Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

Role of Cleaning-in-Place in the Purification of mAb Supernatants Using Continuous Cation Exchange Chromatography

Thomas Müller-Späth[a]; Lars Aumann[ab]; Massimo Morbidelli[a]
[a] ETH Zurich, Institute for Chemical and Bioengineering, Department of Chemistry and Applied Biosciences, Zurich, Switzerland [b] ChromaCon AG, Zurich, Switzerland

To cite this Article Müller-Späth, Thomas , Aumann, Lars and Morbidelli, Massimo(2009) 'Role of Cleaning-in-Place in the Purification of mAb Supernatants Using Continuous Cation Exchange Chromatography', Separation Science and Technology, 44: 1, 1 — 26
To link to this Article: DOI: 10.1080/01496390802581243
URL: http://dx.doi.org/10.1080/01496390802581243

Separation Science and Technology, 44: 1–26, 2009

ISSN: 0149-6395 print/1520-5754 online
DOI: 10.1080/01496390802581243

Taylor & Francis
Taylor & Francis Group

Role of Cleaning-in-Place in the Purification of mAb Supernatants Using Continuous Cation Exchange Chromatography

Thomas Müller-Späth,[1] Lars Aumann,[1,2] and Massimo Morbidelli[1]
[1]ETH Zurich, Institute for Chemical and Bioengineering, Department of Chemistry and Applied Biosciences, Zurich, Switzerland
[2]ChromaCon AG, Zurich, Switzerland

Abstract: Monoclonal antibodies (mAbs) are among the most important therapeutic proteins for the treatment of cancer. They also find wide application in the field of diagnostics. The market for mAbs was US$ 5.4 billion in 2002 and is expected to triple by 2010 (1). MAbs are mainly produced in cell culture and purified by chromatography. Apart from the mAb, the downstream processing is confronted with a multitude of impurities from the upstream process. Some of these impurities bind strongly to the chromatographic stationary phase and can only be removed by separate cleaning solutions. Depending on the type of media used, e.g. affinity or ion exchange, differing cleaning regimes may have to be applied. In addition, sanitization is required to prevent microbial contamination. Both steps are closely tied to the mAb production step and need to be repeated in regular intervals.

In this work, the impact of irreversible impurity adsorption on the separation performance is investigated experimentally; first for a batch column and then for a continuous chromatographic process. At first, the retention time of a model substance is established as a suitable measure to describe the degree of irreversible adsorption. The impact of the presence and the absence of cleaning-in-place (CIP) on the retention time of the model substance is demonstrated.

Since the issue of column cleaning is particularly important if processes are operated in continuous mode, in the second part of this work the introduction

Received 13 April 2008; accepted 21 August 2008.
Address correspondence to Prof. Massimo Morbidelli, Institute for Chemical and Bioengineering, ETH Zurich, Wolfgang-Pauli-Str. 10/HCIF 129, 8093 Zurich, Switzerland. Tel.: ++41-44-632304//++41-44-6323033; Fax: ++41-44-6321082. E-mail: massimo.morbidelli@chem.ethz.ch

of a CIP step in the continuous multicolumn countercurrent solvent gradient purification (MCSGP) process is investigated. The process purifies a mAb from a cell culture supernatant that also contains irreversibly adsorbing impurities—or impurities that desorb in a later cycle of the process as a contamination. Following the same approach as for the batch experiments, the MCSGP process is operated in the presence and the absence of CIP in order to track prospective changes of the process performance caused by changes in the product retention time. Instead of the model substance, a cell culture supernatant, providing a real case impurity profile in an industrial application is used for the process. Although an impact of the product retention time change on the process yield is not observed it is shown that CIP is required for a stable long-term operation in terms of purity and system pressure drop. With the introduction of a CIP step the process was successfully operated for 9000 min (6 days) without interruption.

Keywords: Cation exchange chromatography, CIP, cleaning-in-place, mAb purification, MCSGP, monoclonal antibody, multicolumn countercurrent solvent gradient purification, SMB

INTRODUCTION

Irreversible Adsorption, Column Cleaning, and Sanitization

In the production of biopharmaceuticals, cell culture is widely used. In addition to the product, the clarified cell culture supernatant contains a large number of impurities, such as Host Cell Proteins (HCP), DNA, and media components. Some impurities are product-similar in their adsorption behavior on chromatographic materials, others have totally different adsorptive properties but interfere with the adsorption of the product on the stationary phase, e.g. by competition, accumulation, or deposition on surfaces (fouling). Some impurities are adsorbing very strongly, so that they are not removed from the stationary phase by any combination of the weak and the strong buffer used in gradient chromatography. In the following, these impurities are referred to as "irreversibly" adsorbing components. Non-specific interactions between the impurities and the stationary phase matrix may be responsible for the exceptionally strong binding. The adsorption is not limited to the stationary phase; also the frits or filters of the column may experience irreversible adsorption, precipitate formation, and fouling. An additional step using a suitable cleaning solution is required for the removal of irreversibly adsorbing impurities and for cleaning of the frits or filters.

In ion-exchange chromatography, sodium hydroxide solution is an accepted and very effective cleaning agent. At concentrations of 1 M, it is able to hydrolyze peptide bonds (2). In addition, sodium hydroxide

is also very effective for sanitization of the equipment, i.e. for reduction of microbial load even at low concentrations (3,4). A long residence time is required for sanitization. Depending on the bacterial strain different concentrations and contact times are required (4).

The cleaning effect in cation exchange chromatography through sodium hydroxide is largely due to the generation of negative charges on bound protein. This leads to the induction of strong repulsive forces between the negatively charged ligands and the impurities upon contact with NaOH. Consequently, the residence time of sodium hydroxide solution in the system is usually of minor importance, for cleaning. For industrial processes, regulatory agencies require cleaning and sanitization of all equipment in contact with the biopharmaceutical product in regular intervals. Cleaning and sanitization have become an integral part of good manufacturing practice (GMP) (5). On the other hand, harsh cleaning conditions can lead to changes of stationary phase ligand and matrix properties. For stationary phase manufacturers it is therefore important to provide resins with high chemical stability of matrices, linkers and ligands. Polymer based materials with simple ligands, such as ion exchange resins, have proven to fulfill these conditions while protein-based affinity materials are in general more susceptive against harsh chemical environments (4,6) resulting in the leakage of the proteinaceous ligand as well as in long term reduced capacity. The established affinity materials used in industry for the capture of mAbs from clarified cell culture supernatant ("cCCS") feature Protein A ligands that are incompatible with sodium hydroxide solutions >0.1 M for cleaning and cannot be sanitized with NaOH. For these materials other more expensive agents such as concentrated guanidine hydrochloride solution have to be used (7,8,9) for cleaning. Recently, stationary phases with modified alkaline-resistant forms of Protein A have become available, however, at a higher cost than the earlier materials (10). Still, also the modified Protein A material may experience a loss of dynamic binding capacity under sanitization conditions represented by a prolonged exposure to NaOH solutions (10).

Independently of irreversible adsorption, it is worth noting that the dynamic capacity of the target compound is highly dependent on the feedstock. It has been shown that through competition with cell culture broth components, the dynamic binding capacity of a mAb on an ion-exchange resin was two orders of magnitude lower than the capacity for the pure component (11).

In this work, experiments showing the effect of irreversible adsorption on the performance of batch and continuous chromatographic processes are reported. Irreversible adsorption is a well known effect in chromatographic applications and also the effectiveness of impurity removal through CIP was shown in earlier work with respect to the

dynamic binding capacity (12,13). However, the effect on the retention behavior has not been described yet. In this work, at first retention time measurements were carried out for pulse injections of a model substance in order to track potential changes of the adsorption characteristics for different impurity loads on a cation-exchange column. The irreversibility was visualized using a colored model impurity and transparent columns. The retention time proved to be a suitable indicator for the degree of irreversible adsorption. Retention time measurements have the advantage of fast experimental realization and low material consumption.

A change of the product retention time is expected to have major impact on the performance not only of batch column processes but also of multicolumn continuous processes. The second part of this work deals with the effect of irreversible adsorption on the performance of the MCSGP process in the presence and absence of CIP. Rather than using a model impurity and a trivial purification problem, the experiments are carried out using a clarified cell culture supernatant (cCCS) with a high impurity concentration. Some of the impurities are also expected to bind irreversibly and to have adverse effects on the product retention time and the process performance with increasing accumulation. It is worth noting that, for continuous processes that also incorporate continuous loading, a high product retention factor is more important than a high dynamic capacity: For a Langmuir-type adsorption behavior, which is frequently observed for biomolecules, a low dynamic capacity can be compensated for to a certain extent by changing the operating parameters of the process (switch time) while a low retention factor usually entails a lower product purity. We assume that a cation exchange stationary phase A delivers larger retention factors than material B as a function of the ionic strength (salt concentration) and at a given pH and as a function of the pH at constant ionic strength. This entails that material A can deliver the same retention factor for an antibody but with a pH closer to the pI of the mAb than stationary phase B. Consequently, material A is expected to deliver material of a higher purity when identical buffer and elution conditions (ionic strength) are used. A prerequisite is that the mass transfer properties of the materials are comparable.

MCSGP for Biomolecule Separation

Representing a combination of batch and continuous chromatography, MCSGP (14,15) offers the flexibility of the batch process with respect to gradient design and fractionation. It maintains the efficient stationary phase utilization and therefore the high performance of continuous countercurrent processes such as simulated moving bed (SMB) chromatography (14).

For SMB, CIP was proven to work for the separation of nucleosides and plasmid DNA (16,17). However, designed for two-fraction separations and a single-step gradient, SMB is only of limited use for the purification of biomolecules from a mixture of impurities with similar adsorptive properties. Most SMB applications are in the field of chiral small molecule separations and use feed mixtures with a low impurity load. In addition, mobile phases containing organic solvents are frequently used. Both factors render CIP unnecessary.

MCSGP has proven to be a suitable process for the purification of peptides and proteins where its main advantages, the countercurrent movement of stationary and mobile phase, the continuous production of three fractions and solvent gradient chromatography are best exploited. MCSGP has so far been successfully applied for the purification of the polypeptide calcitonin using reversed phase chromatography (15) and the separation of monoclonal antibody lysine variants using a cation exchange material (18). Also the capture of a monoclonal antibody (mAb) from clarified cell culture supernatant (cCCS) using a three-column MCSGP process has been outlined (14). In the former two separation problems in the product was to be purified from a mixture of very similar components. In the case of the peptide this meant the separation of similar peptides generated by chemical synthesis while for the antibody variants, the molecules differed in only two amino acids. In those cases the product was the main component and present in substantial amounts in the feedstock. Thus the problems treated so far were rather comparable to polishing steps in the downstream purification scheme, requiring no CIP. The capture and purification of a strongly diluted target compound from crude material produced by cell culture techniques poses a new challenge for the MCSGP process. So far, the proof-of-principle for the capture of a mAb from cCCS has been given for the start-up of the MCSGP process without CIP and the achievement of a steady-state with 95% yield (14) but the stable long-term operation with CIP has not been described, which is made up for in this work.

One of the main advantages of MCSGP lies in the fact that it uses linear solvent gradients which are very beneficial in the separation of biomolecules: As mentioned earlier, considering the large number of impurities, the probability is very high that some of the impurities show a retention behavior very similar to that of the desired product on the given stationary phase. It is therefore difficult to separate these components with satisfactory purity using step gradient elutions. Isocratic elutions are better suited to separate these components but the run time needs to be increased leading to a low productivity and a high dilution of the product which makes this technique rather unattractive for large

scale production. Solvent gradient chromatography represents the intermediate state between the two extremes of step gradient elution (gradient slope is infinite) and isocratic elution (gradient slope is zero). With solvent gradient technology it is possible to decrease the run time by decreasing the product retention time, while maintaining a high purity. It is important for gradient optimization that the design of the process is backed up by a suitable mathematical model.

The Separation Problem for MCSGP

The feedstock for the MCSGP process investigated in this study was a cCCS containing mAb in low concentration. Main contaminants in the feed were HCP and DNA. An analysis of a fractionated batch gradient elution on the ion exchange material used in this study showed that HCP and DNA, representing mixtures of impurities themselves, eluted over the whole gradient length with a tendency to early elution (HCP) and late elution (DNA). Thus, with respect to the mAb, HCP and DNA represent both weakly and strongly adsorbing impurities.

The mAb purification target was the capture with a yield of >90% and a reduction of the HCP content of at least 2 orders of magnitude (logs), which represent the minimum values expected for a mAb-capture process in industrial applications. A significant reduction of the DNA-level was also desired.

In order to improve the understanding of the MCSGP process performance, some perfomance parameters are defined at this point:

The process yield Y generally refers to the product outlet and is defined as follows (equation 1):

$$Y = \frac{\dot{m}_3}{\dot{m}_{Feed}} \tag{1}$$

In this equation, $\dot{m}_3$ and $\dot{m}_{Feed}$ denote the mass flow of mAb exiting the process through the product outlet and entering the system through the feed inlet, respectively.

The measure for the product purity P is given by equation 2,

$$P = \frac{c_{imp}}{c_{mAb}} \tag{2}$$

Where c_{imp} and c_{mAb} stand for the product outlet concentrations of impurity and mAb, respectively. In this study, the impurities assessed where DNA and HCP.

MATERIALS AND METHODS

Stationary and Mobile Phases

As stationary phase for batch and continuous experiments, the polymer-based strong cation exchange resin Fractogel EMD HiCap SE (Merck Darmstadt, Germany) was used. This material has been shown to be compatible with 1 M sodium hydroxide as cleaning and sanitization agent by measuring the dynamic binding capacity of a mAb over more than 100 cycles (19).

For batch column and MCSGP experiments, five 2 mL Tricorn 0.5 i.D. × 10 cm columns (GE Healthcare, Uppsala, Sweden) with transparent walls were flow-packed at 800 cm/h.

Four Tricorn columns were used in the MCSGP process. The process was set up with suitably combined commercial equipment (ÄKTA series, GE Healthcare), namely 8-way multiposition valves and pH, conductivity, and UV detectors (pH/C 900 and UV-900) as described in (15). The UV-signal was calibrated with a Gammanorm standard (Octapharma, Germany).

For all batch column experiments, Acetate buffers, 20 mM, pH 5.0 were used. The elution buffer contained 2 M NaCl (buffer B), while the binding buffer (buffer A) contained no NaCl. The ionic strength of the strong buffer was chosen high in order to improve desorption through washes with this buffer.

For all preparative MCSGP runs, acetate buffers, 20 mM, pH 5.0 were used with the elution buffer (buffer C) containing 1 M NaCl while the binding buffer (buffer A) contained no NaCl and was identical to the one used in batch experiments. All buffers were filtered and degassed.

For CIP, sodium hydroxide solution was used (0.1 M in the case of myoglobin and 0.4 M in the case of the cCCS). The stationary phase manufacturer recommends cleaning with 0.1 to 0.5 M NaOH. The removal of myoglobin was tested at NaOH concentration of 0.1 M, 0.25 M, and 0.5 M. It was found that 0.1 M NaOH was most efficient in removing myoglobin from the stationary phase. For the continuous mAb purification process, it was decided to clean the columns with a NaOH solution close to the highest possible concentration recommended by the manufacturer. It was expected that cleaning with this concentration was most effective for the removal of irreversibly bound impurities.

Feedstock

The feedstock to test irreversible adsorption and CIP in batch mode was a myoglobin solution of 1 g/L in 20 mM Acetate-Buffer (buffer A), pH 5,

which gives a clear brown solution. The feedstock for the MCSGP experiments was a clarified chinese hamster ovary (CHO) cell culture supernatant obtained from Excellgene (Lausanne, Switzerland) in the frame of the AIMS project (20). The mAb concentration was c = 0.08 g/L. The mAb exhibited a broad pI range starting at pH 7.5 and reaching beyond pH 9.3, as determined using a Phast System (GE Healthcare, Uppsala, Sweden). A main variant could not be distinguished clearly but the maximum of the intensity of the distribution was around pH 8.5. The pH of the supernatant was 7.3 and the conductivity was 19 mS/cm. This represents non-adsorbing conditions for the mAb on the selected stationary phase. A 2-fold dilution of the feedstock with water was done prior to loading in order to shift to binding conditions. The pH of the diluted supernatant was 7.3 and the conductivity was 10 mS/cm. The supernatant contained 220000 ng HCP/mg mAb and 3200 ng DNA/mg mAb. In order to prevent microbial growth in the supernatant, sodium azide was added with a concentration of 0.5 g/L. For all experiments described in this work, material from the same fermentation batch was used.

Analytics

The process purity with respect to DNA and HCP was not accessible by chromatographic methods since the concentration was either close to or below the detection limit of the UV detectors of the HPLC equipment and, in the former case, the resolution did not allow for the identification of single impurities. This was partially due to the low concentration of HCP and DNA present in the feedstock, but also due to the fact that these impurities actually represent multi-component mixtures themselves. The situation does not change when these impurities are concentrated by ultrafiltration and subsequently run over an analytical column. The purity with respect to HCP was measured with a CHO cell line specific enzyme linked immuno sorbent assay (HCP-ELISA Kit #015, Cygnus Technologies, Southport, NC, USA). The DNA concentration was determined with a fluorescence assay (Quant–iT DNA Assay Kit, High Sensitivity, Invitrogen, Carlsbad, CT, USA).

The quantification of the mAb was done using a Poros A20 analytical Protein A column (Applied Biosystems, Foster City, CA, USA). The binding buffer was 20 mM Phosphate, 0.1 M NaCl, pH 7.0; the elution buffer was 0.1 M Citric Acid adjusted to pH 3.5 with NaOH.

The MCSGP product outlet was sampled for offline analyses. The product outlet and the CIP outlet were equipped with UV-detectors, pH and conductivity probes (ÄKTA UV-900 and pH/C-900, GE

Healthcare, Uppsala, Sweden) for online measurement. Column quality assessment was done through analytical injections and retention time measurements of purified mAb and lysozyme under adsorbing and non-adsorbing isocratic conditions. The presence of charged isoforms of the mAb complicated the determination of the retention time under adsorbing conditions, since some of the mAb isoforms eluted while others were fully retained. Consequently, the column could not be properly characterized using the mAb and the results presented here focus on lysozyme as model substance. It is worth mentioning that, under gradient elution conditions, despite the presence of isoforms, the mAb elutes as a single albeit broader peak.

Single Column Experiments

The purpose of the single column experiments was to show the existence and impact of irreversible adsorption and CIP, respectively, on the product retention for the given buffer/resin system. Myoglobin was used as irreversibly adsorbing model impurity and lysozyme as product. The strategy was to repeat lysozyme retention time measurements for increasing loads of myoglobin and after a final CIP step. Afterwards the Henry coefficient was to be calculated from the retention time data and to be qualified as measure for column quality as a function of the impurity load. All single column experiments were carried out on an Agilent HP1100 series (Agilent, Santa Clara, CA, USA) at 25°C.

A Tricorn column (GE Healthcare, Uppsala, Sweden) with Fractogel HiCap as stationary phase was loaded with 1 g/L myoglobin solution for 50 min at 1 mL/min. Afterwards, the column was alternatingly washed with 100% buffer A and 100% B, for 6 min respectively at 1 mL/min (3 column volumes respectively) in an attempt to remove the myoglobin. The washing was repeated twice. Afterwards the column performance was checked through retention time measurements with lysozyme in buffer A (1 g/L) using analytical injections under isocratic non-adsorbing and adsorbing conditions at 80% B, 60% B, 40% B, 30% B, 25% B, 20% B, and 18% B. The measurements were duplicated.

Subsequently, the column was re-equilibrated with buffer A and additionally 50 mL of myoglobin solution was loaded and the performance tested after washing as described above. In an independent experiment using the column and the flow rate described above the dynamic binding capacity (1%) for myoglobin on Fractogel HiCap was determined to be 116 mg myoglobin/mL column.

After the final wash, the column was reverse-flushed with 0.1 M NaOH at a flow rate of 0.2 mL/min for 30 min, which corresponds to 3 column

volumes. The column was then re-equilibrated with buffer A and the same analysis as before carried out. Summarizing, the lysozyme retention times were measured with a preload of 0, 25 and 50 mg myoglobin/mL column and after cleaning and a prospective complete removal of the myoglobin. The corresponding Henry coefficients were calculated.

Design and Setup of the MCSGP Process

In order to facilitate the understanding of how a CIP-step is realized in the MCSGP process, the basic concept of the process is described here.

A flowchart of the MCSGP process is shown in Fig. 1 including the different tasks (1–8) of the various columns in the process along with the adjustable operating parameters. These are 8 flow rates Q_1–Q_4, Q_{Feed}, Q_6, Q_{CIP}, Q_{equil}, 8 concentrations c_1–c_4, c_{Feed}, c_6, c_{CIP}, c_{equi} and the switch times t_{CC} and t_{BL}. The operating parameters were derived from a linear batch gradient elution. The design procedure of MCSGP to determine the parameters has been described elsewhere in detail for 6- and 3-column systems (15,21). The operating parameters are given in Table 1. As a hybrid process between continuous and batch chromatography, the process comprises of internal recycling states where the columns of the system

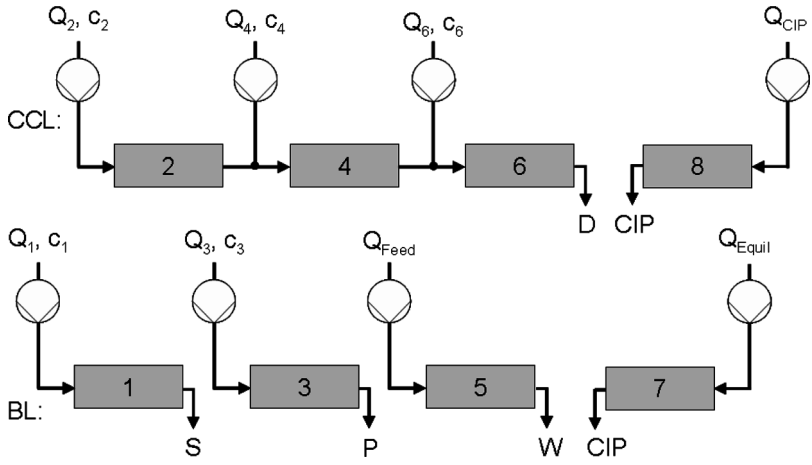

Figure 1. Schematic of an MCSGP process with CIP section including all operating parameters and separation tasks. The latter ones are indicated by the numbers in the columns (see text). The tasks of the interconnected state CCL are shown in the first row; the tasks of the batch state BL are shown in the second row. The pumps, delivering Q_1–Q_4 and Q_6 are able to provide linear gradients, thus the concentrations c_1–c_4 and c_6 are variable over the switch time.

Table 1. Operating parameters for the long duration MCSGP experiment: Percentages given with respect to buffer C. Suffixes: * = reverse flow, a = diluted supernatant feed solution, b = 0.4 M NaOH

t_{CC}	[min]	20
t_{BL}	[min]	3
Q_2	[mL/min]	0.15
Q_1	[mL/min]	1
Q_4	[mL/min]	0.85
Q_3	[mL/min]	0.3
Q_6	[mL/min]	0.0
Q_{Feed}	[mL/min]	1.25
Q_8 (CIP)	[mL/min]	0.2*
Q_7 (equil)	[mL/min]	0.3*
Grad 2	[%]	40.0–43.1
Grad 1	[%]	70.0
Grad 4	[%]	0.2–39.5
Grad 3	[%]	40.0
Grad 6	[%]	0.0
Grad 5	[%]	100.0[a]
Grad 8	[%]	100.0[b]
Grad 7	[%]	70

are interconnected and production states where the columns are operated in batch mode (15).

The three fraction separation and the capability of running solvent gradients in each section of the MCSGP process are made possible through the switching between these states. A separation cycle consists of six different tasks, where three tasks are attributed to the interconnected state (2,4,6) and three to the batch state (1,3,5) (Fig. 1). The batch state tasks are: (1) Elution of the strongly adsorbing impurities S, (3) elution of the product P, (5) loading and elution of the weakly adsorbing impurities W. The interconnected state tasks are: (2) Internal recycling of a portion of the product contaminated with strongly adsorbing impurity, (4) internal recycling of the portion of the product contaminated with weakly adsorbing impurity and (6) re-equilibration of the system. The columns of the system execute these tasks in sequence whereby (1) is followed by (6). The process can use one or multiple columns per section. In this work, the minimal configuration of the continuous process using one column per section was used. While in a 6-column setup, all tasks are performed simultaneously, in a 3-column process, the interconnected state and the batch state tasks are performed sequentially (21). Therefore,

the switch time for the batch state (t_{BL}) and the interconnected state (t_{CC}) can be chosen to be different in the 3–column process while in a 6-column setup the switch times are identical ($t^* = t_{CC} = t_{BL}$), forced by the process design. The MCSGP process offers a large flexibility since further tasks can be easily added to the process. In this study, two further sections were added, namely (7) re-equilibration from Cleaning-in-Place (CIP) and (8) CIP. This corresponds to the addition of one (two) columns to the 3-(6-) column process. Clearly, it is most convenient to place the CIP step after section 1, where the column has been stripped with the strong buffer. The process used in this study, shown in Fig. 1, features 4 columns that perform the tasks of CCL (2,4,6,8) and BL (1,3,5,7) in sequence.

A challenge for the process design is posed by the fact that the switching times of the separation cycle are much shorter than the time period required for CIP and sanitization respectively. Consequently, a section that has entered the CIP mode needs to stay in that mode for longer than one switch. At the same time, the remaining sections need to continue in performing the separation tasks (6)–(1). It is necessary that the column entering the process from the CIP section is devoid of the cleaning agent and equilibrated as the column from section 1 that would regularly enter section 6.

A schematic of the separation and CIP tasks is given in Fig. 2. To get used to the scheme the reader should first focus on the separation cycle represented by a bold rectangular frame (columns 1, 2, 3). This sub-scheme shows the regular separation cycle that would be repeated continuously if the process would be run as a three-column system. The numbers indicate the separation (1–6) or CIP task (7,8) that each chromatographic column is performing at the time (cumulative) given in the sixth column and that correspond to Fig. 1. The fifth column of the scheme shows the duration of the corresponding state (interconnected state). The bold dotted frame represents a CIP subcycle. During this subcycle one column is sanitized (here column 1 in Fig. 2). For the purpose of synchronization of CIP and separation it is necessary to add a synchronization step for the columns that are not in CIP mode. However, during the synchronization, the separation continues so there is no impact on the productivity, i.e. the mass of product purified per time and stationary phase volume. The full scheme in Fig. 2 represents the complete CIP-cycle where every column is sanitized once. The scheme can be modified in such a way that one column remains in the CIP mode for even longer while the others perform the separation.

The separation process was designed based on a batch gradient elution as described in (15) using the same column, buffers and starting material as the MCSGP process. The chosen operating parameters are given in Table 1.

Column 1 section	Column 2 section	Column 3 section	Column 4 section	time, switch [min]	time, cumul. [min]
2	4	6	8	20	20
1	3	5	8	3	23
6	2	4	7	20	43
5	1	3	7	3	46
4	6	2	7	20	66
3	5	1	7	3	69
2	4	6	7	20	89
1	3	5	7	3	92
8	2	4	6	20	112
8	1	3	5	3	115
7	6	2	4	20	135
7	5	1	3	3	138
7	4	6	2	20	158
7	3	5	1	3	161
7	2	4	6	20	181
7	1	3	5	3	184
6	8	2	4	20	204
5	8	1	3	3	207
4	7	6	2	20	227
3	7	5	1	3	230
2	7	4	6	20	250
1	7	3	5	3	253
6	7	2	4	20	273
5	7	1	3	3	276
4	6	8	2	20	296
3	5	8	1	3	299
2	4	7	6	20	319
1	3	7	5	3	322
6	2	7	4	20	342
5	1	7	3	3	345
4	6	7	2	20	365
3	5	7	1	3	368

Figure 2. Switching of the sections for a 4 column MCSGP process with CIP. The minimal CIP cycle, where each chromatography column is cleaned once, is shown. The bold dashed frame represents a CIP subcycle, i.e. the interval of the cleaning of one chromatography column, here column 1. The CIP tasks are highlighted dark grey. The bold solid frame represents a separation subcycle (light grey). Separation tasks performed for the synchronization of the CIP and the separation subcycle are indicated by a white background. The numbers in column 1–4 of the scheme stand for the task (see Figure 1) that each chromatography column is performing starting at the time given in the last column of the scheme. The fifth column of the scheme gives the duration of the respective state (interconnected or batch state).

The CIP step consisted of backflushing of the column at a flow rate of 0.2 mL/min with sodium hydroxide solution for one interconnected and one batch phase ($1(t_{CC} + t_{BL}) = 23$ min), followed by a re-equilibration with 70% buffer C at a flow rate of 0.3 mL/min for three interconnected and three batch states in a row ($3(t_{CC} + t_{BL}) = 69$ min). Thus the total time for one CIP cycle was 92 min ($4(t_{CC} + t_{BL})$). The supply of 70% high-salt buffer C

was required in order to maintain the equivalence between the process with and without CIP in terms of modifier concentrations of the sections that perform the separation. This means that a column entering section 6 from the CIP section needs to contain the salt concentration profile that the column would have after the elution of strongly adsorbing impurities in section 1 without CIP.

Operation of the MCSGP Process

In a first experiment, in order to demonstrate the effect of CIP as in the batch experiment, a run was performed with the MCSGP unit that used Acetate buffer A with 1 g/L myoglobin (pH 5.0) as feed solution. In a second experiment, the process was run with the clarified cell culture supernatant as feedstock. The aim of the experiment was to run the process until the achievement of cyclic steady state (phase A), to continue the process with the cleaning agent replaced by buffer C (i.e. CIP was essentially switched off) and to run until reaching a new cyclic steady state if necessary (phase B). The experiment was then to be continued after turning CIP back on (phase C) until reaching the initial steady state, i.e. the one corresponding to phase A. During the experiment, the column configuration and all gradient conditions and flow rates were exactly the same, except for the CIP step for which the NaOH solution was replaced by buffer C during phase B. Thus, the conditions in phases A and C were identical. Consequently, if irreversible adsorption would be present (that could be prevented by CIP with NaOH) during phase B a decline of the process performance in terms of yield and purity due to the absence of CIP, would be expected. This prospective decline was to be recovered in phase C due to re-activation of CIP and the performance was to reach the values of phase A. Since the experimental run was expected to last several days, a low load and thus a low productivity was selected in order to save feed material.

RESULTS AND DISCUSSION

Single Column Experiments

The loading of the cation exchange column with myoglobin solution led to irreversible adsorption. The contamination of the column was clearly visible as brown color and the color front progressed along the column with increasing load. The color could not be removed through repeated washes with 100% buffer A and B and the UV-detector (A_{280}) did not measure any myoglobin elution. However, when the column was

flushed with the sodium hydroxide solution, the colorization of the column could be removed and the A_{280} signal confirmed the removal of the myoglobin.

The reason for the irreversible adsorption of myoglobin is not known; judging solely by the isoelectric point, myoglobin (pI 6.8 and 7.2) should be less strongly adsorbing than e.g. lysozyme (pI 9–11).

Lysozyme was chosen as a model product substance for retention time measurements since it has a similar molecular weight as the model impurity myoglobin (14.4 kDa vs. 17.0 kDa) and should therefore have a similar accessibility of the stationary phase pores. Thus, strong effects on retention behavior were expected.

In order to check the reproducibility of the retention time measurements, duplicate runs were performed for all measurements and the extent of experimental scatter was evaluated: In rows 1 and 2, Table 2 shows the results for a retention time measurement ($t_{R0,1}$) and duplicate run ($t_{R0,2}$) before myoglobin was loaded for non-adsorbing (row 1, 80% B) and adsorbing conditions (row 2, 18% B) respectively. The results shown here were the ones with the largest deviation among experiment and repetition, showing the worst case (row 2 of Table 2). The retention time values of 14.5 min and 14.8 min correspond to an absolute error of 18 s and a relative error of $E_{rel} = 2\%$. In general, the relative error for the retention time measurement was below $E_{rel} = 2\%$. For the measurements under non-adsorbing conditions (row 1), the error was below $E_{rel} = 1\%$.

Table 2. Results for retention time measurements of lysozyme at different myoglobin loads. "Conc" stands for the concentration of the isocratic experiment. $t_{R0,1}$ indicates the first retention time measurement, $t_{R,2}$ indicated the repetition of this experiment. The average of $t_{R0,1}$ and $t_{R0,2}$ is expressed by $t_{R0,avg}$. $t_{R50,avg}$ stands for the average of measurement and repetition after a load of 50 mg myoglobin/mL column. Now $t_{R0,2}$ is compared to $t_{R0,1}$. E_{abs} is the absolute error between the measurements, E_{rel} is the corresponding relative error with respect to $t_{R0,1}$. The measurement $t_{R50,avg}$ is compared to $t_{R0,avg}$ in the same way. Δ_{abs} denotes the absolute difference, Δ_{rel} the relative difference

Row	Conc [%B]	$t_{R0,\ 1}$ [min]	$t_{R0,\ 2}$ [min]	E_{abs} [s]	E_{rel} [%]
1	80	2.30	2.32	2	1
2	18	14.5	14.8	18	2
Row	Conc [%B]	$t_{R0,\ avg}$ [min]	$t_{R50,\ avg}$ [min]	Δ_{abs} [s]	Δ_{rel} [%]
3	80	2.31	2.42	6	4
4	18	14.7	10.9	225	26

In rows 3 and 4 of Table 2, the average retention times of experiment and duplicate run under non-adsorbing and adsorbing conditions are shown for the unloaded column ($t_{R0,\ avg}$) and the column loaded with 50 mg myoglobin/mL column ($t_{R50,\ avg}$). Under adsorbing conditions, the decrease of retention time of lysozyme for the unloaded and the loaded column was from 14.7 min ($t_{R0,\ avg}$) to 10.9 min ($t_{R50,\ avg}$), row 4. This corresponds to a difference Δ_{rel} of 26% which is one order of magnitude larger than the relative experimental error E_{rel} of measurement and repetition. For non-adsorbing conditions, the corresponding difference Δ_{rel} was 4% or larger. In general, the difference in retention time became larger for decreasing modifier concentrations. These results indicate that the effect of myoglobin loading on lysozyme retention is clearly superior to the experimental error.

Concentrations of 80% B and 60% B represent non-adsorbing conditions for lysozyme. They were used to determine the lysozyme-specific porosity of the column using the following equation:

$$\varepsilon_{Lys} = \frac{(t_{R,na} - t_{dead}) \cdot Q}{CV} \tag{3}$$

The retention time measurements under adsorbing conditions were used to calculate the Henry coefficient as a function of the salt concentration, according to the following equation:

$$H = \left(\frac{(t_R - t_{dead}) \cdot Q}{\varepsilon_{Lys} \cdot CV} - 1 \right) \cdot \frac{1}{F} \tag{4}$$

For ε_{Lys} (and F), the value determined under non-adsorbing conditions was employed (i.e. equation 3). It is worth noting that the term in parentheses in equation 4 is identical to the retention factor k_i', which is often found in the literature. In Fig. 3, the Henry coefficients of lysozyme are shown as a function of the salt concentration for increasing myoglobin load. A clear decrease of lysozyme retention time was observed for increasing myoglobin load, resulting in a lowering of the Henry coefficients at a given salt concentration.

The Henry values were fitted with power functions (slope α and exponent β). The values for these parameters are given in Table 3. The obtained values confirm the previous observation that the relative difference in retention time becomes larger as the modifier concentration decreases.

The difference in the retention time cannot be explained by a change of the porosity alone: Taking the porosity from the column loaded with myoglobin and calculating the theoretical peak retention time using the Henry

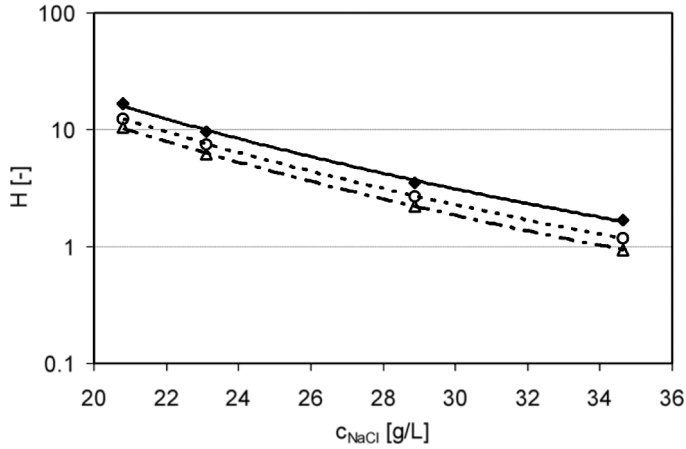

Figure 3. Henry coefficients for lysozyme as determined by isocratic elutions of analytical injections at different modifier concentrations and myoglobin loads. Diamonds denote the values for the unloaded column, circles stand for a load of 25 and triangles for a load of 50 mg myoglobin/mL column. The lines represent calculations through the power functions fits reported in Table 3 (continuous and dashed lines).

coefficient of the unloaded column, the peak retention time should be 13.9 min against the measured value of 10.9 min. This corresponds to a difference of 28% and is significantly larger than the relative difference $\Delta_{rel} = 4\%$ that was observed for non-adsorbing conditions (Table 2, row 3).

This means that, apart from the porosity that can be regarded as a geometric parameter, the Henry-coefficient and consequently the thermodynamics of the system change with increasing impurity load. These results show that the Henry coefficient is a suitable parameter to assess column quality under different impurity loads.

This change of the system properties over time due to irreversible adsorption of impurities is undesired in process chromatography both

Table 3. Porosity and Henry function parameters from single column batch experiments

Load [mg]	ε [-]	α [10^7]	β [-]
0	0.83	1.25	−4.47
50	0.80	1.53	−4.62
100	0.78	1.61	−4.70
After CIP	0.81	1.28	−4.51

in batch and continuous mode. Obviously, shorter product retention times under the same elution conditions entail worse separation and, consequently, a lower purity. The underlying mechanisms are not well understood and far from being modeled. To eliminate the effects of "irreversible" adsorption, CIP is required.

The effect of CIP in batch column mode is shown in Fig. 4, where the fitted Henry coefficients as a function of the salt concentration at loads of zero and 50 mg myoglobin/mL column as well as the values obtained after CIP are shown. The curves of the unloaded column and the cleaned column nearly match, indicating that the original retention behavior could be re-established to a great extent. Measurements under non-adsorbing conditions demonstrated that the original porosity was also regained. Table 3 shows the values of the parameters α and β also for the cleaned column. A good agreement with the values of the unloaded column is apparent.

MCSGP Experiment

The functioning of MCSGP in combination with CIP in terms of piping and valve-switching was demonstrated in a mock run by visualizing the removal of the brown myoglobin-derived color from the columns.

According to phases A, B, C described earlier, the MCSGP process was started up using the mAb cCCS as feedstock and the operating

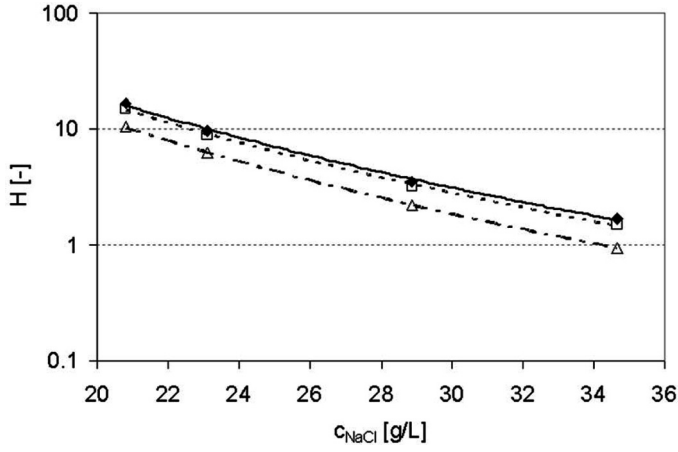

Figure 4. Henry coefficients for lysozyme as determined by isocratic elutions of analytical injections at different modifier concentrations and myoglobin loads. Diamonds denote the values for the unloaded column, triangles stand for a load of 50 mg myoglobin/mL column and squares stand for the values obtained with the NaOH-cleaned column. All data were fitted with power functions as in Fig. 3.

parameters given in Table 1. The results are shown in terms of online and offline analyses in Fig. 5 as a function of time. The black solid line represents the calibrated online A_{280} signal of the product outlet that is proportional to the protein concentration. The triangles stand for the mAb concentration determined by offline analyses. The ratio DNA/mAb is represented by diamonds while the ratio HCP/mAb is represented by squares.

During phase A, the MCSGP process including CIP was started up and reached cyclic steady state, which was confirmed by a leveling of the online A_{280} signal and offline mAb concentration measurements. In phase A, the DNA concentration was measured with a large scatter giving values between 1000 and 4000 ng DNA/mg mAb. The HCP content was around 1500 ng HCP/mg mAb at cyclic steady state. The system pressure drop was constant at 1 bar.

As cyclic steady state was confirmed after 1500 min of operation, the CIP was switched off by replacing the NaOH solution with buffer C, marking the beginning of phase B. During the entire run, the A_{280}, the pH, and conductivity values of the CIP outlet were monitored. In Fig. 6, these values are shown for the last two CIP-subcycles (bold dashed

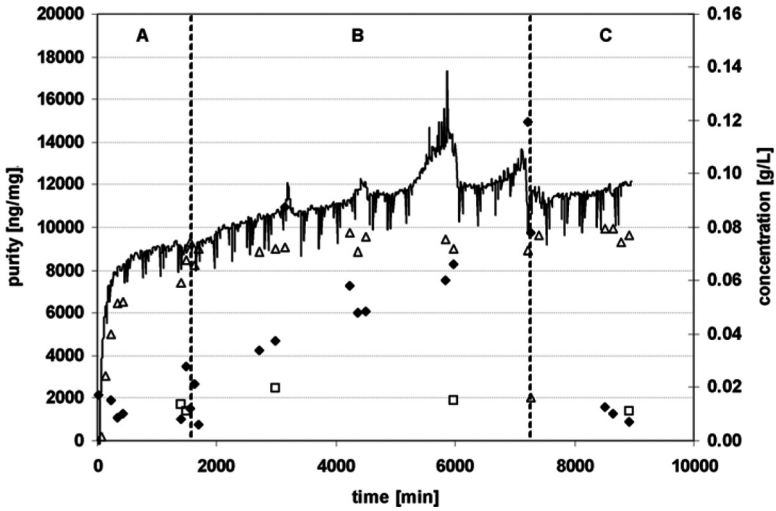

Figure 5. MCSGP run with mAb cCCS as feedstock. As a function of time are shown: The calibrated online UV-signal (black line), mAb concentration (empty triangles), HCP concentration (empty squares) and DNA concentration (filled diamonds) as determined by offline analyses. The run is separated into phases A, B and C marked by the dashed vertical lines. In phases A and C, CIP is employed while in phase B it is disengaged.

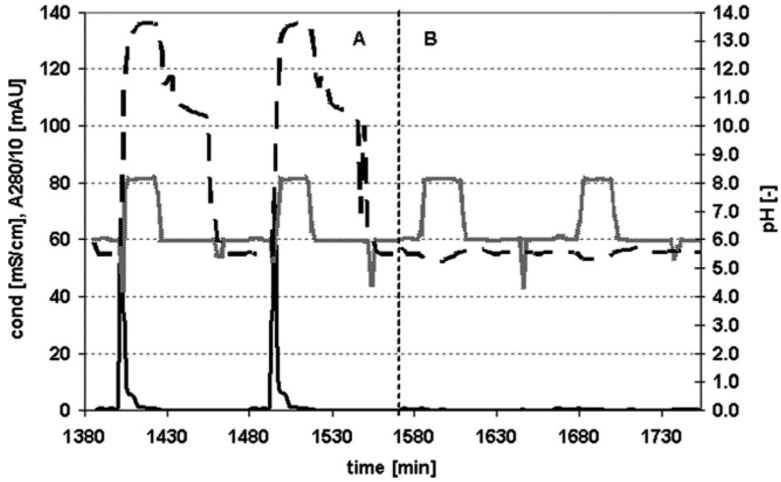

Figure 6. Transition of phase A and B: Solid black line: A280 UV signal scaled by 1/10, Solid grey line: conductivity, dashed black line: pH. The vertical dashed line marks the point in time where CIP is switched off, i.e. the boundary between phase A and B.

frame in Fig. 2) of phase A and the first two CIP-subcycles of phase B. At 1385 min, the column entering the CIP step has just left section 1, so its liquid volume contains 70% buffer C, corresponding to a conductivity of 60 mS/cm at pH 5. As NaOH breaks through the column, conductivity and pH rise to 80 mS/cm and 13.5, respectively. During this rise, irreversibly adsorbing components, visible as a peak in the A_{280} signal, are eluted from the system. The pH stays above 12 for about 20 min. As the CIP agent is removed from the system and the column is re-equilibrated, the conductivity decreases rapidly and the pH follows more slowly. With a safety time period of 10 min before the column leaves the CIP subcycle, the pH and conductivity reaches the starting values again. The CIP subcycle is completed after 92 min which is given by the time of four switches of 23 min duration each ($t_{CC} = 20$ min, $t_{BL} = 3$ min). At 1477 min, the next CIP-subcycle starts and the cleaning procedure is repeated. Again, the A_{280} signal shows a peak as pH and conductivity rise. After the completion of this subcycle, at 1569 min, phase A is terminated and the CIP agent is replaced by buffer C. As buffer C breaks through at constant pH of 5 and the conductivity rises, the A_{280} signal remains constant, showing that no impurities are eluted at this point. Thus, an elution with 100% buffer C can not substitute the clearance of proteins by NaOH. At 1569 min, the next CIP-subcycle is initiated and again, no impurity is eluted while backflushing just with buffer C.

Inevitably, the impurities that were previously eluted by NaOH now accumulate inside the unit due to irreversible adsorption. A negative impact on the process performance during phase B is expected i.e. a lowering of the yield due to a decreased Henry coefficient as seen in the case of lysozyme in batch mode for increasing myoglobin load.

As phase B progressed (Fig. 5) the UV signal started to show an upward drift which was however not due to a change in the mAb concentration. The DNA content was analyzed offline and a clear increase could be observed over time. The DNA level reached 15000 ng DNA/mg mAb at 7200 min. HCP values were 2500 and 1900 ng HCP/mg mAb at 3000 and 6000 min of operation, respectively.

Excursions of the UV signal could be linked to the feed properties. When the feed exhibited turbidity due to precipitation after a couple of hundred minutes, excursions in the UV signal occurred and the feed was exchanged which led to a partial reversion of the excursion. It was shown in earlier experiments that the precipitation of the feed followed a slow kinetics and was initiated by the dilution of the feed. The precipitation could not be linked to mAb concentration. A solution to avoid excessive precipitation which was implemented recently is the inline dilution of the supernatant directly before entering the column.

The pressure drop curve of the MCSGP run is shown in Fig. 7 as a function of time. The pressure sensor is located at the pump that delivers Q_2, giving a measure of the system backpressure for the interconnected state. In the course of the experiment while CIP was absent (phase B),

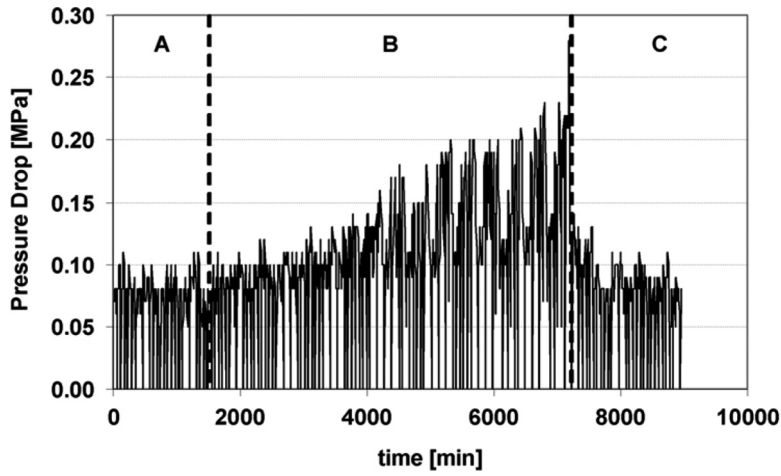

Figure 7. Development of the pressure drop measured at pump 1 (delivers Q1 and Q2) over time in phases A, B, and C.

a pressure drop increase from 1 bar to 2 bar was observed. After ca. 7200 min of operation, a head space was observed in one of the columns (ca. 2 mm, picture not shown). The head space formation and the pressure drop increase was due to a blockage of the inlet frit, possibly with irreversibly adsorbing contaminants, precipitate components, or smaller cell debris that was not removed by clarification of the supernatant. The column is designed in such a way that the inlet frit lies on top of the bed. If the frit is blocked it may function as a piston and compress the bed, certainly changing the column porosity. The increasing backpressure can be seen in direct analogy to a dead-end filtration. The larger the build-up of irreversible impurities on the filter (frit), the larger is the backpressure as the volume flow is constant. In addition, head spaces represent undesired extra-column mixing volumes that have a negative impact on the column performance.

Head space formation and pressure drop increase served as good reason to turn on the CIP again using the sodium hydroxide solution (phase C).

With the first cleaning of the respective column in phase C, the headspace disappeared and did not develop again in the remainder of the experiment. Within one CIP-cycle, the pressure drop returned back to the initial value of 1 bar (Fig. 7). The A_{280} signal of the CIP outlet gave a large peak upon breakthrough of NaOH, indicating the removal of a large amount of impurities (data not shown). The mAb concentration of the product outlet did not change showing that the contamination with irreversibly adsorbing components did not affect the process yield in the considered time frame. The HCP value was 1400 ng HCP/mg mAb at 8900 min. When taking into account the scatter of the ELISA, an effect of CIP on the product purity with respect to HCP could not be confirmed. However, as a consequence of the CIP the DNA levels dropped back from ca. 10000 ng DNA/mg mAb to ca. 1000 ng DNA/mg mAb. This shows that DNA was among the irreversibly adsorbing components that could not be desorbed by buffer C. Consequently, the DNA was carried over and accumulated in the system. A fraction of the DNA then co-eluted with the product, leading to the increased DNA concentration in the product stream. A control elution in batch mode including fractionation (with cCCS as load) indicated that DNA eluted over the whole range of the linear gradient with increasing concentrations (data not shown). This shows that in this case DNA does not adsorb due to charge interaction on the ion-exchange material, which would have been surprising due to the generally low pI of DNA. The elution behavior indicates that unspecific adsorption is involved. It is also possible that the DNA in the supernatant consists of a multitude of DNA fragments which are not distinguished by the DNA assay but have different adsorptive

properties or that it follows slow desorption kinetics. The practical consequence is that CIP is necessary to clear DNA from the system, ensuring a low DNA content of the product. The MCSGP run was terminated after 9000 min. The process yield was not affected significantly by the presence or the absence of CIP and was between 90 and 94%. In other words, it could not be shown that the retention behavior of the mAb is influenced by the irreversibly adsorbing impurities in such a way that the process yield is affected. The reasons for this can be the robust operating parameters that were chosen or that irreversibly adsorbing impurities with negative impact were present in too small amounts. However, the irreversible adsorption had a dramatic impact on product purity and system pressure drop.

CONCLUSIONS

In single column batch experiments, the effects of irreversible adsorption of myoglobin on lysozyme adsorption on a strong cation exchange resin were examined as a model system. It was found that the bound myoglobin reduces the specific porosity of lysozyme and decreases its retention, resulting in a decrease of the Henry coefficients. The Henry coefficient was shown to be a suitable measure to determine the degree of irreversible adsorption. The effectiveness of NaOH solution as a cleaning agent for the cation exchange resin was demonstrated and the original lysozyme retention behavior in the model system could be restored.

This part of the work shows the effect of irreversible adsorption and CIP on the thermodynamic properties of the system.

In the future it would be worth investigating the effects of system properties such as protein size and charge on irreversible adsorption to understand more about the underlying mechanisms.

The second part of the work shows the effect of irreversible adsorption and CIP on the physical properties of the system and the product purity in continuous biomolecule chromatography.

In order to examine the impact of irreversible adsorption and CIP on the performance of a continuous process, a CIP step was introduced to the MCSGP process for the purification of mAb from cCCS. Given the large number of impurities in the mAb supernatant, the observation that some of them were irreversibly adsorbing, was not surprising. When CIP was switched off, no significant impact on the mAb retention times, potentially resulting in a decrease of the yield was seen. However, adverse effects on the product purity of the continuous process were observed with respect to DNA when CIP was not present. It was shown that CIP is necessary to prevent pressure drop increase and head space formation

caused by fouling impurities over time. CIP is therefore absolutely required to ensure a stable long-term operation of the continuous process when dealing with high impurity loads such as in a mAb capture step, it prevents the negative impact of irreversibly adsorbing impurities on thermodynamic and physical system properties.

ACKNOWLEDGEMENTS

The authors greatly acknowledge the delivery of cCCS and funding by the European Integrated Project AIMS (Advanced Interactive Materials by Design), Sixth Research Framework Program of the European Union (NMP3-CT-2004-500160).

NOMENCLATURE

$c_1 - c_4, c_6$	[g/L]	Gradient concentrations
c_{imp}	[g/L]	Impurity concentration
c_{mAb}	[g/L]	MAb concentration
c_{Mod}	[g/L]	Liquid phase modifier (e.g. salt) concentration
CV	[mL]	Column volume
E_{abs}	[s]	Absolute Error between run and repetition
E_{rel}	[%]	Relative Error between run and repetition
F	[-]	Phase ratio $F = (1-\varepsilon_{Lys})/\varepsilon_{Lys}$
H_i	[-]	Henry coefficient for component i
k_i'	[-]	Retention factor
$\dot{m}_3$	[g/min]	Mass flow of product leaving the system through the product outlet
$\dot{m}_{Feed}$	[g/min]	Mass flow of product entering the system through the feed inlet
Q	[mL/min]	Flow rate
$Q_1 - Q_4, Q_6$	[mL/min]	Flow rates for the sections 1–4, 6 of the MCSGP process
Q_{CIP}	[mL/min]	Flow rate of CIP step
Q_{Equil}	[mL/min]	Flow rate of equilibration step
Q_{Feed}	[mL/min]	Feed flow rate for MCSGP process
t_{BL}	[min]	Time period length of MCSGP batch state
t_{CC}	[min]	Time period length of MCSGP interconnected state
t_R	[min]	Retention time
$t_{R0,1}$, $t_{R0,2}$	[min]	Retention time value at 0 myoglobin load, 1st measurement and 2nd measurement

$t_{R0,avg}$	[min]	Average retention time value after 0 and
$t_{R50,avg}$		50 mg myoglobin/mL column load
$t_{R,na}$	[min]	Retention time under non-adsorbing conditions
t_{dead}	[min]	Dead time
α	[-]	Empirical constant for Henry function
β	[-]	Empirical constant for Henry function
Δ_{abs}	[-]	Absolute difference between measurements
Δ_{rel}	[%]	Relative difference between measurements
ε_{Lys}	[-]	Specific porosity for lysozyme, constant

REFERENCES

1. Walsh, G. (2005) Biopharmaceuticals: recent approvals and likely directions. *Trends in Biotechnology*, *23* (11): 553–558.
2. Anspach, F.B.; Spille, H.; Rinas, U. (1995) Purification of recombinant human basic fibroblast growth factor: stability of selective sorbents under cleaning in place conditions. *Journal of Chromatography A*, *711*: 129–139.
3. Burgoyne, R.F.; Priest, M.C.; Roche, K.L.; Vella, G. (1993) Systematic development and validation of sanitization protocols for a chromatographic system designed for biotherapeutics purification. *Journal of Pharmaceutical & Biomedical Analysts*, *2* (11/12): 1317–1325.
4. Girot, P.; Moroux, Y.; Duteil, X.P.; Nguyen, C.; Boschetti, E. (1990) Composite affinity sorbents and their cleaning in place. *Journal of Chromatography A*, *510*: 213–223.
5. U.S. Department of Health andHuman Services Food andDrug Administration. (2001) Q7A Good manufacturing practice guidance for active pharmaceutical ingredients.
6. Hale, G.; Drumm, A.; Harrison, P.; Phillips, J. (1994) Repeated cleaning of Protein-A affinity column with sodium-hydroxide. *Journal of Immunological Methods*, *171* (1): 15–21.
7. GE Healthcare, rProtein A Sepharose Fast Flow instructions 71-5000-09 AC.
8. GE Healthcare (2002) MabSelect instructions 71-5020-91.
9. GE Healthcare (2005) MabSelect Xtra instructions 11-0026-02 AB.
10. GE Healthcare (2005) MabSelect SuRe instructions 11-0026-01 AB.
11. Staby, A.; Johansen, N.; Wahlstrøm, H.; Mollerup, I. (1998) Comparison of loading capacities of various proteins and peptides in culture medium and in pure state. *Journal of Chromatography A*, *827*: 311–318.
12. Siu, S.C.; Baldascini, H.; Hearle, D.C.; Hoare, M.; Titchener-Hooker, N.J. (2006) Effect of fouling on the capacity and breakthrough characteristics of a packed bed ion exchange chromatography column. *Bioprocess Biosyst. Eng.*, *28*: 405–414.
13. Levison, P.R.; Badger, S.E.; Jones, R.M.H.; Toome, D.W.; Streater, M.; Pathirana, N.D.; Wheeler, S. (1995) Validation studies in the regeneration of ion-exchange celluloses. *Journal of Chromatography A*, *702*: 59–68.

14. Ströhlein, G.; Aumann, L.; Müller-Späth, T.; Morbidelli, M. (2007) The multicolumn countercurrent solvent gradient purification process. *Biopharm. International*, 42–48 Suppl. S.
15. Aumann, L.; Morbidelli, M. (2007) A continuous multicolumn countercurrent solvent gradient purification (MCSGP) process. *Biotechnology and Bioengineering*, *98* (5): 1043–1055.
16. Abel, S.; Baebler, M.; Arpagaus, C.; Mazzotti, M.; Stadler, J. (2004) Two-fraction and three-fraction continuous SMB separation of nucleosides. *Journal of Chromatography A*, *1043*: 201–210.
17. Paredes, G.; Mazzotti, M. (2005) SMB operation for three-fraction separations: purification of plasmid DNA. *Adsorption*, *11*: 841–845.
18. Müller-Späth, T.; Aumann, L.; Melter, L.; Ströhlein, G.; Morbidelli, M. (2008) Chromatographic separation of three monoclonal antibody variants using multicolumn countercurrent solvent gradient purification (MCSGP). *Biotechnology and Bioengineering*, *100* (6): 1166–1177.
19. Jacob, L.R.; Frech, M.. (2004) Scale-up of antibody purification: From laboratory scale to production, Antibodies Volume 1, Production and Purification, Chapter 4, Subramanian, G. (Editor), Kluwer Academic/Plenum Publishers.
20. Homepage of the European Integrated Project AIMS (''Advanced Interactive Materials by Design''), www.aims-eu.de
21. Aumann, L.; Morbidelli, M. 2008. A semicontinuous 3-column countercurrent solvent gradient purification (MCSGP) process. *Biotechnology and Bioengineering*, *99* (3): 728–733.