Universität für Bodenkultur Wien

Department für Biotechnologie

Institut für angewandte Mikrobiologie
Vorstand:
Ao.Univ.Prof. Dipl.-Ing. Dr.nat.techn. Florian Rüker

Betreuer:
Ao.Univ.Prof. Dipl.-Ing. Dr.nat.techn. Alois Jungbauer

CONTINUOUS PRECIPITATION OF THERAPEUTIC PROTEINS, WITH AN EMPHASIS ON MONOCLONAL ANTIBODIES

Dissertation
zur Erlangung des Doktorgrades
an der Universität für Bodenkultur Wien

Eingereicht von
Ralf Sommer

Wien, Dezember 2013

Abstract

A capture step for direct capture of recombinant antibodies from clarified culture supernatant was developed. This method is a combination of $CaCl_2$ and polyethylene glycol PEG precipitation. For separation of HMWI such as dsDNA and aggregates $CaCl_2$ precipitation was the method of choice and PEG precipitation was used for mAbs capturing to separate LMWI, mainly HCP. Precipitation tests were performed with five different CHO cell culture supernatants and showed yields of at least 80 up to 95 %. Purity was roughly comparable to protein A purification. For further improvement of purity, a combination of caprylic acid and PEG precipitation was developed and showed equal values compared to protein A purification. For caprylic acid/PEG precipitation combination, a continuous operable lab scale precipitation reaction was designed and constructed. Different precipitation methods were combined, to replace the whole state of the art discontinuous mAb purification process with a continuous column free process. This combination consists of four different precipitations (CA, PEG, $CaCl_2$ and CEP), aligned in a certain way to be able to drive the process continuously. Yield and purity of this process reach almost drugs substance values with a HCP concentration of 270 ppm, no HMWI and approximately 70 % yield. The implementation of an additional step (anion exchange filter of monolith) would lead to the achievement of equal purities compared to biopharmaceuticals. However, it was shown that there is the possibility to replace the state of the art column based mAbs purification with a novel continuous purification process, which has equal yield and purity with a far smaller economical footprint.

Zusammenfassung

Im Zuge dieser Arbeit wurde ein Verfahren, für die Aufreinigung von rekombinanten Antikörpern aus CHO Zellkulturüberstand entwickelt, welches als Ersatz für den Aufkonzentrierungsschritt mittels Protein A Affinitätschromatographie verwendete werden kann. Dieses Verfahren besteht aus der Kombination einer $CaCl_2$ und Polyethylenglycol (PEG) Präzipitation. Wobei $CaCl_2$ zur Abtrennung von höher molekularen Verunreinigungen (Aggregate, DNS) und PEG als Präzipitationsmittel des Zielproteins (Antikörper) eingesetzt wird. Das Präzipitationsverfahren wurde mit fünf verschiedenen Antikörperzellkulturüberständen ausgetestet. Die Ausbeute lag zwischen 80 und 95 % und die Reinheit war annähernd vergleichbar mit der einer Protein A Affinitätschromatographie. Zur Erhöhung der Reinheit wurde die PEG mit einer Caprylsäure (CA) Präzipitation kombiniert, welches zu einer Verbesserung führte und somit die Reinheit einer wie bei der Affinitätschromatographie erreichte. Für diese Kombination wurde ein kontinuierlich betreibbarer Präzipitationsreaktor im Labormaßstab entworfen und gebaut. Um den gesamten diskontinuierlichen Antikörperaufreinigungsprozess zu ersetzen, wurden verschiedene Präzipitationsmethoden kombiniert. CA, PEG, $CaCl_2$ und die kalte Ethanol Präzipitation wurden aneinander gereiht um einen kontinuierlichen Prozess zu erhalten. Mit einer Ausbeute vom ~ 70 % und ohne höher molekulare Verunreinigungen sowie einer Wirtszellproteinkonzentration von ~ 270 ppm zeigt der neue kontinuierliche Aufreinigungsprozess seine Konkurrenzfähigkeit im Vergleich zum aktuellen Standartprozess. Durch die Implementierung eines zusätzlichen kontinuierlichen Prozessschrittes (Anionentauscher Filter oder Monolith), würde höchstwahrscheinliche der Reinheitsstandard eines fertigen biopharmazeutischen Produktes erreicht werden. Jedenfalls wurde gezeigt, dass der diskontinuierliche Stand der Technik Antikörperaufreinigungsprozess durch einen neuen kontinuierlichen Aufreinigungsprozess ersetzt werden kann, mit gleicher Reinheit, jedoch einem kleineren ökonomischen Fußabdruck.

Danksagung

Herzlichen Dank an alle, die mich bei meiner Arbeit unterstützt haben.
Besonderer Dank gilt meinem Betreuer, Prof. Alois Jungbauer, der mir meine Dissertation ermöglicht hat und mir stets mit Rat und Tat zur Seite stand.

Der Firma Novartis Pharma AG danke ich für die Finanzierung des Conprec Projektes.

Bei meinen Projektkollegen Peter Satzer, Nikolaus Hammerschmidt und Maciej Kandúla, sowie Bernhard Helk und Henk Schulz von Novartis möchte ich mich für die äußerst gute Zusammenarbeit bedanken.

Ein ganz besonderer Dank gilt Anne Tscheliessnig, die mir jederzeit mit ihrer fachlichen Kompetenz und stets motivierenden Worten zur Seite stand.

Meiner Arbeitsgruppe Downstream Processing danke ich für das tolle Arbeitsklima, die kollegiale Hilfe und für die wissenschaftlichen und nicht-wissenschaftlichen Gespräche während Kaffeepausen, Festen oder Büro-Brainstormings. Danke an Martin Wellhöfer, Petra Steppert, Svjetlana Medjakovic, Elisabeth Schmöger und Silke Neff.

Herzlichen Dank gebührt meinen Eltern die mir dies alles erst ermöglicht haben, insbesonders meinem Vater der mich beim Bau des Präzipitationsreaktors tatkräftig und mit technischem Fachwissen unterstützte. Weiters danke ich meiner Frau Carmen die mich immer in meinem Tun bestärkte, obwohl sie viele Wochenenden allein verbringen musste.

Contents

Introduction

Demand of monoclonal antibodies as biopharmaceuticals

The pharmaceutical market is one of the biggest and most profitable branches. A look on the sales figures of this market show annual revenue of 500 billions. Most of this turnover is generated with small molecule products, but the sale values of small molecules are stagnating over the last view years. Compared to that, increases the turnover of therapeutic proteins, monoclonal antibodies and vaccines constantly and forecasts till 2015 divines further increase. Whereby, monoclonal antibodies show the steepest increase (Table 1).

Table 1: Forecast sales (in millions) of monoclonal antibodies to 2015 (Elvin et al. 2013)

Products	2009	2010	2011	2012	2013	2014	2015
Small molecule	396,663	396,442	400,035	388,985	386,222	387,360	390,296
Therapeutic protein	63,107	64,893	67,725	70,036	72,327	74,232	75,429
Monoclonal antibody	36,398	40,346	44,914	49,979	54,130	58,647	62,658
Vaccine	20,937	23,807	24,438	25,917	27,302	28,769	30,159
Total	517,105	525,488	537,140	534,916	539,981	549,008	558,541

One reason for such prognoses is the top ten list of biopharmaceutical blockbuster which shows that five of the top five and six of the top ten products are mAbs or mAb derived biopharmaceuticals (Table 2). Certainly, the product ranking varies a bit from year to year but major changes do not happen.

Table 2: Biopharmaceutical blockbuster products of 2009; six of ten are mAbs or mAb fragments (Walsh 2010)

Products	Sales value ($ billions)	Company
Enbrel (etanercept)	6.58	Amgen, Wyeth, Takeda Pharmaceuticals
Remicade (infliximab)	5.93	Centocor (Johnson & Johnson), Schering-Plough, Mitsubishi Tanabe Pharma
Avastin (bevacizumab)	5.77	Genentech, Roche, Chugia
Rituxan/mabThera (rituximab)	5.65	Genentech, Biogen-IDEC, Roche
Humira (adalimimab)	5.48	Abbott, Eisai
Epogen/Procrit/Eprex/ESPO (epoetin alfa)	5.03	Amgen, Ortho, Janssen-Cilag, Kyowa Hakko Kirin
Herceptin (trastuzumab)	4.89	Genentech, Chugai, Roche
Lantus (insulin glargine)	4.18	Sanofi-aventis
Neulasta (pegfilgrastim)	3.35	Amgen
Aranesp/Nespo (darbepoetin alfa)	2.65	Amgen, Kyowa Hakko Kirin

Further reason for increasing turnover prognoses of mAb products are the sales data of the top 25 actively marketed mAb. The first four are above 6 billion and the first nine are at least above 1 billion dollar (Table 3).

Table 3: Sales of 25 actively marketed monoclonal antibody therapeutics in 2010 (Elvin et al. 2013)

mAb Brands	2010 sales ($ m)	Market (%)
Avastin	6973	16.02
MabThera/Rituxan	6859	15.76
Humira	6548	15.04
Remicade	6520	14.98
Herceptin	5859	13.46
Lucentis	3106	7.14
Erbitux	1791	4.12
Tysabri	1230	2.83
Synagis	1038	2.38
Xolair	750	1.72
Soliris	541	1.24
Actemra	464	1.07
Vectibix	402	0.92
Stelara	393	0.9
Cimzia	283	0.65
Simponi	226	0.52
ReoPro	205	0.47
Simulect	110	0.25
Campath/MabCampath	67	0.15
Arzerra	48	0.11
Prolia/XGEVA	41	0.09
Zevalin	29	0.07
Ilaris	26	0.06
Bexxar	10	0.02
Removab	4.3	0.01

Also the constantly increasing amount of recent approved therapeutic mAbs casts a positive light on mAb products and gives an outlook about the future trends in pharmaceutical industry. Furthermore, the comparison of mAb indications show a broad application field which extends from cancer over inflammatory diseases right up to asthma and rheumatic disease. However, an additional evidence for increasing market of therapeutic mAbs is the homogeneous distribution of different mAb products over several pharmaceutical companies (Table 4). This indicates that a big part of the pharmaceutical industry is working on the development of novel mAb products. Consequently, there is a demand on improved mAb production and purification processes to reduce the economical footprint.

Table 4: Monoclonal antibody therapeutics approved in either the US or EU that are actively marketed as of June 2011 (Elvin et al. 2013)

Brand name	Industrial name	Company(s)	Form	Construct	Isotype	Target	Indication
Orthoclone OKT3	Muromonab-CD3	Janssen Biotech/Johnson & Johnson	Murine	IgG	IgG2a	CD3	Transplantation rejection
ReoPro	Abciximab	Eli Lilly / Johnson & Johnson	Chimeric	Fab	IgG1κ	GPIIb/IIIa	Ischemia/angina
Rituxan/MabThera	Rituximab	Roche	Chimeric	IgG	IgG1κ	CD20	NHL<comma> CLL<comma> RA
Simulect	Basiliximab	Novartis / Cerimon Pharmaceuticals	Chimeric	IgG	IgG1κ	CD25	Acute organ rejection renal transplants
Synagis	Palivizumab	Abbott Laboratories / AstraZeneca / MedImmune	Humanized	IgG	IgG1κ	RSV F protein	Respiratory Syncytial Virus (RSV)
Remicade	Infliximab	Johnson & Johnson/Merck & Co./Mitsubishi Tanabe Pharma	Chimeric	IgG	IgG1κ	TNF-α	Crohn's disease<comma> ulcerative colitis<comma> RA ankylosing spondylitis<comma> psoriatic arthritis
Herceptin	Trastuzumab	Roche	Chimeric	IgG	IgG1κ	HER-2	Breast cancer<comma> gastic/gastroesophageal carcinoma
Campath/MabCampath	Alemtuzumab	Sanofi / Genzyme / Bayer	Humanized	IgG	IgG1κ	CD52	B-cell chronic lymphocytic leukemia (B-CLL)
Zevalin	Ibritumomab tiuxetan	Spectrum Pharmaceuticals / Bayer	Murine	111In or 90Y radio-labeled IgG	IgG1κ	CD20	Radiotherapy for NHL
Humira	Adalimumab	Abbott Laboratories	Human	IgG	IgG1κ	TNF-α	Rheumatoid arthritis<comma> juvenile idiopathic arthritis<comma> psoriatic arthritis<comma> ankylosing spondylitis<comma> Crohn's disease<comma> plaque psoriasis
Xolair	Omalizumab	Novartis / Roche	Humanized	IgG	IgG1κ	IgE	Asthma
Bexxar (US)	Tositumomab-iodine131	GSK	Murine	Radio-labeled IgG	IgG2a	CD20	Non Hodgkin's lymphoma
Erbitux	Cetuximab	Eli Lilly / Bristol-Myers Squibb / Merck Serono	Chimeric	IgG	IgG1κ	EGFR	Head and neck cancer<comma> KRAS-ve colorectal cancer
Avastin	Bevacizumab	Roche	Humanized	IgG	IgG1κ	VEGF	Colorectal cancer<comma> non squamous non-small-cell lung cancer<comma> renal cell carcinoma<comma> glioblastoma
Tysabri	Natalizumab	Biogen Idec / Elan	Humanized	IgG	IgG4κ	alpha-4 Integrin	Multiple sclerosis<comma> Crohn's disease
Lucentis	Ranibizumab	Novartis / Roche	Humanized	Fab	IgG1κ	VEGF	Wet age-related macular degeneration<comma> macular oedema (retinal vein occlusion)
Vectibix	Panitumumab	Amgen / Takeda Bio	Human	IgG	IGg2κ	EGFR	Metastatic colorectal carcinoma
Soliris	Eculizumab	Alexion Pharmaceuticals	Humanized	IgG	IgG2/4κ	C5	Paroxysmal nocturnal hemoglobinuria
Cimzia	Certolizumab pegol	UCB	Humanized	Pegylated Fab'	IgG1κ	TNF-α	Crohn's disease<comma> rheumatoid arthritis
Removab (EU)	Catumaxomab	Fresnius / Trion	Murine	"Trispecific" IgG	mouse IgG2a/rat IgG2b	EpCAM and CD3	malignant ascites<comma>(EU only)
Simponi ankylosing spondylitis	Golimumab	Johnson & Johnson / Merck & Co. / Mitsubishi Tanabe Pharma	Human	IgG	IgG1κ	TNF-α	RA psoriatic arthritis
Ilaris	Canakinumab	Novartis	Human	IgG	IgG1κ	IL-1-beta	Cryopyrin-associated periodic syndromes<comma> familial cold autoinflammatory syndrome<comma> Muckle-Wells syndrome
Stelara	Ustekinumab	Centocor Ortho Biotech Johnson & Johnson	Human	IgG	IgG1κ	IL-12/23 p40	Plaque psoriasis
Arzerra	Ofatumumab	GSK / Genmab	Human	IgG	IgG1κ	CD20	Chronic lymphocytic leukemia
Actemra RoActemra (EU)	Tocilizumab	Roche / Chugai Pharmaceutical	Humanized	IgG	IgG1κ	IL-6R	Rheumatoid Arthritis
ProliaXgeva	Denosumab	Amgen / GSK / Daiichi-Sankyo / AstraZeneca	Human	IgG	IgG2	RANKL	Bone metastases from solid tumors
Benlysta (US)	Belimumab	Human Genome Sciences / GSK	Human	IgG	IgG1λ	BLyS.	systemic lupus erythematosus
Yervoy (US)	Ipilimumab	Bristol-Myers Squibb / Medarex	Human	IgG	IgG1κ	CTLA4	Metastatic melanoma

State of the art production and purification of mAbs

Production of biopharmaceuticals consists of an upstream (fermentation / cell culture) and a downstream part (purification and polishing). Due to ecological issues main focus of the improvements has to be on downstream processing because it is more costly and compared to the upstream processing the development was neglected in the last decades. For improvement of the mAb purification an overview of the state of the art process sequence (Birch and Racher 2006; Marichal-Gallardo and Álvarez 2012) is shown in Figure 1. After the upstream part, consisting of fermentation, cooling and centrifugation (cell removal), the downstream starts with a product capture step usually realized with protein A. Subsequently a purification step and a polishing step with IEX (ion exchange) columns is needed and prior formulation a virus separation step (ultra / diafiltration) has to be performed. All these downstream steps have properties which influences the production costs negatively. The most important issues are the prize of protein A (Kelley, 2007) and that there is actually no method implemented to drive this process continuous.

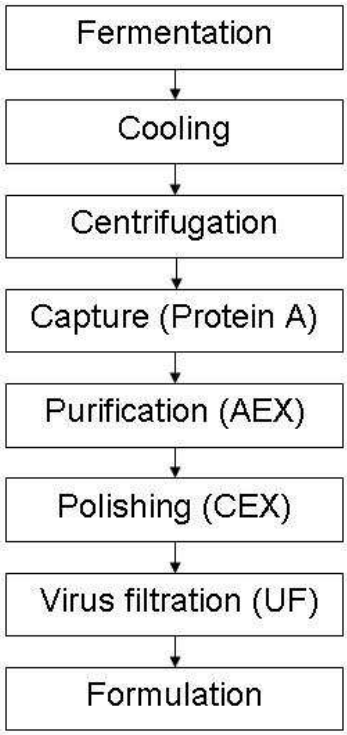


Figure 1: Schema of state of the art monoclonal antibody purification sequence

To get an impression about a state of the art commercial downstream process, the purification sequences of some mAb products are shown in Figure 2. The examples consist of blockbuster products like Rituxan and Remicade as well as less successful products like MabCampath (Sommerfeld and Strube 2005). Comparison of the different products shows that all have a similarity in their process sequence, with minor difference. The processes consists of purification and polishing steps realized with IEX, SEC and HIC as well as virus inactivation steps and a capture step usually realized with protein A. This leads to the outcome that all biopharmaceutical production process of mAbs are discontinuous and most of them need protein A for antibody capturing.

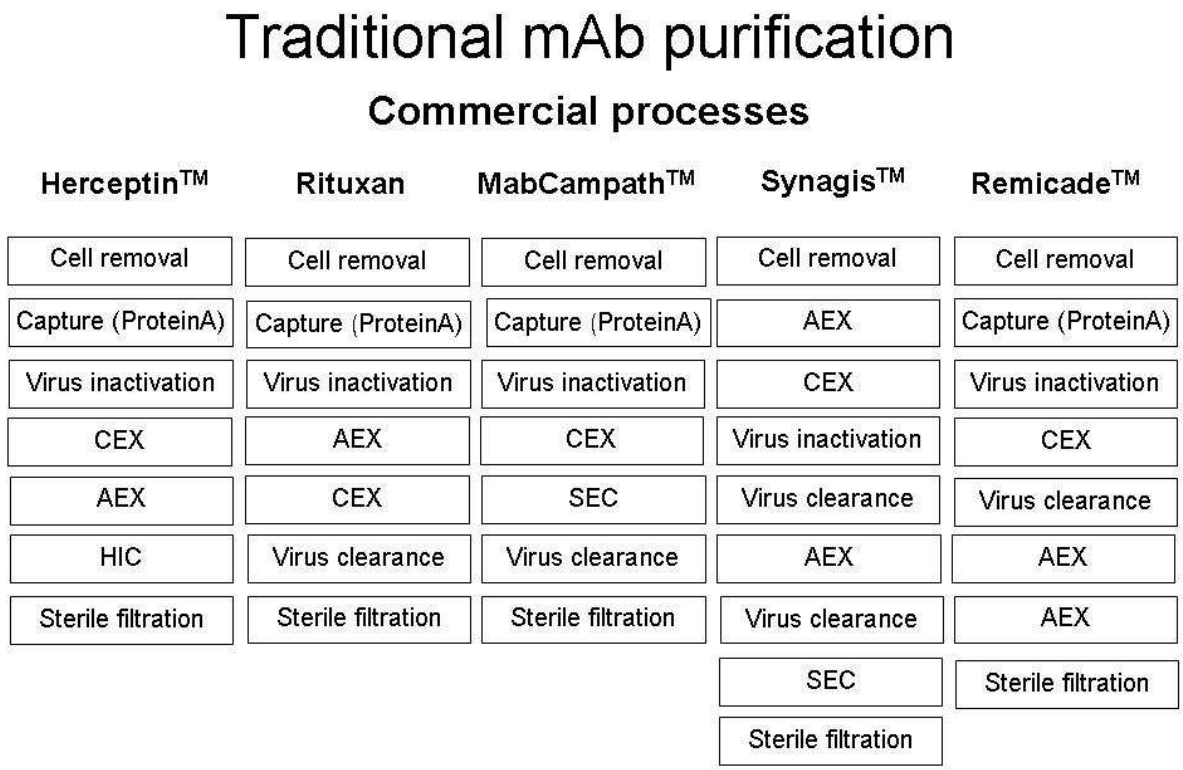


Figure 2: State of the art monoclonal antibody purification processes of common mAb products (Sommerfeld and Strube, 2005)

Appearance of recombinant monoclonal antibodies

For mAbs purification, properties like shape, size, net - charge and mAb binding areas are important (Figure 3). Conventional purification methods rather dependent on the binding areas and net - charge of the mAb, for example in case of affinity and ion exchange chromatography. The separation mechanism for precipitation bases on protein size and net - charge. Due to the large size of IgG (5-10nm), volume (water) exclusion precipitation is the mechanism of choice because with this kind of precipitation method large molecules precipitate prior small ones and molecules with a lower solubility precipitates prior molecules with higher. Purification of mAb can also be carried out via impurity precipitation. Common ways are the co-precipitation with insoluble salts and fatty acids precipitation. Mechanisms for these precipitation methods are based on the molecule net - charge. Usually, net - charge and consequently the isoelectric point of mAbs are above pH 7, which allows them to stay in solution during a fatty acids or salt co-precipitation.

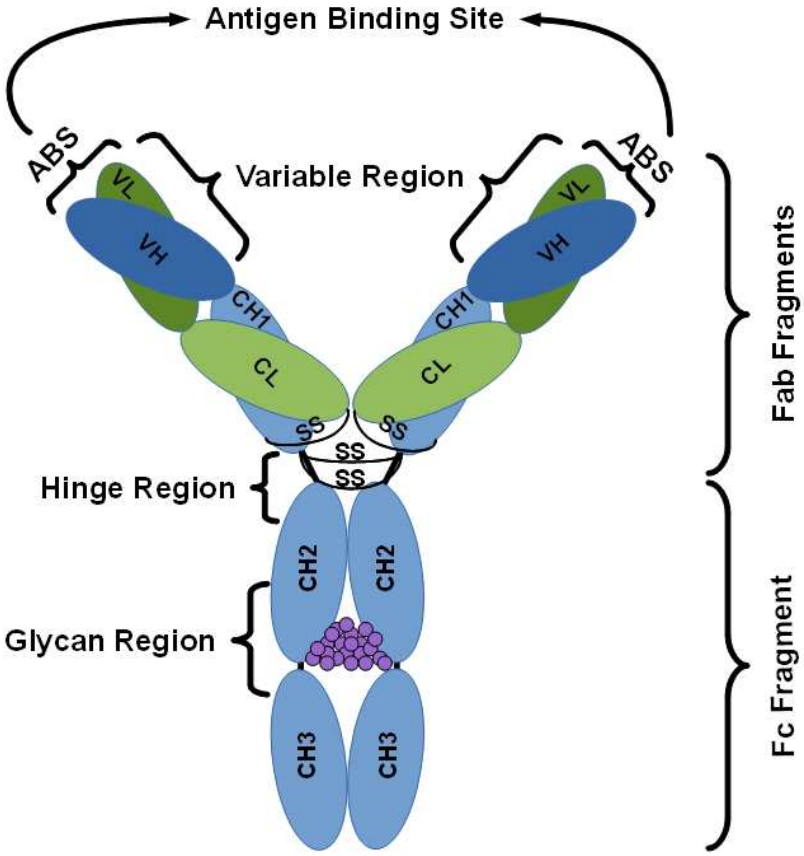


Figure 3: Schematic figure of a monoclonal antibody with ~150kDa and ~5-10nm; Heavy chain in blue (CH3, CH2, CH1 and VH) and light chain in green (CL and VL); Constant regions in light blue and light green (CH3, CH2, CH1 and CL); Variable region in dark blue and green (VH and VL);

Precipitants for protein separation

For protein precipitation several methods with corresponding precipitants are available. In this work four different precipitation methods were used and further developed to get an adequate mAb purification method. These four precipitation methods and their precipitants are described below.

Polyethylene glycol

Polyethylene glycol (PEG) is also known as polyethylene oxide (PEO) or polyoxyethylene (POE) depending on the molecular weight (Kahovec et al. 2002). It is a polymer of ethylene oxide and is available in different geometric shapes. Generally, the linear PEG version (Figure 4) is used for biopharmaceutical issues such as PEGylation (Pasut and Veronese 2012; Veronese and Harris 2002).

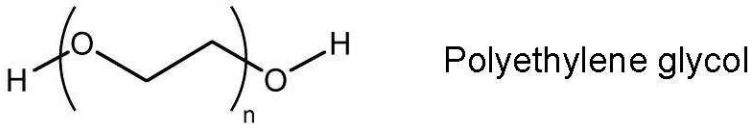


Figure 4: Structure formula of polyethylene glycol (PEG)

In the early 1960s Polson screened different linear polymers for their ability of precipitating gamma globulin and fibrinogen and developed a fractionation of human plasma with PEG (Polson et al., 1964). Juckes showed that solubility of proteins in presence of PEG follows a semi-logarithmic behaviour (Juckes, 1971). Further research on precipitation and the solubility curves of various proteins were performed (Atha and Ingham, 1981; Haire et al., 1984; Hasko and Vaszileva, 1982; Middaugh et al., 1979). These findings lead to a general model of protein precipitation by non-ionic polymers, which assume that the PEG causes volume (water) exclusion and this lowers the solubility of antibodies (Mahadevan and Hall, 1990; Mahadevan and Hall, 1992). Figure 5 show schematic a precipitation of proteins with PEG as water excluding precipitant. PEG precipitation increases with increasing hydrodynamic radius of PEG rather than molecular mass (Sim et al., 2012a; Sim et al., 2012b). Furthermore, PEG is not able to separate HMWI from proteins because they have to differ in molecular mass by at least a factor of 2 to get approach 100% separation (Lis and Schleif, 1975). Separation of proteins by PEG is relatively strictly related to the size of the proteins. Due to this reason further precipitation methods are needed for mAb purification.

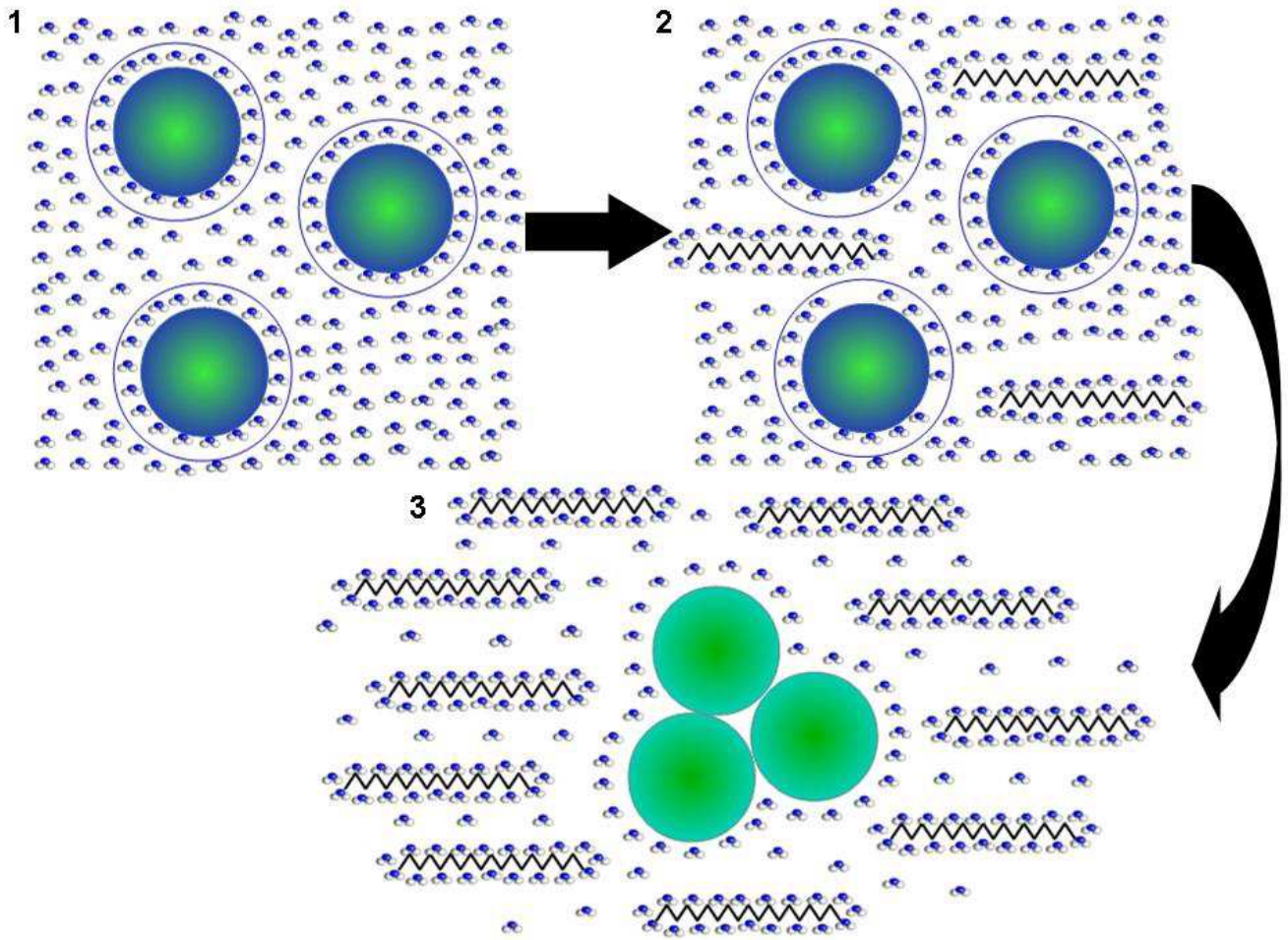


Figure 5: Precipitation of protein with volume (water) exclusion; **1**: Proteins solved in water and surrounded with a water shell. **2**: Proteins solved in water at low PEG concentration. PEG takes up water and start draining the protein water shell. **3**: Proteins agglomerated and precipitated at higher PEG concentrations. PEG fixed most of the water and drained the protein water shell.

Calcium chloride

Minor drawback of PEG precipitation is the missing ability to separate HMWI (Lis and Schleif, 1975). The co-precipitation with $CaCl_2$ as precipitant is a convenience way for separation of HMWI (Wilson et al., 1995). Mixing of $CaCl_2$ with PO_4^{3-} causes a formation of different kinds of insoluble calcium phosphate precipitates (Figure 6). These insoluble precipitates have the capability to co-precipitate large and negatively charged molecules. Due to the size and the negatively charged phosphate backbone of dsDNA is this method predestinated for DNA precipitation. So, adding of $CaCl_2$ to a cell culture supernatant with a certain PO_4^{3-} concentration causes the precipitation of aggregates and dsDNA. However, mAbs and smaller impurities stay in solution during $CaCl_2$ precipitation and have to be separated in a different manner.

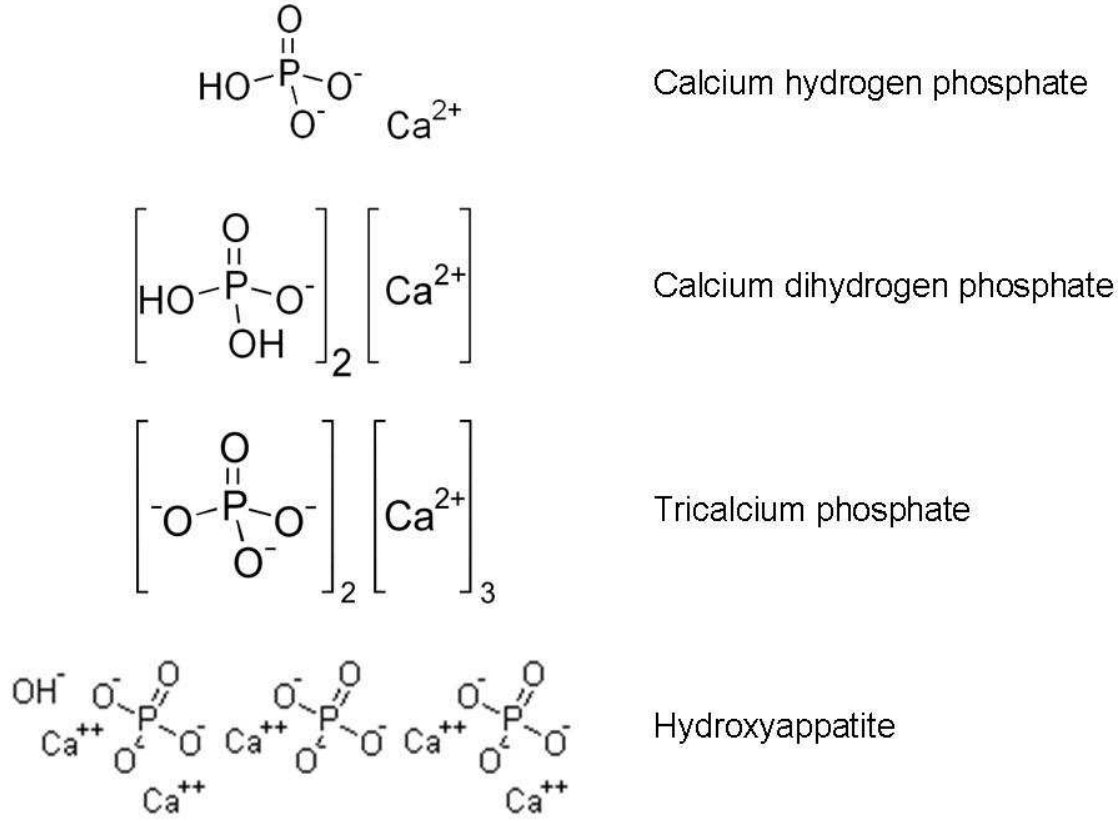


Figure 6: Structure formulas of different possible calcium phosphate precipitate appearances

Caprylic acid

Caprylic (octanoic) acid is an eight-carbon saturated fatty acid (Figure 7). It is found naturally in the milk of various mammals, coconut oil and palm kernel oil (Beare-Rogers et al. 2001). Caprylic acid is an oily liquid and minimally soluble in water.

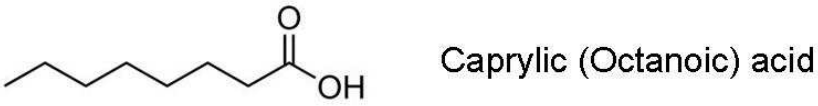


Figure 7: Structure formula of caprylic acid (CA)

Precipitation with caprylic acid can be used for host cell protein (HCP) reduction because it is a common way for separating venom out of horse plasma (Rojas et al., 1994). Precipitation parameters are described in several publications (McKinney and Parkinson, 1987; Mohanty and Elazhary, 1989; Rosa et al., 2013; Russo et al., 1983). CA precipitation was combined with several purification methods like ammonium sulphate precipitation to purify human immunoglobulins (Perosa et al., 1990) or with ion-exchange chromatography to separate equine antivenom (Raweerith and Ratanabanangkoon, 2003). Also, a combination of CA precipitation of Chinese hamster ovary HCP and an additional cation-exchange chromatographic step was performed (Wang et al., 2009). It was found that the effect of CA is due to direct interaction with the precipitating protein. This means that the mechanism of CA precipitation bases on the binding of CA to specific sites of the protein, thereby inducing partial unfolding of the protein, which exposes additional binding sites. More CA molecules incorporate into all sites in the form of mixed micelles. Thus, the interfacial protein surface becomes highly hydrophobic and increases protein-protein attraction, causing association and precipitation of the macromolecular complexes (Morais and Massaldi 2012). All these data lead to the assumption that the CA precipitation has the ability to reduce the HCP concentration.

Ethanol

Ethanol precipitation is a historical method for the separation of different blood components (Buchacher and Iberer 2006; Cohn et al. 1946; Moure et al. 2003). Most important components are immunoglobulins which were produced from collected human plasma using a series of cold ethanol precipitations (CEPs) known as the Cohn process (Buchacher and Iberer 2006; Burnouf 2007; Cohn et al. 1950; Cohn et al. 1946; Moure et al. 2003; Radosevich and Burnouf 2010). This process is based on the variation of different process parameters: pH, conductivity, ethanol concentration, protein concentration and temperature. Parameters were several times modified for yield and purity improvements (Buchacher and Iberer 2006; Deutsch and Gosting 1946; Kistler and Nitschmann 1962; Oncley et al. 1949). Due to the reason that cold ethanol precipitation can selectively purify a broad range of different immunoglobulins from a complex medium like blood plasma, it is clear that this process could separate mAbs from cell culture supernatant and therein present impurities (HCP, dsDNA). Mechanisms of cold ethanol and PEG precipitation are equal. Both induce precipitation of proteins via volume (water) exclusion (Figure 5). Advantages of cold ethanol precipitation compared to other methods are the simplicity, cost-effectiveness and the long safety record. Intravenous immunoglobulins are produced using human plasma from a large pool of donors. Usually, strict control of potential contaminations with viruses and prions should be necessary (Buchacher and Iberer 2006; Radosevich and Burnouf 2010). But it has been shown that the cold ethanol precipitation is able to reach the required safety standards (Buchacher and Iberer 2006; Cai et al. 2002; Foster 2000; Foster et al. 2000). Hence, cold ethanol precipitation is a reliable method to ensure virus clearance for a combination of orthogonal precipitation methods.

Objectives

Aim of this study was the development of a novel downstream process for purification of recombinant monoclonal antibodies derived from chinese hamster ovary cells. Main focus was on reduction of the ecological footprint. For reaching this target a total change of mind about setup and applied purification methods of the antibody downstream process was needed. Most important amendment was to divert the mAb purification away from the state of the art discontinuous driven process and orient it towards a fully continuous process.

Initial goal was the replacement of the expensive first capture step, usually realized with protein A affinity chromatography, via implementation of a monoclonal antibody precipitation. After development of this novel precipitation method in small scale, next task was planning and construction of a continuous lab scale reactor. In addition, further state of the art for purification and polishing process steps had to be replaced with precipitation or other continuous purification steps. Ultimate objective during these developments were to stay competitive with yield and purity compared to the traditional process, for getting a fare inexpensive downstream process with equal or higher purity.

Material and Methods

All chemicals were purchased from Merck (Darmstadt, Germany). Solution were prepared using HQ-H_2O and filtered through a 0.22 µm filter Buffers for analytical HPLC runs were additional degassed prior use.

CHO cell culture supernatants

Clarified CHO cell culture supernatants of four mAbs were provided by Novartis Pharma AG (Basel, Switzerland). They were stored at -20°C for long-term storage or 4°C for short-term storage. Prior to use the supernatants were filtered (0.22 µm). MAb1 and mAb2 are antibodies of the same amino acid sequence but expressed in different cell lines. MAb3 tends to aggregation and was used for evaluation of the effects that precipitation have on such antibodies. Further properties of all mAbs are shown in Table 5.

Table 5: Properties of all monoclonal antibodies

Antibody properties	Antibodies				
	mAb1	mAb2	mAb3	mAb4	mAb5
mAb concentration in supernatant [mg/mL]	3.44	1.31	1.71	4.61	4.25
mAb pI [-]	9.2	9.2	6.8	6.8	8.5
Phosphate concentration in supernatant [mM]	7.8	3.0	0.2	0.7	0.0

PEG precipitation kinetics

The kinetic tests were performed in 96 deep well plates (2mL, NUNC, NUN-278752, NUN-276005) with cell culture supernatants of mAb1, mAb2, mAb3 and mAb5. PEG solution (1.0 mL) in different sizes (PEG2000, 4000, 6000 and 20000) with different pH values (5.0, 7.0, 8.5) and certain concentrations for each size (2.5%, 7.5%, 10.0%, 12.5%) were pipetted into the wells. To start the reaction cell culture supernatant (0.5 mL) was added. Four well plates were incubated on an end-over-end rotator (Stuart) at 5 rpm. Each of these well plates was incubated for a different period of time (15 min. 30 min, 60 min and 180 min) and then centrifuged at 3700 rpm for 10 min in an Eppendorf centrifuge. Supernatant was discarded and precipitates were resuspended in 1.5mL PBS buffer for yield determination with analytical affinity chromatography (Staphylococcal Protein A).

PEG precipitation screening

For the PEG precipitation screening 1.0 mL PEG solution with different PEG (2000, 4000, 6000, 20000) sizes, concentrations (1.0%, 2.5%, 5.0%, 7.5%, 10.0%, 12.5%, 14.0%) and pH values (5.0, 7.0, 8.5) were pipetted in a 96 deep well plates (2mL, NUNC, NUN-278752, NUN-276005) well plate (NUNC, material: ethylene vinyl acetate). 0.5 mL supernatant (mAb1, mAb2, mAb3 and mAb5) was added and incubated for 60 min on an end-over-end rotator at 5 rpm.

After incubation the well plates with PEG2000, 4000 and 6000 were centrifuged (Eppendorf 5804) at 800 rpm for 15 min. Due to the high density and viscosity of PEG20000 well plate was centrifuged at 2250 rpm for 30 min. Supernatant was discard and precipitates were resuspended in 1.5mL Histidine buffer per well (20 mM Histidine; 100 mM NaCl). Yield and purity were determined with analytical affinity chromatography (Staphylococcal Protein A).

Solubility curves of purified mAbs and total protein solution

Material for solubility determination was obtained by affinity chromatography of clarified CHO cell culture supernatant. Solubility curves were made of total protein (flow through of supernatant) and purified mAb (eluted antibody). Curves were determined using PEG6000 in 15mL Greiner tubes with a working volume of 7mL. The mixture consists out of 2mL PEG6000 solution and 5mL purified antibody (in Britton Robinson buffer pH 7.0) or flow-through. Samples with an final concentration of 5.0%, 6.0%, 7.5%, 9.0%, 10.0%, 12.5% and 15.0% PEG6000 for purified mAbs and 10.0%, 12.5%, 14.0%, 15.0%, 16.0%, 18.0% and 20.0% PEG6000 for total proteins were prepared. During the 60 min incubation time the Greiner tubes were mixed on a rotator. After reaction the precipitates were centrifuged (Centrifuge, Beckmann) and IgG and total protein concentration of the supernatant was measured. Yield for mAbs were analyzed with analytical spA chromatography and the total protein concentration was analyzed with a dye based protein assay (Bradford 1976).

$CaCl_2$/PEG precipitation in the automated lab reactor (EasyMax)

Precipitation experiments in the automated lab reactor (EasyMax) were performed in two reaction vessel for 60min reaction time, at 25°C reaction temperature, with 100mL

reaction volume and stirring speed of 400rpm. For calcium chloride precipitation 69.5mL mAb cell culture supernatant were kept at 25°C and 10.5mL calcium chloride stock solution was added to reach a concentration of 350mM in 80mL reaction volume. After 60min precipitation time the suspension was collected and centrifuged (Centrifuge Multifuge X3 FR, Thermo Scientific). The precipitate was discarded and the supernatant used for next purification step. For PEG precipitation 70mL cell culture supernatant and 30mL of PEG6000 stock solution were mixed to reach a final concentration of 14% (v/v) PEG6000.

Optimization of $CaCl_2$/PEG precipitation

Optimization of $CaCl_2$/PEG precipitation was performed in 15mL Greiner tubes. Samples with low or no phosphate (mAb3, mAb4, mAb5; shown in Table 5) were mixed with phosphate buffer (pH 8.0) to get a concentration of 5mM phosphate (9.9mL cell culture supernatant with 0.1mL phosphate stock solution). $CaCl_2$ precipitation was performed with 100mM $CaCl_2$ in solution (9.75mL cell culture supernatant with 0.25mL calcium chloride stock solution) over 60 minutes reaction time and mixed with a rotator (Stuart) at 5 rpm. Occurred precipitate was centrifuged (Centrifuge Multifuge X3 FR, Thermo Scientific) with 4000g for 15 minutes and discarded. Subsequently PEG precipitation was performed with 7mL $CaCl_2$ precipitation supernatant and 3ml PEG6000 stock solution (final concentration 14% (v/v) PEG6000). The originated mAb precipitate was centrifuge (Centrifuge Multifuge X3 FR, Thermo Scientific) with 4000g for 15 minutes. A pictorial illustration of the precipitation setup is given in Figure 14. Yield and purity of all samples were measured with analytical affinity chromatography (Staphylococcal Protein A) and size exclusion chromatography.

CA/PEG precipitation screening

Pre-screening of CA/PEG precipitation

For pre-screening a cell culture supernatant stock solution was prepared. Sodium acetate solution (4M) was added to the clarified cell culture supernatant for reaching a concentration of 50 mM sodium acetate and pH value was adjusted to 4.0 with acetic acid. For pH value screening, stock solution was separated in four parts and adjusted to pH 4.5, 5.0, 5.5 and 6.0. These solutions were mixed with 1 % CA at a rotator with

40 rpm for 60 minutes. Precipitates were centrifuged (Avanti J-25, Beckmann Coulter, Brea, USA) at 4 °C with 10000 g and filtered (Syringe filter 25 mm GD/X, Cat.No. 6872-2502, Whatman, Maidstone, UK) through 0.22 µm filter prior PEG precipitation was performed at the actual pH value. Yield and purity data were determined after CA and PEG precipitation. Due to influences of low pH values on data measured with analytical affinity liquid chromatography, yield was determined with SEC (Bio SEC 3, Agilent, Santa Clara, USA) by comparing IgG peaks of cell culture supernatant and precipitation samples. Purity was monitored via measuring HCP (HCP ELISA, Cygnus, Rockville, USA) and HMWI (TSKgel G3000SWxl, Tosoh, Tokyo, Japan).

CA/PEG precipitation screening

For improvement of combined CA/PEG precipitation the parameters of CA precipitation were screened. Precipitations were performed at different pH values (4.5, 5.0 and 5.5) with different salts (sodium acetate and sodium citrate), salt (5, 10, 20, 40, 50, 100, 125 and 200 mM) and CA concentration (0.5, 1.0, 2.0, 4.0, 6.0 and 10.0 %) as well as precipitation parameters like mixing time (15, 30, 45, 60 minutes) and speed (10, 20, 30 and 40 rpm). For all screenings the parameters were fix at certain values (pH 5.5, 50 mM salt, 1 % CA, 14% PEG, 60 minutes reaction time, mixing speed 40 rpm for CA and 5 rpm for PEG) and only the parameter selected for screening was changed. After CA precipitation separation of precipitates were performed as described in “Pre-screening of CA/PEG precipitation”. Only difference to pre-screening was the pH value prior PEG precipitation which was adjusted to approximately 7 (between 6.9 and 7.1). Yield (Bio SEC 3, Agilent, Santa Clara, US and CIM Protein A HLD, BIA Separations, Ljubljana, Slovenia) and HMWI (TSKgel G3000SWxl, Tosoh, Tokyo, Japan) were determined after both precipitation steps. HCP concentration (HCP ELISA, Cygnus, Rockville, USA) was only measured after CA/PEG precipitation.

Combination of different precipitation methods

Combinations of different precipitation methods were performed with caprylic acid (CA), polyethylene glycol (PEG), $CaCl_2$ and cold ethanol precipitation (CEP). These precipitation methods were aligned in different manner with varying amounts of precipitations steps. CA precipitation was performed with a sodium citrate

concentration of 100 mM at pH 4.5 on a rotator (SB-3, Stuart, Staffordshire, UK) with 30 rpm for 45 minutes. Precipitates were centrifuged (Avanti J-25, Beckmann Coulter, Brea, USA) at 4°C with 10000 g and filtered (Syringe filter 25 mm GD/X, Cat.No. 6872-2502, Whatman, Maidstone, UK) through 0.2 µm filter. Clarified supernatant was adjusted to a pH of approx. 7 (between 6.9 and 7.1). PEG precipitation was performed at an ambient pH value (approx. 7) with 14 % PEG on a rotator (SB-3, Stuart, Staffordshire, UK) with 5 rpm for 60 minutes. Precipitated mAb was centrifuged (Heraeus Multifuge X3, Thermo Fisher Scientific, Waltham, USA) at 20 °C with 4000 g and afterwards resolved in His-buffer (20 mM histidine and 100 mM NaCl, pH 6.5) for analytical measurements and further precipitation steps. $CaCl_2$ precipitation and CEP were performed according to Anne Tscheliessnigs work (“A purification platform based on cold ethanol precipitation for mAbs from CHO culture supernatant”).

Analytics

Analytical mAb determination

Analytical affinity chromatography slightly modified as described by Tscheliessnig et al (Tscheliessnig and Jungbauer 2009) was used for mAb determination. A convective interactive media (CIM) monolithic disk with Staphylococcus Protein A (sProtein A) as ligand (CIM Protein A HLD, BIA Separations, Ljubljana, Slovenia) was connected to an Agilent 110 Series (Agilent Technologies Waldbronn, Germany). Concentration of mAb was calculated by integration of the elution peak and comparison to a calibration curve prepared from a polyclonal antibody (Octagamma, gift of Octapharma, Vienna, Austria).

Analytical SEC

If not mentioned otherwise all determinations of yield were performed using a Bio-SEC3 column (Agilent, Santa Clara, USA).

Analytical SEC for yield determination (Bio-SEC3)

A Bio-SEC3 column (Agilent) with the respective guard column (Agilent) was connected to an Agilent 1100 Series (Agilent Technologies Waldbronn, Germany). The column was equilibrated with running buffer (0.2 M sodium phosphate, 0.1 M

potassium sulphate, pH 6.0) at 1.2 ml/min flow rate. For measurements 100 µl of filtered (0.2 µm, Millipore, Carrigtwohill, Ireland) sample was injected and the UV absorbance monitored at 280 nm. The IgG peak appears at an elution time between 7 and 9 minutes. Yield was determined by comparing IgG peak area of cell culture supernatant and precipitation samples.

Analytical SEC for HMWI determination (TSK G3000 SWxl)

For high molecular weight impurity (HMWI) determination an analytical size exclusion chromatography (SEC) with a TSKgel G3000SWxl (Tosoh, Tokyo, Japan) column and the respective guard column (Tosoh) were connected to an Agilent 1100 Series (Agilent Technologies) and tempered to 30 °C. SEC was performed using 150 mM potassium phosphate, pH 6.5 at 0.4 mL/min. 10µl of filtered (0.2 µm, Millipore syringe filter) sample was injected and the UV absorbance monitored at 210 nm. Aggregates and HMWI are defined as peaks found at an elution time lower than 20 minutes, mAb peak is located from 20 to 25 minutes and remaining impurities (HCP) are defined as peaks at an elution time higher than 25 minutes.

2D DIGE

Samples were buffer exchanged into 4 M urea using NAP-5 columns (GE Healthcare, Uppsala, Sweden) according to manufacturer's protocol. For all samples the protein concentration was determined with analytical affinity chromatography (Staphylococcal Protein A) and adjusted so that concentration is ±10% of the average of all samples. Then total protein concentration was determined by photometric analyse (ND-1000 UV-Vis Spectrophotometer, NanoDrop Technologies Inc, Wilmington, USA.) for calculating the dye amount to be applied. Further steps of 2D DIGE were performed according to Grzeskowiak et al (Grzeskowiak et al. 2009). Spot determination was realized with the DeCyder Software (GE Healthcare). Selection criteria were slope (<1), volume (>20000) and height (50 to 100000) of the spot. For spot classification a 2.5 fold change compared to the drug substance gel was selected as the indication of increased (more spots than DS) or decreased spots (less spots than DS).

HCP ELISA

A protein-binding microtiter plate (Immuno 96 MicroWell Plates, MaxiSorb, NUNC, Roskilde, Denmark) was coated overnight at 4 °C with a diluted (1:400 in 200 mM sodium carbonate, pH 9.4) goat anti-CHO HCP antibody (3G-0016-AF, Cygnus, Southport, NC, USA). Then the microtiter plates were blocked with 3 % bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, USA) in TBS/Tween (500 mM Tris, 1500 mM NaCl, 0.05 % (v/v) Tween 20, pH 7.4) for 1 h at 37 °C. Two standard curves were prepared for each plate (1.6-200.0 ng/ml) by serial dilution (1:2) of a CHO HCP stock (F553H, Cygnus) in 1% BSA in TBS/Tween. Samples were diluted in the same manner and transferred with the standards into the microtiter plate and incubated for 1 h at 37 °C. After incubation with diluted (1:2000 in TBS/Tween) goat anti-CHO HCP HRP concentrate (3G-0016-AF, Cygnus) for 1 h at 37 °C, the TMB Peroxidase EIA Substrate Kit (Bio-Rad, Hercules, USA) was used for staining. The enzymatic reaction was stopped by adding of 50 µl of 1 N H_2SO_4 into each well. Absorbance at 405 nm was measured with a Tecan Infinite F500 plate reader (Tecan, Salzburg, Austria).

dsDNA quantification

Quantification of dsDNA was performed with Quant-iT PicoGreen DNA kit (Invitrogen, Paisley, UK) in microtiter plates. Reagents and buffers were prepared according to manufacturer's instructions. Samples and standards (diluted to either 100 or 1000 µg/ml Lambda DNA standard) were 1:2 serial diluted with TE-buffer in a 96-well microtiter plate. From each well 100 µl diluted sample were transferred into a black U96 MicroWell Plates (Nunc, Wiesbaden, Germany) and mixed with 100 µl of the diluted Quant-iT PicoGreen DNA reagent. The fluorescence intensity was measured with an excitation of 485 nm (20 nm bandwidth) and an emission of 535 nm (25 nm bandwidth) using the Genius Pro plate reader (Tecan, Grödig, Austria).The calibration curve was fitted linear and used for calculation of the dsDNA concentration of the samples.

Results

Development of an antibody precipitation method with Polyethylene glycol

First the optimal conditions for precipitation were determined in small scale and then scaled to 100 ml stirred tank reactor. These conditions have been further compared to protein A affinity chromatography; a protocol used in industry.

In order to find the optimal parameters a high throughput screening of antibody precipitation out of CHO (Chinese Hamster Ovary) cell culture supernatant was carried out This high throughput screening was performed in 96 deep well plates and allowed the determination of optimal polyethylene glycol (PEG) precipitation parameters such influence of PEG size, PEG concentration, pH value of the supernatant and time to reach equilibrium.

PEG precipitation kinetics

Figure 8 shows the precipitation behaviour of four different mAbs (mAb1, mAb2, mAb3 and mAb5) with different PEG sizes at certain concentration (PEG2000 12.5%, PEG4000 10.0%, PEG6000 7.5%, PEG20000 2.5%) and different pH values (5.0, 7.0, 8.5). Precipitation screening was performed in 96 deep well plates. At time 15, 30, 60 and 180 samples were taken for analysis. Yield of precipitated protein did not change over the whole reaction time but was dependent on (i) antibody, (ii) PEG size and (iii) concentration. We assume that precipitation has reached equilibrium after 15 minutes. In this setting we are not able to get kinetic data at the very beginning of the reaction after addition of PEG.

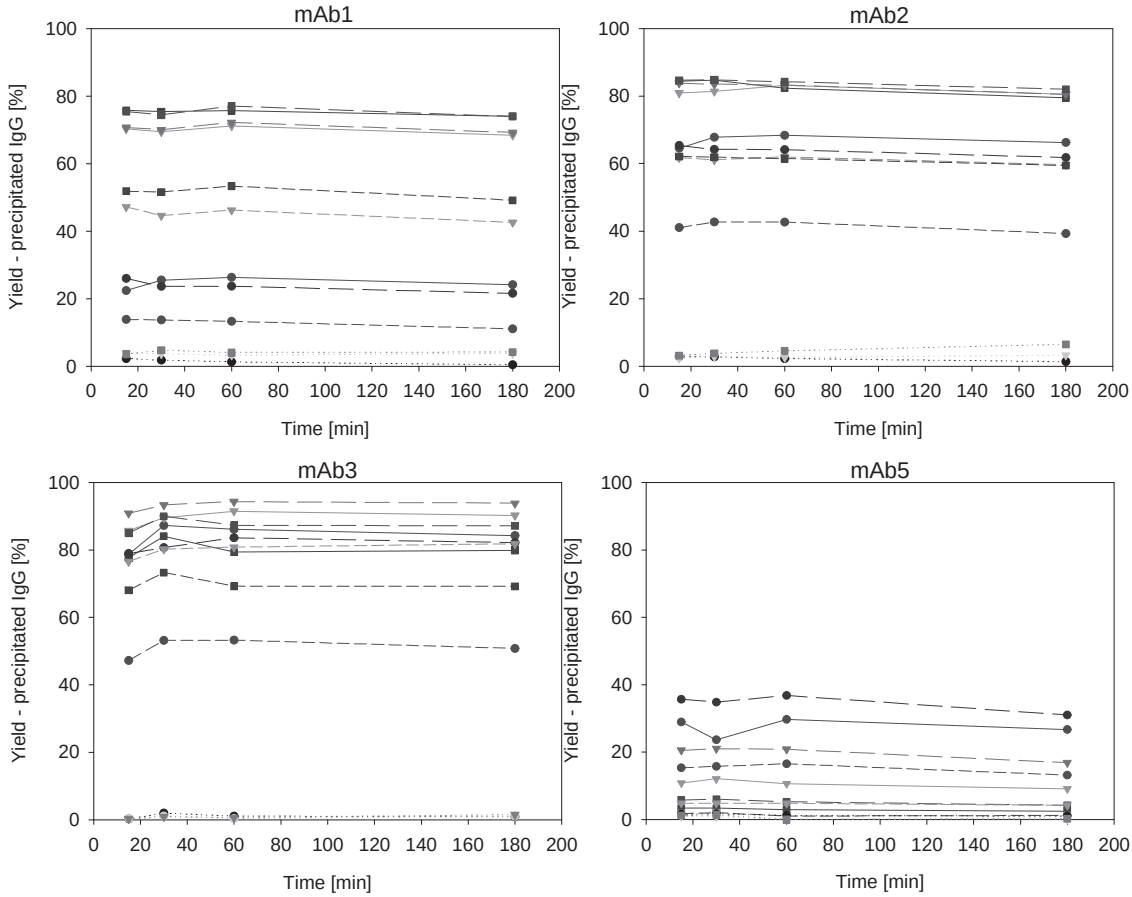


Figure 8: Screening of mAb precipitation kinetics from 15 to 180 minutes reaction time with different PEG sizes and concentrations (solid line 12.5 % PEG2000, dashed line 10.0 % PEG4000, short dashed line 7.5 % PEG6000 and dotted line 2.5 % PEG20000) at different pH values (samples with dots were precipitated at pH 5.0, with triangles at 7.0 and with squares at 8.5) and with four different mAbs (mAb1, mAb2, mAb3, mAb5)

PEG size, concentration and pH value screening

In further screening experiments, a reaction time of 60 minutes was used to ensure that equilibrium has been reached. For screening of effective PEG concentration and optimal pH value precipitations were performed with (i) four different mAbs (mAb1, mAb2, mAb3, mAb5), (ii) at different pH values (5.0, 7.0, 8.5) as well as (iii) various PEG sizes (2000, 4000, 6000, 20000) and (iv) concentrations (1.0%, 2.5%, 5.0%, 7.5%, 10.0%, 12.5%, 14.0%) in 96 deep well plates. All screening samples were analysed with analytical affinity chromatography (Staphylococcal Protein A) for IgG content and purity (Figure 9). Increasing of PEG size has a higher impact on yield than increasing of PEG concentration. The pH value (5.0-8.5) had no significant influence on yield at higher PEG sizes (PEG4000 to PEG20000) or concentrations (10 to 14% PEG (v/v)). Based on these observations we selected 14% (v/v) PEG6000 as optimal precipitation parameters for reaching almost highest yield. Only with PEG20000 a higher yield was reached on the expense of a much higher viscosity. Furthermore, purity (Figure 9, purity - right figures) decreased with increasing PEG size. Whereby, with PEG20000 at concentrations from 12.5% to 14.0% (v/v) maximal purity was reached. Certainly, at lower PEG concentrations lower purities were obtained this is, explained by the lower yield because purity is calculated by comparing peak areas of the analytical affinity chromatograms. Purity was not influenced by different pH values except precipitations with lower PEG concentration showed little pH influence.

Screening showed that a purification method can be established which is not influence by the pH. PEG precipitation with 14% (v/v) PEG6000 can be used as a platform process for mAbs without adjusting pH to the pI of the target antibody.

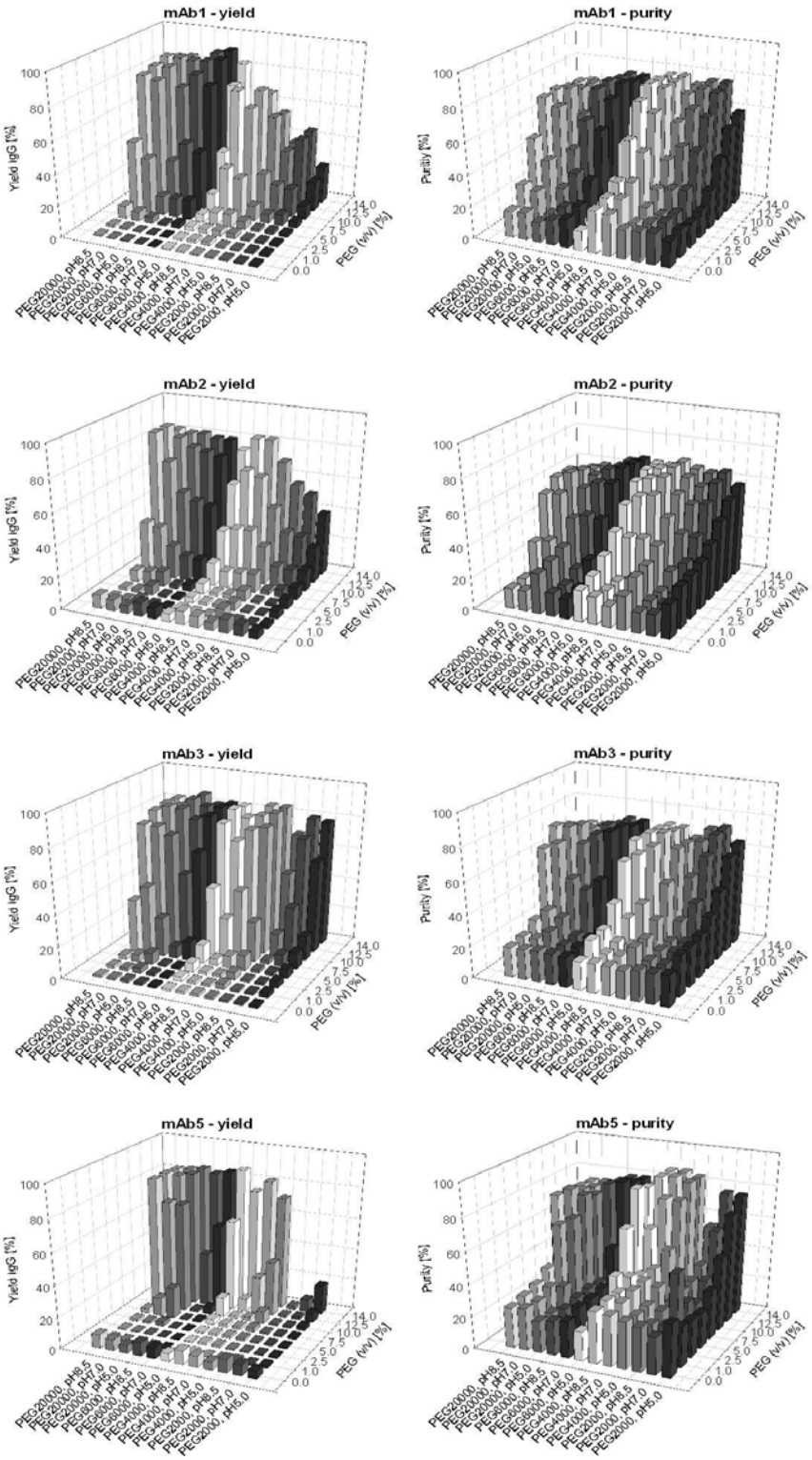


Figure 9 Screening values of different mAbs (mAb1, mAb2, mAb3, mAb5) precipitated with PEG2000, PEG4000, PEG6000, and PEG20000 at the pH values 5.0, 7.0, 8.5 versus different PEG concentration up to 14.0% (v/v) versus the IgG yield and purity of the resuspended precipitate

Influence of temperature on yield and purity

Also the influence of temperature on yield and purity was screened (Figure 10). Precipitation (14% (v/v) PEG6000, pH ~ 7) was performed with three different mAb (mAb1, mAb2 and mAb3) at different temperature values (5°C, 25°C and 35°C). Yield and purity was determined with analytical affinity chromatography and showed no significant differences. Higher or lower temperatures were not tested because denaturation out of this temperature range may occur.

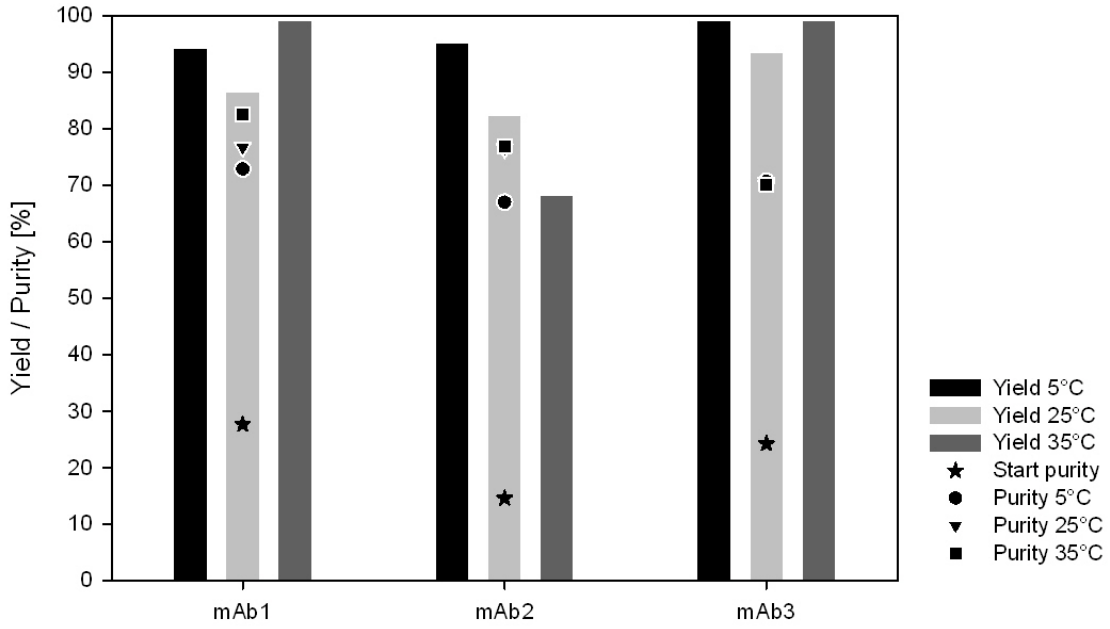


Figure 10: Yield (5°C black bar, 25°C light gray bar and 35°C dark gray bar) and purity (5°C black dot, 25°C black triangle and 35°C black square) comparison of three different tempered PEG precipitations (14% PEG) with cell culture supernatant (purity: black star) and performed with three mAbs

Yield and purity in small scale

To confirm the screening results PEG precipitation with 14% (v/v) and PEG6000, at pH ~ 7 was performed in 15 mL scale. Yield determined with analytical affinity chromatography (Staphylococcal Protein A) and showed values above 95 % (data not shown) which are approximately 20 % higher than the values of the 96 deep well plates. This is explained by the liquid film which remains at the wall of the 96 deep well plate wells after discarding precipitation supernatant. Purity of PEG precipitated and protein A purified (affinity chromatography) mAbs were determined with size exclusion. Comparison of the size exclusion chromatograms are shown in Figure 11 (dashed light grey - PEG precipitation; short dashed dark grey - Protein A purified) and

have for all five mAbs nearly equal shapes. Only high molecular weight impurities (dsDNA and aggregates) as well as some low molecular weight impurities (host cell proteins) were not separated with PEG precipitation.

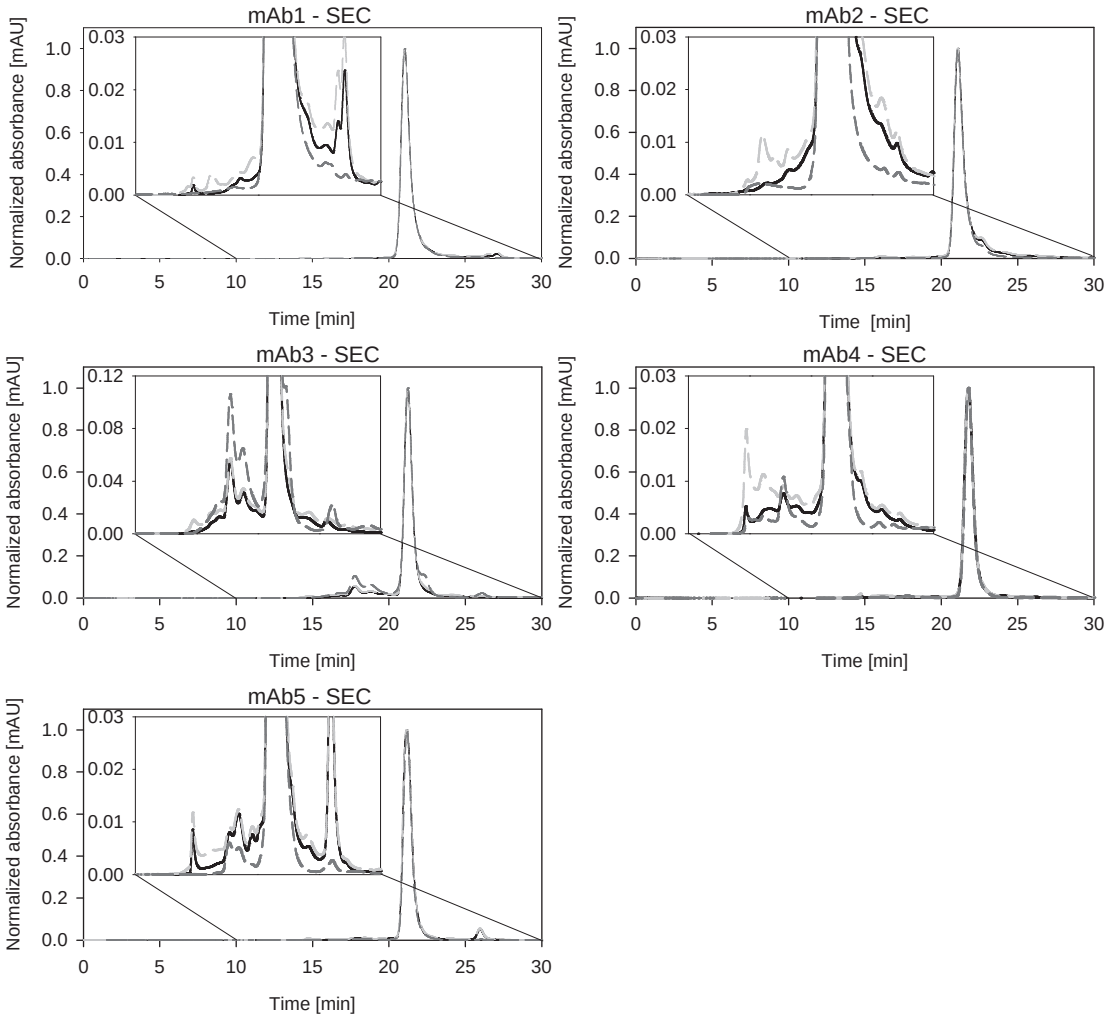


Figure 11: Size exclusion chromatogram (TSK3000 SW XL, Tosoh) comparison of $CaCl_2$/PEG (dark solid line), protein A (gray dashed line) and PEG (light gray short dashed line) purified mAbs (mAb1 to mAb5)

Solubility of recombinant mAbs and total protein impurities

For improvement of the PEG precipitation method, solubility of the recombinant produced mAbs and the attendant protein impurities (total protein impurities) was determined. Solubility (S_0) was ascertained via extension of the half logarithmic solubility curve to the zero point of the x-axis (PEG concentration). Solubility curve is an important tool to characterise the precipitation behaviour of desired proteins.

Influences of mAb and PEG concentration on precipitation

Figure 12 show the solubility curve and the linear equation of purified mAb2. Above the curve mAbs will precipitate and below they are in solution. For better understanding were courses of two precipitation examples mapped. First example show remaining mAb in cell culture supernatant (mAb2) by use of 14% PEG6000. Starting from point A to B at the solubility curve and going from there to C, where we get the mAb2 solubility in presents of 14 % PEG6000, which is with 1.8 µg/mL (log S = -2,75), approx. 0.1 % of the yield. Second example shows how much PEG6000 is needed when cell culture titer is at 10 mg/mL. From point D to E only PEG concentration increases. At point E mAb precipitations starts and at F a log S of - 1 is reached, which means that 0.1 mg/mL (0.1 %) mAb is left in solution. Point G shows the needed PEG concentration (approx. 9 %) to have a yield of 99.9%. These examples pointed out the characteristics of mAb precipitation behaviour and show how important a solubility curve for the setup of a precipitation process is.

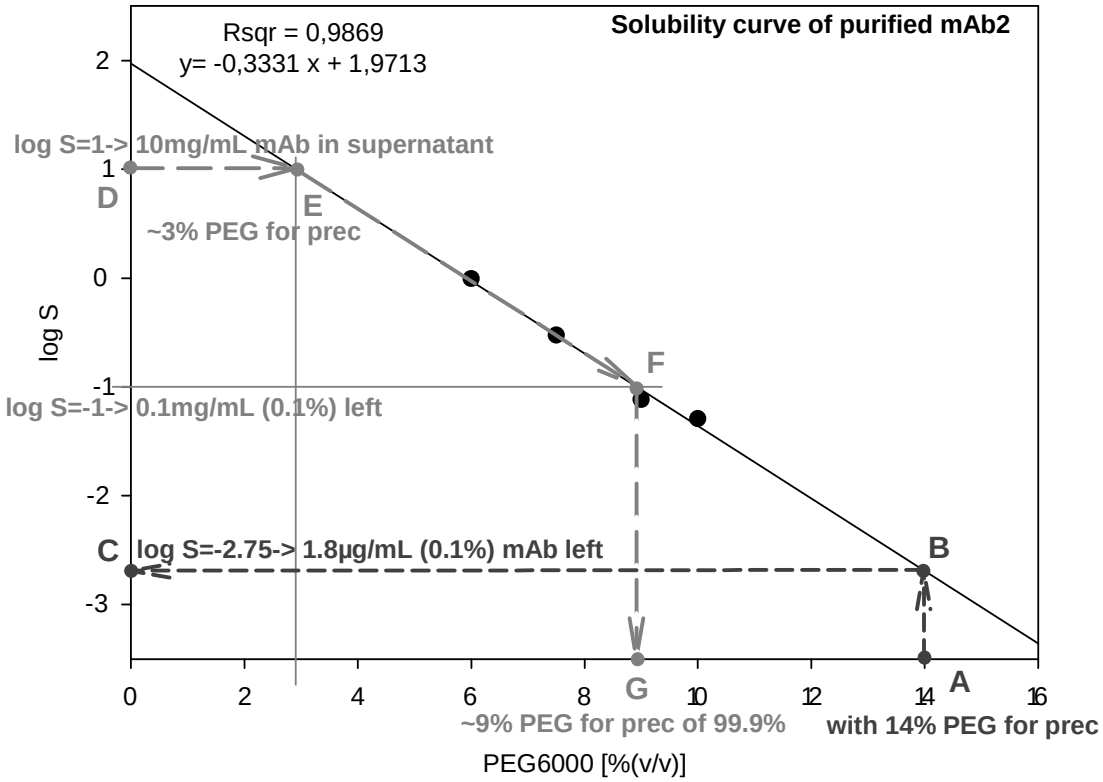


Figure 12: Solubility curve of purified mAb2 (black dots and line) with explanation of PEG and mAb concentration correlation; A to C (dark gray dots and short dashed line) show remaining mAb in solution by use of 14% PEG; D to G (light gray dots and long dashed line) show needed PEG concentration to precipitate 99.9 % of 10 mg/mL mAb

Solubility curves of purified mAbs and total proteins

For evaluation of separation behaviour of mAb and protein impurity from cell culture supernatants, solubility curve were determined. Comparison of purified mAb and total protein impurity curves showed different precipitation characteristics. Solubility curve of the purified mAb are steeper and have a higher solubility compared to total protein impurities, which are flat with low solubility. Reason for such a total proteins solubility curve pattern is the low amount of precipitable protein. Precipitation of impurities without co-precipitation of pure mAb can be performed in the area above the solubility curve of total protein impurities and below the solubility curve of purified mAb. These precipitation areas only exist for mAb1 and mAb2. For mAb3 is the area too narrow to precipitate the impurities (Figure 13). Consequently, the precipitation of total protein impurities with PEG is for some antibodies possible, but for realisation solubility of total protein impurities have to be low and even thereby a small amount of impurities will remain in solution.

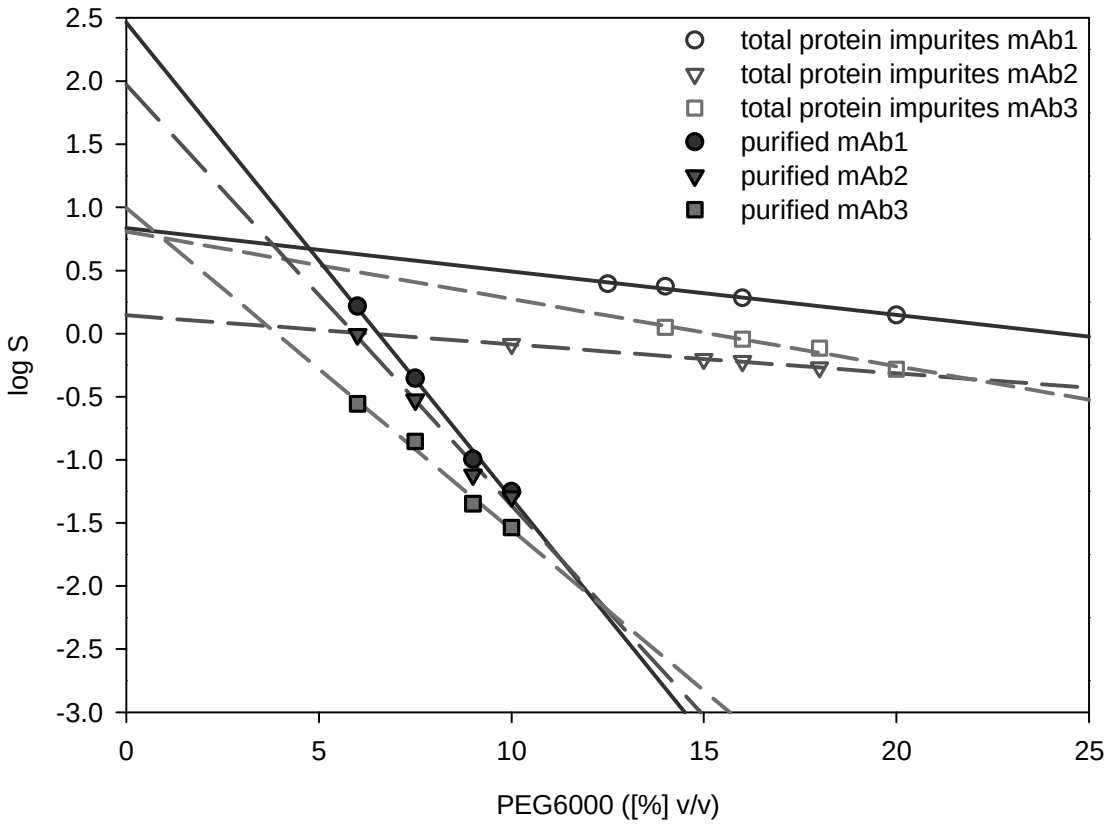


Figure 13: Solubility curves of purified mAbs and total protein impurities from all cell culture supernatants. Solubility curves in black are for mAb1, dark gray are for mAb2 and light gray are for mAb3. Solubility curves with circles, triangles, squares are for total protein impurities and curves with filled circles, triangles, squares are for purified mAbs

Solubility of total protein impurity and purified mAb are derived by a linear regression (Figure 13) describe with the Juckes equitation (Juckles 1971). Slope and interception of purified mAbs and total protein impurities from this linear equitation are show in. Comparison show a ten time higher slope for purified mAb and 2 to 60 times higher solubility calculated from interception. Solubility of purified mAbs is higher compared to total protein impurities. This confirms PEG precipitation as mAb purification method.

Table 6: Solubility curve slope, interception and S_0 from purified mAbs and total protein impurities of three recombinant mAbs

Protein	Supernatant	β	$\log S_0$	S_0 [mg/mL]
purified mAb	mAb1	-0.3767	2.4643	291.27
	mAb2	-0.3331	1.9713	93.61
	mAb3	-0.2549	0.9962	9.91
total protein impurities	mAb1	-0.0343	0.8360	6.85
	mAb2	-0.0230	0.1459	1.40
	mAb3	-0.0532	0.8083	6.43

Combination of $CaCl_2$ and PEG precipitation

For improvement of purity a combination of PEG and $CaCl_2$ precipitation was developed in 10 mL scale plastic tubes. $CaCl_2$ can form an insoluble precipitate with phosphate which co-precipitates HMWI. HMWI are visible in the SEC of the PEG precipitations (Figure 11); compare 10 to 20 minutes retention time.

Optimization and scale up of $CaCl_2$/PEG precipitation

Initial $CaCl_2$ precipitation tests showed that precipitation only occurs with cell culture supernatant of mAb1 and mAb2. Supernatant of the other antibodies (mAb3, mAb4 and mAb5) stayed clear after $CaCl_2$ addition. Reason was the absence of phosphate (Table 5). However, for optimization of CaC_2/PEG precipitation the adding of phosphate to the supernatants was performed (Figure 14). Figure 11 show the comparison of protein A purified (affinity chromatography), PEG precipitated and $CaCl_2$/PEG precipitated mAbs. Highest purity was achieved with protein A purification closely followed by $CaCl_2$/PEG precipitation which nearly has the same high molecular weight impurity (HMWI) reduction but some low molecular weight impurities (LMWI). Poorest purity resulted by single PEG precipitation. Yields were determined with analytical affinity chromatography (Staphylococcal Protein A) and the target values of approximately 80 % were achieved with $CaCl_2$/PEG precipitation (Table 5). For a further confirmation of these results precipitation was performed in a stirred tank reactor, the automated lab reactor (Easymax, Mettler-Toledo). The working volume of this stirred reactor was at 100 ml and both precipitations were sequently performed in this stirring tank. During precipitations the mixing speed and temperature were controlled and turbidity was

online measured (Figure 15). Turbidity data of $CaCl_2$ and PEG precipitation for all mAbs showed that precipitation is completed after 2 to 5 minutes for $CaCl_2$ and after 5 to 10 minutes for PEG precipitation. The combined $CaCl_2$/PEG precipitation is a fast platform process for mAb purification which may replace the affinity capture step.

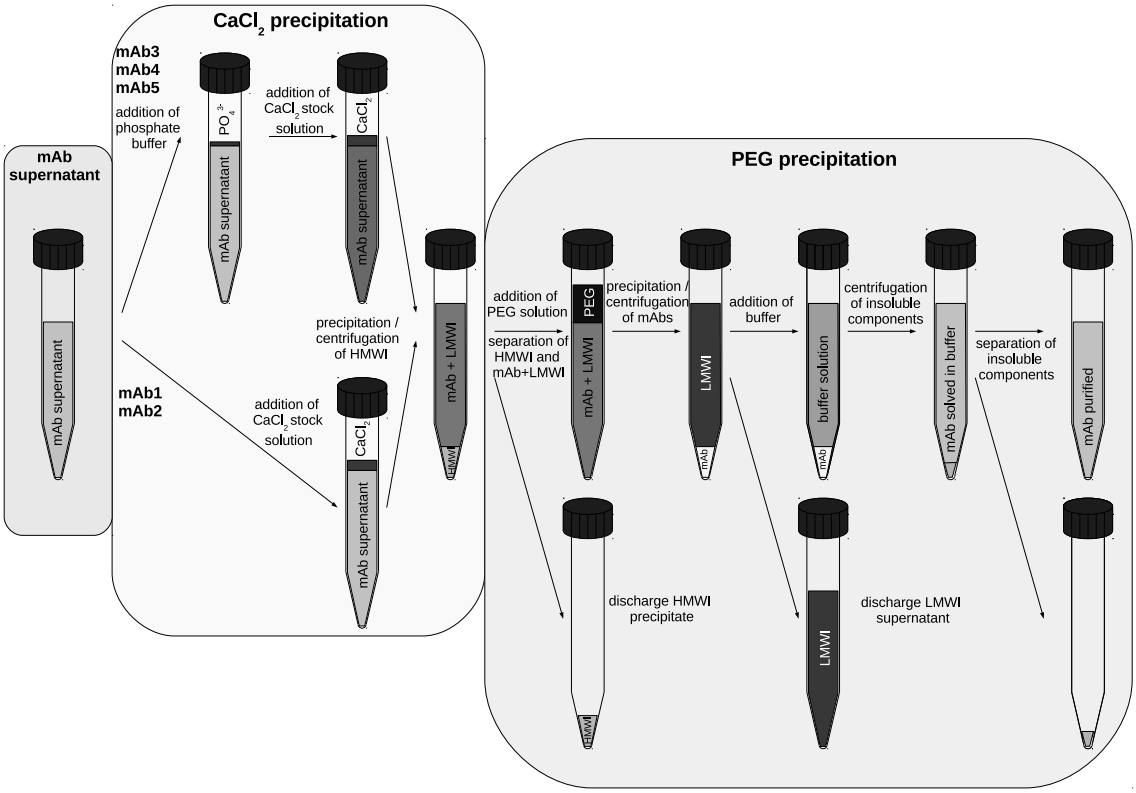


Figure 14: Alignment of the purification steps ($CaCl_2$ and PEG) for all mAbs

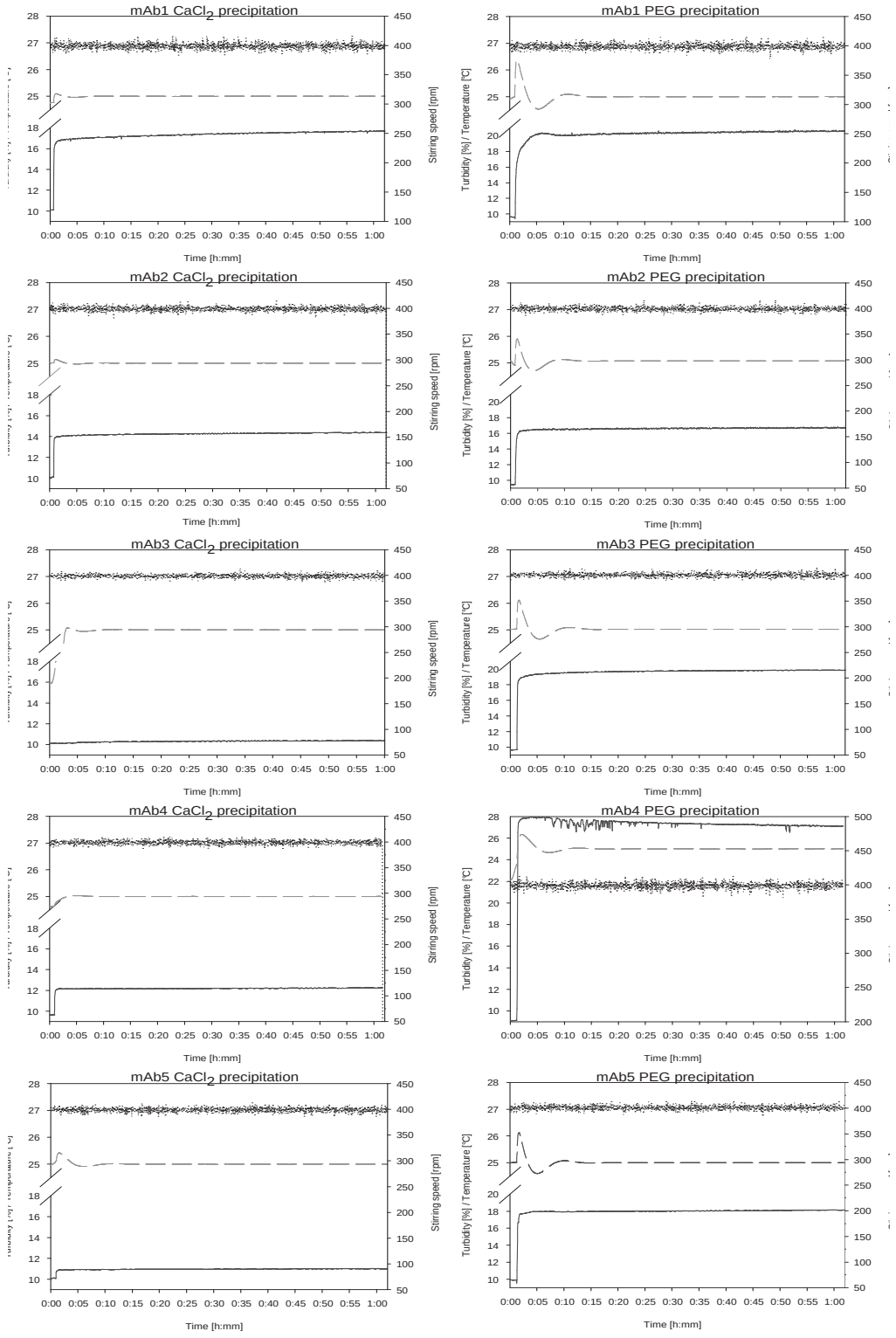


Figure 15: Automated Lab Reactor (EasyMax, Mettler-Toledo) online data (solid line is turbidity, dashed line is temperature, spotted line is stirring speed) of $CaCl_2$ and PEG precipitation of all mAbs (mAb1 to mAb5)

Yield and purity: $CaCl_2$/PEG precipitation versus affinity chromatography

To further confirm the competitiveness of $CaCl_2$/PEG precipitation to staphylococcus protein A affinity chromatography, yield and purity were compared with affinity chromatography (protein A purification). Five different antibodies were used to be able to make a broader conclusion (Table 7). A comparable yield data of $CaCl_2$/PEG precipitation and affinity chromatography was observed. The yield is always in the range of 80 to 95 %. Purity data measured with SEC and HCP ELISA are also summarized in Table 7: Yield and purity parameter comparison between cell culture supernatant, affinity chromatography and CaCl2/PEG purified mAbs (mAb1 to mAb5)Table 7. SEC analysis of HMWI showed for mAb1 and mAb2 equal reduction for both purification methods. The other antibodies (mAb3 to mAb5) showed lower HMWI reduction for $CaCl_2$/PEG precipitation compared to affinity chromatography. HCP reduction with $CaCl_2$/PEG precipitation was between 3 and 13 fold. For being competitive to affinity chromatography the HCP reduction has to be between 30 and 900. However, direct comparison of SEC does not show such differences in purity. Reason for inconsistency of the purity analysis by SEC and HCP ELISA might be interference of Ca^{2+} in HCP ELISA.

Table 7: Yield and purity parameter comparison between cell culture supernatant, affinity chromatography and $CaCl_2$/PEG purified mAbs (mAb1 to mAb5)

Yield and purity parameters	Antibodies				
	mAb1	mAb2	mAb3	mAb4	mAb5
Antibody yield					
Yield after affinity chromatography [%]	83	100	75	79	96
Yield after $CaCl_2$/PEG precipitation [%]	81	78	96	90	94
High molecular weight impurities (HMWI) / aggregates					
HMWI cell free harvest [%]	1.34	3.82	18.28	5.03	1.90
HMWI after affinity chromatography [%]	0.50	0.60	5.10	0.80	0.70
HMWI after $CaCl_2$/PEG precipitation [%]	0.40	0.50	11.50	2.30	2.30
HMWI comparison (harvest/affinity) [fold]	2.7	6.4	3.6	6.3	2.7
HMWI comparison (harvest/prec) [fold]	3.4	7.6	1.6	2.2	0.8
Host cell proteins (HCP)					
Cell free harvest HCP [ppm]	255637	388296	160115	676304	275377
Affinity chromatography HCP [ppm]	8170	5645	1440	5000	299
$CaCl_2$/PEG precipitation [ppm]	-	35929	46072	53312	73700
HCP comparison (harvest/affinity) [fold]	31.3	68.8	111.2	135.3	921.0
HCP comparison (harvest/prec) [fold]	-	10.8	3.5	12.7	3.7

In order to clarify this question a 2D DIGE of mAb1 purified with $CaCl_2$/PEG precipitation and affinity chromatography was performed (Figure 16). For identification of HCP and mAb spots on the 2D gel drugs substance (DS) was used as reference sample. All spots which are not present on the DS gel were considered as impurity spots. Comparison of the different stained samples showed less increased impurity spots for protein A purified than for $CaCl_2$/PEG precipitated samples compared to DS (Table 8). Both samples show an equal decrease of spots compared to DS which is most properly caused by the use different batches. However, 2D DIGE demonstrates that protein A purification reduces more HCP than $CaCl_2$/PEG precipitation, but exact purities can not be determined. Despite unclear purity values, $CaCl_2$/PEG precipitation show advantageous properties for mAb purification. Due to this reason further research on this purification method should be done.

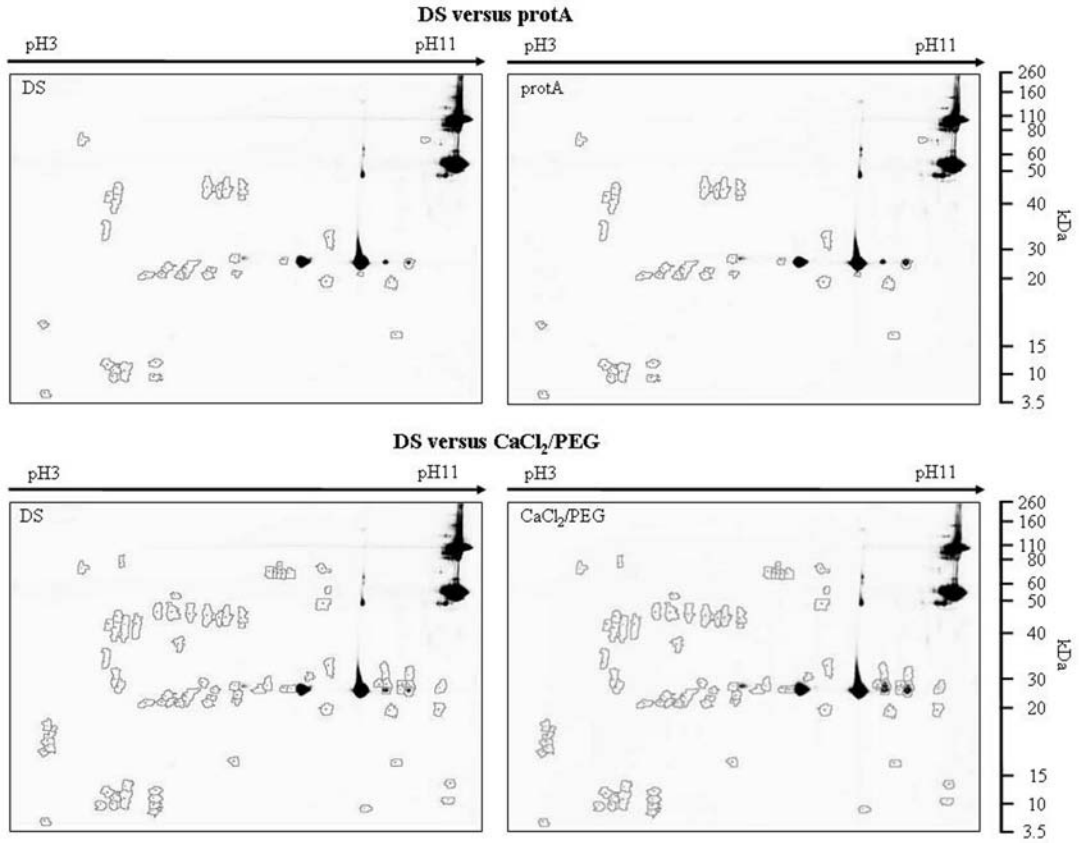


Figure 16: 2D-DIGE gel of $CaCl_2$/PEG precipitated and protein A purified samples versus dugs substance

Table 8: Data evaluation of 2D-DIGE gel from comparison of $CaCl_2$/PEG precipitated and protein A purified samples vs. drug substance (DS); in- or decrease means more or less impurity than DS

spot behavior	DS vs protA		DS vs $CaCl_2$/PEG	
	spots	spots [%]	spots	spots [%]
decreased	15	5.8%	15	5.8%
similar	208	80.6%	160	62.0%
increased	35	13.6%	83	32.2%

Combination of caprylic acid and PEG precipitation

The separation of impurities and antibodies out of plasma or cell culture supernatant was frequently done with caprylic acid (CA) and PEG precipitation (Atha and Ingham 1981; Hasko and Vaszileva 1982; Mahadevan and Hall 1992; Mohanty and Elazhary 1989; Perosa et al. 1990; Polson et al. 1964; Rojas et al. 1994; Rosa et al. 2013; Russo et al. 1983). Solely the combination of both precipitation methods was not researched. Due to this reason a screening of a precipitation combination with CA and PEG was performed with a clarified CHO cell culture supernatant. The first purification step with CA is for separation of HCP and dsDNA and the second step is for mAb capturing which causes an additional reduction of HCP.

Pre-screenings for CA/PEG precipitation

Based on data from literature most frequently applied precipitation conditions for polyclonal IgG are pH values in the range about 4.5, sodium acetate concentration in the range of approx. 60 mM, four fold increase of volume by addition of a dilute salt solution, and caprylic acid concentration in the range of approx. 1 %. Especially, with focus on continuous manufacturing, reduced material input is the major aim and connection of caprylic acid precipitation with PEG precipitation, without pH adjustment between precipitation steps. The caprylic acid precipitation was performed with 50 mM sodium acetate, which was added as 4M sodium acetate stock solution in order to reduce the working volume. After addition of sodium acetate the solution was aliquoted and the pH was adjusted to 4.5, 5.0, 5.5, or 6.0, respectively and precipitations were performed with 1 % caprylic acid. Precipitates were separated by centrifugation and with supernatant PEG precipitation by addition of 14 % PEG was performed without pH adjustment. At lower pH purity is higher and yield lower (Figure

17). Low pH of the protein solutions is not compatible for direct injection to analytical affinity chromatography, thus analytical size exclusion was used for determination of yield and purity. Yield after caprylic acid precipitation step decrease from 80 to 60 % with increasing pH from 4.5 to 6.0. HCP reduction was in the range of 10. In further screenings, HCP reduction was not measured after the caprylic acid precipitation, because the reduction was not significant. After second precipitation a yield drop-off for all pH values was measured. But with an increasing pH value, the drop-off occurs in reduced extent (10 to 40 %). Solubility of IgG at low pH seems to be higher. Highest HCP reduction 120 to 160 fold was observed for mAb1 and 50 to 60 fold for mAb2 at pH values of 4.5 and 5.0.

In summary, at lower pH values yield after first precipitation step and HCP reduction after second precipitation step matches our requirements. Only yield after second precipitation was to poor at lower pH values. Hence, for further precipitation screenings pH has to be adjusted to an ambient value (approx. 7) prior second precipitation step.

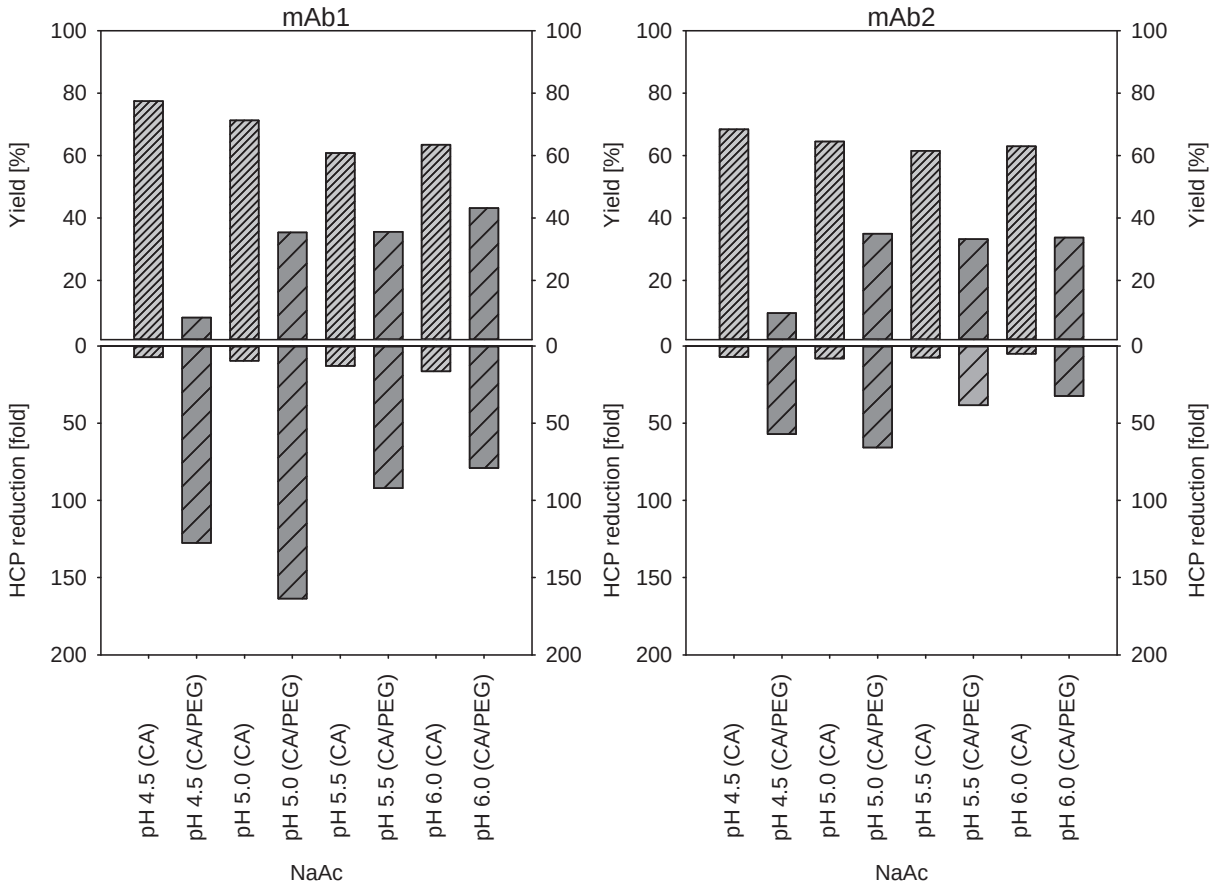


Figure 17: Screening of CA/PEG precipitation of two different mAbs with 1 % CA, 40 rpm, 60 min with 50 mM sodium acetate at pH 4.5, 5.0, 5.5 and 6.0 for CA precipitation and with 14 % PEG - 5 rpm - 60 min without pH adjustment for PEG precipitation was performed and analysed with SEC

Salt and pH value optimization

Upon the previously obtained findings a screening was preformed, to figure out the optimal precipitation conditions for first purification step (CA) of combined CA/PEG precipitation. The pH values 4.5, 5.0 and 5.5, salt concentration of 5, 10, 20, 40, 50, 100, 125 and 200 mM and two different salts (sodium acetate and sodium citrate) were tested in all combinations. Figure 18 and Figure 19 show the screenings of both supernatants with different salts. For Figure 18 sodium acetate and for Figure 19 sodium citrate was used. Yield was determined after first and second precipitation step and purity only after second precipitation step. pH value screening show a decrease of yield with increasing pH (4.5 to 5.5) after first (approx. 85 to 50 %) and second precipitation step (approx. 50 to 35 %). This trend was equal for both salts with the exception that the uses of sodium citrate causes higher yield after second precipitation (approx. 60 to 40 %). Purity determination showed HCP reductions between 50 and 350

fold, depending on pH value and kind of salt. Sodium citrate cause slightly lower purity but higher yield. Due to the reason that the reached HCP reduction of approx. 100 fold is state of the art for common mAb purification processes, a higher yield and consequently the use of sodium citrate was more suitable for the novel process. Screening of salt concentrations showed with sodium acetate (Figure 18) highest yield after both precipitation steps and a sufficient purity at low salt concentrations (5 to 10 mM). These values occurred because of the pH drop-off from approx. 5.5 to 5.0 which are caused by deficient buffer ability at low salt concentration. Under consideration of these data, highest yield with adequate purity was reached with 100 mM sodium citrate (Figure 19). Higher salt concentration causes increased HCP reduction but a decrease of yield. Whereby, lower salt concentrations (below 100 mM) show slightly lower yield and HCP reduction. All these findings indicate that pH 4.5 and 100 mM sodium citrate are the most promising CA precipitation parameters for the combined CA/PEG precipitation.

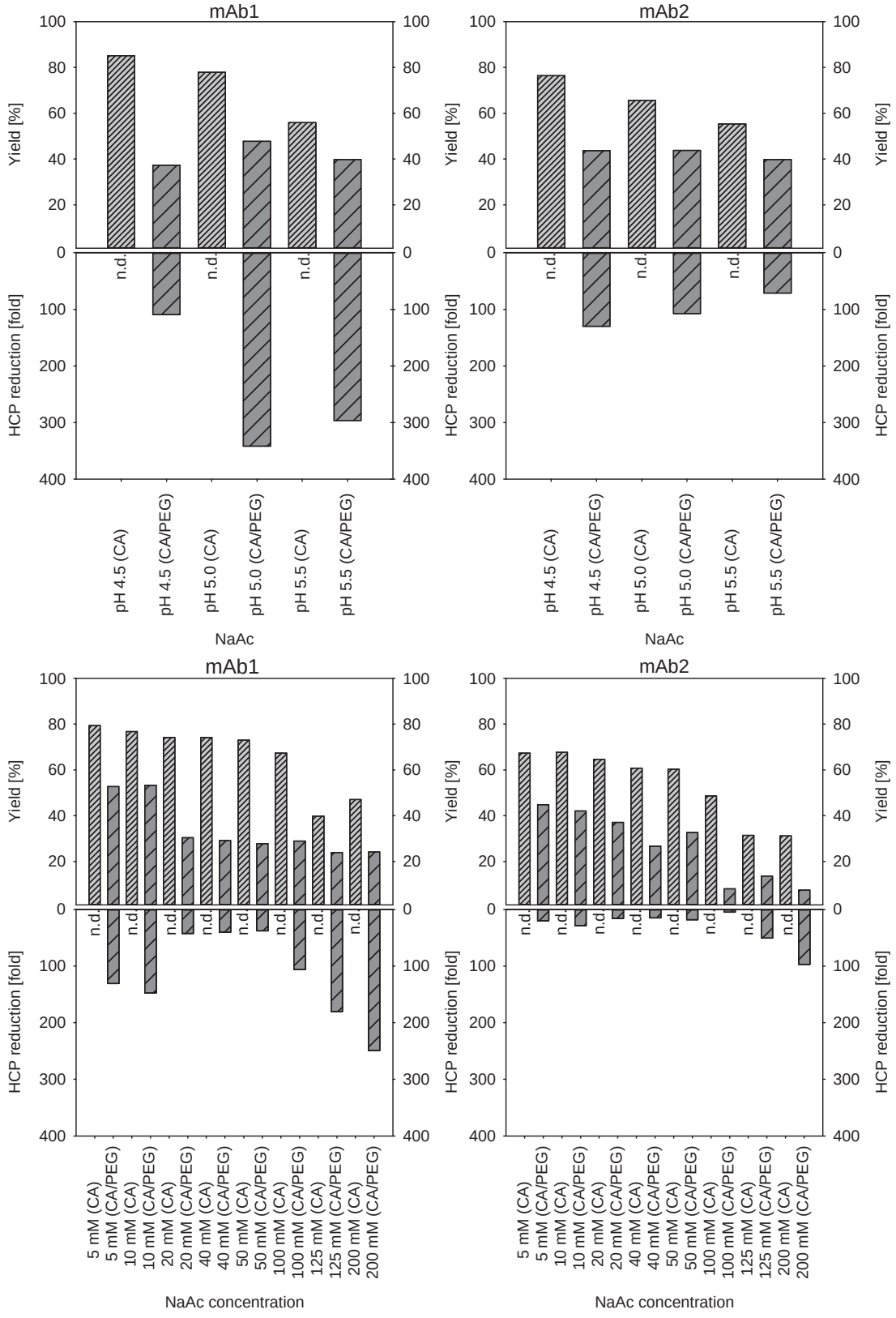


Figure 18: Screening of CA/PEG precipitation of two different mAbs with 1 % CA, 40 rpm, 60 min with different sodium acetate concentrations (5 to 200 mM) at pH 4.5, 5.0 and 5.5 for CA precipitation and with 14 % PEG, 5 rpm, 60 min with approx. 7 pH for PEG precipitation was performed and analysed with SEC

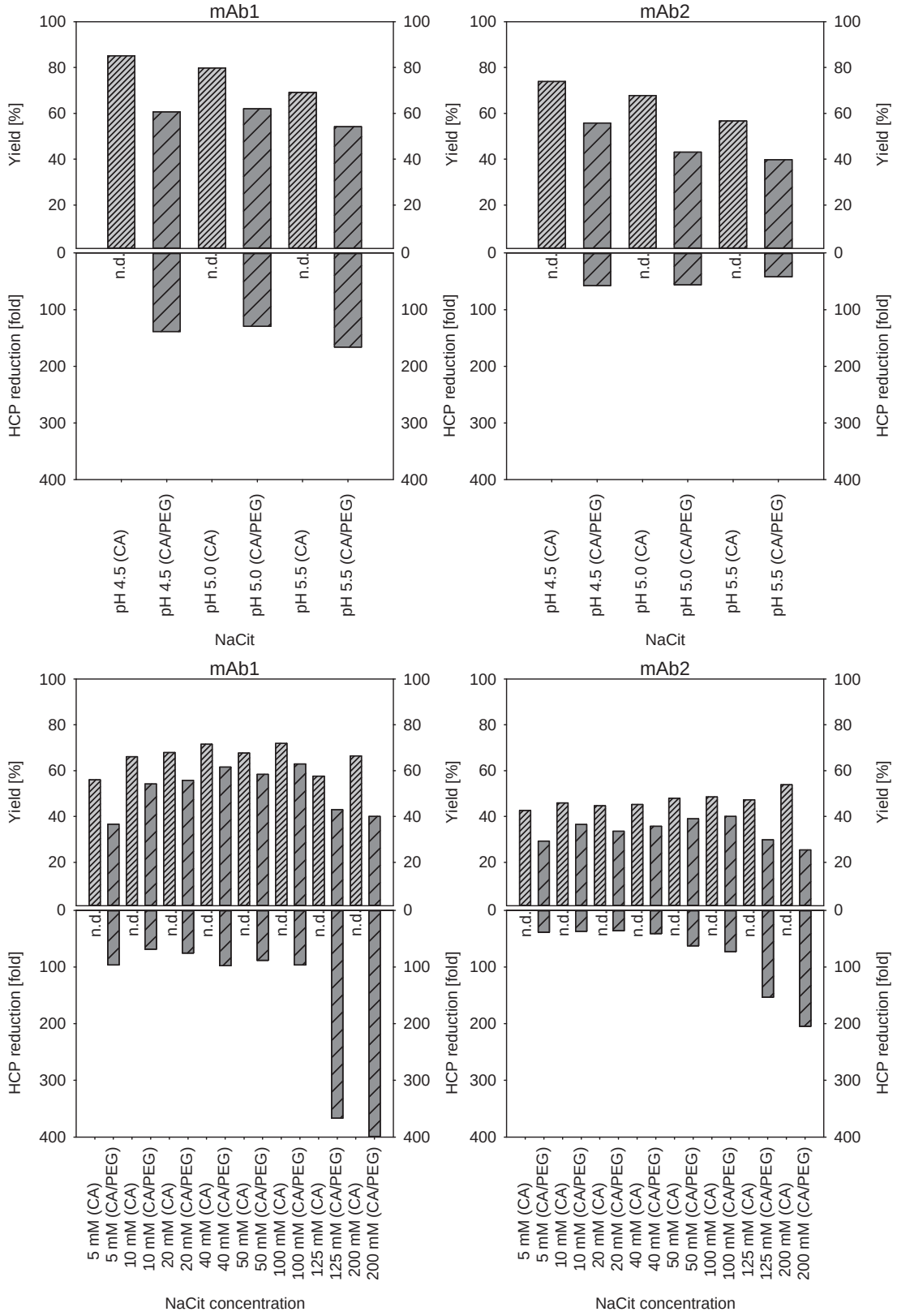


Figure 19: Screening of CA/PEG precipitation of two different mAbs with 1 % CA, 40 rpm, 60 min with different sodium citrate concentrations (5 to 200 mM) at pH 4.5, 5.0 and 5.5 for CA precipitation and with 14 % PEG, 5 rpm, 60 min with approx. 7 pH for PEG precipitation was performed and analysed with SEC

Screening of CA concentration and mixing parameters

Effect of mixing time (15, 30, 45 and 60 min) and mixing speed (10, 20, 30 and 40 rpm) as well as CA concentration (0.5, 1.0, 2.0, 4.0, 6.0 and 10.0 %) was evaluated for mAb1 and mAb2. Both mAb screenings of mixing time (pH 4.5, 100 mM NaCit, 1 % caprylic acid - 40 rpm, 14 % PEG - 5 rpm - 60 min) showed improved HCP reduction after second precipitation step (caprylic acid/PEG) but an decrease of yield with longer caprylic acid precipitation times (Figure 20). Increase of CA precipitation mixing speed (pH 4.5, 100 mM NaCit, 1 % CA - 60 min, 14 % PEG - 5 rpm - 60 min) causes also decrease of yield but the HCP reduction followed no certain trend (Figure 20). Obviously 45 min mixing time and a mixing speed of 10 rpm during the CA precipitation should be appropriate for optimal mAb purification. But due to the reason that CA forms a two phase system with the cell culture supernatant, a higher mixing speed has to be selected for getting an optimal dispersion. Hence a mixing speed of 30 rpm has highest possible dispersion, prior yield decrease after first precipitation step (CA). All these findings lead to the conclusion that for optimal CA precipitation best parameters are 45 minutes reaction time and 30 rpm mixing speed.

Then effect of caprylic acid concentration (0.5, 1.0, 2.0, 4.0, 6.0 and 10.0 %) was evaluated while other conditions were kept constant such as pH (4.5), sodium citrate concentration mixing speed (30 rpm) and reaction time (45 min) The second precipitation was performed with 14 % PEG at 5 rpm and 60 min. Caprylic acid concentration did not effect on yield and HCP reduction (Figure 21). Sole exception is the precipitation of mAb2 with 1 % CA, which show higher yield after second precipitation step (CA/PEG) then the other screened concentrations. Due to these differing values all samples were additional measured with analytical affinity chromatography (Figure 21). Data showed also no convincing trend in yield and lead to the conclusion that an increase of CA concentration has no significant positive impact on yield and purity. Therefore the CA concentration of 1 % CA for first precipitation step (CA) will be retained.

Furthermore, CA concentration screening data were used for yield comparison of analytical size exclusion and analytical affinity chromatography, to get a correlation between all prior performed yield measurements (cell culture supernatant as reference material) and the affinity chromatography values (calibration curve with Octagamma).

Novel data of both mAbs showed approx. 30% higher yield values after first (CA) and approx. 40% after second precipitation step (CA/PEG) compared to the SEC data (Figure 21). Reason for these differences was the use of cell culture supernatant as reference material for analytical SEC. The peak assigned to the IgG in size exclusion may also contain other impurities, when injecting the crude supernatant. These yield differences has no influence on the heretofore postulated assumptions, because for the precipitation parameters screenings only yield and purity trends and not exact values are important.

However, data showed that the use of higher CA concentration for the first precipitation step has no positive influence on yield or purity. Furthermore, the comparison of the analytical methods for yield determination showed that the initially measured yields were calculated too low and reflects only the screening data trends. Due to this reason the analytical affinity chromatography has to be used for future yield determination.

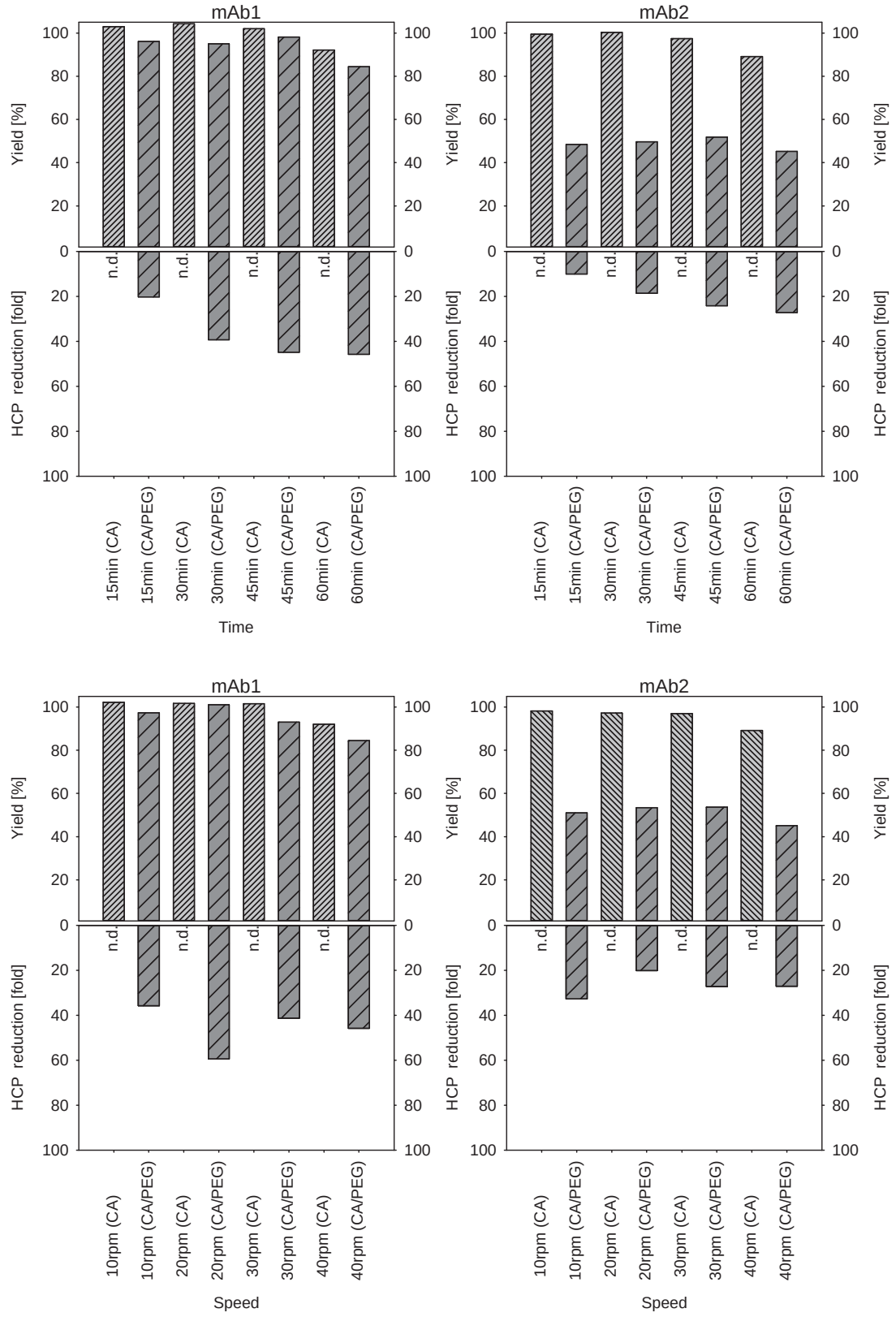


Figure 20: Screening of CA/PEG precipitation of two different mAbs with 1 % CA, 100 mM sodium citrate, pH 4.5 with different mixing speed (5 to 40 rpm) and mixing time (15 to 60 min) for CA precipitation and with 14 % PEG, 5 rpm, 60 min with approx. 7 pH for PEG precipitation was performed and analysed with SEC

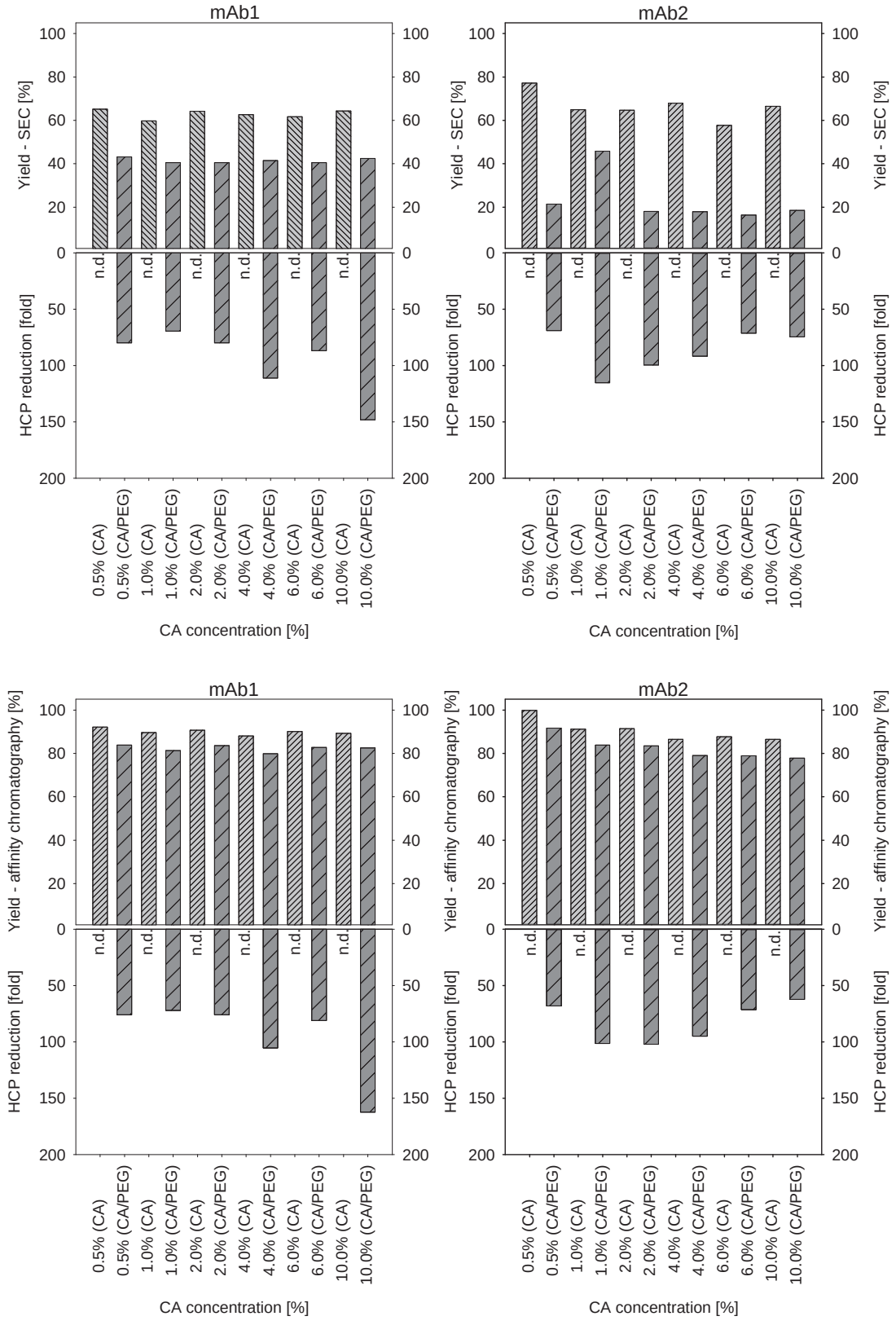
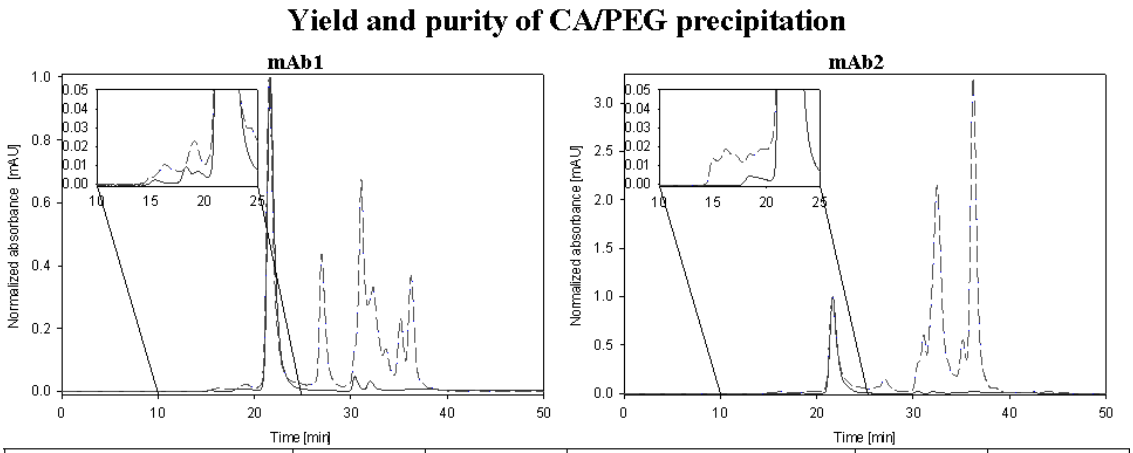


Figure 21: Screening of CA/PEG precipitation of two different mAbs with 100 mM sodium citrate, 30 rpm, 45 min, pH 4.5 with different CA concentrations (1 to 10 %) for CA precipitation and with 14 % PEG, 5 rpm, 60 min with approx. 7 pH for PEG precipitation was performed and analysed with SEC and affinity chromatography

Yield and purity data of CA/PEG precipitation

To verify all the screening assumptions, a two-step precipitation (CA/PEG) of both mAbs was performed. For the CA precipitation a concentration of 1 % CA with 100mM sodium citrate at pH 4.5 was used and the precipitation lasted for 45 minutes with 30 rpm mixing speed on a rotator. After first purification step PEG precipitation was performed with 14 % PEG6000 at approx. pH 7 for 60 minutes reaction time with 5 rpm mixing speed. Prior analytical measurements, IgG precipitate was washed with PEG solution and resolved with histidine buffer. For getting a general view about the stage of mAb yield and purity after CA/PEG precipitation, values were determined wit analytical affinity chromatography (protein A CIM disk), HCP ELISA (Cygnus) and analytical SEC (TSK G3000 SWxl). Figure 22 shows all yield and purity data in comparison to the values of conventional protein A affinity chromatography purification step. The SEC chromatograms show a comparison of protein A and CA/PEG purified cell culture supernatant of two mAbs (mAb1 and mAb2). Recognizable is the comparable reduction of low molecular weight impurities (LMWI), starting after the IgG peak (20 to 23 min retention time). The high molecular weight impurities (HMWI), which are in the area prior the IgG peak, looks nearly similar for protein A and CA/PEG purification. But zooming in, showed that protein A purification has a higher reduction of HMWI. For protein A purified samples reduction was below 1 % and for CA/PEG purified samples between 1 to 2 %. Determination of HCP showed a higher fold reduction (between 70 and 100 fold) with CA/PEG precipitation compared to the protein A purified samples (between 40 and 60 fold). The absolute HCP values of the CA/PEG purification are with approx. 4000 ppm in a common range of protein A purification. Yield determination was performed with analytical affinity chromatography and gave for both samples values above 80 %, which are sufficient for first capture step.

Comparison of CA/PEG precipitation and affinity chromatography showed the competitiveness of the novel purification method. There are minor differences in yield and purity. However, the ability to drive the precipitation processes fully continuous exceeds this yield and purity difference.



mAb1	PEG/CA	protein A	mAb2	PEG/CA	protein A
Monomers [SEC] (%)	97.9	99.0	**Monomers [SEC] (%)**	98.9	99.3
HMWI [SEC] (%)	2.1	0.5	**HMWI [SEC] (%)**	1.1	0.6
Yield [ALC](%)	81.0	83.3	**Yield [ALC](%)**	84.0	99.9
HCP [ELISA] (ppm)	3711	8170	**HCP [ELISA] (ppm)**	3038	5645
HCP reduction (fold)	72	40	**HCP reduction (fold)**	102	62

Figure 22: Yield and purity values of two different CHO cell culture supernatants (mAb1 and mAb2) purified with CA/PEG precipitation. Determination were performed with SEC and affinity chromatography

Design and construction of a tubular precipitation reactor

After establishing the CA/PEG precipitation combination for mAb purification in small scale (15mL batch; Greiner tubes), a setup for a continuous lab scale process was needed. To reach this aim, a tubular reactor was designed and constructed.

Design of the tubular CA/PEG precipitation reactor

For reactor designing a P&I flowchart was prepared with a student AutoCAD version (Figure 23). In the flowchart the tubular reactor consists of two precipitation areas, first for CA precipitation and second for PEG precipitation. For the CA precipitation four different reservoirs for adding of sodium citrate buffer, sodium hydroxide and caprylic acid to the cell culture supernatant were planned. After a mixing line with a residents time of 15 to 45 minutes a separation area for CA precipitates was scheduled. For the following PEG precipitation a reservoir with citric acid for pH adjustment and the PEG solution reservoir were planned. Second mixing line with a residents time of 15 minutes for PEG precipitation was planned. In connection to the PEG precipitation area a separation system with three filter units was designed. Separation, washing, back flushing and solving of the PEG precipitate is possible.

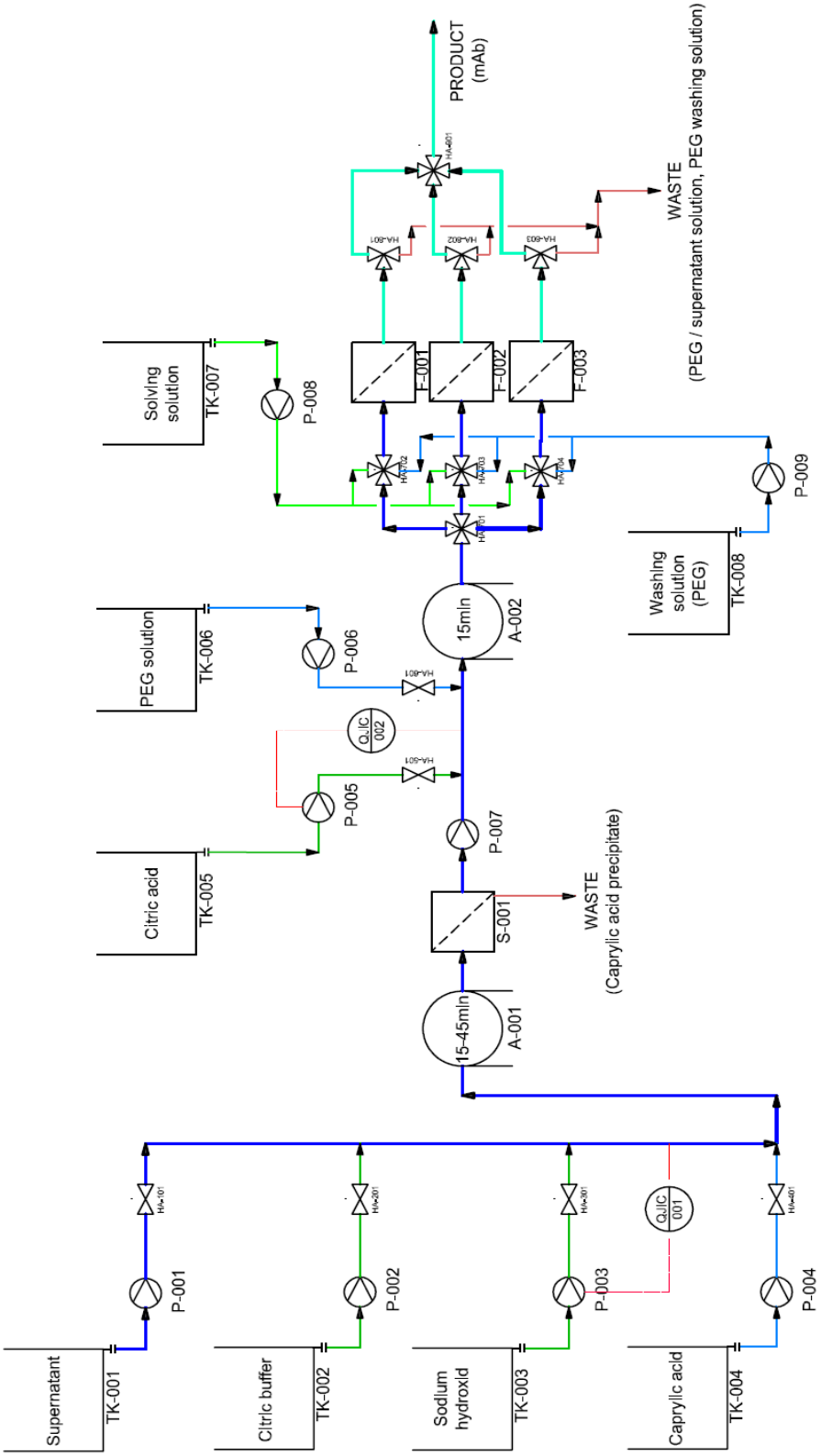


Figure 23: P&I diagram for the tubular precipitation reactor with two continuous precipitation areas for CA and PEG precipitation

Construction of the tubular CA/PEG precipitation reactor

After designing of the tubular reactor with a P&I flowchart the reactor was constructed. Setup of the reactor was realized with a metal rack, whereby the precipitation areas were installed on above the other (Figure 24). For tubular reactor lines silicon tubes (d_i = 5 mm) were used and the precipitate separation areas were realized with magnetic valves and filter units. Both separation areas were constructed with a black flush mode (Figure 26 and Figure 28). For fast intervening during precipitation run, system control was realized with an interconnected analogous system, with the possibility to switch from one to the next filter state by hand. Additionally, an analogous pressure alarm system with a sonic alert was established, to know when it is time for a switch to the next filter state (Figure 29). Both precipitation lines can be run independent, for having the option of single precipitation tests (Figure 25 and Figure 27). In future, after development of an intertwining precipitation procedure, a digital process control system will be established. The reactor volume amounts approx. 2L, depending on the length of precipitation lines and the size of filter units. Actual precipitation line length derives from needed residents time and flow-rate of solutions which was assumed with 20 mL/min. For realizing this solution stream, the tube pump Ismatec REGLO digital was selected as pump for our lab scale precipitation reactor. The flow rate of 20 mL/min and the lab scale reactor sizing can manage a continuous throughput of approx. 200 L cell culture supernatant. This leads, with 80 % yield and 2 mg/mL mAb in supernatant, to an IgG output of approx. 320 kg/week, which fits to production scale amounts.

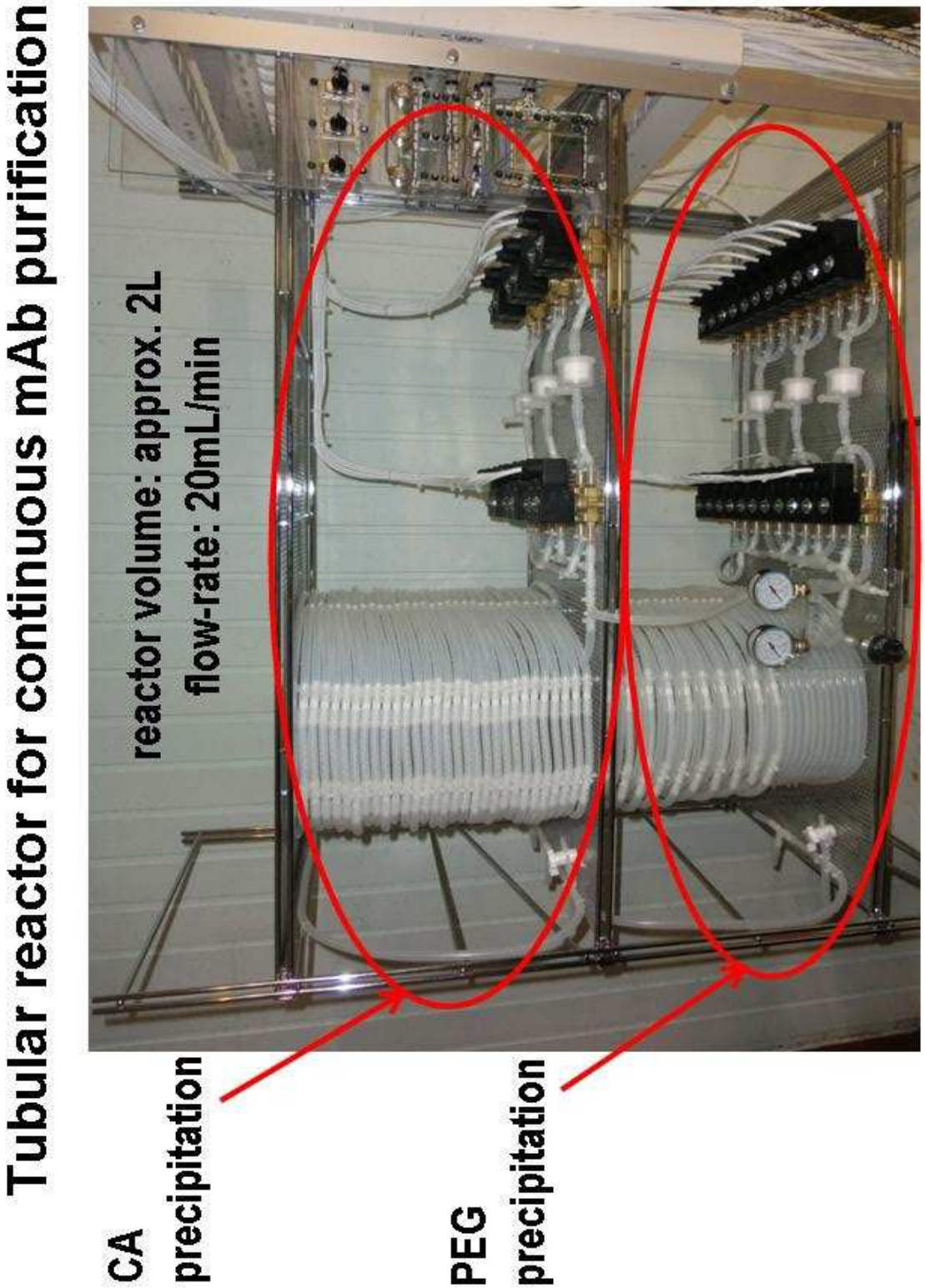


Figure 24: Picture of the tubular reactor for continuous mAb purification

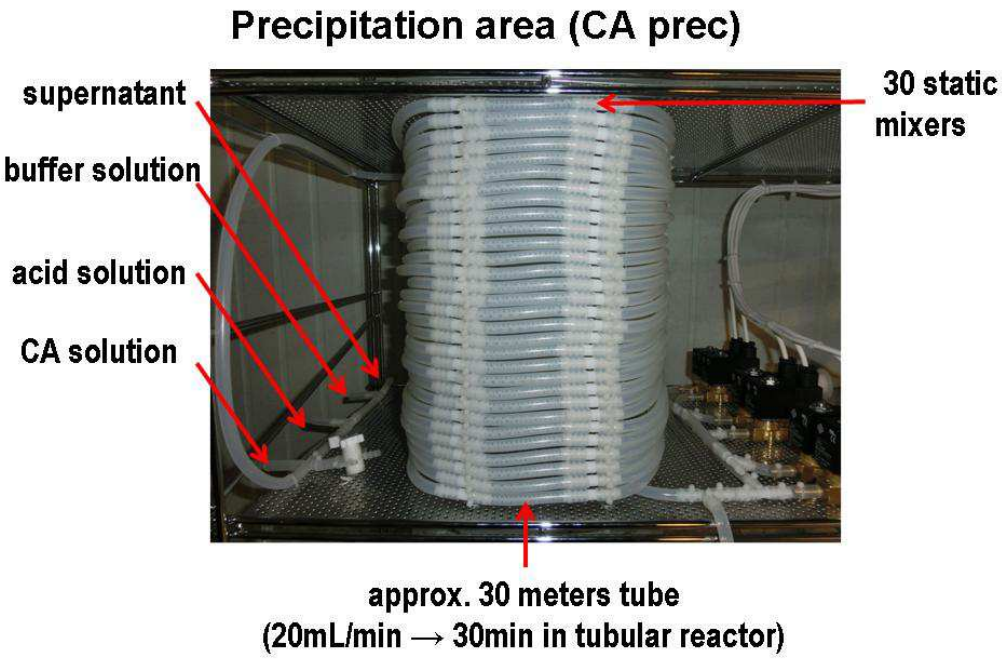


Figure 25: CA precipitation area for impurity precipitation with four different solution inlets for pH adjustment and CA adding

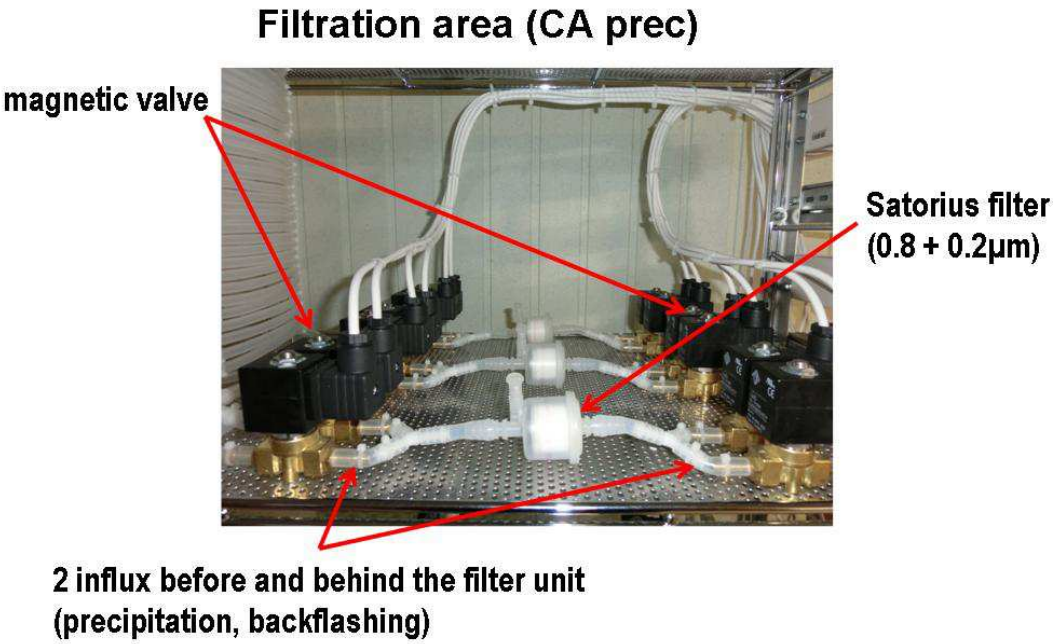


Figure 26: CA filtration area for separation of precipitated impurities

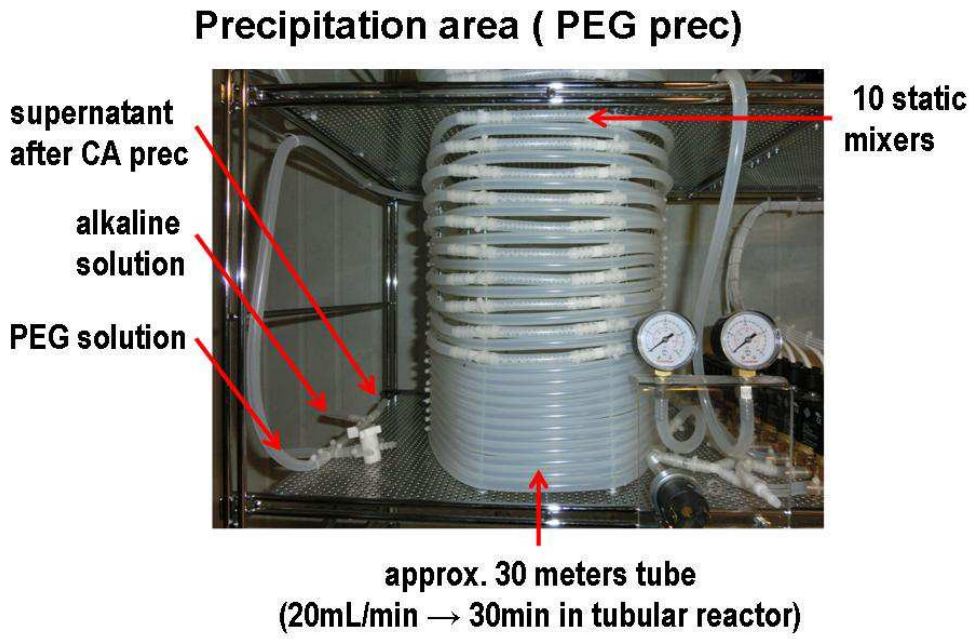


Figure 27: PEG precipitation area for precipitation of mAbs with three different solution inlets for pH adjustment and PEG adding

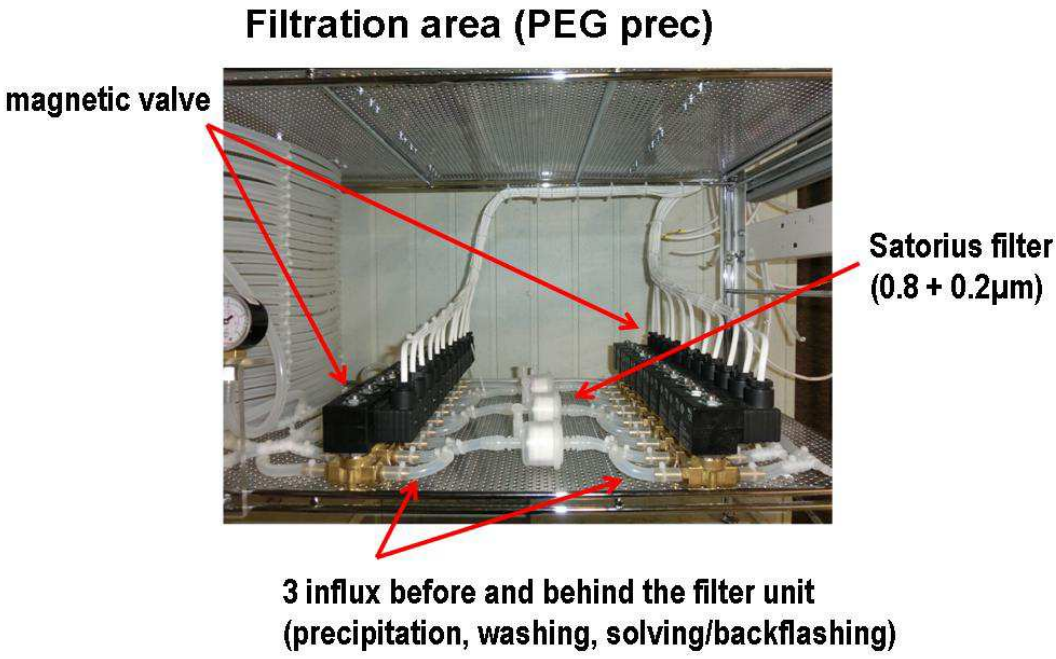


Figure 28: PEG filtration area for collection, washing and solving of mAb precipitates

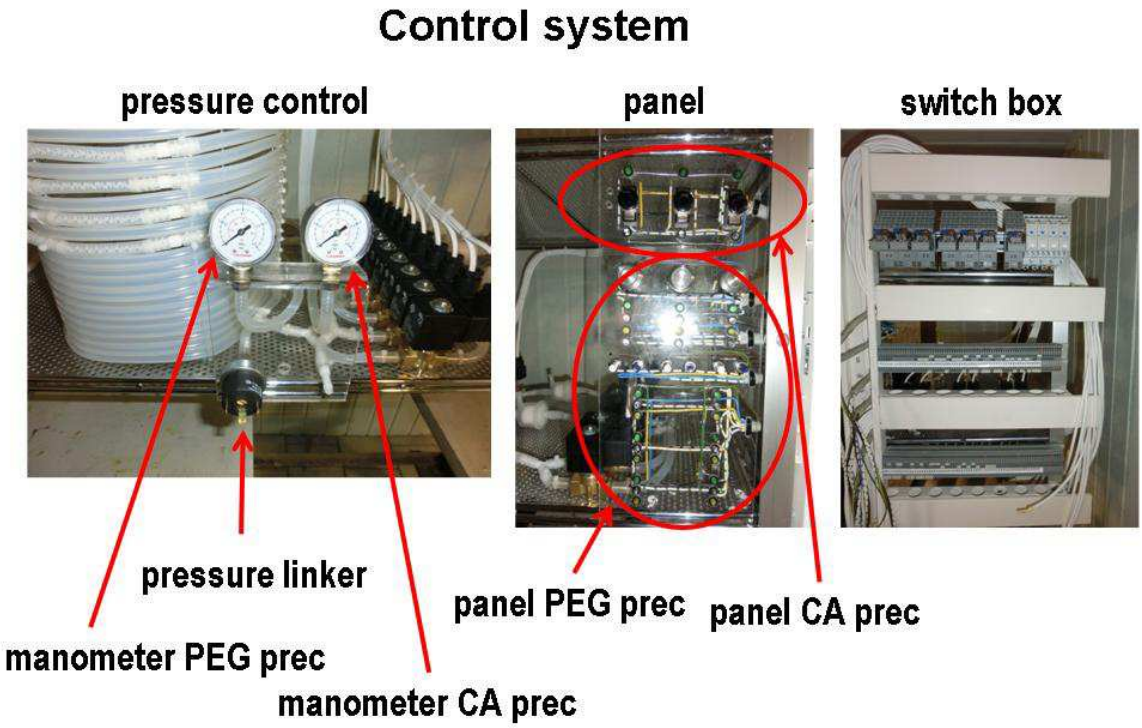


Figure 29: Control system for the tubular precipitation reactor with a panel for PEG and CA precipitation area and an additional pressure alert system

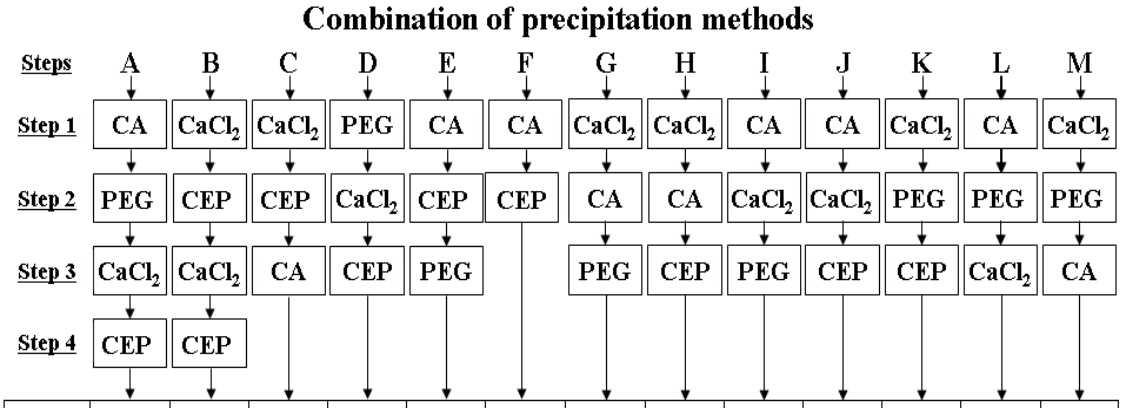
Combination of 4 different precipitation methods

Aim of this screening was the development of a precipitation step alignment, which achieves the purity of drug substance. The above described precipitation method (CA/PEG) as well as prior developed precipitations, like $CaCl_2$/PEG (Sommer et al) and $CaCl_2$/CEP (Tscheliessnig et al) were applied for this screening.

Precipitation combinations

For determination of the most promising precipitation combinations, sequences of two to four precipitation steps were conceived. The precipitation methods with caprylic acid (CA), calcium chloride ($CaCl_2$), polyethylene glycol (PEG) and ethanol (CEP) as precipitant were used for the precipitation sequence screening of one cell culture supernatant (mAb1). Figure 30 show thirteen different precipitation combinations (from A to M) and their yield and purity values after the last precipitation step. Certainly, more then thirteen precipitation combinations were possible but combination with CA or $CaCl_2$ as last precipitation step were eliminated, for having the product in solid state. We did not want to have caprylic acid or $CaCl_2$ as last step. In addition we wanted the product as precipitate and not in supernatant. Sequential precipitation of caprylic acid and $CaCl_2$ lead to the formation of a high amount of insoluble precipitate (Figure 30, sequence I and J). Some precipitation sequences (Figure 30, sequence D, G, K, L and M) had yields from 50 to 70 % but with HCP reduction between 4000 and 8000 ppm. Better HCP reductions (1000 to 1700 ppm) were reached with the precipitation sequences C, E and H. However, the yields of these sequences were rather low with values from 55 to 65 %. Nevertheless, one precipitation sequence consisting of only two precipitation steps (CA and CEP, Figure 30, sequence F) and had yield values above 80 % with a HCP reduction down to 1500 ppm. However, the precipitation sequences A and B showed highest yield (60 to 70 %) combined with HCP reductions from 170 to 250 ppm and HMWI from non detectable to 0,012 %, nearly the required purity target of a biopharmaceutical was reached. Reason for such values might be the deployment of four precipitation steps. Furthermore, explanation for the high purity of sequence A is that orthogonal methods have been used.

In summary, it can be stated that the combination of various precipitation methods lead to highly remarkable yield and purity values, which are comparable with the current used purification methods.



	A	B	C	D	E	F	G	H	I	J	K	L	M
Yield [%]	69	59	57	50	64	82	66	60	n.d.	n.d.	53	52	69
HCP [ppm]	277	590	1.733	4.002	1.208	1.511	6.006	995	n.d.	n.d.	8.676	5.472	4.990
HCP [fold]	253	170	56	24	81	65	16	98	n.d.	n.d.	10	18	20
HMWI [%]	0,000	0,012	0,057	0,093	0,110	0,315	0,177	0.000	n.d.	n.d.	0,069	1,155	1,549
dsDNA [ng/mL]	94	245	6	250	175	297	169	6	n.d.	n.d.	120	#NV	#NV

Figure 30: Comparison of thirteen different precipitation sequence consisting CA, PEG, $CaCl_2$ and CEP performed with CHO cell culture supernatants of mAb1. Yield and purity values were determined with SEC, affinity chromatography and PicoGreen dsDNA assay

Most prominent precipitation combinations

For the most promising precipitation sequences (A and B) yield purity, DNA, HMWI were determined after each step (Figure 31). When the first two steps of sequence A (CA/PEG) were preceded, yield and HMWI fit to the final values of the CA/PEG precipitation screening (Figure 22). Only HCP reduction was with 12000 ppm in comparison to the screening values of 3000 to 4000 ppm to low. However, due to the additional precipitation steps ($CaCl_2$/CEP) a HCP concentration of 277 ppm was reached and the HMWI were entirely separated with approx. 70 % yield. DNA concentration of 94 ng/mL was reached. Sequence B showed similar values. Yield values of approx. 60 % HCP concentration of 570 ppm, 0.0012 % HMWI and 245 ng/mL dsDNA.

These data lead to the assumption that there are more precipitation combinations to reach yield and purity of state of the art mAb purification methods.

Data of most successful experiments

A	Yield [%]	HCP [ppm]	HCP [fold]	HMWI [%]	dsDNA [ng/mL]
Clarified cell culture supernatant	100	97.608	0	2,931	6.374
pH & buffer adjustment **CA precipitation**	93	27.357	4	2,134	1.363
pH adjustment **PEG precipitation**	86	12.158	8	1,343	258
pH & PO_4^{3-} adjustment **$CaCl_2$ precipitation**	77	1968	29	0,000	#NV
pH adjustment **CEP precipitation**	69	277	253	0,000	94

B	Yield [%]	HCP [ppm]	HCP [fold]	HMWI [%]	dsDNA [ng/mL]
Clarified cell culture supernatant	100	100.120	0	2,931	6.374
pH & PO_4^{3-} adjustment **$CaCl_2$ precipitation**	97	58.956	2	0,973	56
pH adjustment **CEP precipitation**	76	14.957	7	0,000	#NV
pH & PO_4^{3-} adjustment **$CaCl_2$ precipitation**	65	2.884	35	0,000	80
pH adjustment **CEP precipitation**	59	590	170	0,012	245

Figure 31: Yield and purity comparison of the two most successful precipitation sequences performed with CHO cell culture supernatants of mAb1. Determination were performed with SEC, affinity chromatography and PicoGreen dsDNA assay

Discussion

The biopharmaceutical market is growing in the last years continuously. Main reasons were the development of novel therapeutic mAbs and their broad application field. Six of the top ten top-selling biopharmaceuticals in 2009 were therapeutic mAbs (Walsh 2010). Whereby, most of these products are with 4000 USD per dose in the median price range, like Enbrel and Rituxan (Hagel et al. 2007; Kelley 2007). In 2010 sales of mAb therapies exceeded 40 billion USD and for 2015 a value of 70 billion USD are expected (Chon and Zarbis-Papastoitsis 2011). Due to the reason of the fast growing biopharmaceutical market and the demand for fast, easy cheap mAb purification processes the development of a precipitation processes was the logical issue. Our study is an attempt to explore at laboratory scale, how existing methods can be used for replacement of existing steps. The precipitation of mAb or impurities out of a cell culture supernatant, for getting purified antibodies, was done several time in past. But it was unrewarding because of low antibody titers. Only, the improvement of the upstream processing led to cell culture supernatants with titers of 2 g/L or higher. Due to such high product titers, loos of yield during precipitation dropped below a value of 10 %.

PEG has been always considered as an option for antibody purification. In the early days of monoclonal and recombinant antibodies the concentration the supernatant was way to low in order to get a reasonable purity and yield. The substantial improvement of upstream processing allows the reconsideration of old methods. PEG is definitely an inexpensive method but lacks in selectivity. Model studies have shown that precipitation is clearly related to size of protein. Thus it is difficult to remove high molecular weight impurities. Indeed, as expected from the theory, with a single PEG precipitation a purity, not sufficient for replacement of a protein A step, was obtained. Such impurities are most likely IgG aggregates and dsDNA will always co-precipitate. Due to this reason a combination with other precipitation methods like $CaCl_2$ and caprylic acid precipitation is a further opportunity to improve purity.

High throughput screening in small microtiter plates is a valuable tool for process optimization in downstream processing (Konstantinidis et al. 2012; Łacki 2012; Nfor et al. 2012; Sanaie et al. 2012; Treier et al. 2012). In the high throughput screening of

PEG precipitation we could show that pH from 5.5 to 8.5 did not have a significant influence on yield and purity. The salt concentration during precipitation is high enough to compensate for electrostatic effects. Furthermore high throughput screening methods allow rapid adjustment of working conditions. We have tested five different antibodies and all of them could be purified. We think the method is in principle suited to serve as platform process. First the process is independent of pH. This small variations in pH as always observed in cell culture do not affect yield and purity. If desired the concentration of PEG can be fine tuned by high throughput experiment in the microtiter plates, but we do not think that it would have an impact on yield and purity. Furthermore the high throughput experiments have a certain limitation. We clearly observed that a film of antibody solution is retained in the well are removing the liquid. This prevents accurate determination of yield and prediction to laboratory and pilot scale. On the other hand our high throughput method combined with the rapid quantification of IgG and impurities can be highly automated and thus pone can rapidly screening conditions for IgG purification form other host cells (Dietmair et al. 2012) than CHO, because selective interaction of HCP with IgG has been observed (Sisodiya et al. 2012).

Additional tool to evaluate PEG precipitation behaviour of recombinant mAb and the corresponding total protein impurity is the preparation of solubility curves. Not only solubility (S_0) can be derived from such a curve, also the relation between PEG and protein concentration can be determined. Usually, the higher protein concentration the lower PEG concentration is needed for precipitation without loss of yield. Furthermore, solubility curve comparison of pure mAb and the corresponding total protein impurities gives information of about the reachable purity. Due to the reason that PEG precipitation mechanism is size dependent impurities which have higher molecular weight (HMWI) than IgG were not separated.

Introduction of $CaCl_2$ precipitation at a certain phosphate concentration was successful and the HMWI could be also removed. Both PEG and $CaCl_2$ are inexpensive and perfectly suited for the design of a platform process. $CaCl_2$/PEG precipitation showed remarkable yield and purity values with a competitiveness to the affinity chromatography. Results in stirred and shaken system are similar and thus both may be suited for prediction of large scale, but engineering of large scale PEG antibody

purification with recombinant antibodies is still pending. We could imagine that our method is competitive to other methods such as counter current loading of chromatography columns or aqueous two phase extraction (Rosa et al. 2013). Precipitation equilibrium is reached after 5-10 min. This is approximately the mixing time in large scale stirred tank reactors. Further mixing does not harm yield and purity. After 160 min still same purity and yield was observed. Although the fast reaction would suggest a continuous process. Thus another dimension could be reached. Besides replacing a chromatography step the footprint of the unit could be substantially reduced.

Based on these former developed purification methods, additional precipitation combinations, like CA with PEG precipitation were examined, to improve yield and purity. For the development of CA/PEG precipitation several parameters (pH value, salt kind, salt concentration, mixing time, mixing speed and CA concentration) were screened, to get adequate yield and purity values. Findings of this work showed improved purity values compared to $CaCl_2$/PEG precipitation and competitiveness to the protein A affinity chromatography. Certainly, a direct comparison of a precipitation purification processes and the protein A affinity chromatography is not that simple because the purification methods are based on different mechanisms. Therefore, the precipitation process has somewhat lower yield (80 to 85 %) and HMWI reduction (1 to 2 % aggregates) but obviously higher HCP reduction (3000 to 4000 ppm). Due to the reason that the precipitation process was performed with two mAb cell culture supernatants this novel purification method can be seen as platform process. However, a further benefit of a precipitation process is the short performance time and the easy operability, which makes this process to an optimal candidate for continuous manufacturing of antibodies.

Due to these reasons, a continuous tubular lab scale reactor was designed and constructed for proving prior performed small scale batch test. Reactor setup was designed to perform caprylic acid and PEG precipitation sequently by hand, with the option of installing a process control system later on. Continuous tubular lab scale reactor dimensions were, with a reactor volume of approx. 2 L and a solution stream of 20 mL/min, able to produce production scale amounts over a longer period of time. This small reactor size permits the possibility to construct a fully disposable reactor.

Due to the high demand on cell culture supernatant, first reactor test were not yet performed, but this is the next milestone for future.

All prior performed precipitation examinations, aimed for replacement of the column based protein A affinity chromatography. Next target was to develop a column free purification process which has the ability to reached yield and purity of a whole state of the mAb purification process. Due to this reason, several precipitation methods ($CaCl_2$, CA, PEG and CEP) were aligned in different manner and thereby yield and purity were compared. Out of this screening, three precipitation sequences emerge with exceptional yield and purity values. One of these three purification methods was a two step precipitation with CA and CEP. Values of this precipitation sequence were with 80 % yield and approx. 1500 ppm HCP remarkable because usually a third or fourth step is needed to reach such purity. Second precipitation was a consecutively performance of two $CaCl_2$/CEP precipitations (four steps) with quite good yield (approx. 60 %) and purity (590 ppm HCP, 0.012 % HMWI, 245 ng/mL dsDNA) values. However, highest yield (approx. 70 %) and purity (277 ppm HCP, 0.000 % HMWI, 94 ng/mL dsDNA) was reached with a sequence of all four precipitation methods. Starting with CA followed by PEG and $CaCl_2$ and finally a CEP was performed. Reason for yield and purity comparable to drugs substance, might be the differences of the methods mechanisms, which makes this precipitation sequence to an orthogonal purification process. Furthermore, due to the reason that all purification steps are precipitations, the implementation of this sequence as a continuous process is obvious.´

Conclusion

We developed a precipitation method where PEG and $CaCl_2$ were combined. They can be used as first capture step in recombinant antibody purification. The $CaCl_2$ precipitation serves for separation of HMWI such as dsDNA and aggregates and PEG precipitation separates mAbs from LMWI mainly HCP. All precipitation tests were performed with five different CHO cell culture supernatants and showed yields between 80 and 95 %. Purity was determined with SEC, HCP ELISA and 2D DIGE. Chromatography and electrophoreses data of $CaCl_2$/PEG precipitation were roughly comparable to protein A purification. We conclude that the combination of $CaCl_2$/PEG precipitation could be the basis for a platform process for capturing antibodies from a clarified culture supernatant without any further conditioning. This purification method provides enough selectivity and recovery to be competitive to the staphylococcus protein A affinity chromatography.

Combination of CA and PEG precipitation for mAb purification is based on a prior invented $CaCl_2$/PEG precipitation. Reason for this further development was the improvement of purity, for reaching values to replace protein A affinity chromatography. The CA precipitation was used for HMWI as well as HCP reduction and PEG precipitates IgG, which causes a further reduction of HCP. Finally a yield between 80 and 85 % with a HCP concentration from 3000 to 3700 ppm and HMWI (aggregates) between 1 and 2 % were reached. These values show that the CA/PEG precipitation is highly competitive to affinity chromatography.

Upon these values a continuous lab scale reactor was designed and constructed, which has the ability to verify the lab scale batch test. Dimension and solution stream of the reactor, permit in continuous operation production scale performance. Furthermore, reactor size allows a disposable constriction.

Screening of different precipitation sequences was performed, to figure out if it is possible to reach purity values approaching to drugs substance, without a column purification step. The combination of CA, PEG, $CaCl_2$ and CEP was the most prominent sequence and lead to a HCP concentration of 277 ppm with no HMWI, 94ng/mL dsDNA and a yield of approximately 70 %. Those values are quite close to drugs substance purities. For reaching equal values only the implementation of an anion

exchange step might be enough. Due to the reason that this precipitation process can be driven continuously, an anion exchange filter or monolith would be the material of choice, to have a fully non-chromatographic continuous purification process for antibodies.

In summary, a novel purification process for recombinant mAbs was developed. This process can be driven continuously and fully non-chromatographic. Further benefit ere shown by the construction of a lab scale reactor, which has the ability to cover production scale demands and could be constructed as disposable reactor.

Tables

Figures

References

Atha DH, Ingham KC. 1981. Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume. Journal of Biological Chemistry 256(23):12108-12117.

Beare-Rogers J, Dieffenbacher A, Holm JV. 2001. Lexicon of lipid nutrition. Pure and Applied Chemistry 73(4):685-744.

Birch JR, Racher AJ. 2006. Antibody production. Advanced Drug Delivery Reviews 58(5-6):671-685.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72(1–2):248-254.

Buchacher A, Iberer G. 2006. Purification of intravenous immunoglobulin G from human plasma - Aspects of yield and virus safety. Biotechnology Journal 1(2):148-163.

Burnouf T. 2007. Modern Plasma Fractionation. Transfusion Medicine Reviews 21(2):101-117.

Cai K, Miller JLC, Stenland CJ, Gilligan KJ, Hartwell RC, Terry JC, Evans-Storms RB, Rubenstein R, Petteway Jr SR, Lee DC. 2002. Solvent-dependent precipitation of prion protein. Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology 1597(1):28-35.

Chon JH, Zarbis-Papastoitsis G. 2011. Advances in the production and downstream processing of antibodies. New Biotechnology 28(5):458-463.

Cohn EJ, Gurd FRN, Surgenor DM, Barnes BA, Brown RK, Derouaux G, Gillespie JM, Kahnt FW, Lever WF, Liu CH and others. 1950. A system for the separation of the components of human blood: Quantitative procedures for the separation of the protein components of human plasma. Journal of the American Chemical Society 72(1):465-474.

Cohn EJ, Strong LE, Hughes Jr WL, Mulford DJ, Ashworth JN, Melin M, Taylor HL. 1946. Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. Journal of the American Chemical Society 68(3):459-475.

Deutsch HF, Gosting LJ. 1946. Biophysical studies of blood plasma proteins; recovery of gamma-globulin from human blood protein mixtures. The Journal of biological chemistry 164:109-118.

Dietmair S, Nielsen LK, Timmins NE. 2012. Mammalian cells as biopharmaceutical production hosts in the age of omics. Biotechnology Journal 7(1):75-89.

Elvin JG, Couston RG, Van Der Walle CF. 2013. Therapeutic antibodies: Market considerations, disease targets and bioprocessing. International Journal of Pharmaceutics 440(1):83-98.

Foster PR. 2000. Prions and blood products. Annals of Medicine 32(7):501-513.

Foster PR, Welch AG, McLean C, Griffin BD, Hardy JC, Bartley A, MacDonald S, Bailey AC. 2000. Studies on the removal of abnormal prion protein by processes used in the manufacture of human plasma products. Vox Sanguinis 78(2):86-95.

Grzeskowiak JK, Tscheliessnig A, Toh PC, Chusainow J, Lee YY, Wong N, Jungbauer A. 2009. 2-D DIGE to expedite downstream process development for human monoclonal antibody purification. Protein Expression and Purification 66(1):58-65.

Hagel L, Jagschies G, Sofer G. 2007. Handbook of Process Chromatography, Second Edition: Development, Manufacturing, Validation and Economics. 2, editor: Academic Press.

Hasko F, Vaszileva R. 1982. Solubility of plasma proteins in the presence of polyethylene glycol. Biotechnology and Bioengineering 24(9):1931-1939.

Juckles IRM. 1971. Fractionation of proteins and viruses with polyethylene glycol. Biochimica et Biophysica Acta (BBA) - Protein Structure 229(3):535-546.

Kahovec J, Fox RB, Hatada K, Kahovec J. 2002. Nomenclature of regular single-strand organic polymers (IUPAC Recommendations 2002). Pure and Applied Chemistry 74(10):1921-1956.

Kelley B. 2007. Very large scale monoclonal antibody purification: The case for conventional unit operations. Biotechnology Progress 23(5):995-1008.

Kistler P, Nitschmann H. 1962. Large scale production of human plasma fractions. Eight years experience with the alcohol fractionation procedure of Nitschmann, Kistler and Lergier. Vox Sanguinis 7:414-424.

Konstantinidis S, Kong S, Chhatre S, Velayudhan A, Heldin E, Titchener-Hooker N. 2012. Strategic Assay Selection for analytics in high-throughput process development: Case studies for downstream processing of monoclonal antibodies. Biotechnology Journal 7(10):1256-1268.

Łacki KM. 2012. High-throughput process development of chromatography steps: Advantages and limitations of different formats used. Biotechnology Journal 7(10):1192-1202.

Mahadevan H, Hall CK. 1992. Experimental analysis of protein precipitation by polyethylene glycol and comparison with theory. Fluid Phase Equilibria 78(C):297-321.

Marichal-Gallardo PA, Álvarez MM. 2012. State-of-the-art in downstream processing of monoclonal antibodies: Process trends in design and validation. Biotechnology Progress 28(4):899-916.

Mohanty JG, Elazhary Y. 1989. Purification of IgG from serum with caprylic acid and ammonium sulphate precipitation is not superior to ammonium sulphate precipitation alone. Comparative Immunology, Microbiology and Infectious Diseases 12(4):153-160.

Morais V, Massaldi H. 2012. A model mechanism for protein precipitation by caprylic acid: Application to plasma purification. Biotechnology and Applied Biochemistry 59(1):50-54.

Moure F, Rendueles M, Díaz M. 2003. Coupling process for plasma protein fractionation using ethanol precipitation and ion exchange chromatography. Meat Science 64(4):391-398.

Nfor BK, Ripić J, van der Padt A, Jacobs M, Ottens M. 2012. Model-based high-throughput process development for chromatographic whey proteins separation. Biotechnology Journal 7(10):1221-1232.

Oncley JL, Melin M, Richert DA, Cameron JW, Gross Jr PM. 1949. The separation of the antibodies, isoagglutinins, prothrombin, plasminogen and β1-lipoprotein

into subfractions of human plasma. Journal of the American Chemical Society 71(2):541-550.

Pasut G, Veronese FM. 2012. State of the art in PEGylation: The great versatility achieved after forty years of research. Journal of Controlled Release 161(2):461-472.

Perosa F, Carbone R, Ferrone S, Dammacco F. 1990. Purification of human immunoglobulins by sequential precipitation with caprylic acid and ammonium sulphate. Journal of Immunological Methods 128(1):9-16.

Polson A, Potgieter GM, Largier JF, Mears GEF, Joubert FJ. 1964. The fractionation of protein mixtures by linear polymers of high molecular weight. BBA - General Subjects 82(3):463-475.

Radosevich M, Burnouf T. 2010. Intravenous immunoglobulin G: Trends in production methods, quality control and quality assurance. Vox Sanguinis 98(1):12-28.

Rojas G, Jimenez J, Gutierrez J. 1994. Caprylic acid fractionation of hyperimmune horse plasma: Description of a simple procedure for antivenom production. Toxicon 32(3):351-363.

Rosa PAJ, Azevedo AM, Sommerfeld S, Mutter M, Bäcker W, Aires-Barros MR. 2013. Continuous purification of antibodies from cell culture supernatant with aqueous two-phase systems: From concept to process. Biotechnology Journal.

Russo C, Callegaro L, Lanza E, Ferrone S. 1983. Purification of IgG monoclonal antibody by caprylic acid precipitation. Journal of Immunological Methods 65(1-2):269-271.

Sanaie N, Cecchini D, Pieracci J. 2012. Applying high-throughput methods to develop a purification process for a highly glycosylated protein. Biotechnology Journal 7(10):1242-1255.

Sisodiya VN, Lequieu J, Rodriguez M, McDonald P, Lazzareschi KP. 2012. Studying host cell protein interactions with monoclonal antibodies using high throughput protein A chromatography. Biotechnology Journal 7(10):1233-1241.

Sommerfeld S, Strube J. 2005. Challenges in biotechnology production - Generic processes and process optimization for monoclonal antibodies. Chemical Engineering and Processing: Process Intensification 44(10):1123-1137.

Treier K, Berg A, Diederich P, Lang K, Osberghaus A, Dismer F, Hubbuch J. 2012. Examination of a genetic algorithm for the application in high-throughput downstream process development. Biotechnology Journal 7(10):1203-1215.

Tscheliessnig A, Jungbauer A. 2009. High-performance monolith affinity chromatography for fast quantitation of immunoglobulin G. Journal of Chromatography A 1216(13):2676-2682.

Veronese FM, Harris JM. 2002. Introduction and overview of peptide and protein pegylation. Advanced Drug Delivery Reviews 54(4):453-456.

Walsh G. 2010. Biopharmaceutical benchmarks 2010. Nature Biotechnology 28(9):917-924.