the unclarified *E.coli* cell lysate of q_{max}: 0.168 g/g with a k_D of 0.063 g/L. For the following calculations the particle mass m_p was set to 316 g, accounting for 80 % of the 1 % breakthrough for high viscous feedstocks. The separator volume V_{sep} is fixed at 0.98 L and the initial his-GFP concentration is set to 8 g/L. With a variation of the capacity ratio CR which involves a variation of V_{batch} the equilibrium concentration c* can be calculated. As described, protein purification by MP in combination with a magnetic separation device uses a batch binding step in a stirred external tank. Consequently, the achievable protein loading of the MP depends on the remaining protein concentration in the supernatant after equilibrium is reached. This results in the known interrelation that high binding yields (low equilibrium concentrations left in solution) correspond to low protein loadings of the MP and vice versa. The equilibrium conditions are influenced by the ratio of the protein amount that can be bound by the mass of MP used and the protein amount offered in the feed volume of the batch. In previous publications we showed how this CR and the isotherm parameters of the considered purification task influence the predicted yield, purity and productivity of protein purification using magnetic separation [7] (see supporting information). Productivity and resulting yield of the described model process are illustrated in dependence of the CR in Figure 17. The yield increases up to 95 % in a linear manner up to a CR of approx. 1, after which the further yield increase is quite small with increasing CR. In

the case of MP with high target affinity, the productivity also reaches its maximum close to this point, in our case at a CR of 1.1, corresponding to a batch volume of approx. 5.5 dm^3.

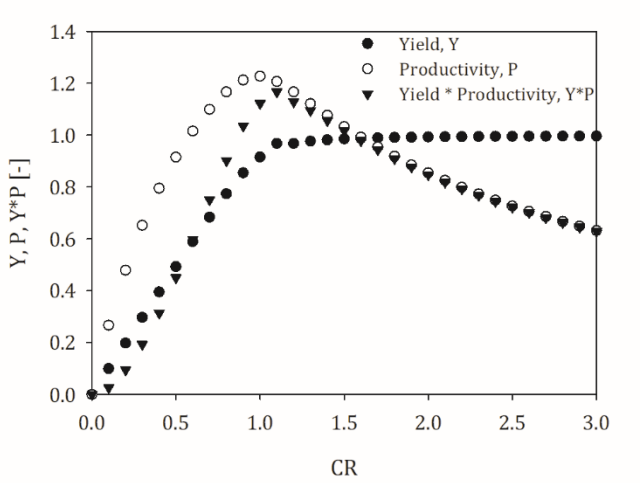

Figure 17: Dots: Yield; inverse triangle: Productivity; squares: Yield*Productivity as function of the capacity ratio CR for the target protein his-GFP. Yield*Productivity depending on CR indicates an optimal process point at CR 1.2.

To process the batch volume, a cycle time of 37 min has been experimentally determined. The cycle time is mainly influenced by the pump speed and includes the time for all process steps, such as loading, washing, elution, particle cleaning, equilibration and recovery. In summary, in the case of the described model system, a yield of 96.7 % is predicted with a productivity of 1.17 g/min*L. In 24 h the simulated process is able to treat 210 L of fermentation broth while capturing and purifying more than 1.6 kg of his-GFP.

4.5 Discussion

The separation performance test with the novel GMP-compliant 'rotor-stator' magnetic separator revealed that separation capacity as well as breakthrough behaviour strongly depends on the properties of the MP. Decisive in this regard are the significant differences in size, magnetization and agglomeration properties. Despite the high magnetization, the size and weight of the MagPrep MP is crucial for the separation step. For protein purification processes, the particle amount loaded to the separator should not exceed 80 % of the maximum loading to ensure an optimal washing of the MP in the chamber. The viscous feed solution was chosen to simulate natural and sticky feedstocks such as blood serum. The shearing effect of the viscous feed solution resulted in a lower separation capacity as well as efficiency. Lower pump speeds should allow the particles to settle fast enough on the separation matrix and achieve separation results comparable to the ones with aqueous feed solutions. The separation device presented here meets or even exceeds ideal specifications for a magnetic separation device compiled by Franzreb et al. [73]. At the moment there is no comparable system on this scale on the market. Separation efficiencies are comparable to smaller systems optimized for particle separation without taking GMP guidelines and cleanability into account [79,113]. The outstanding MP recovery performance due to the matrix design allows an economic process design with optimal MP washing results during the process. Furthermore, theoretical results of the process simulation are in good correlation with process results for a his-GFP purification using a previous version of a 'rotor-stator' magnetic separator from Gracìa et al. [79]. The simulation in combination with the results for the two strongly differing types of MP show the high efficiency and flexibility of the developed magnetic separator. Nevertheless, suitable MP have to be chosen carefully for every process. Besides the affinity and selectivity for the target molecule, the magnetization, size as well as size distribution, structure and availability are crucial factors.

4.6 Concluding Remarks

In this work, a GMP-complained 'rotor-stator' high-gradient magnetic separator ready for the use in biopharmaceutical purification processes was introduced. The device, whose commercialization was launched at the beginning of the year 2017, allows the expansion of applications for magnetic separation from analytic and small laboratory scale to the integration of this elegant direct capturing tool to biopharmaceutical production processes. An approved matrix design combined with a reengineered separation chamber and an advanced sealing concept showed excellent separation capacities while overcoming difficulties in MP recovery and cleanability of previous designs of magnetic separators. It was shown that the breakthrough behaviour of MP is widely independent of the solution but strongly depends on the type of MP. Simulated operation data based on small scale experimental results showed promising process performance, capable of treating more than 200 L of crude fermentation broth a day with the direct capturing unit operation presented here.

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5 One-step Integrated Clarification and Purification of a Monoclonal Antibody Using Protein A Mag Sepharose Beads and a cGMP-compliant High-gradient Magnetic Separator

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5.1 Abstract

Monoclonal antibodies dominate the biopharmaceutical market today. Typically, the purification process is performed by classical platform purification processes. High costs and rising demands require the development of efficient and flexible integrated purification processes. Until now, high-gradient magnetic separation as a direct capturing tool has been suffering from the lack of suitable GMP-compliant separation equipment for industrial purification processes. As a solution for this bottleneck we present a purification process for a monoclonal antibody directly from CHO cell culture by use of protein A functionalized magnetic particles and the first pilot-scale GMP-compliant 'rotor-stator' high-gradient magnetic separator. We perform five consequent purification cycles achieving constant yields of over 85 % and purities of over 95 %. Stable cell viabilities during the magnetic separation process enable integration of the device as an *in situ* product removal tool. A comparison with state-of-the-art protein A column-based purification processes reveals a three times higher process productivity per milliliter of applied resin and demonstrate the great potential of magnetic separation in downstream processing.

Keywords: direct capturing; GMP; high-gradient magnetic separation; industrial biotechnology; mAb downstream processing

Running Title: GMP pilot-scale mAb purification via HGMS

5.2 Introduction

Biopharmaceuticals have grown to be among the main products of the pharmaceutical industry with a market above 200 billion dollars and a growth rate beyond 15% [150]. Monoclonal antibodies dominate the market as blockbusters and will do so for the next years with more than 50 candidates currently in late-stage development [17,34,35]. Established companies invested in large production capacities of stainless steel plants to keep up with the demands. Standardization and platform processes have been established using structural similarities, which has improved productivity significantly [34]. These technologies consist typically of a clarification using centrifuges and filtration methods followed by product capturing via protein A chromatography, virus inactivation and filtration, and finally one or two polishing steps by anion and or cation exchange column chromatography [18,29,151]. The use of disposables as well as more flexible equipment allowed also smaller companies and a growing number of contract manufacturers to set up more flexible production capacities and secure shares from the growing demands [152]. With rising markets for individualized medications and smaller dynamic production sites on the rise, high investment costs for large stainless steel capacities and traditional costs of down-stream purification methods have to be reconsidered [153]. One approach are integrated purification methods which combine classical process steps such as clarification and capturing to save time and costs and prevent product losses. Typical representatives are expanded bed adsorption (EBA) or aqueous two-phase systems (ATPS). Both process steps are well described and have proven their applicability in numerous examples. EBA offers the opportunity to process fermentation broth without clarification as capturing step but is often limited in throughput and prone to resin fouling [43,46,47]. ATPS as a particle-free method is not susceptible to fouling and offers similar opportunities compared to EBA as capturing step for the separation of target proteins, impurities, and solids in different liquid phases. Drawbacks are elaborate investigations of suitable ATPS and the introduction of impurities for phase separation such as poly-ethylene glycol (PEG) and high salt concentrations in the purification process [21,31,153]. Another approach is to integrate clarification into fermentation processes. Techniques such as perfusion stirred-tanks or hollow-fiber bioreactors offer the possibility for constant product removal while proceeding with fermentation in order to create a continuous processes [35]. In recent years, high-gradient magnetic separation (HGMS) technology has emerged as an additional approach to whole-broth treatment. In combination with functionalized magnetic particles

(MP), the technology allows target-orientated capturing of the molecule of interest (MOI) directly from the fermentation broths. The technique has proven its benefits in numerous approaches [74,124]. In the analytical scale, HGMS is routinely used for cell sorting or DNA purification [66,103]. Easy scale-up from laboratory to preparative scale was proven by process examples as described by [76,97]. Advantages such as fast binding kinetics, short process times due to much higher flowrates than in column chromatography, and an easy adaption of MP amounts to batch sizes makes magnetic separation particularly interesting. Furthermore, HGMS batch adsorption approaches are easy to integrate in the fermentation process. This process variant has already been reported [21,59]. The use as *in situ* product removal (ISPR) tool creates new opportunities for integrated process operations. However, the lack of suitable cGMP-compliant HGMS equipment and limited availability of large quantities of MP prevented this technique from being used in biopharmaceutical production processes until today. Since the beginning of the year 2017, this gap has been closed by the development and commercialization of a cGMP-compliant 'rotor-stator' HGMS device. Previous non-GMP versions of 'rotor-stator' magnetic separators have been used for a wide range of purification tasks [72,79]. Technical details and validation data of the latest cGMP-compliant version can be found elsewhere [125]. In this study, we prove the process-relevant purification power of the combination of high-performance MP and the cGMP-compliant HGMS device. Protein A Mag Sepharose were used to bind a monoclonal antibody (mAb) directly from CHO cell culture. A mAb was chosen as target molecule due to its relevance to industry and the possibility to show purification performances in comparison to well established platform antibody purification processes. Five purification cycles were performed using Protein A Mag Sepharose and the HGMS system to show the stability and robustness of the process combination. Purity, yield, and host cell protein (HCP) reduction level were determined and compared to common purification methods.

5.3 Materials and Methods

5.3.1 Introduction of a Magnetic Separation Process

A process scheme of a typical purification process using a HGMS system such as the MES 100 RS and functionalized MP is described in Figure 18. Incubation of functionalized MP with fermentation broth, as the first process step, is performed externally in order to handle larger amounts of fluids. Fermentation broth can be, for example, unclarified CHO feedstocks or disrupted *E.coli* feedstocks. Also natural sources such as blood serum are possible. A constant mixing is required to avoid particle sedimentation.

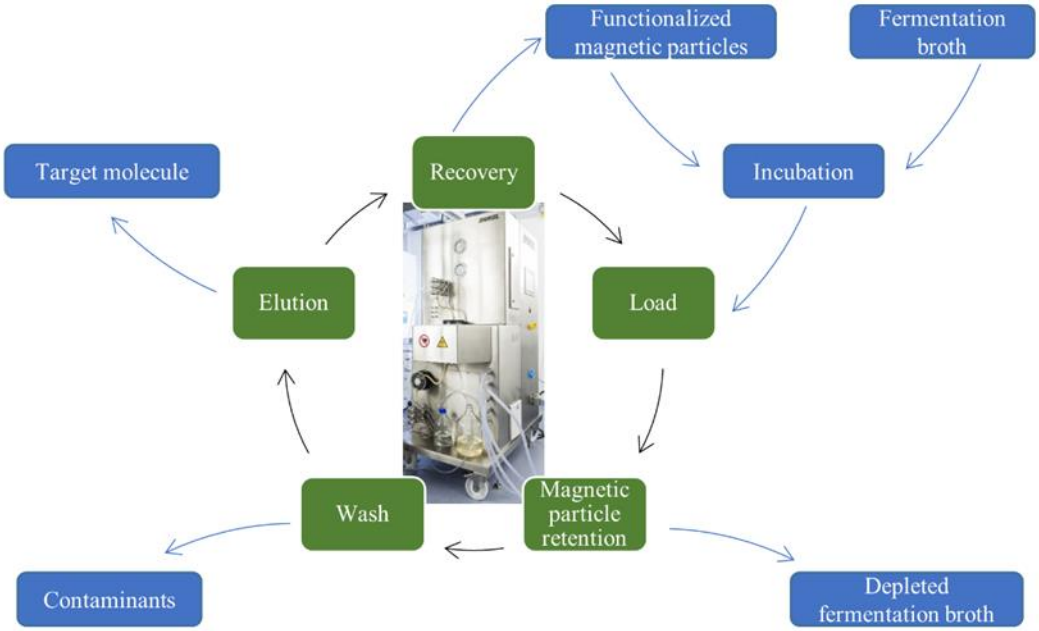

Figure 18: Process scheme of a HGMS process. Green boxes represent process steps conducted inside the HGMS device. Blue boxes represent process steps or fluid streams conducted in external vessels or leaving the device.

After incubation, the feedstock-MP suspension is pumped into the HGMS system. MP with the bound MOI are separated by an external magnetic field at the magnetized separation matrix whereas the fermentation broth passes through the separation device. The magnetic separation can be described as a deep-bed filtration, selectively removing MP from fermentation broth [73]. After separation, MP are resuspended in a wash solution to remove weakly bound protein and impurities derived from the fermentation process. After every washing cycle, MP have to be rebound to the separation matrix in order to replace the liquid phase. The elution procedure typically also takes place in the HGMS device. Therefore, MP

are suspended in the elution buffer, which is collected after particle separation. Finally, MP have to be cleaned from heavily bound impurities, equilibrated and recovered from the HGMS device for further use or storage.

5.4 Materials

Salts and chemicals for buffer preparation and pH adjustments were purchased from Merck (Darmstadt, Germany) in analytical grade. Mag Sepharose functionalized with protein A was used for small and pilot-scale purification experiments, HiTrap MabSelect SuRe column for bind and elute mAb titer analysis and two Superdex 200 Increase GL 5/150 columns for purity analysis (GE Healthcare, Uppsala, Sweden). Small-scale MP separation was performed using a MagRack 6 (GE Healthcare, Uppsala, Sweden). For all pilot-scale experiments, a 'rotor-stator' high-gradient magnetic separator (MES 100 RS) was used (Andritz KMPT, Vierkirchen, Germany). Before column analytics, all samples were centrifuged to ensure absence of solids.

5.4.1 Magnetic Particles

In this study, Mag Sepharose prototype (supplied by GE Healthcare, Uppsala, Sweden) were used for all experiments. The MP are based on a highly cross-linked agarose matrix with magnetite inclusions and have a mean diameter of 54 µm. Magnetic behaviour can be described as ferrimagnetic with a maximal magnetization of 47 Am^2/kg and a remanence of 14% determined by an alternating gradient magnetometer (Micromag 2900 Princeton Measurements). For purification of mAb from an unclarified crude CHO cell culture, the MP were functionalized with a protein A ligand by GE Healthcare (Uppsala, Sweden).

5.4.2 'Rotor-Stator' High-Gradient Magnetic Separator MES 100 RS

The latest cGMP-compliant, commercially available version of a `rotor-stator` HGMS (MES 100 RS) was developed in close cooperation of Karlsruhe Institute of Technology and Andritz KMPT GmbH. The unique design of the separation chamber together with an advanced sealing concept enables a pilot-scale production under GMP guidelines. The setup of the MES 100 RS consists of a two-way peristaltic pump with a maximal pump rate of 2.7 L/min at the current setting. Two pressurized air-controlled valve blocks with six connections, one below and one above the separation chamber, control the process streams. The separation chamber is a one-liter cylinder surrounded by a water cooling jacket and an electro magnet, which generates a magnetic field of 0.25 T in the bore. Inside the separation chamber, a stack of perforated magnetizable stainless steel discs serves as separation

matrix. The holes within the discs allow process liquors to pass the separation chamber unhindered, whereas the metal ligaments between the holes get highly magnetized by the external magnetic field to strongly attract and separate MP. The discs are alternately fixed to an inner rotatable shaft (rotor-discs) or the outer housing (stator-discs). In order to detach MP from the matrix discs or to clean the separation chamber, the inner shaft together with the connected 'rotor'-discs can be rotated at 1500 rpm to create shear forces in the gap between 'rotor'- and 'stator'-discs. Polyetheretherketone (PEEK) elements serve as spacers and sealing between the matrix elements. The MES 100 RS is controlled and programmed via an integrated graphical user interface. The software is highly flexible and offers the operator three different modes, from total automatic, semi-automatic to a complete manual control of the device. A closer description of the system setup, handling and performance can be found elsewhere Ebeler et al [125].

5.5 Methods

5.5.1 Cell Culture

CHO cultivation was performed in the fed-batch mode using a ReadyToProcess WAVE 25 Bioreactor and ActiPro cultivation media (GE Healthcare, Uppsala, Sweden) expressing the mAb applied here. Cell harvest was split into day 11 and 12 of the cell cultivation process.

5.5.2 MAb Concentration Determination

The mAb contents of all collected samples from small-scale binding studies and process-scale experiments were analyzed by a MabSelect SuRe bind-and-elute method. For calibration, a pure mAb standard was used. All samples were centrifuged to ensure the absence of solids. A 1 mL HiTrap MabSelect SuRe column was equilibrated with 10 column volumes (CV) of PBS+0.05 % Tween 20 (137 mM NaCl, 2.7 mM KCl and 12 mM Phosphate, pH 7.4). 100 µL sample was injected to the column followed by a 5 CV wash with PBS+0.05 % Tween 20, a 3 CV elution using 0.1 M sodium phosphate pH 2.9 and a 5 CV equilibration step. The flowrate was kept constant at 1 mL/min. For detection, a 10 mm UV cell at a wavelength 280 nm was used.

5.5.3 MAb Purity Determination

The purity of the eluted samples was analyzed by size exclusion chromatography (SEC). Two coupled Superdex 200 Increase 5/150 GL columns (GE Healthcare, Uppsala, Sweden) were equilibrated with 2 CV 200 mM sodium phosphate pH 6.8. Either 5 µL in the case of elution fractions one and two, or 10 µL in the case of elution fraction three was injected at a flow rate of 0.5 mL/min. All peaks, from impurities and mAb, were integrated. The purity was calculated by dividing the area of the main peak by the total area of all peaks.

5.5.4 Cell Viability Determination

Samples for cell viability testing were drawn from fresh CHO cell culture after 30 min and 60 min of incubation with MP and after processing the cell culture-MP-suspension with the magnetic separator. Cell counts were determined by a VI-Cell XR cell counter using VI-Cell

XR 2.0 software (Beckman Coulter Life Science, Krefeld, Germany). HCP levels were detected using a CHO HCP 3rd Generation ELISA kit (Cygnus Technology, Southport, North Carolina) customized for a Gyrolab™ xP workstation (Gyros AB, Uppsala, Sweden) using Gyrolab Bioaffy 200 CD disks.

5.5.5 Small-Scale Binding Studies

Langmuir model-based maximal binding capacity Q_{max} and dissociation constant K_D were determined for binding of the mAb onto Protein A Mag Sepharose by small-scale adsorption experiments. Varying amounts of functionalized MP (10-200 µL sedimented bed) were incubated with mAb containing CHO cell culture in 1.5 mL sample tubes at constant mixing for one hour. Subsequently, the MP were separated from the cell culture by magnetic separation and the solids were removed by centrifugation. The supernatant was analyzed for the mAb content by the MabSelect SuRe assay as described above. The bound protein amount was calculated by subtracting the protein concentration in the supernatant after binding from the initial concentration and calculating the protein loading of the MP by help of a mass balance. Equilibrium loading and concentration data were fitted using the Langmuir model.

5.5.6 Pilot-Scale Magnetic Separation

In the following, an exemplary sequence of a pilot-scale HGMS purification process is outlined. Five mAb purification runs from CHO cell culture were performed on two consecutive days in order to demonstrate process stability and reproducibility. On the first day, three batches were processed showing an mAb titer of 2.1 g/L. On the second day, the last two runs were performed with an mAb titer of 2.5 g/L. All buffer storage tanks were connected via tubing to the valve blocks as illustrated (Figure 19). Tubings were prefilled and the separation chamber was equilibrated using PBS to avoid air inclusions. At the outlet valves, sufficient containers for collecting wash and elution fractions for further analysis were provided.

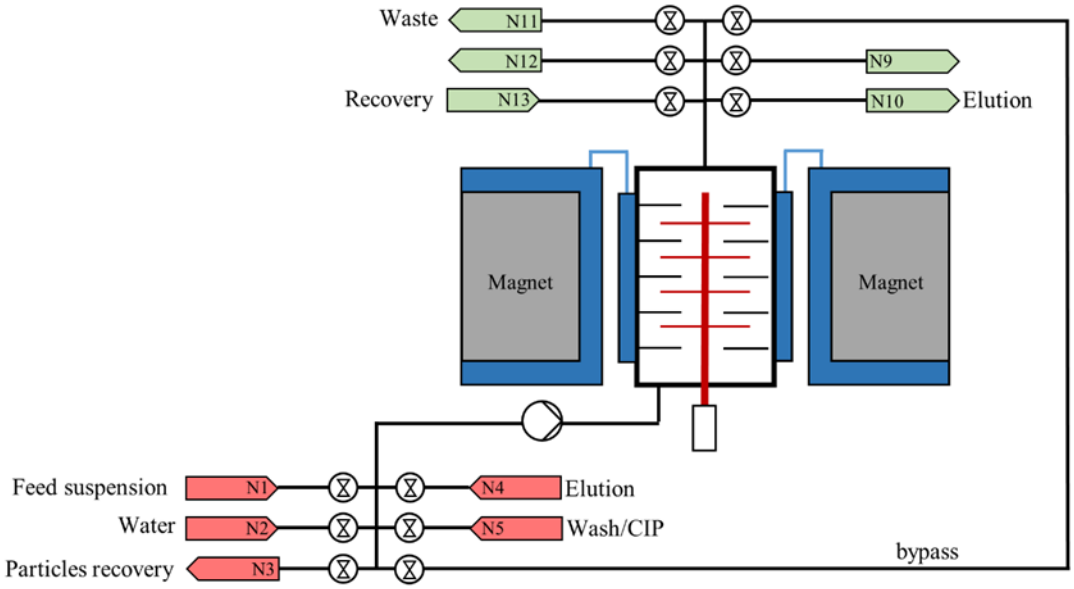

Figure 19: Schematic flow sheet of the separator including the valve blocks and connections of the buffer solutions.

For every purification process cycle, the available batch of 200 mL of Protein A Mag Sepharose was incubated with CHO cell culture in an external stirring vessel. No pretreatment of the feed was applied. A constant mixing was ensured for the time of incubation. During incubation, 1 mL samples were drawn after 0, 2, 5, 10, 20, 30, 40, 50, and 60 min in order to monitor mAb uptake. After incubation, the MP-CHO cell culture suspension was passed through the separator at a flow rate of 2.2 L/min from valve 1 to valve 10**.** MP were collected at the separation matrix by applying a magnetic field while impurities were passed through the separator and collected for further analysis. MP were washed three times using 1.5 L PBS per wash. In the course of these washing steps, the remaining depleted cell culture feed in the separation matrix was completely replaced by PBS. During each washing step, the external magnetic field was switched off and the MP were resuspended in the new buffer system by rotating the inner shaft at 1500 rpm. To recollect the suspended MP, the external magnetic field was applied again and the process liquor was bypassed through the separation chamber from valve 6 to 8 for recollection of all MP before replacement of the liquid phase. The wash procedure was finalized by a wash step using pure water. For eluting mAb from MP, the separation chamber was filled with 1 L 0.1 M sodium-acetate solution pH 2.9. Higher elution pH is often utilized in Protein A chromatography, but in this study it was of main interest to investigate product yields and secure that all mAb elutes off in one step. Elution pH optimization has therefore not been

included in this study. MP were resuspended as described for 5 min before recollection. In total, three elution steps were performed. After elution, MP were washed three times with PBS before three cleaning cycles with 0.1 M NaOH were performed. In order to reuse MP, they were equilibrated using PBS solution and recovered from the separator as described previously [125]. All process streams leaving the separator were collected and weighed. Eluates were adjusted to pH 5 by 3 M tris-base before storage at 4 °C until analytics were performed. MAb content of the feed, wash, elution and equilibration fractions was determined via the MabSelect SuRe assay. Purities of the elution fractions were determined by SEC. All process protocols were run in a semi-automatic mode in which the MES 100 RS runs all pre-programmed process steps such as loading MP, wash, elution or recovery independently.

5.6 Results and Discussion

5.6.1 Small-Scale Binding Studies

Small-scale binding studies with Protein A Mag Sepharose and mAb containing CHO cell culture were performed to test particle binding performance for the used mAb. Fitting the results to the Langmuir isotherm, a maximum binding capacity Q_{max} of 87 mg/mL and a K_D of 0.06 mg/mL were determined (Figure 20A). Maximum binding capacity as well as equilibrium dissociation constant were in the top range of conventional protein A chromatographic resin [154]. Therefore, the presences of solid impurities as cells and cell debris seemed not to affect the binding capacity. However, the working capacity for the batch chromatography in this case is depended on the desired yield. To ensure minimal process losses, a yield of 98 % should be achieved and therefore low equilibrium concentrations must be adjusted. In the presented case, start concentrations of 2.5 mg/mL in the fifth cycle were obtained. As a consequence, the working binding capacity reduced to 39.5 mg/mL.

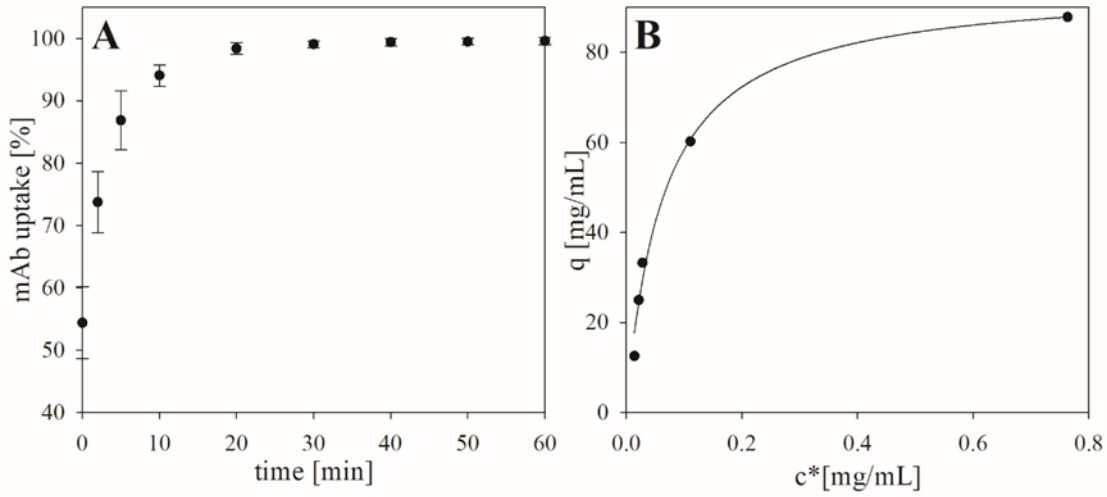

Figure 20: (A) Kinetics of the mAb binding on Protein A Mag Sepharose MP of the five process cycles. Over 90 % mAb binding in the first 10 min of the process. The binding equilibrium is reached after 20 min. (B) Langmuir adsorption isotherm for the binding of a mAb in a cell culture to Protein A Mag Sepharose. A maximum binding capacity q_{max} of 87 mg/mL and a dissociation constant k_D of 0.06 mg/mL have been determined.

5.6.2 Pilot-Scale Process Studies

Five purification cycles of mAb containing CHO cell culture were performed using Protein A Mag Sepharose and the HGMS system to prove stable process results and reusability of MP. Start conditions of all cycles are displayed in Table 5. Variations of the actual feed volume were due to leftover from the previous purification cycle to avoid air being pumped into the system. Differences in mAb titer between cycles 1-3 and 4, 5 were caused by the ongoing fermentation process from day one to day two of the harvest. Feed volume and titer variations influenced the mAb loading on the MP.

Table 5: Process feed volume, used particle amount, mAb titer in the feed, amount of mAb loaded to MP, and feed pumped through the separator for all five process cycles.

	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
Feed volume [mL]	2766	2268	1980	2799	2305
MP volume [mL]	200	200	200	212	170
mAb titer [mg/mL]	2.1	2.1	2.1	2.5	2.5
MP loading [mg/mL]	31	29	26	33	38
Feed loaded [mL]	2258	2261	2995	2486	3039

Values between 26 and 38 mg mAb/mL beads were calculated. During incubation of Protein A Mag Sepharose with mAb containing CHO cell culture, samples were taken to monitor the protein binding and adjust incubation duration. Figure 20B illustrates the mAb uptake during a period of 60 min for all five process cycles. The instant product binding of approximately 50 % was due to the batch adsorption process. All particles will be in instant contact with the mAb. As a consequence, short process times can be realized. After 10 min, already 95 % of the total binding took place, after 30 min, the equilibrium was reached. This time point represented the optimal start point for pumping the particle suspension into the separator.

5.6.3 MAb Process Yield

MAb mass and corresponding yield for all five process cycles and every process step are listed in Table 6. A nearly complete mAb binding from the CHO cell culture and negligible

losses during three washing cycles were observed due to the high affinity of the ligand and the excess of binding sites.

Table 6: MAb mass and corresponding yields for all process fractions and cycles.

	Cycle 1		Cycle 2		Cycle 3		Cycle 4		Cycle 5	
Process steps	mAb [mg]	Yield [%]	mAb [mg]	Yield [%]	mAb [mg]	Yield [%]	mAb [mg]	Yield [%]	mAb [mg]	Yield [%]
Start	4657	100	4610	100	6047	100	5831	100	7107	100
Flow-through	0	0	0	0	0	0	0	0	37.2	0.5
Wash 1-3	0	0	14.5	0.3	0	0	25.3	0.8	31.2	0.5
Water wash	58	1.1	100.3	2.2	68	1.1	64.5	1.1	70.4	1
Elution 1-3	4028	86.5	4319	93.7	5266	87.1	5000	85.8	6053	85.2

The change of the buffer system to pure water led to mAb losses of 1 % to 2 % of the total yield. This step was necessary to bring the MP in a system with no buffering properties. By this, the following pH drop for elution took place more rapidly whereby smaller elution volumes could be applied. Marginal mAb amounts were detected in the flow-through of the fifth process cycle. This can be explained by the particle loading of 38 mg/mL in this cycle, which is close to the working capacity of the batch process explained above. However, constant high total elution yields of at least 85.2 % were achieved. Significantly more than 60 % of the total elution yield was found in the first elution fraction, approximately 20 % in the second, and only around 5 % was found in elution fraction three (Figure 21A). Therefore, more than three elution fractions were not useful as they would lead to a dilution of the mAb without a significant increase in total mAb amount eluted. In this process, elution times of 5 min per elution were applied. Longer elution times should even shift mAb distribution to the first elution fraction and allow higher concentrations of the product as long as the dissociation equilibrium is not reached.

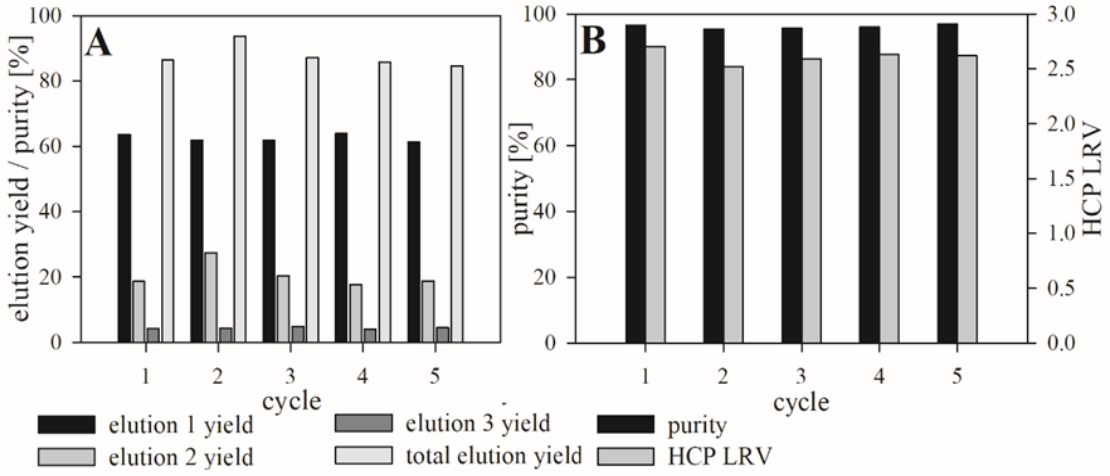

Figure 21: (A) Single yields for elution fractions 1-3 as well as total elution yield for all five process cycles. (B) Purity an HCP reduction level of the pool elution fractions from all five process cycles.

MAb concentrations from CIP and equilibration fractions could not be detected due to pH conditions and the denaturation of antibodies. The amounts of mAb in these fractions were assumed to close the mass balance. Conventional purification processes show similar yields to this magnetic separation process. Although centrifugation and filtration steps for harvest of cell culture can reach yields of up to 98 % [155], they contribute to the product loss of conventional capturing process schemes. Together with a yield of approximately 90 % for the protein A column chromatography capturing steps, yields of 85-88 % can be estimated for the conventional capturing process [27]. The constant high elution yield of over 85 % showed the robustness of the HGMS process and the reusability of the MP.

5.6.4 MAb Process Purity

Eluates from all process cycles were analyzed by SEC. A typical SEC chromatogram from a mAb eluate fraction with a purity of 96.34% was observed (Figure 22A). From the chromatogram became obvious that small volumes of impurities of 3.36% of the total peak area were low molecular weight impurities that elute around 10 minutes (Figure 22B). This fraction includes impurities deriving from the fermentation process such as HCPs. A smaller fraction of approximately 0.3 % likely formed by antibody aggregates elutes directly prior to the main peak. The negligible small amount of aggregates in eluates from the magnetic separation purification process is an evidence for a product-gentle process. It is commonly reported that shear stress has an impact on aggregation behaviour [27].

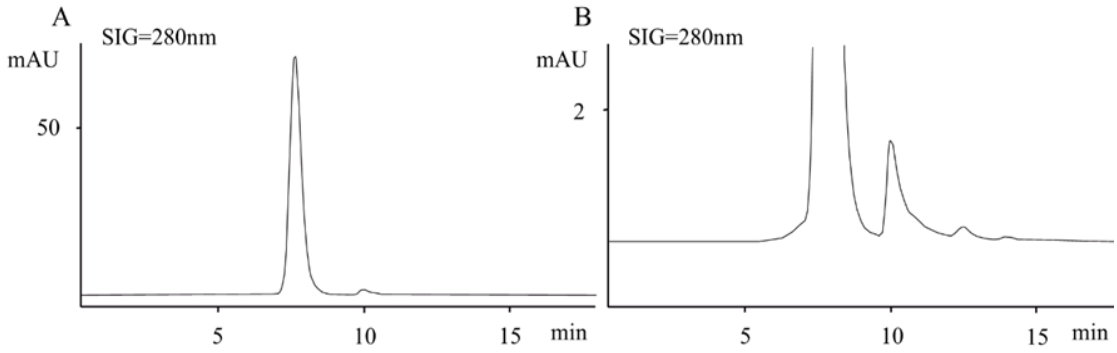

Figure 22: (A) A typical chromatogram for an elution sample from the mAb purification process. The impurity peaks and the main peak were integrated. The purity of mAb was calculated by dividing the area of the main peak with total area of impurity peaks and main peak. (B) The zoomed chromatogram

For the reported process, shear forces due to particle suspension by the matrix discs seemed not to affect the product quality. Furthermore, high local product concentrations as occurring during elution from conventional column chromatography which favor antibody aggregation could not be observed during batch elution in magnetic separation processes. Constant high purities of over 95 % were reached in all process cycles (Figure 22). Although this magnetic separation process was performed for the first time, it showed comparable results regarding the purity of the product compared to common purification processes. For column protein A chromatography applied as capturing step in an industrial setting, purities above 95 % have been reported [18,123]. These processes, however, have been intensively optimized over the years in contrast to the magnetic separation process here presented.

5.6.5 Cell Viability

To access cell viability, samples were drawn from fresh CHO cell culture, after 30 and 60 min of incubation with MP and after passing it through the separator. Constant cell viability of 93.5 % was obtained in all samples taken during the process which indicates that the cells were not influenced by the presence of MP, incubation conditions or shear stress during pumping through the separator. This makes the separator suitable for continuous harvesting operations as ISPR described for magnetic separation previously [21,59] or as an alternative for continuous perfusion or hollow-fiber reactors [35]. Furthermore, applications in large-scale cell separation are a possible scenario, due to the fact that magnetic separation is already routinely used in this field in laboratory scale [156].

5.6.6 Host Cell Protein Reduction

Stable HCP reductions of over 2.5 log reduction units and HCP/mAb weight fractions between 2430 and 1614 ppm in the elution fractions could be achieved (Table 7). These reduction levels are slightly lower than common reduction levels from conventional protein A resins in column chromatography where more than 3 log reduction units can be expected [157]. FDA has not specified an exact concentration for HCPs allowed in the final product but most biotech products reviewed positively by the FDA showed HCP concentrations between 1-100 ppm [158]. However, common mAb purification schemes include further chromatographic steps following protein A easily reducing the HCP level to the required levels. In addition, it should be possible to reach higher HCP reductions with the presented system by adding further wash steps to the protocol. The required additional process times due to more wash cycles would be only in a range of a few minutes per cycle.

Table 7: HCP reduction and mAb concentration for the five pooled elutions of the HGMS mAb purification process.

cycle	mAb conc. [mg/ml]	HCP conc. [ng/ml]	Log reduction value HCP	ppm
Start feed cycle 1-3	2.1	1700682		
Start feed cycle 4,5	2.5	1796024		
1	1.26	2034	2.7	1614
2	1.33	3232	2.52	2430
3	1.60	3323	2.59	2076
4	1.48	2819	2.63	1904
5	1.79	3504	2.62	1957

5.6.7 Overall Process Performance

Looking at the process as performed in this case study, an obvious disadvantage was a rather low mAb concentration in the elution fractions, which is about 60 % to 70 % of the mAb concentration in the feed. This dilution was mainly caused by the system setup and the applied process parameters. First, each elution step volume was fixed to one liter,

corresponding to one time the size of the separation chamber. In the presented process, three elution cycles were carried out leading to an elution batch of three liters. Second, maximal performance of the system has not been reached during the trials due to the limited amount of affinity beads available. Only 200 mL of MP were used, corresponding to less than 50 % of the working particle loading capacity of the separator. The maximum MP loading of the separator was approximately 600 mL for Mag Sepharose. In order to ensure optimal washing results, the working capacity should not exceed 80 % of the maximum loading capacity, which still would mean that more than double the amount of MP could be used resulting in at least a doubling of the product concentration. To demonstrate the advantages of the cGMP-compliant separator, a performance comparison of the HGMS purification process and a conventional column chromatography process is presented. The process data is based on the results of the case study as well as general rules for the design of magnetic protein purification processes presented by Franzreb et al. [124] and applied for the here presented separator by [125]. The maximum capacity of the used Protein A Mag Sepharose was determined to be 87 mg/ml, as described earlier. For the process performance calculations, a required binding yield of 95 % and a mAb start concentration of c_0 = 2.5 g/L were assumed resulting in a working capacity of Q^* = 58.8 mg/mL according to the measured isotherm. In comparison to our case study it should be noted that in order to optimize working capacity and productivity we reduced the aimed binding yield from practically 100% to 95%. With these numbers and a MP volume of 480 mL (80 % of the maximum capacity) a capacity ratio CR of 1.4 and in consequence a batch volume of 11.9 L can be determined. The estimated cycle time needed for this batch size includes the equilibration of the MP, particle loading to the separator, wash, elution, CIP, re-equilibration, and recovery of the MP, summing up to 35 min. The process time does not include the incubation, since this process step is performed in an external stirring vessel and does not occupy the separator. With this process, a mAb production of 28.32 g per cycle with a concentration factor of 4 and an overall process productivity of P = 1.72 mg mAb/ml beads * minutes is reached. For comparison of the HGMS process to a column process data from a MabSelect Sure capturing step by a ReadyToProcess™ product (GE Healthcare, Uppsala, Sweden) were used. Equal amounts of chromatographic material, start batch, and working capacity were assumed. Furthermore, a flow velocity of 500 cm/h and a bed height of 20 cm were given. The process time with the given volumes for equilibration, bind, wash, elution, CIP, and re-equilibration sum up to 1 h and 48 min, which results in the same production as in the HGMS process with a concentration factor of 4.9

and a process productivity for the capturing step of 0.58 mg mAb/ml beads * minutes. The used cycle time for the conventional process does not include the time necessary for clarification of the fermentation broth, which is already included in the HGMS process. Even without taking into account this extra time, the HGMS process is nearly three times more productive due to its short process step times and fast sorption times which are performed in an external vessel and therefore do not occupy the HGMS system.

5.7 Conclusion

The combination of commercially available Protein A Mag Sepharose with our GMP-compliant magnetic separator, which is now commercially available too, opens new possibilities in down-stream processing. The combination has been used to replace several traditional process operations in harvesting, such as centrifugation and filtration and column chromatography for capturing by one-unit operation without a reduction in performance. In the presented process, the purification of a mAb from CHO cell culture reached average yields of 87.7 % and purities above 95 %. Furthermore, robustness of the process has been proven over five consecutive process cycles with constant high yield and purity. The direct purification of an antibody from CHO cell culture allows implementation of this process as an ISPR method. Cost and time savings due to the absence of single-use filters and process times for clarification offers a unique advantage of the magnetic separation process. In addition, productivity calculations predict the HGMS process being three times as productive as a comparable column process, even without taking into account the saved time for clarification. On the other hand, the presented process shows slightly lower reduction levels for HCP compared to the conventional purification process. Furthermore, the mAb concentration in the eluate will reach only around 8 g/L and therefore about half the concentration of conventional protein A columns. However, to our opinion the advantages of omitting clarification and increasing productivity while achieving comparable yields and purities clearly prevail the stated small disadvantages.

Acknowledgement

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6 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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6.1 Abstract

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 (CIP) 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 (eCG) 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 contaminates by reliably meeting the 10 ppm criteria.

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

6.2 Introduction

Magnetic separation is known for many decades in biotechnological separation processes [8,64]. 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 [3,7,64]. Magnetic separation is a highly flexible technique that has been applied for various applications in biotechnology. Typically, magnetic separation is used for protein, enzyme or cell purification, recycling of immobilized enzymes, as well as cell labeling and sorting [65,66,69,70]. The use of MP is well established and routinely used in industry for analytical-scale processes [159]. For large-scale protein purification purposes magnetic separation is virtually unknown in industry although there have been most different applications and processes described in literature reaching from milliliter to 100 L scales [73,76,77,79,92]. 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 [125]. The device allows direct capturing and purification of MOI from crude feedstocks such as cultivation broth or natural sources such as blood or blood serum [21,72]. By the reduction of process steps, higher yields as well as time and cost savings are expected [160]. As an example for the advantages of HGMS processes the purification of the glycoprotein 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 [161–164]. 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 [78,94]. 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 [95]. 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.

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 represents another obstacle to a successful introduction of magnetic separation in industrial purification processes [73,93]. Cleanability – in the best case without disassembling but as automated routine in a CIP procedure – is an indispensable prerequisite for the industrial application of process equipment in biopharmaceutical industry and demanded by the FDA [126,130,134,135]. 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 [132,133]. 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 (API), if used as multiproduct equipment [139,140]. 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 high performance liquid chromatography or enzyme-linked immunosorbent assays (ELISA) also non-specific analytical methods gain importance [27,136]. Total organic carbon (TOC) analysis is simple to implement as it does not require the development of a dedicated method to identify specific contaminants. 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 [137,165,166].

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 [125]. 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.

6.3 Materials and Methods

6.3.1 equine Chorionic Gonadotropin 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 [167]. 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 pregnancy. 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 Langensalzer, Germany) according 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) JANUS™ workstation equipped with a Varispan™ 4 fixed tip arm (Perkin Elmer, Waltham, Massachusetts, 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.

6.3.2 '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 electro magnet. 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 alternatingly mounted to the hosing 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 (PEEK) elements were used. Fluid streams are moved by a hose 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 are 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 [125,168].

6.3.3 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 & Co. 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 were used. As cleaning solution 0.5 M sodium hydroxide solution (Carl Roth GmbH & Co. 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.

6.3.4 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 g/L – 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 µL. 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 mL and 15 mL total volume with consistent particle concentrations.

Long-term recycling studies were performed by the help of the LHS. 2.5 g/L MP were incubated with 200 µL 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.

6.3.5 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 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 23) 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.

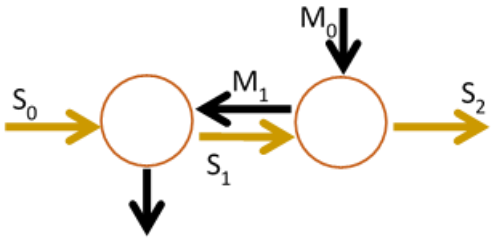

Figure 23: Schematic illustration of a counter-current batch adsorption process. The feedstock is pumped countercurrently to the magnetic particles. Fresh feedstock (S_0) is contacted with pre-loaded magnetic particles (M_1) and subsequently used in a second binding process as once used feedstock (S_1) 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.

6.3.6 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 [168]. 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.

6.3.7 Cleaning in Place 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. 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 (WFI).

6.3.8 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, United Kingdom). The TOC of the sample was oxidized and passed a CO_2 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 ± 2 %. The calibration was linear in a range from carbon free samples up to 1.5 ppm carbon.

6.3.9 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.

6.3.10 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. 200 µL of 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.

6.3.11 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. 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.

6.3.12 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 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.

6.4 Results and Discussion

6.4.1 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 and, despite their porous structure (Figure 24A). 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 [73]. 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 µL as well as 2 mL and 15 mL scales were observed, however besides the expected Langmuir isotherm shape with a steep slope at the beginning and saturation of binding capacity in case of higher equilibrium concentrations an unexpected feature shows. Even at high concentrations of adsorption particles applied, a fraction of around 20 % of the original eCG activity remains in solution (Figure 24B). 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. 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 processes due to the low number of separation stages. In a 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 fraction of 22 % non-binding eCG variant is substracted from all ELISA results of binding tests with serum, resulting in a corrected Langmuir isotherm which is shown in Figure 24B. 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.

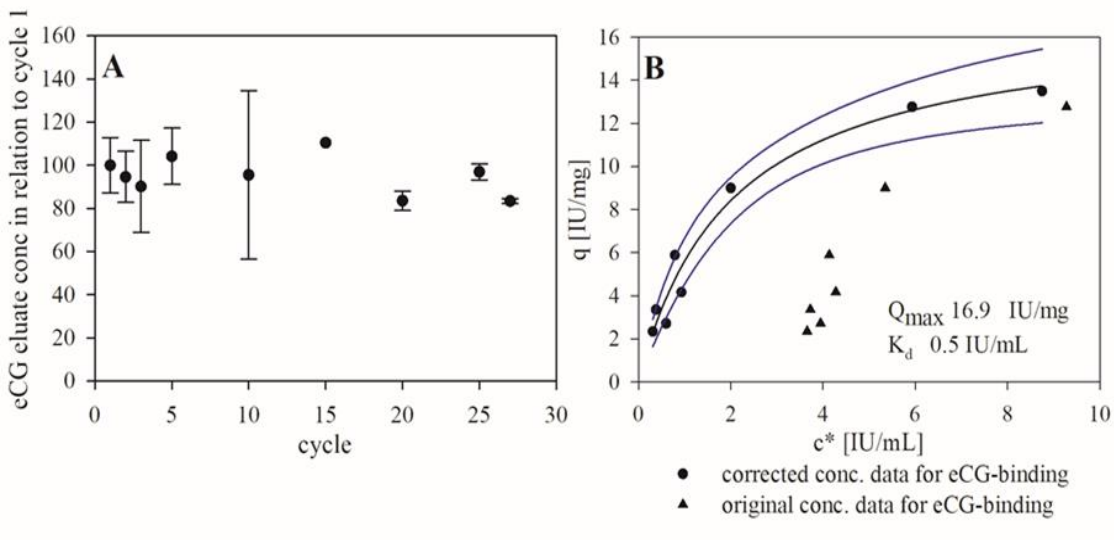

Figure 24: (A) eCG eluate concentrations in relation to the concentration determined in the eluate of the first cycle for 30 reuses of the particles. (B) Corrected and original concentration data from eCG binding studies to anti-eCG functionalized Mag Sepharose fitted with the Langmuir model. A maximum binding capacity of 16.9 IU/mL and a dissociation constant of 0.5 IU/mL were determined

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 trade-off 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 mL 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 25). 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 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.

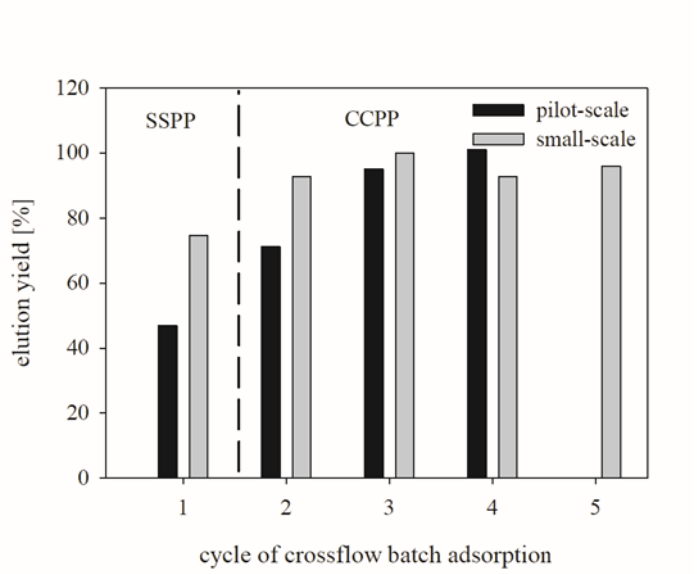

Figure 25: Yield of the eCG elution fractions of the four pilot-scale CCPP cycles is plotted in black. eCG elution yields of small-scale CCPP cycles are plotted in grey.

6.4.2 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 25 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 increases 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 an 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.

6.4.3 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. 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.

6.4.4 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 26A). 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 mg to 2 mg showed a linear correlation over the tested range (Figure 26B).

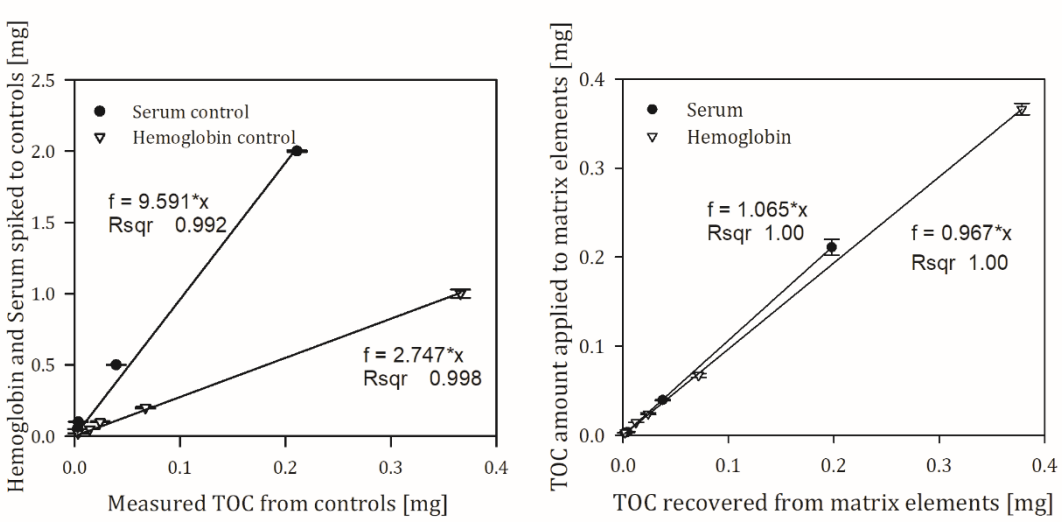

Figure 26: (A) Determination and validation of the accuracy of the chosen analytic for hemoglobin (inverted triangles) and serum (dots), y-axis: Hemoglobin and Serum amount applied to control, x-axis: Measured TOC after dissolving procedure. (B) Recovery efficiency of organic carbon from the matrix elements for hemoglobin (inverted triangles) and serum (dots), y-axis: TOC amount applied to matrix elements, x-axis: Measured TOC after swabbing and dissolving procedure.

6.4.5 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).

Table 8: 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	Hemoglobin				Horse serum			
Cleaning agent	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

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 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 off 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 contaminates 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 follows that the 10 ppm criterion is reached for all cases. Beyond that, the TOC content of all scenarios is below the allowed TOC level for WFI with 500 ppb which can be considered as an acceptable level, accept the TOC amount after the hemoglobin – COSA CIP 92 combination which was discussed already.

6.5 Conclusion

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 a 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 the whole range of industrial requirements.

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7 General Conclusions and Outlook

This doctoral thesis deals with the key aspects of a new GMP-compliant 'rotor-stator' high-gradient magnetic separator. After accompanying the development and construction of the new device, the commissioning, characterisation and validation were a main part of the work. System parameters like separation capacity, recovery rates and reusability of magnetic particles were studied and quantified. First process prediction studies based on these system parameters showed encouraging promising results which had to be verified by implementing a protein purification process. This was done by the successful implementation of a mAb purification process using protein A functionalized magnetic particles along with the new separation device. Furthermore, a specific bottleneck of magnetic separation processes was addressed with the optimization of the eCG purification from horse serum. Lower affinities between ligand and MOI lead to lower protein loadings on the particles due to the batch adsorption process. Developing a multi-stage counter-current process lead to a significant rise in yields. Finally, the development of CIP protocols and their validation was presented. The cleaning validation is a crucial factor in process equipment design and was demonstrated here for a magnetic separation device for the first time.

The validation of the cleaning process employed a surface swabbing test with a following TOC analysis. For both model contaminants (horse serum and haemoglobin) linear relationships between the concentration of the contaminant and the TOC measured laid the foundation for the analytic technique. The TOC analytics is recommended by the FDA and has been preferred to a HPLC analytics due to its simpler and timesaving development, faster sample turnover and lower detection limit. The handling of the samples is still a point requiring further optimization. To resolve the samples from the swab, plastic sampling tubes were used. TOC solved from these tubes might increase the base line level of TOC and consequently might result in an increase of the detection limit. Special pre-cleaned glass tubes will lower the blank and thus also the detection limit. In this study plastic tubes have been used due to storage conditions. Time between sampling and analytic demanded freezing of the samples. Nevertheless, the cleaning protocol was carried out successful.

Protein masses below 2 mg were detected for the whole product touching the surface of the separation device. Even lower amounts were achieved by applying pure sodium hydroxide solution as cleaning agent. This effect can be attributed to the complex nature of the commercial cleaning agents which also contain organic substances traces of which might show up in the TOC analytics. The successful cleaning validation proved the GMP conform design of the new HGMS device. Above all the separation functionality of the GMP design was not compromised. Franzreb et al. [73] defined key parameters a magnetic separation device should meet. Among others these include a separation capacity of at least 100 kg particles/m^3 chamber volume. This could be easily reached and exceeded with both types of magnetic particles tested, while maintaining separation efficiencies of 99.9 % and recovery rates above 98 %. The separation capacity experiments also reveal a high degree of independency of the filter capacity from the fluid phase. A viscous feedstock showed a slight reduction in separation capacity but no major impact. The choice of magnetic particle turned out to be quite significant. The small, highly magnetic Mag Prep particles showed lower filter capacities compared to the larger less magnetic M-PVA particles. In addition, the higher remanence and the back-mixing behaviour as well as the tendency to stick to surfaces of the smaller particles lead to more recovery cycles required to recover a sufficient amount of particles. Therefore, it is evident that the choice of particle is a decisive step for successful process development. Moreover, it has a major influence on the process performance of the presented magnetic separator. In addition to selecting the appropriate type of the magnetic particle the choice of the ligand binding the MOI is crucial. A major advantage of magnetic separation processes over ATPS systems is the availability of a large number of well described ligands omitting the need for expensive ligand screenings and developments. Nevertheless, the cost of magnetic particles is still high. However, it seems reasonable to expect that the increasing demand of magnetic particles for the implementation of large-scale purification processes will scale also the particle production and therefore drive down the costs.

The successful development and implementation of a mAb purification process from cell culture using protein A functionalized Mag Sepharose proved the robustness, scalability and efficiency of this technique as requested for a successful process alternative by Shukla et al. [28]. The presented integrated mAb purification process showed stable yields over 85 % in all five consecutive process cycles. Furthermore, purities over 95 % could be shown by SEC for all elution fractions. As one of the major impurities, a 2.5 log reduction of HCP was

proven. Even without elaborate process optimization the presented magnetic separation process delivers process figures comparable to a highly optimized mAb platform purification process. Purity and HCP reduction levels might need some further improvements in the future. This should be easily possible by a wash buffer screening and adding further wash step. A disadvantage that must be taken into account is the lower elution concentrations when comparing magnetic separation to column chromatography. The minimal elution volume corresponds with the separation chamber volume. Therefore, one liter is the smallest possible elution volume for the new magnetic separator. In the presented process three elution steps were performed resulting in an elution volume of three liters for the mAb process. However, 60 % of the mAb eluted in the first fraction. This percentage has to be increased by longer elution times, but even in this case the achievable degree of elution is limited by the respective equilibrium conditions. For higher eluate concentrations new elution strategies have to be developed such as the temporary transfer and packing of the particles into a small external column for elution.

The affinity purification of eCG from horse serum illustrated the disadvantage of batch adsorption processes when the binding affinity between ligand and MOI is not optimal. Lower binding affinities and low protein start concentrations leading to low protein loadings on the particles due to application of only one equilibrium stage. As a result, large amounts of particles or small batch volumes must be chosen for the process in order not to lose a substantial part of the product in the flow through. To partly overcome this challenge of batch wise processes a multi-stage counter-current binding process was applied and optimized. Due to the multiple use of magnetic particles in consecutive batch adsorption steps, more equilibrium stages could be realized and therefore more effective use of the binding capacity of the particles was made. However, the enhanced efficiency comes at the price of slightly longer process times. Due to fast binding kinetics in batch processes and nearly no limitation in flow velocities in the magnetic separation process longer process times are negligible in comparison with the gain of productivity.

The described magnetic separation process can compete in regard of purity and yield with modern column based purification processes. In addition, further advantages have not been taken into account yet. The integration of several process steps like harvest, capturing and purification into a single process step opens up new possibilities in process design. Process operations like centrifugation and filtration for product harvest can be skipped completely. Even an integration of the magnetic separation process in the fermentation process should

be possible. The reduction of the number of unit operations goes along with a reduction of product losses and investment cost. 25 % of the production cost in a mAb process are due to harvest process operations. Therefore, the integration of harvest into the protein A step represents a unique opportunity for massive cost savings. Furthermore, the reduction of process times will have an influence on the labour cost.

To summarize, this thesis discusses the opportunities a GMP-compliant high-gradient magnetic separator can offer in protein purification processes. For the first time ever a magnetic separation process, from design of the equipment over the process development until the cleaning in place and the cleaning validation, is described in one manuscript. This thesis demonstrates the possibility to integrate magnetic separation processes in biopharmaceutical production, which was previously prevented by the lack of suitable equipment conforming to GMP. However, this thesis can only lay a foundation, open up opportunities and arouse the interest of the industry. An industrial implementation is not possible within the university framework and it is now up to the industry to take up the idea of magnetic separation processes with the GMP-complaint equipment to develop new customized purification strategies.

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9 Appendix

9.1 Supporting Information to Section 4

In order to find the optimal working conditions with respect to maximum yield and purity various parameters can be applied to evaluate the performance of the new developed 'rotor-stator' high-gradient magnetic separator. First the dimension less capacity ratio CR can be calculated.

$$CR = \frac{m_p * Q_{max}}{c_0 * V_{batch}} \qquad 9.1$$

Where m_p is the mass of particles used, Q_{max} is the maximal loading, c_0 is the initial concentration of target molecules and V_{batch} is the volume of the batch. CR describes the ratio between the theoretical maximum amount of target molecules which can be bound by the MP mass used and the amount of target molecules provided in the actual batch volume.

In reverse, using a given CR of e.g. 120 %, eq. 1 can be transformed in order to calculate the maximum batch size processable in dependence of a limiting factor, such as the available amount of magnetic particles or the filtration capacity σ of the device:

$$V_{batch} = \frac{\sigma * V_{sep.device} * Q_{max}}{CR * c_0} \qquad 9.2$$

As described by Franzreb et al. the yield increases with increasing CR values. To reach yields higher than 90 % it can be necessary to use larger amounts of particles than expected if the simple adoption C = 1 is assumed. The final yield Y of the process depends on the ratio of the starting concentration c_0 of the target protein and the concentration of the target molecule in solution after the binding equilibrium was reached c*.

$$Y = 1 - \frac{c^*}{c_0} \qquad 9.3$$

With

$$\frac{c^*}{c_0} = \frac{1}{2} * \left[1 - \frac{K_D}{c_0} - CR + \sqrt{4 * \frac{K_D}{c_0} + \left(CR - 1 + \frac{K_D}{c_0}\right)^2}\right] \qquad 9.4$$

Eq. 9.4 assumes that a simple Langmuir model $Q^* = \frac{Q_{max} * c^*}{K_D + c^*}$ is used to describe the batch adsorption of the target protein onto the MP, including the Langmuir parameters Q_{max} and K_D. Besides yield, the productivity of the separation device is of interest and can be defined as:

$$P = \frac{m_{target}}{t_{cycle} * V_{sep.device}} \qquad 9.5$$

Where m_{target} describes the mass of purified target molecules, $V_{sep.device}$ is the volume of the separation chamber, and t_{cycle} is the required time for the process including the loading time as well as the operation time of the separator for the purification steps including washing, elution, cleaning of the particles, equilibration and recovery. While the loading time of the MP suspension into the separator is linearly dependent onto its initial batch volume, the time needed for the remaining process steps is constant.

9.2 Abbreviations

Abbreviation	Meaning
AEC	Anion exchange chromatography
API	Active pharmaceutical ingredients
ASME	American society of mechanical engineers
ATPS	Aqueous two phase system
CCPP	Counter-current purification process
CEX	Cation exchange chromatography
CHO	Chinese hamster ovary
CIP	Cleaning in place
COP	Cleaning out of place
DSP	Down-stream process
eCG	Equine chorionic gonadotropin
EBA	Expanded bed adsorption
EHEDG	European hygienic engineering and design group
ELISA	Enzyme-linked immunosorbent assays
ESEM	Environmental scanning electron microscope
FDA	Food and drug administration
GMP/cGMP	Good manufacturing practice
HCP	Host cell protein
HGMF	High-gradient magnetic fishing
HGMS	High-gradient magnetic separation
HIC	Hydrophobic interaction chromatography
ISPR	*In situ* product removal
LHS	Liquid handling station
mAb	Monoclonal antibody
MOI	Molecule of interest
MP	Magnetic particles
M-PVA	Magnetic polyvinyl alcohol
PEEK	polyetheretherketone
SEM	Secondary electron microscope
SIP	Sterilization in place
SSPP	Single step purification process
TOC	Total organic c

USP	Up-stream process
WFI	Water for injection

9.3 Publications and Patents

J. Kittelmann, F. Hammerling, M. Ebeler, J. Hubbuch, *Light extinction and scattering by agarose based resin beads and applications in high-throughput screening*. J. Chromatogr. A. 1397 (2015) 52–58. doi:10.1016/j.chroma.2015.04.013.

M. Ebeler, F. Pilgram, K. Wolz, G. Grim, M. Franzreb, *Magnetic Separation on a New Level: Characterization and Performance Prediction of a cGMP Compliant "Rotor-Stator" High-Gradient Magnetic Separator*, Biotechnol. J. (2017) 1700448. doi:10.1002/biot.201700448.

M. Ebeler, O. Lind, N. Norrman, R. Palmgren, M. Franzreb, *One-step integrated clarification and purification of a monoclonal antibody using Protein A Mag Sepharose beads and a cGMP-compliant high-gradient magnetic separator*, N. Biotechnol. 42 (2018) 48–55. doi:10.1016/j.nbt.2018.02.007

M. Ebeler, T. Wellhöfer, K. Frankenfeld, M. Franzreb, *First Comprehensive View on a Magnetic Separation based Protein Purification Processes: from Process development to Cleaning Validation of a GMP-ready Magnetic Separator.* J. Chromatogr. A.(submitted)

M. Franzreb, M. Ebeler, A. Tschöpe, *Vorrichtung und Verfahren zur selektiven Fraktionierung von Feinstpartikeln* (patent application)

9.3.1 Conference Contributions

Ebeler, M; Pilgram, F; Franzreb, M. *Development of a "fit-for-purpose" cGMP compliant magnetic separation device* (talk and poster award). Dechema Himmelfahrtstagung*:* New Frontiers for Biotech-Processes (Mai 2016, Koblenz)

Ebeler, M; Franzreb, M. *A 'fit-for-purpose' cGMP compliant magnetic separation device: Cleaning validation and first protein purification results* (talk). European Symposium on Biochemical Engineering Sciences (ESBES) (September 2016, Dublin, Ireland)

Ebeler, M; Franzreb, M. *Magnetic separation on a new level: first cGMP compliant magnetic separation device* (talk). European Congress on Biotechnology (ecb) (July 2016, Krakow, Poland)

Ebeler, M; Franzreb, M. *"Fit-for-purpose" cGMP compliant magnetic separation device: Cleaning validation and protein purification results* (talk). ACS 253rd National Meeting & Exposition (April 2017, San Francisco, CA, US)