Influence of capture step on critical quality attributes of monoclonal antibodies in biopharmaceutical production

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II. Table of content

III. List of abbreviations

AIEX	Anion exchange chromatography
CD	Circular dichroism
CHO	Chinese hamster ovary
CIEX	Cation exchange chromatography
DBC	Dynamic binding capacity
DSP	Downstream processing
DSC	Differential scanning calorimetry
DSF	Differential scanning fluorimetry
EBC	Equilibrium binding capacity
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
EMA	European Medicines Agency
HCP	Host cell proteins
HMWI	High molecular weight impurities
RMP	Reference medicinal product
FDA	Food and Drug Administration
HCP	Host cell proteins
HPLC	High-pressure liquid chromatography
ITC	Isothermal titration calorimetry
mAb	Monoclonal antibody
PAT	Process analytical technology
PEG	Polyethylene glycol
ScFv	Single chain variable fragment
SEC	Size exclusion chromatography

IV. Abstract

Antibodies and their derivatives constitute the lion's share of the biopharmaceutical market. With demands rising, manufacturers are challenged to develop new, efficient and safe processes. Downstream processing of antibody products – at least of the ones containing a Fc part – is conventionally based on protein A chromatography which is renowned for high yields and efficient host cell protein (HCP) clearance. However, protein A chromatography media are cost-intensive and contribute greatly to overall process costs. Furthermore, there is the need for harsh elution conditions which can lead to product losses. Therefore, several non-chromatography based antibody capture technologies have been developed in recent years. The feasibility of a continuous PEG precipitation in combination with a two-stage tangential flow filtration for antibody capturing was recently shown by Burgstaller et al.

In this doctoral thesis, protein A based capture chromatography was compared to PEG-precipitation in regard of their effect on critical quality attributes of an antibody product. In the first part, three commercially available protein A chromatography media were compared in regard of their binding capacities at different temperatures. By use of differential scanning calorimetry, it was shown that antibodies undergo transient, structural alterations while being adsorbed to the materials. In the second part of this thesis, protein A chromatography and PEG precipitation were compared in regard of structural effects on the antibody product as well as effects on the storability of the antibody intermediate. During protein A chromatography, an additional, acidic isoform was formed which reconverted to the main charged variant within several days. Thermal stress testing showed that PEG precipitation leads to a decrease in antibody stability compared to the protein A purified material.

V. Kurzfassung

Antikörper und -derivate dominieren den biopharmazeutischen Markt. Mit steigender Nachfrage vergrößert sich auch der Druck auf die Hersteller, neue, effiziente und sichere Prozesse zu entwickeln. Das Aufreinigungsverfahren basiert – sofern das Fc-Fragment des Antikörpers vorhanden ist – auf Protein-A-Chromatographie, welche sich durch hohe Ausbeuten und effiziente Wirtszellproteinabreicherung auszeichnet. Als Nachteile sind die hohen Kosten des Materials zu nennen, welche signifikant zu den Gesamtprozesskosten beitragen. Weiters werden zur Elution harsche Bedingungen benötigt, welche zu Produktverlusten führen können. Daher wurden in den letzten Jahren verschiedene nicht-chromatographische Aufreinigungsmethoden entwickelt. Kürzlich wurde von Burgstaller et al. in einer Machbarkeitsstudie die Aufreinigung eines Antikörpers basierend auf PEG-Präzipitation mit anschließender 2-stufiger Tangentialflussfiltration demonstriert.

In dieser Doktorarbeit wurde die Protein-A-Chromatographie mit der PEG-Präzipitation verglichen, um den Einfluss auf die kritischen Qualitätsmerkmale (CQA) eines Antikörpers zu untersuchen. Im ersten Teil wurden drei kommerziell verfügbare Protein-A-Chromatographiemedien hinsichtlich ihrer Bindungskapazitäten bei verschiedenen Temperaturen verglichen. Auf Basis von Differential-Scanning-Kalorimetrie wurde gezeigt, dass Antikörper transiente, strukturelle Veränderungen erfahren, während sie an den Medien adsorbiert sind. Im zweiten Teil wurden Protein-A-Chromatographie und PEG-Präzipitation im Hinblick auf strukturelle Effekte auf das Antikörperprodukt sowie Effekte auf die Lagerfähigkeit des Antikörperzwischenprodukts verglichen. Protein-A-Chromatographie führt zur Bildung einer zusätzlichen, sauren Isoform, die sich innerhalb weniger Tage in die Hauptladungsvariante zurückbildet. Thermische Stresstests zeigten, dass die PEG-Präzipitation zu einer Abnahme der Antikörperstabilität im Vergleich zu dem Protein A-Material führt.

1 Introduction

1.1 Antibodies

As of July 2018, a total of 285 distinct biopharmaceutical active ingredients are available on European and/or US markets with roughly 45% of them having been released within the preceding four years (*1, 2*). More than 50% of the new releases of the years 2014 to 2018 are accounted to the class of antibody products and antibody based products. The majority of licensed antibody based products are full-length antibodies of the IgG1 or IgG2 subclass but in recent years, a great variety of alternative molecular formats has been developed as well, such as bispecific antibodies, IgGs appended with an additional antigen binding site, antibody fragments, antibody fusion proteins and antibody conjugates (*3*). Also, the interest in biosimilars continues to grow; these copycat drugs show no clinical differences from a *reference medicinal product* (RMP) and are brought on the market soon after the patents of the RMP's manufacturer has expired. Structurally speaking, antibodies are glycoproteins composed of four polypeptide chains, two heavy and two light chains, connected by a varying number of disulfide bonds and non-covalent forces (Figure 1) *(4, 5).*

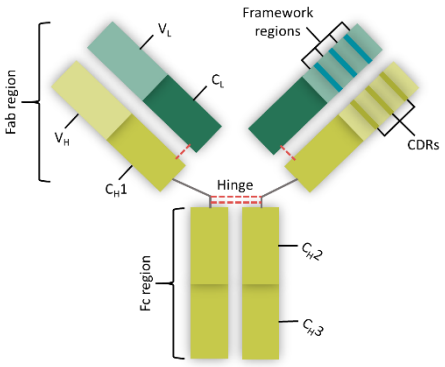

Figure 1: Schematic representation of an IgG molecule.

With a molecular weight around 150 kDa, they are approximately three times the size of an average protein in an eukaryote (*6*). In mammals, five antibody classes can be distinguished based on their heavy chains. The heavy chains are denoted by the greek letters α, δ, ε, γ, and μ and the immunoglobulins are denoted IgA, IgD, IgE, IgG and IgM respectively. There are two types of light chains which are denoted as λ or κ. Both, heavy and light chains, consist of a variable and a constant region. While the light chain has only one variable (VL) and one constant domain (CL), the heavy chain consists of one variable domain (VH) and three constant domains (CH1, CH2, CH3). Each domain comprises about 110 amino acids that fold into two β-sheets joined by loop regions. The loop regions of the variable domains contain three complementary determining regions (CDR) which determine the antibody-antigen interaction and four framework regions. The intramolecular distance between the two antigen

binding sites is ~ 9 nm (*7*). VL, CL, VH and CH1 together are referred to as Fab (antigen binding fragment) while the CH2 and the CH3 domains together are denoted as Fc (fragment crystallizable) region.

In humans, antibodies are produced in B cells exclusively and act as part of their normal immune response to infectous antigens. After the first contact with an antigen, the naïve B-cell undergoes differentiation and proliferation and starts antibody secretion. In humans, the major serum immunoglobulin is IgG constituting ~80% of immunoglobulins in serum of a healthy adult. At position N297, all IgG molecules carry an N-linked glycan moiety which occurs in several dozen different compositions and has a significant impact on the quaternary structure of the protein (*8*). IgA is the second most common in serum with ~15% and plays an important role in local, mucosal immunity as it is the predominant immunoglobulin in secretions. IgM forms a pentamer and constitutes ~5% of human serum. The primary mechanism of action of IgM and IgG is the activation of the host's complement system via the classical pathway which leads to the formation of the proteolytic membrane attack complex (MAC) and lysing of the target cell. IgD is hardly found as a soluble molecule in serum but is located on the B cell surface where it acts as receptor for antigens. IgE plays an important role in parasite clearance and is present only in trace amounts in the serum. In addition to the immunoglobulin classes, several subclasses exist in all members of a particular animal species. For example, IgG has four subclasses in human: IgG1, IgG2, IgG3 and IgG4 (in order of decreasing abundance in serum). Despite many IgG derivatives currently being in the pipeline, the majority of licensed products are full-length antibodies of the IgG1 and IgG2 subclasses. The two subclasses show a sequence homology of ~90% but variations in the variable regions, upper CH2 domains, as well as the hinge-region are responsible for differences in antigen binding affinities, pharmacokinetic half-life and effector functions (*9, 10*). The hinge region is of particular interest as it determines the flexibility of the IgG by restricting the possible conformations of the Fab arms relative to each other as well as in relation to the Fc part. IgG1 has a longer hinge region than IgG2 (15 and 12 amino acids respectively) and is therefore considered more flexible (*11*). However, IgG2 shows a higher degree of disulfide heterogeneity than IgG1. So far, three structural isoforms of IgG2 with different disulfide connections in the hinge region have been detected in human (*12*). The composition of the IgG2 subtypes does not stay constant and disulfide shuffling is observed in patients' blood when observed over several days (*13*).

1.1.1 Antibody Folding and Assembly

Antibodies consist of multiple domains which are structurally highly similar to each other (*4, 14*). The characteristic composition consists of two parallel β-sheets which give the domains a sandwich-like appearance known as Ig fold. Variable domains consist of 9 strands

(*abcc'c''defg*) while constant domains are made up by 7 strands (*abcdefg*). In most antibodies, the strands of the β-sheets are connected by disulfide bonds which increase the stability of the domain structure. α-helices are present only in small quantities (*15*). Up to 10% of the amino acids are proline residues – these high proline concentrations have implications for the folding process. At the ribosome, proline is exclusively synthesized as the *trans* form. Proline isomerization, which is catalysed by prolyl isomerase enzymes, often constitutes the rate-limiting step in antibody folding (*16*). The structural similarities of the domains could lead to the belief that all domains fold according to one distinct folding pathway, but actually, three different folding pathways have been identified (*17*). Folding of the individual domains is most easily followed by a dissection of the antibody into its domains, quenching experiments in vitro and analysing the quenched intermediates. C_L and C_H2 domains have similar pathways; in a first step, nonpolar amino acids withdraw themselves from the aqueous surrounding and start adopting the characteristic β-sheet structure. A disulfide bond between the *b* and *f* strands stabilizes this folding intermediate (*18, 19*). After isomerisation of a proline residue in the loop between strands *b* and *c*, the domain can obtain its final monomeric conformation. C_H3 domains fold in a similar matter but after proline isomerization, two C_H3 domains dimerize. The C_H1 domain also folds via two intermediate steps (*20*); first, it loosely forms a dimer with an already folded C_L domain, then a proline residue in the loop between the *b* and *c* strands needs to isomerize before folding is completing by the formation of an interchain disulfide bond of the C_H1/C_L dimer.

1.1.2 Effect of Temperature and PH on Antibodies

When stressed by elevated temperature or extreme pH, conformational changes are observed in proteins. While changes in the tertiary structure are usually reversible, changes in the secondary structure lead to irreversible denaturation (*21*). With the elaborate folding pathways described in the last chapter in mind, it is hardly surprising that antibody domains undergo unfolding in independent processes as well. Under stressed conditions, complex unfolding behaviours can be observed and the individual domains are unfolded stepwise which leads to the formation of intermediates with partly denaturated domains (*22*). Vermeer et al. observed that the domains show different sensitivity towards heat and pH treatments. While the Fab arms reacted more sensitive to heat, the Fc part was more sensitive to low pH treatments *(5)*.

In equilibrium, proteins usually have access to three conformation: the folded state, the unfolded state and the aggregated state. However, antibodies can adopt another stable conformation, the so-called "*alternatively folded state*" or "*molten globule*" which is observed at pH < 2.0 *(23)*. This alternative conformation shows similar secondary structures as the native state but is less stable in stress-induced tests, e.g. thermal unfolding. It is believed that molten globules take a key role in antibody aggregation acting as intermediates in the

aggregation pathway (*24*). Acid-induced aggregation depends highly on antibody subclass and glycosylation state of the C_H2 domain (*25*). Antibodies of the IgG1 subclass were resistant to aggregation at pH 3.5 while IgG2 aggregated readily under the tested conditions. The differences are believed to be a result of differences in C_H2 stability. This was verified by two-dimensional NMR analysis where it was shown that a pH shift from 4.7 to 3.5 had no effect on the C_H3 domain but already led to partial denaturation of C_H2. Further lowering of the pH to 3.1 led to major losses of secondary and tertiary structures in the C_H2 domain. Unfolding of the C_H3 domain was observed upon further lowering of the pH from 3.1 to 2.5 (*26*). Heat leads to irreversible denaturation and aggregation of antibodies which can visually be followed by the formation of precipitate once the denaturation temperature is reached. Temperatures above 70 °C, sometimes even above 80 °C are necessary for antibody denaturation. Heat-induced stress tests are used to measure the temperature sensibility of molecules and infer a general stability of the protein structure. For antibodies, they are performed by heating the sample to a temperature 5 – 10 °C below the precipitation temperature for several minutes. At increased temperature, partial unfolding occurs and hydrophobic patches of the inner domain are exposed which leads to formation of small self-associated structures consisting of a few monomers which gradually grow into larger structures up to several thousand nanometers (*27-29*). In a study published by Ishikawa et al. (*30*), the effect of pH on heat-induced aggregate was studied for antibodies of the subclasses IgG1, IgG2 and IgG4. Independent of the subclass, the least aggregation was found between pH 5.0 and 5.5. IgG4 showed the highest susceptibility for aggregate formation at low pH (4.0) while IgG1 and IgG2 were most likely to form aggregates at pH 7.0. Despite the high sequence homology among all antibody subclasses, they depict a greatly varying behaviour in structural stability. These structural differences between IgG subclasses as well as isoforms of the same subclass and their impact on stability complicate manufacturing where antibodies are subjected to pH levels in the acidic and neutral range. As a result, process parameters have to be adjusted for each antibody product.

1.1.3 Antibody Manufacturing

1.1.3.1 Upstream Processing

Full-length antibodies have been successfully[a] produced in a variety of different expression systems such as plants (*31*), insect cells (*32*), bacteria (*33*), yeasts (*34*), filamentus fungi (*35*),

[a] In the above listed examples, correct folding of the antibodies was induced by the hosts themselves and additional refolding steps after expression were not required. Of the above mentioned expression systems, expression in bacterial and fungal hosts failed to produce antibodies with human-like N-glycosylation patterns. The antibodies produced in the *E. coli* cytoplasm by Robinson et al. showed in vitro Fcγ-receptor affinities despite the lack of the glycan moiety. The N-glycans derived from *A. awamori* by Ward et al., contained partly glycosylated, partly unglycosylated heavy chains; ADCC activity was shown in vitro.

cell-free systems (*36*) as well as mammalian cells (*37*). For production of therapeutic proteins at industrial scale, a number of variables determines the choice of the expression system: (a) high productivities, (b) correct protein folding and posttranslational modifications to ensure pharmacokinetic activity, (c) product safety, (d) easy genetic modifiability of the host, (e) scalability and (f) overall costs including downstream processing. Currently, most therapeutic glycoproteins are produced in mammalian cell culture, especially Chinese Hamster Ovary (CHO) cell lines have been shown to be highly efficient (*38*). The first approved antibody product produced in CHO cells was *Rituxan*, an anti-CD20-mAb for the treatment of Non-Hodgkin's lymphoma that was brought onto the market in 1997 by *IDEC Pharmaceuticals*. Due to improvements in media composition and reactor design, cell densities over 20 millions cells/mL and titers > 10 g/L are nowadays achieved in fed-batch fermentations (*37*). To cover the increasing demands for antibody products, pharmaceutical companies are investing in large scale manufacturing plants containing multiple 15000 L stainless steel bioreactors with overall capacities above 100000 L (*39*).

Ideally, cells are cultivated under a set of stable and controllable physicochemical conditions such as temperature, pH, oxygenation rate and nutrients. Any changes in the cells' environment can have an impact on their metabolism and consequently impact the final product quantity and quality. Several different bioreactor designs have been used for cell cultivation. The easiest operational mode is a batch process, where the medium is inoculated and cells grow until an exhaustion of media components leads to a decrease of viable cells. Fed-batch processes are advanced batch processes, where nutrient limitations are overcome by sequential addition of concentrated media during cultivation. As a result, cell count and productivity are improved compared to the batch process but also the bioreactor volume increases consecutively and harvests need to be performed batch-wise. To achieve high cell densities at a constant bioreactor volume, it is necessary to remove fluid from the bioreactor continuously. The most straightforward way to achieve a continuous operating scheme is known as chemostat (from *chemical environment is static*) process where fresh media is added at a constant flow rate and cell suspension is continuously removed at the same flow rate without any cell retention. A more efficient and elegant operational mode is a perfusion process where cells are continuously supplied with fresh medium and cell supernatant is removed continuously with the cells being retained within the bioreactor. Cell retention is usually based on microfiltration, using either tangential flow filtration (TFF) or alternating flow filtration (ATF) *(40).* Additionally, cell-bleeds are required to keep the cell density constant.
Figure 2 shows a schematic batch-based (A) and a perfusion-based (B) antibody fermentation in stirred-tank reactors. Figure 2C-E show the resulting cell density in the bioreactor, the mAb concentration in the bioreactor and the total amount of produced mAb respectively (dashed

line: batch process, solid line: perfusion process). Once the perfusion culture reaches a steady-state, product can be harvested continuously for up to 60 days while batch based fermentations are usually run for 10-14 days only, as the cell viability starts to drop afterwards (*41*).

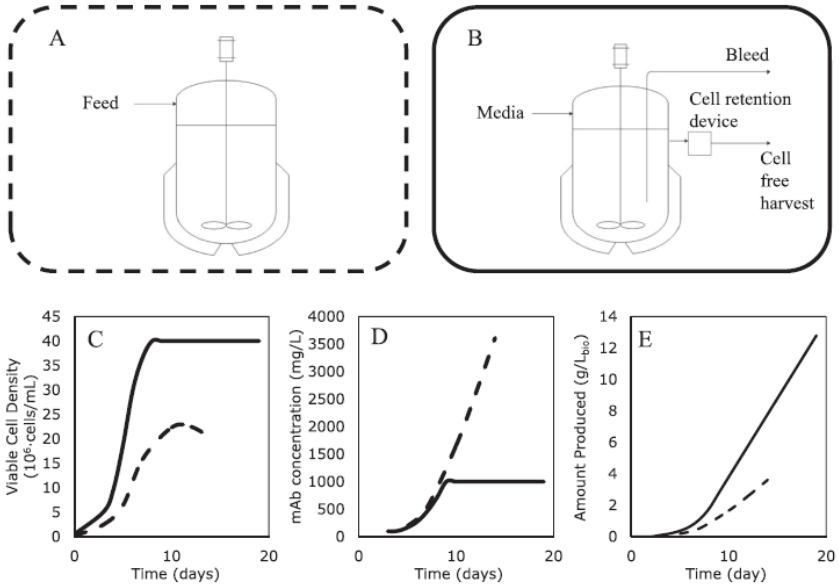

Figure 2: Schematic drawings of a batch-based (A) and perfusion cell culture (B). In Figures C-E, the differences of the processes are shown in regard to cell density (C), mAb concentration (D) and production amounts (E). Reprinted from (40) (published under Creative Commons license CC BY-NC-ND 4.0).

Originally, perfusion was only used for proteins with stability issues that needed to be processed as quickly as possible such as the blood coagulation factors VIII and XIV (*42*). However, in recent years, perfusion has gained much in popularity, was used for a wide range of products and is more and more seen as a viable alternative to fed-batch fermentations. One reason for this development is the high volumetric productivity of perfusion processes and therefore the fact that the bioreactor size can be reduced if the same amount of product is being produced in a perfusion culture. While fed-batch cultures are routinely run at 1000-10000 L scale, most perfusion bioreactors are run in the range of 50 to 500 L for the same volumetric productivity. Perfusion does however have higher medium consumption as 1.5 – 2 volume exchanges per day are carried out and the mAb concentration in the harvest is lower. Overall, it has been shown that switching from fed-batch fermentation to continuously operated upstream decreases total process costs (*43*).

The use of single-use, disposable equipment has also grown in popularity in recent years due to increased flexibility that the manufacturer is being provided with. Their usage enables quicker adaptions to unpredicted changes on the rapidly changing biopharmaceutical market, for example, when a biosimilar enters the market and therefore the RMP demands are downsized or a new blockbuster drug is approved. They are also a viable option for high-

throughput screening and pre-commercial, small productions (*44*). Especially for small companies, the use of disposable technologies brings the advantage of decreased demands in regard of floor space and lowers investment costs while however driving up consumable costs.

1.1.3.2 Downstream Processing

As outlined in the previous chapter, therapeutic antibodies are nowadays most frequently produced in CHO cell lines. Platform processes have been developed for downstream processing of antibodies produced in such a mammalian cell culture (*45*) but the actual execution still depends on a variety of variables like upstream titer, scale, availability of equipment and product characteristics. These properties will dictate the design of the downstream unit operations. The top panel in Figure 3 depicts a conventional, batch based antibody process. The lower panel shows an alternative format operated in a fully continuous mode.

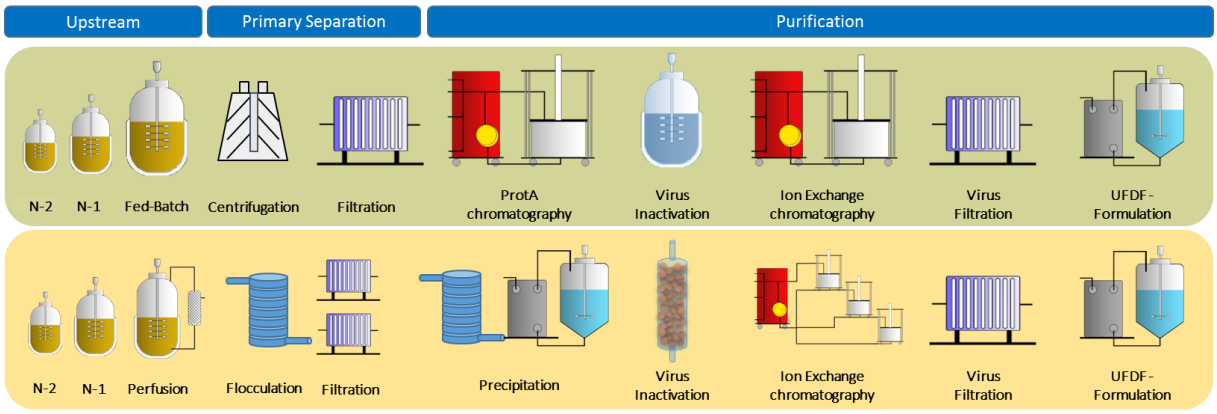

Figure 3: Schematic representation of the unit operations typically used in an antibody process (top: batch, bottom: continuous mode).

Since mammalian cells secrete the product into the surrounding extracellular fluid, the cells are removed after the fermentation in the primary recovery step. This is conventionally achieved by centrifugation with disk stack centrifuges (*46*) or, alternatively, cells can also be removed by flocculation and subsequent filtration (*47*) or by gravity settlement in plate settlers (*48*). In case of a perfusion cell culture, the cells are retained in the bioreactor by micro-filtration (ATF or TFF) and a cell removal step is rendered unnecessary. Cell removal is followed by a capture step which is commonly achieved by protein A chromatography resulting in yields >95% (*45*) and purities >98% (*49*). Protein A is originally derived from the bacterial cell wall of *Staphylococcus aureus* and depicts high selectivity towards the Fc-part of IgGs of the subclasses 1, 2 and 4 (*50*). More specifically, it binds between the CH2 and CH3 domain and can therefore not be used for antibody products that lack the Fc part like ScFvs or Fab fragments. Native staphylococcal protein A has five highly homologous IgG-binding domains

designated as E, D, A, B and C. The immobilized protein A ligands used currently in chromatography are derivatives of modified B domains of the native protein. Compared to the wild type, these engineered variants depict higher binding capacities, prolonged media lifetime, alkaline stability and/or improved elution characteristics. Protein A is produced recombinantly, usually in *E. coli*, and is therefore also a product of an up- and downstream process. As a result, protein A chromatography media contribute significantly to the overall process costs (*51*), especially during early stage development where the media lifetime is not utilized to full extend.

Due to the high affinity of protein A to IgG, harsh elution conditions are required with pH as low as 3.0, which may cause aggregation or/and precipitation (*52*), especially when working with acid-labile antibodies. Therefore, multiple protein A chromatography media enabling milder elution conditions have been developed. Pabst et al. (*53*) developed two chromatography media based on derivatives of the "Z4" ligand of GE Healthcare used for the MabSelect SuRe material. The newly engineered ligands carried one and two point mutations respectively and enabled elution at pH 4.0 for five full-length antibodies, one Fc-fusion and one bispecific antibody. Losses in dynamic binding capacity were observed only in case of the Fc fusion protein. Koguma et al. (*54*) developed a thermo-responsive protein A material denoted as *Byzen Pro* that enables elution by a change in temperature. While loading and washing are performed at a temperature <10 °C, the elution is carried out at an elevated temperature of 40 °C. Thereby, the pH is kept constant during the whole process but the use of this ligand comes at the expense of decreased dynamic binding capacities. The most recent addition to the list of materials that enable mild elution is a calcium-dependent ligand where elution is possible at pH 5.5 in the presence of EDTA (*55*). Dynamic binding capacities of the new ligand were not determined but interaction to human and rabbit IgG1 are weaker compared to the non-engineered type.

Usually, the low pH after elution is used for viral deactivation too but if the low pH step is eliminated, viral deactivation has to be accomplished by a different technique, e.g. addition of detergents (*56*). Therefore, other approaches are based on alterations of the elution buffer instead of the ligand itself. By addition of arginine, the dissociation of the ligand-antibody complex is facilitated and antibody-antibody interactions are repressed. It was shown that thereby the aggregation rates are lowered (*57*). Also, aggregation rates are decreased when the process is run at decreased temperature *(67).*

To reach final drug product purity, the low pH deactivation is followed by one or two chromatographic polishing steps (CIEX/AIEX), a viral filtration and an ultra-/diafiltration for final formulation (*45*). Generally, protein A chromatography is efficient with HCP removal as the

ligand binds antibodies only. Still, some HCP species are difficult to remove as they bind to the immobilized antibodies; these HCP-mAb complexes can be weakened by the use of chaotropic wash buffers in the alkaline pH range (*58*). Protein A chromatography is also highly efficient in DNA clearance but, if necessary, further DNA depletion can be achieved by an anion exchange polishing step. High molecular weight impurities are removed in a cation exchange or hydrophobic interaction chromatography step (*49*). Mixed mode media are gaining attention due to their high selectivity which makes them applicable in polishing steps for removal of host cell proteins and aggregates (*59*). Proof-of-concept as an alternative capture chromatography medium for antibodies in clarified cell culture harvest has also been provided for mixed-mode material (*60*).

Due to constant advances in upstream processing, the need for improved performance and more flexible unit operations is present in downstream processing as well. For downstream processing, it has been shown that a shift from batch-wise operation to continuous and single-use operation offers cost saving potential for certain production scales (*43*). Chromatography can be operated truly continuously by the use of simulated moving bed but, for practical reasons, usually periodic counter-current chromatography (PCCC), a semi-continuous method, is used instead. While the inflow in PCCC is continuously, the outflow is discontinuous which leads to a fluctuation in product concentrations and intermitted flow downstream. Several alternative formats, that avoid protein A media and even chromatography entirely, have been demonstrated in recent years. Hammerschmidt et al. implemented a continuous antibody precipitation using polyethylene glycol (*61*) as well as one based on cold ethanol precipitation (*62*). Burgstaller et al. combined continuous PEG precipitation with a two-stage tangential flow filtration (*63*) enabling washing, concentration and dissolution of the precipitate in a continuous matter.

Since the supernatant is easily removed from the precipitate, the material can be reconstituted in a buffer of choice and at a desired concentration. The removal of the supernatant also adds the advantage of cutting down storage space and therefore costs. Another advantage is that precipitation scales only by volume but is independent of the upstream titer (*64*). Consequently, varying titers in the upstream process do not require any adjustments in the process parameters and precipitation can be used for highly concentrated feed streams where protein A chromatography becomes uneconomical.

Despite a growing number of alternative capture steps, protein A chromatography remains the workhorse of antibody purification but there is a need for flexible capture technologies that can keep up with the steadily increasing bioreactor outputs and can be operated in continuous and single-use mode. It has also been noted that only a handful of antibody products actually

require very large production volumes (*49*); smaller batch sizes become increasingly important because of an interest in personalized medicine and they enable more efficient screening for best conditions in early drug development. On the other hand, it is obvious that companies are reluctant to change to new processes if this renders existing facilities obsolete, especially if high capital costs were allocated for the instruments in the past. Large-scale centrifuges and tanks are particularly cost-intensive in this regard (*49*). Another reason for the conservativeness of the industry towards process alterations is the uncertainness regarding critical quality attributes of the product and the resulting constraints of the regulatory authorities.

2 Critical Quality Attributes of Antibody Products

Biopharmaceuticals are associated with a larger degree of product variability than small molecule drugs as they are produced in living organisms that introduce a variety of host-dependent post-translational modifications. Furthermore, product related derivatives can be introduced during each unit operation, during hold steps or storage (*46*). While some modifications seem to have no effect at all, others affect the product in regard of storability, efficacy or even lead to immunogenic responses in the patient (*65, 66*). During product and process development, it is therefore important to identify critical quality attributes (CQA) which have an impact on the biological activity of the molecule. For antibodies, the case is especially difficult due to their large size and the high degree of microheterogeneity that results from the covalent linkage of four subunits by multiple disulfide bonds. Common modifications of antibody products are sialyation, glycosylation, deamidation, oxidation, N-terminal pyroglutamine cyclization, C-terminal lysine cleavage, aspartate isomerisation and disulfide bond shuffling (*67*).

2.1 The Effect of Protein A Acid Elution on Antibody CQAs

As outlined in chapter 1.1.3.2, antibodies are conventionally purified by protein A chromatography which requires a low pH elution. In chapter 1.1.2, the effect of low pH on antibodies was discussed. It was shown that antibodies adopt a thermodynamically stable but non-native conformation at low pH that represents a precursor in the aggregation pathway. These alternative folded antibodies are found at pH < 2.0 but in a conventional antibody process, where low pH steps are required for protein A elution and viral deactivation, the pH is set to 3.0 – 4.0 (*29*). However, for some antibodies, a pH of 4.0 is already sufficient to increase aggregation susceptibility (*30*). An increase in aggregation rates was observed for antibodies after protein A chromatography while the mere incubation of the antibodies in low pH elution buffer did not increase the aggregation potential any further (*52*). This shows that low pH in combination with adsorption on chromatography media has different effects on

antibody properties than mere incubation at low pH. The fact that proteins undergo conformational changes upon adsorption is also made use of in case of matrix-assisted-refolding) where unfolded proteins are adsorbed on a stationary phase and refolding is thereby initiated (*68*).

Gagnon et al. showed conformational plasticity as a result of protein A purification of three antibodies of the subclass IgG1. Their hydrodynamic radii were reduced from 11.5 to 5.5 nm when eluted in a step elution by 100 mM acetic acid pH 3.5. If the antibodies were solely titrated to pH 3.5 without any purification step, the size of the molecules remained at 11.5 nm (*69*). In a later study (*17*), a large heterogeneity across the protein A elution peak was found; elution started at pH 6.0 where the molecules had a size of 9.4 nm, the minimum was found at pH 3.9 with 2.2 nm. Upon further acidification during elution to pH 3.5, the radius increased again to 10.4 nm. Most drastic changes in secondary structure were detected at the lowest pH. Experiments at varying DBCs and sodium chloride concentrations revealed that the IgG size is not only dependent on pH but also on conductivity and antibody concentration.

Other antibodies are seemingly unaffected by protein A chromatography. Ejima et al. tested the effect of protein A elution at pH 2.7 - 3.9 on two human and one mouse IgGs and observed small conformational changes but no aggregations or adoption of the alternatively folded state were observed (*70*). At low pH, the antibodies were more sensitive to heat and unfolded at lower temperatures compaired to antibodies kept at neutral conditions. Only upon re-titration, aggregation of the antibodies eluted at pH 2.7 were higher than the ones eluted at pH 3.9.

As outlined in chapter 1.1.3.2., several strategies to lower aggregation rates in protein A eluates have been used, e.g. the use of alternative protein A chromatography materials that do not rely on low pH elution or the addition of stabilizing agents to the elution buffer.

2.2 Analytical Tools for Detection of CQAs

Due to the high degree of microheterogeneity in antibody products, robust analytical techniques are required to detect changes during production. As a result, the FDA published a guideline in 2004 that supports the implementation of process analytical technologies (PAT) in all unit operations of pharmaceutical development, manufacturing, and quality assurance (*71*). PAT is used for measurement of critical process parameters (CPP) which affect CQAs during processing. Ideally, CPPs are determined in- or on-line by analytical techniques with short measurement times and can be used for process control in real-time. Spectroscopic methods, like UV, Raman or near-infrared spectroscopy, fulfill the requirements in regard of response time and can be implemented in-line. In combination with chemometric tools, they guarantee stable product quality during processing (*72*).

Detailed product characterization e.g. characterization of the final drug product, is based on offline analytics. Usually, these methods are HPLC based with the most widely used techniques being size exclusion chromatography (SEC), cation-exchange chromatography (CIEX) or affinity chromatography. Such methods capture the aggregate formation, degradation/clipping of the antibody to a smaller molecular variant and formation of charge variants.

Changes in the secondary structure, folding and binding properties can best be observed by circular dichroism (CD) (*73*) or surface plasmon resoncance (SPR). The measurement by CD is based on the unequal absorption of left-handed and right-handed circularly polarized light by chiral molecules. As outlined in chapters 1.1.2 and 2.1, the secondary structure of antibodies is affected by pH and heat. Therefore, these changes can be easily followed by CD measurements. It has also been used to study antibody-antigen interaction (*74*) and to detect antibody aggregates (*75*). A difficulty in CD measurements is to find the correct protein concentration that lies between 0.005 to 5 mg/mL, depending on the adsorption characteristics of the specific molecule and the instrumental setup. The concentration needs to be high enough to get a sufficient signal but at too high concentrations, the sample absorbs too much light and the detector is unable to acquire any signal. A drawback of CD measurements is that the samples need to be relatively pure (~95% purity) and several common buffer components, e.g. sodium chloride, already compromise the data quality in moderate concentrations.
As outlined in chapter 1.1.1, antibodies belong to the group of multi-domain proteins and their domains can (un)fold independently. Thermal stability of domains is best studied by differential scanning calorimetry (DSC). In DSC experiments, the temperature is slowly increased until the unfolding temperature of a domain is reached. The protein unfolding results in a higher heat capacity and therefore in a higher uptake of energy per degree Celsius. The unfolding temperatures of the individual domains result in a thermogram which is characteristic for the tested protein. Multi-phasic unfolding transitions are oftentimes not completely resolved in DSC experiments and the individual peaks overlap. It is commonly used in the biotechnological industry to estimate protein stabilities in different formulation buffers and batch-to-batch variability (*76-78*). Fortunately, this method allows in-situ measurements, e.g. where the protein is adsorbed to chromatography material. Thereby, it can be tested if the domains are stabilized or destabilized upon adsorption.

Classically, product characterization is focused on final drug formulations (*79-81*) but with the current push of Quality by Design (QbD), it is clear that the quality of the final product depends on all the individual steps in the manufacturing process. An EMA guideline from 2016 (*82*) recommends evaluating the impact of hold steps, mid-manufacturing storage and transportation on process intermediates. Studies under worst case conditions or non-standard

conditions, e.g. unexpected process interruption, need to be included. Time limits for hold steps need to specified for each production step to avoid the risk of product deterioration. Intermediate characterization plays also a vital role when multiple research facilities work together to develop a single process and shipping of material is frequently required from one site to the next. Obviously, the interest in intermediate characterization is especially high in case of product alterations caused by a change in a unit operation, e.g. a switch from batch to continuous mode or the switch from a chromatography-based purification to a precipitation based purification.

3 Objectives

In the first part of this doctoral thesis, the temperature sensitivity of two conventional protein A materials (*MabSelect SuRe* from GE Healthcare and *TOYOPEARL AF-rProtein A HC-650F* from Tosoh Bioscience LLC) is compared to a thermosensitive protein A chromatography material (*Byzen Pro* from Nomadic Bioscience Co.). To achieve that, the equilibrium binding capacities of two antibody subclasses (IgG1 and IgG2) are determined at five temperatures ranging from 4 to 40 °C. The dynamic binding capacities are then determined for the IgG2 subclass at three temperatures (4 °C, room temperature and 40 °C). Furthermore, it is investigated whether the use of different protein A materials leads to structural alterations of the antibody upon binding; these alterations are determined by size exclusion chromatography (SEC), circular dichroism (CD) and differential scanning calorimetry (DSC). Isothermal titration calorimetry (ITC) is used to measure the heat of adsorption of the antibody.

In the second part of the doctoral thesis, the intermediate stability of a neutralized IgG2 eluate – purified with *MabSelect SuRe* – is compared to polyethylene glycol precipitate over a period of 13 months at 3 temperatures (-20 °C, 5 °C and room temperature). Monomer content, high molecular mass impurities, product loss and changes in the composition of the charge variants are determined at six time points during the storage. Structural alterations are determined at the beginning and end of the study by CD, DSC and differential scanning fluorimetry (DSF). Figure 4 contains a graphical summary of this thesis' work.

Based on that, the following objectives were formulated for the thesis:

- Determination of the temperature sensitivity of three commercially available protein A materials including the thermo-responsive material Byzen Pro
- Determination of structural alterations resulting from the interaction with protein A ligands by CD, DSC and SEC
- Determination of structural alterations occurring during a 13 month storage period of a neutralized protein A eluate and PEG-precipitated antibody by SEC, CIEX-HPLC, CD, DSC and DSF

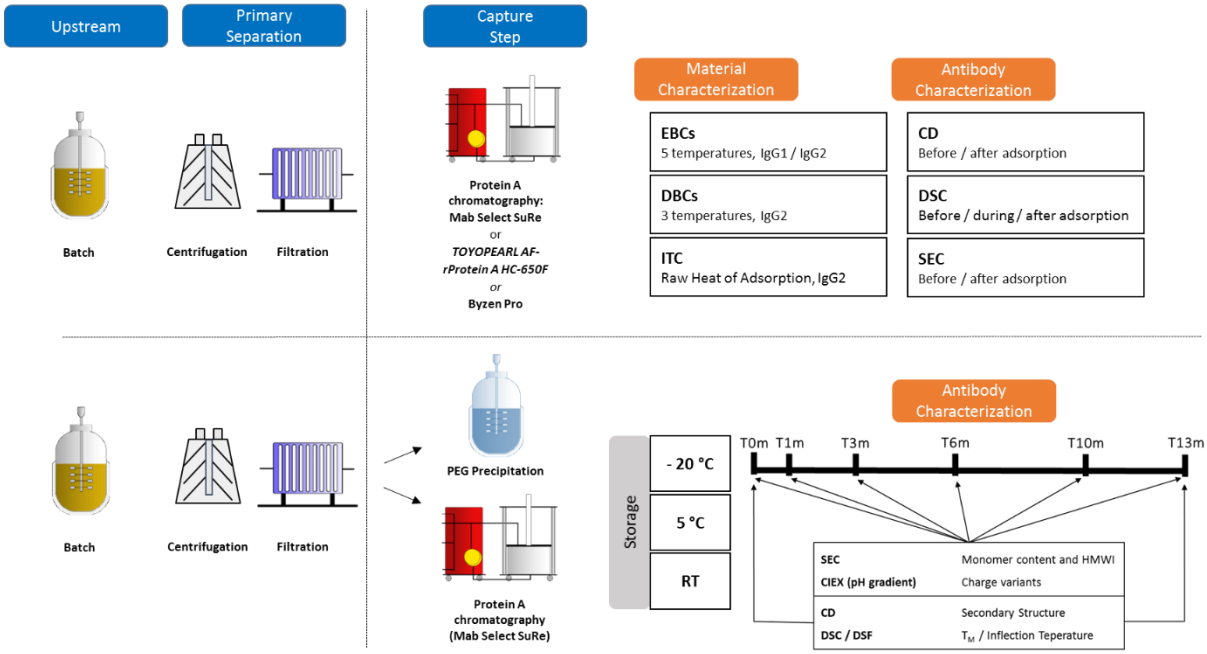

Figure 4: Graphical abstract of thesis. Top: Three protein A chromatography materials were compared in regard to their binding capacities and influence on antibody structure. Bottom: Comparison of storability of neutralized protein A eluate and precipitated material.

4 Discussion

The main results of this work are presented in this thesis and two publications, one published in Journal of Chromatography A with the title “Temperature dependence of antibody adsorption in protein A affinity chromatography” (*83*), the other one published in Biotechnology Progress with the title “Mid-manufacturing storage: Antibody stability after chromatography and precipitation based capture steps” (*84*)

In the first part of the thesis, three commercially available protein A materials were characterized in regard of their binding capacities at different temperatures. Important properties of the selected materials are listed in Table 1. The ligands of MabSelect SuRe and Byzen Pro are both based on the B domain of the native protein A material. The ligand of the

MabSelect SuRe is a tetramer of this modified B-domain. Additional mutations were introduced to improve the alkaline resistance of the material to support common cleaning-in-place methods after usage (*85*). According to the manufacturer, the ligand of Byzen Pro is sensitive to temperature: at low temperatures (<10 °C), antibodies are bound, while at elevated temperatures (40 °C), the ligand becomes unstable and the antibodies are released, thereby the target molecules can be recovered without a change in pH. It was not disclosed by the manufacturer whether the ligand is composed of a monomer or an oligomer. The ligand of TOYOPEARL AF-rProtein A HC-650F has a hexameric structure and is derived from the C domain. The material is alkaline stable as well. The ligand densities were not disclosed for any of the three materials.

Table 1: Characteristics of tested protein A materials

	MabSelect SuRe	**TOYOPEARL AF-rProtein A HC-650F**	**Byzen Pro**
Bead Size [µm]	85	45	70
Ligand Composition	Tetramer	Hexamer	Undisclosed
Backbone	Cross-linked agarose	Polymethacrylate	Cross-linked polyvinyl alcohol
Notable Characteristics	Alkaline stable	Alkaline stable	Low pH not required for elution; heat elution possible

The equilibrium binding capacities (EBCs) were determined for antibodies of the IgG1 as well as the IgG2 subclass at five different temperatures: 4, 12, 22, 30 and 40 °C. Obtained data was fitted using the Langmuir isotherm (*86*) given in Equation 1. The capacity at a given concentration C in mg/mL is denoted as q, q_{max} is the maximum binding capacity in mg/mL and K_L is the affinity constant in mL/mg.

$$q = q_{max} \frac{K_L * \mathrm{C}}{1 + K_L * C}$$

Equation 1

Byzen Pro showed the highest temperature variance in EBC curves but also both conventional media showed variations in q_{max} at different temperatures. For all three materials, the q_{max} variations were greater for the IgG1 subclass than for the IgG2 subclass. This is assumed to be a result of the longer hinge region of IgG1 molecules that lead leads to an increase in flexibility. Binding capacities for the IgG1 subclass were higher than for the IgG2 subclass. Due to the high affinity of protein A towards antibodies, EBC curves usually have a rectangular

shape. In case of Byzen Pro, the curves were however not rectangular at 40 °C, indicating a decreased affinity of the material at this temperature. EBC curves do however also show that the ligand is not fully denatured at 40 °C as antibodies are still bound. In regard to process efficiency, this means that product losses will occur when temperature elution is used. For both antibody subclasses, the affinity constants of Byzen Pro were found to be at their minima at 40 °C while the q_{max} did not differ greatly from the other temperatures. To determine the implications of the differences in the affinity constants and $q_{max,}$ we determined dynamic binding capacities (DBC) of the materials as well. While EBC depends on the available surface area, ligand density and adsorption equilibrium, the DBC takes the flow dispersion into account as well. Since the adsorption process is diffusion limited,the experiments were performed at two flow rates, 125 and 250 cm/h. As the flow rate is increased, the dynamic binding capacity decreases as there is less time for the molecules to diffuse into the pores. DBCs were determined for the IgG2 subclass at three different temperatures. For Byzen Pro, it was expected that the $DBC_{10\%}$ would be highest at 4 °C and lowest at 40 °C – this trend was however only observed at low flow rates while the capacities at high flow rates were almost identical for all three tested temperatures. It follows that the process is dominated by mass transfer limitations at high flow rates and that the change in decreased affinity of the material at 40 °C has a subordinate role in the binding capacities. This means that the flow rate of the chromatography step will have to be decreased if temperature elution is desired. For MabSelect SuRe, the $DBC_{10\%}$ rises as the temperature is increased independent of the flow rate. This shows that the process under these conditions is not yet entirely governed by mass transfer limitations and diffusivity is affected by temperature changes. The relation of diffusivity and temperature is described by the Stokes Einstein equation given Equation 2, where D is the diffusion constant, k_B is the Boltzmann's constant, T is the absolute temperature, η is the viscosity of the medium and r is the radius of the spherical particle.

$$D = \frac{k_B * \mathrm{T}}{6\pi * \eta * r}$$

Equation 2

The $DBC_{10\%}$ of TOYOPEARL AF-rProtein A HC-650F was also found to be independent of flow rate. The lowest $DBC_{10\%}$ was found at 4 °C while the $DBC_{10\%}$ at 22 and 40 °C were similar. The increased diffusivity at 40 °C seem to be leveled out by mass transfer effects. Speaking in absolute numbers, the EBC as well as the $DBC_{10\%}$ were highest for TOYOPEARL AF-rProtein A HC-650F, followed by MabSelect SuRe and Byzen Pro.

Next, different elution strategies for the three materials were tested. Conventional acid elution was performed for MabSelect SuRe and TOYOPEARL AF-rProtein A HC-650F at pH 3.0 and 4.0 and for Byzen Pro at 4.0 since it is not recommended to use the material at a pH lower than 4.0. Product recovery of 99% was achieved for Byzen Pro at pH 4.0, while the interaction

between antibody and MabSelect SuRe and TOYOPEARL AF-rProtein A HC-650F was too strong for quantitative elution at pH 4.0. For MabSelect SuRe the step yield was 77%, for TOYOPEARL AF-rProtein A HC-650F it was 83%, which is not sufficient for a capture step for monoclonal antibodies. However, product recoveries >99% were achieved for both materials once the elution pH was lowered to 3.0. It is therefore very clear that for conventional protein A chromatography a very low pH of 3.0 is absolutely necessary for an efficient process and cannot be omitted. Therefore, conventional protein A chromatography material is incompatible with antibody products that are sensitive to low pH conditions, even when efforts are made to optimize the elution conditions. The only way for an efficient process avoiding product degradation for pH sensitive antibody products is therefore to use alternative protein A chromatography media, or use alternative technologies that are not based on chromatography and/or low pH conditions. While the use of ByzenPro is not optimal as complete elution from the column was not possible through the use of temperature induced elution, it at least presents a possibility for antibody products that are stable while adsorbed on a column and unstable under low pH conditions. Yet, one still would have to accommodate for a lower productivity due to reduced binding capacity under those conditions.

During process development, it became clear that Byzen Pro is sensitive to salt, therefore elution by a sodium chloride pulse was tested. Buffers with high concentration of sodium chloride are frequently used for wash steps in protein A chromatography and it is desirable that the interaction between ligand and antibody is not significantly impeded in the presence of sodium chloride. For Byzen Pro, a quantitative product recovery was achieved by using a buffer containing 1 M of sodium chloride for elution. For pH sensitive antibody purifications, the salt sensitivity of Byzen Pro is advantageous as it allows elution at neutral pH. For MabSelect SuRe, the salt elution led to a recovery of 55% while in case of TOYOPEARL AF-rProtein A HC-650F only 11% were recovered in the elution peak.

The salt sensitivity of Byzen Pro was proven to be significant also for the heat elution; heat elution was first probed with a 20 mM sodium phosphate buffer at pH 6.9 but there was no product recovered under these conditions. Only the addition of 0.05 M NaCl to the buffer enabled elution upon a temperature change from 4 to 40 °C. For MabSelect SuRe and TOYOPEARL AF-rProtein A HC-650F, the change in temperature under neutral pH did not result in product recovery. Product recoveries (%) for the tested conditions are summarized in Table 2. Findings of this study were published in the publication (*87*).

Table 2: Product recovery (%) for the different elution strategies

	MabSelect SuRe	TOYOPEARL AF-rProtein A HC-650F	Byzen Pro
Acid [pH 4.0]	77%	83%	99%
Acid [pH 3.0]	>99%	>99%	-
Salt	55%	11%	98%
Heat	No Elution	No Elution	96%

It is known that proteins can change their conformation upon adsorption (*88*), therefore it was investigated whether the interaction with protein A material leads to any structural alterations of the antibody. This was achieved by probing the structure of the IgG2 subclass with several analytical techniques, namely differential scanning calorimetry, circular dichroism and size exclusion chromatography. DSC experiments give an insight in the thermal stability of the probed molecule. CD is a non-invasive method to determine the secondary structure and folding properties of proteins (*73*). SEC is used to determine the hydrodynamic radii of molecules by molecular sieving and can be used to determine aggregations, oligomerizations and molecule clipping.

In DSC, antibodies unfold in a biphasic process where the C_H2 and Fab domains unfold at slightly lower temperatures and then a second unfolding process is observed for the unfolding of the C_H3 domain (*89*). This biphasic transition was observed for the unfolding of the antibody before and also after elution (and neutralization) from all three materials. DSC can however also be used to probe the thermal stability during adsorption to the material. The materials were saturated with antibody and then loaded into the DSC chamber. Thereby it can be observed if the thermal stability of the antibody changes during adsorption to the media. When adsorbed on Byzen Pro, the antibody still unfolds in a biphasic transition but the unfolding temperatures are decreased by 3.3 °C for T_M1 and 1.3 °C for T_M2 compared to the unbound state (Table 3) indicating a stability decrease in the adsorbed state. When adsorbed to MabSelect SuRe or TOYOPEARL AF-rProtein A HC-650F, the antibody unfolds in a single transition process at 78.4 and 78.5 °C respectively. In the unbound state, the C_H3 domain unfolds at similar temperatures, therefore, the stabilization effect of interaction with protein A seems to affect the C_H2 and Fab regions to a greater extent than the C_H3 domain. It follows that the adsorption on conventional protein A media is favorable for the antibody due to an increase in stability.

Table 3: Unfolding temperatures (T_M) in differential scanning calorimetry

	T_M1	T_M2
MabSelect SuRe	78.4	-
TOYOPEARL AF-rProtein A HC-650F	78.5	-
Byzen Pro	70.3	77.7
Before / after Elution	73.6	78.4

CD and SEC was performed before and after adsorption. Results show that the secondary structure and also monomer composition are identical for the antibodies before/after adsorption and that the changes observed in DSC experiments are not of a permanent nature but transient and reversible. This is in line with the findings of Gagnon et al. (*69, 90*) who showed that the hydrodynamic radii of antibodies are altered by the interaction with protein A material. While exposure of the antibodies to the acidic elution buffer alone did not have an effect on the molecule size, the radius decreased from 11.5 nm to 2.2 nm when proteins were eluted off the protein A material at pH 3.9. After neutralization, the proteins went back to their original size. Size measurements by dynamic light scattering (DLS) were performed with the IgG2 in our study too but there were no differences in regard of hydrodynamic radii detected comparing the material before and after protein A chromatography.

The results of this thesis supply further proof that antibodies are not rigid but undergo transient, conformational changes during purification. Despite the fact that all the observed changes were reversible, the conformational flexibility of antibodies should be kept under review until the impact of these transient forms on product safety is clear. The results show that the antibody conformation can be affected in different ways depending on the choice of protein A material.

In the second part of this doctoral thesis, two antibody capture methods were compared in regards to their effects on antibody quality, including a storability study over 13 months. Antibodies were captured from the clarified cell culture fluid (CCCF) either by protein A chromatography (MabSelect SuRe) or by PEG-precipitation. The protein A eluted antibodies were incubated at the elution pH of 3.6 for one hour, then the pH was readjusted to 6.5 and the material was stored for 13 months at three temperatures (-20 °C, 5 °C and room temperature). Alternatively, antibodies were precipitated by addition of 13.2% PEG (w/w). After decantation of the liquid, the precipitate was stored under the same conditions as the protein A purified material. The same IgG2 molecule as for the experiments in the first part was used.

In Figure 5, an important finding of this work is visualized: the use of protein A chromatography leads to the formation of an additional charge- variant which is not present in the clarified cell

culture fluid. This isoform is not produced when the antibodies are captured by precipitation. Incubation of the precipitated and resolubilized antibodies in the acidic elution buffer for one hour does not lead to the formation of the variant either.

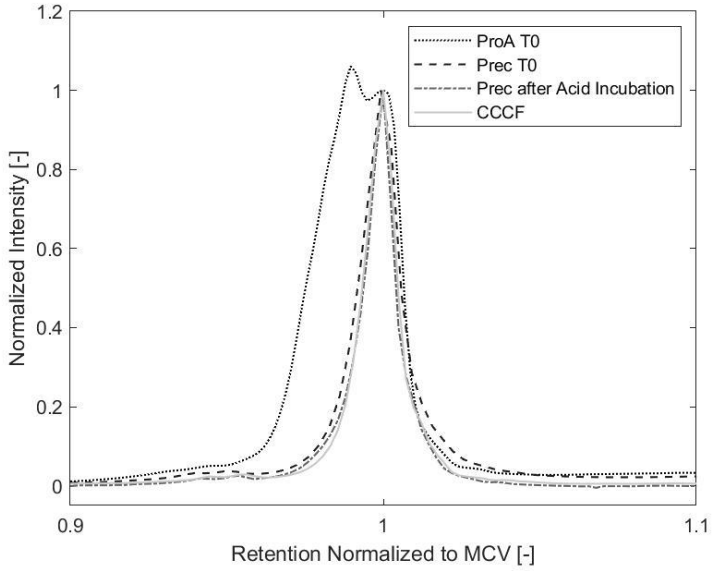

Figure 5: Antibody charge variants separated by pH gradient of IgG2 after protein A chromatography (ProA T_0), after PEG-precipitation (Prec T_0), the precipitate after acid incubation (Prec after Acid Incubation) and the clarified cell culture fluid. Reproduced from (87)

The acidic variant formed during protein A chromatography has a transient nature which was shown by incubation of the purified material at different temperatures (5 °C, room temperature and 40 °C) by charge-variant analysis at 0, 24, 48 and 120 hours. The reformation of the acidic variant to the main charged variant (MCV) was shown to be temperature dependent: at 40 °C, the acidic variant reverts back to the MCV within 48 hours while the same process takes 120 hours at 5 °C.

Monomer content, high molecular weight impurities (HMWI) and product losses were determined at six time points during the storage period by HPCL measurements based on size exclusion chromatography. The starting material is referred to as T_0 material. Table 4 comprises the relevant data.

In the protein A purified samples, the monomer content at the beginning is at 99.8% and decreases by 2% over the storage period at all tested temperatures (Table 4). At the same time, we see an increase of HMWI from 0.6% to 2.2 – 2.5%. Interestingly, there is not much difference between the storage temperatures, the samples stored at room temperature show only slight increases in HMWI compared to the samples stored at – 20 °C. Also, there are practically no product losses detectable for any storage condition.

For the precipitated material, the picture looks quite different. In the precipitated T_0 sample, there are 5.5% HMWI present, that means, the amount of HMWI present at the beginning is already higher compared to protein A purified material. When stored at -20 °C, the initial HMWI concentration of 5.5% stays constant for 13 months, at 5 °C an increase by about 1% is

observed. The monomer concentration stays constant at -20 °C but decreases by 5% under 5 °C storage. In regard of product losses, it is observed that the precipitate stored at -20 °C or 5 °C needs to be reconstituted within the first month of storage to achieve products yields >90% compared to the concentration at T_0. Afterwards, the product losses increase to ~30% and stay constant for the rest of the storage. At room temperature, the precipitated material is not stable. Due to the results regarding monomer content, HMWI and charged variants, it is assumed that the antibody is degraded after 3 months of storage where we see the formation of numerous new charge variants in the charge isoform pattern. Furthermore, product losses are observed during the first 3 months in storage but afterwards, the losses start to decline again, resulting in final product losses of less than 0% which is virtually impossible. Therefore, it is assumed that aromatic amino acids, that are buried in the hydrophobic core of the native protein, become accessible due to a conformational change, thereby falsifying the UV measurements. At the same time, we see a rise in HMWI in the precipitated material which also indicates that conformational changes are occurring leading to self-association and subsequent formation of aggregates.

At the beginning and end of the study, further analytical tests were performed, namely CD, DSC and DSF. In CD spectra, the precipitated antibodies have an observed maximum at 198 nm while the protein A purified material has its maximum 202 nm. All samples have a minimum at 218 nm but the signal of the precipitated material is a bit stronger than the one of the protein A purified material. Deconvolution of the spectra shows that the precipitated material contains higher amounts of random coil. The main unfolding event in DSC which accounts for the unfolding of the CH2 domains and the Fab domains is approximately 0.4 °C higher for the protein A purified material than for the precipitate. In DSF experiments, we see a difference of approximately 1 °C in the inflection temperature. In summary, it can be concluded that the precipitated material shows a slightly decreased stability compared to the protein A purified material. The differences are already present at the beginning and storage time seems to have no altering effect on the stabilities.

Table 4: Monomer content (%), HMWI (%) and product loss (%) data for storage at -20 °C, 4 °C and room temperature.

Storage at -20 °C

	Monomer Content ± St.Dev. (%)		HMWI ± St.Dev. (%)		Concentration compared to T_0 ± St.Dev. (%)	
	ProA Purified Material	Precipitated Material	ProA Purified Material	Precipitated Material	ProA Purified Material	Precipitated Material
At T_0	99.8 ± 0.2	85.2 ± 3.6	0.58 ± 0.08	5.54 ± 0.68	100.0 ± 0.69	100.0 ± 0.88
At T_1M	99.5 ± 0.2	85.5 ± 3.6	0.75 ± 0.02	5.93 ± 0.66	100.5 ± 1.13	90.2 ± 1.42
At T_3M	97.8 ± 1.4	83.1 ± 0.4	1.41 ± 0.20	5.74 ± 0.16	99.3 ± 0.30	77.7 ± 7.16
At T_6M	98.0 ± 0.4	83.2 ± 1.6	1.76 ± 0.04	5.12 ± 0.19	96.8 ± 0.68	73.0 ± 1.95
At T_{10}M	99.1 ± 0.2	82.9 ± 0.4	2.54 ± 0.28	6.42 ± 0.10	100.7 ± 0.23	72.6 ± 2.66

At $T_{13}M$	98.2 ± 0.3	84.3 ± 1.8	2.20 ± 0.28	5.6 ± 0.04	100.0 ± 0.23	67.3 ± 3.45

Storage at 5 °C

	Monomer Content ± St.Dev. (%)		HMWI ± St.Dev. (%)		Concentration compared to T_0 ± St.Dev. (%)	
	ProA Purified Material	Precipitated Material	ProA Purified Material	Precipitated Material	ProA Purified Material	Precipitated Material
At T_0	99.8 ± 0.2	85.2 ± 3.6	0.58 ± 0.08	5.54 ± 0.68	100.0 ± 0.69	100.0 ± 0.88
At T_1M	100.0 ± 0.0	84.8 ± 1.8	0.81 ± 0.06	6.05 ± 0.24	97.9 ± 0.67	97.0 ± 3.98
At T_3M	97.5 ± 0.4	80.4 ± 2.4	2.21 ± 0.44	5.89 ± 0.58	100.4 ± 3.42	68.4 ± 4.59
At T_6M	97.9 ± 0.0	82.0 ± 1.6	2.00 ± 0.40	4.89 ± 0.20	98.6 ± 0.04	70.5 ± 2.13
At $T_{10}M$	98.2 ± 0.8	77.7 ± 0.4	2.32 ± 0.64	5.87 ± 0.48	102.4 ± 1.82	71.4 ± 0.25
At $T_{13}M$	98.3 ± 0.6	79.8 ± 1.6	2.32 ± 0.26	6.24 ± 0.02	99.2 ± 0.64	66.5 ± 1.91

Storage at Room Temperature

	Monomer Content ± St.Dev. (%)		HMWI ± St.Dev. (%)		Concentration compared to T_0 ± St.Dev. (%)	
	ProA Purified Material	Precipitated Material	ProA Purified Material	Precipitated Material	ProA Purified Material	Precipitated Material
At T_0	99.8 ± 0.2	85.2 ± 3.6	0.58 ± 0.08	5.54 ± 0.68	100.0 ± 0.69	100.0 ± 0.88
At T_1M	99.4 ± 0.8	79.0 ± 3.6	1.05 ± 0.04	5.06 ± 0.32	98.9 ± 1.16	94.8 ± 3.95
At T_3M	97.3 ± 0.0	74.0 ± 5.6	2.03 ± 0.18	4.40 ± 0.62	99.0 ± 1.17	85.9 ± 6.23
At T_6M	96.7 ± 0.4	70.1 ± 7.2	2.75 ± 0.02	4.33 ± 0.52	100.6 ± 1.40	95.4 ± 1.29
At $T_{10}M$	97.7 ± 0.2	64.0 ± 4.2	2.54 ± 0.28	7.44 ± 5.14	99.8 ± 0.97	104.2 ± 0.49
At $T_{13}M$	98.0 ± 0.4	60.5 ± 2.2	2.54 ± 0.18	15.57 ± 0.20	101.4 ± 0.30	119.0 ± 1.33

5 Conclusion

Protein A chromatography is the golden standard for antibody purification but several alternative methods have been developed in recent years. While an implementation at industrial scale is pending, it has been shown that these methods constitute robust replacements for protein A chromatography at pilot scale. The focus of this doctoral thesis was to compare different capture methods in regard of their influence on antibody quality.

At first, let me conclude the results of the three protein A materials that were compared in regard of binding capacities. Byzen Pro is an alternative protein A material that enables elution at neutral pH by a change in temperature from 4 to 40 °C. Temperature elution is possible with this material but it was implemented at the expense of decreased binding capacities. The claim of the manufacturer that Byzen Pro can be used with any neutral buffer of choice is only partially true as the temperature elution did not work with a salt free buffer. Generally, Byzen Pro was found to be highly sensitive to the salt concentration in the buffer and antibodies could even be eluted by a salt pulse. This is a benefit for acid-labile antibodies and non-commercial small scale purifications as they can use salt or heat elution. At industrial scale, both methodologies seem unlikely. Elution by temperature is difficult to scale up; with increasing column diameters, it becomes more and more difficult to temper the chromatography material evenly and temperature gradients between the inner and outer part of the column are likely formed. Besides temperature sensors at the column in- and outlet, it would also be necessary to measure the temperature inside the column at several locations. Salt elution on the other side is easy scalable but adds the disadvantage of having the product in a high salt buffer after protein A elution. This might require a buffer exchange before the next unit operation. In both cases, the virus inactivation needs to be achieved by an alternative method, e.g. by the use of detergents. Both conventional materials, MabSelect SuRe and TOYOPEARL AF-rProtein A HC-650F, also show temperature dependence in binding capacities but a change in temperature from 4 to 40 °C was not sufficient to elute the product. Overall, the highest binding capacities were found for TOYOPEARL AF-rProtein A HC-650F followed by MabSelect SuRe and Byzen Pro.

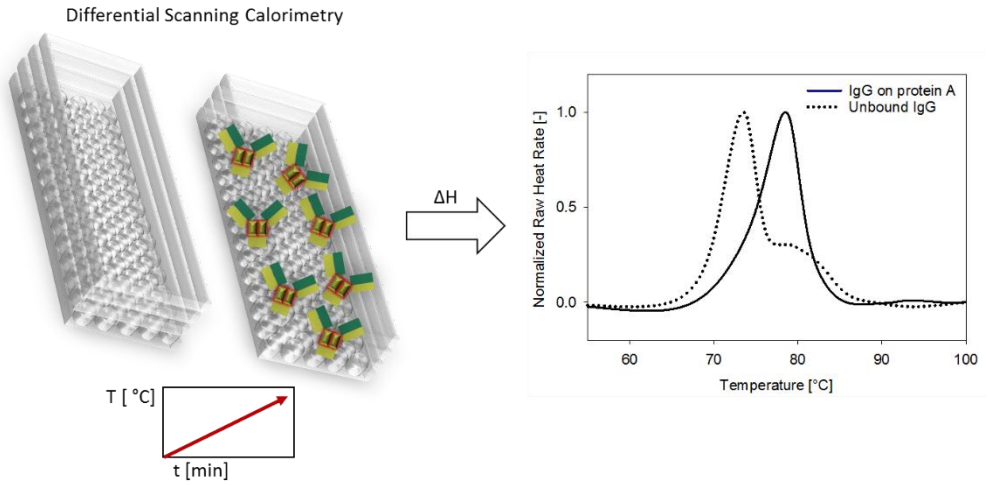

Figure 6: In this thesis it was shown that antibodies undergo transient, structural changes upon binding to protein A chromatography material. These changes were detected in-situ by differential scanning calorimetry measurements.

It was shown that upon binding to protein A materials, antibodies undergo transient structural change (Figure 6). A newly formed acidic variant was detected after protein A elution by HPLC CIEX with a pH gradient. The reconversion of this isoform to the main charge isoform is temperature dependent and took between 48 hours (40 °C) and 120 hours (5 °C). Commonly observed post translational modifications which lead to the formation of acidic variants are deamination, sialyation and glycation (*91*) but these modifications are irreversible. Reversible modifications that lead to acidic variants are observed too (disulfide bond variants, trisulfides) but in the published studies, these variants are stable and do not revert to the main charged variant. Wypych et al. (*92*) observed disulfide heterogeneity of IgG2 in a CHO supernatant and performed RP-HPLC before and after protein A chromatography. They did not detect differences before and after the low pH protein A elution. It is clear that the acidic variant formed during protein A chromatography of our study must have a different conformation which alters the surface charges of the protein and therefore leads to different interaction on the CIEX column. The newly formed variant is not stable at neutral conditions but reverts back to the main charged variant. It has been observed that antibodies adopt alternative folded states upon acidification (*23, 93*) but the conformational transformations occur at pH < 2 and have so far not been observed at typical protein A elution pH of 3.6. Even more importantly, it needs to be pointed out that the acidic isoform was not found in the precipitated and resolubilized material after low pH incubation. The conformation change is therefore not solely pH dependent but occurs only in connection with protein A chromatography. The resulting changes are not detected by circular dichroism experiments since the CD spectra for the antibodies immediately after elution (T_0) were identical to the protein A purified antibodies after long-term storage, which means significant changes in the secondary structure can be ruled out. Gagnon et al. (*69, 90*) observed a change in hydrodynamic radii after protein A elution.

We measured the hydrodynamic radius of our material at T_0 by dynamic light scattering but could not detect any differences in radii compared to material without the acidic isoform.

For the precipitated material, the results are quite different. Firstly, we do not observe any changes in the isoform pattern. An overlay with the CCCF shows an identical pattern. However, T_M in differential scanning calorimetry and fluorimetry are slightly decreased for the precipitated material compared to the protein A purified material. Circular dichroism revealed differences in secondary structure with a higher amount of random coil when compared to the protein A purified material. These results are in contrast with previous findings in our group where an antibody of the IgG1 subclass was tested; based on CD spectra, the structure of the IgG1 antibody after protein A chromatography and the precipitated material were found to be identical. Potentially, molecules of the IgG2 subclass have therefore a higher susceptibility for structural alterations. The differences in thermal unfolding and secondary structure are believed to be the reason for different stabilty properties too. If resolubilized within the first month of storage, the product losses for the precipitated product stored at -20 or 5 °C are below 10% but after three months in storage, ~30% of the antibodies cannot be resolubilized anymore as the material seem to undergo an ageing process but there are no new variants appearing in the isoform analysis. The product losses could be caused by a compression of the precipitated material during storage which leads to a decrease in resolubilisation yields. Potentially, it is necessary to use a different buffer for the resolubilisation of longer stored material to decrease the product losses. The altered storability behavior of the precipitated intermediate could also be caused by residual amounts of PEG or the co-presence of HMWIs and HCPs. These are either absent or present in lower concentrations in the protein A purified samples. Protein A purified material was found to be remarkable stable, even at room temperature, there are no product losses or conformational changes observed after a storage period of 13 months. Based on the storability study, we can conclude that precipitated antibodies are stable at least for storage up to a month which gives enough time to ship the material between different manufacturing sites. Nowadays, where processes are oftentimes developed by multiple research institutes, this issue is of strategic importance.

6 Outlook

Newly emerging capture technologies struggle for recognition on the highly competitive and cost-driven antibody downstream processing market. On the one hand, this is caused by the conservativeness of the biotech industry which is a result of regulatory constraints and intellectual property issues. On the other hand, protein A chromatography is very well established and difficult to compete with due to its robustness and high specificity.

Nevertheless, efforts to develop flexible, easy-to-scale and titer-independent downstream technologies will continue to be made as long as demands and titers of antibody products continue to rise. In order to get new technologies established on the market, it is indispensable to prove that efficacy and safety of the active pharmaceutical ingredient are at least equivalent to the existing technologies. Therefore, future research activities need to be focused on the identification of process parameters with an influence on CQAs. In regard to emerging antibody capture technologies, precipitation is a promising candidate as it scales only with volume but not with titer *(64)*, yields similar purities as protein A chromatography and can easily be implemented in a continuous set-up. Upcoming projects should focus on a proof-of-concept of continuous precipitation at industrial scale and determine the exact structural composition of the precipitates by e.g. high-resolution microscopy or HDX-MS to ease the way for quick regulatory approvals. The findings of this work show that the CQAs of an antibody product can be significantly altered based on the choice of capture method and potentially have to be monitored differently than the established procedures used for protein A material.

7 List of Tables

8 List of Figures

9 Publications

9.1 First author publications

Krepper W., Satzer P, Beyer BM, Jungbauer A. Temperature dependence of antibody adsorption in protein A affinity chromatography. Journal of Chromatography A 2018; 1551:59-68.

Krepper, W., Burgstaller, D., Jungbauer, A., and Satzer, P. (2019) Mid-manufacturing storage: Antibody stability after chromatography and precipitation based capture steps. Biotechnology Progress. n/a, e2928.[b]

First author publications have been added to the appendix (chapter 11).

[b] As of 29th of November, 2019, this publication is available online as an Early View version before an inclusion into an issue.

9.2 Contributions to other publications

Burgstaller D, **Krepper W**, Haas J, Maszelin M, Mohoric J, Pajnic K, et al. Continuous cell flocculation for recombinant antibody harvesting. Journal of Chemical Technology & Biotechnology 2018; 93:1881-90.

In this publication, pDADMAC - a polycationic flocculation agent – was used for a continuous flocculation in combination with depth filtration as the primary recovery step in an antibody purification process. I contributed to this work by doing analytical experiments on the antibody before and after flocculation. Charged antibody variants were separated by a pH gradient in HPLC-CIEX. It could be shown that the use of pDADMAC as flocculant has no impact on the composition of the charged variants.

Satzer P., Burgstaller D., **Krepper W**., Jungbauer A. „Fractal dimension of antibody PEG-precipitate: Light microscopy for the reconstruction of 3D precipitate structures“. Manuscript accepted for publication to Engineering in Life Sciences.

In this publication, the 3D structure and the fractal dimension of precipitate generated under batch and continuous operation was determined by light microscopy tomography. It could be shown that the generation of precipitates under different conditions has significant impact on the structure and fractal dimension of the resulting precipitate particles.

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11 Appendix

11.1 Publication 1

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Temperature dependence of antibody adsorption in protein A affinity chromatography

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ABSTRACT

Staphylococcal protein A affinity chromatography is a well-established platform for purification of clinical-grade antibodies. The wild type ligand has been mutated to improve caustic stability, elution behavior, and/or to increase binding capacity. Several modified protein A ligands are nowadays commercially available, one of them being the thermosensitive chromatography medium Byzen Pro from Nomadic Bioscience Co., Ltd. According to the manufacturer, Byzen Pro has the ability to release IgG upon a change in temperature. It is based on a thermosensitive mutant of protein A which should allow elution at neutral pH by changing the temperature from binding at 5 °C to elution conditions at 40 °C. We determined equilibrium binding capacities of the thermosensitive protein A medium (Byzen Pro), MabSelect SuRe (GE Healthcare), and TOYOPEARL AF-rProtein A HC-650F (Tosoh Bioscience LLC) for antibodies of the subclass IgG1 and IgG2 at five different temperatures from 4 °C to 40 °C to elucidate the temperature effect. We also observed a temperature dependence of the dynamic binding capacities which were determined for the subclass IgG2 at three temperatures from 4 °C to 40 °C. However, for Byzen Pro, the temperature dependence was only present at a low flow rate and vanished at high flow rates indicating that pore diffusion is the rate-limiting step. Binding of the antibody to MabSelect SuRe and TOYOPEARL AF-rProtein A HC-650F stabilized the conformations as shown by an increase in melting temperature in differential scanning calorimetry measurements. The antibody conformation was slightly destabilized upon binding to the thermosensitive ligand. The conformation change upon binding was fully reversible as shown by circular dichroism, differential scanning calorimetry and size exclusion chromatography. Isothermal titration calorimetry was used to measure the raw heat of adsorption for the IgG2 molecule. The thermosensitive ligand can also be used for antibodies with low stability, because elution can also be effected by salt.

1. Introduction

Staphylococcal protein A affinity chromatography is the workhorse for purification of antibodies and Fc-γ fusion proteins [1]. Due to its high efficiency in regard to HCP clearance, capacity, and yield, it is popular at the laboratory as well as the industrial scale and is considered a platform process for antibodies [2]. Native staphylococcal protein A (SpA) has five highly homologous IgG-binding domains, designated as E, D, A, B and C. Commercial chromatography media are nowadays based on engineered protein A mutants which are often derived from the B domain. Compared to the wild type, these chromatography media have higher binding capacities, prolonged media lifetime, alkaline stability, or better elution behavior. The high affinity of protein A ligand for antibodies requires harsh elution conditions with pH as low as pH 3.0, which may cause aggregation or/and precipitation [3]. Several chromatography media have been designed to address this problem [4,5], one of them is based on a thermosensitive protein A ligand where elution is achieved upon an increase of temperature. Due to amino acid alterations introduced in the hydrophobic backbone of this ligand, it is claimed to become unstable at elevated temperatures of 40 °C, thereby causing the release of the bound analytes [4]. This invention was the motivation to investigate the temperature dependence of conventional protein A ligands to compare them to the temperature sensitive variant. A change in temperature does, however, not only affect the ligand but also the antibody [6,7]. This can cause conformational changes, (partial) unfolding and degradation that lead to fundamental changes in the efficacy of the antibody. Many

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commercially available chromatography media have been characterized in regard to their affinities and binding capacities for whole antibodies or antibody fragments of various sources [8]. These studies are often conducted at the common operating temperature, i.e., room temperature, only and deviations thereof are not being evaluated. Our study focuses on changes in structural stability of three commercially available engineered protein A chromatography media upon temperature change and the resultant effects on antibody adsorption kinetics. We selected the following three protein A chromatography media that are commercially available. The temperature-sensitive protein A material Byzen Pro from Nomadic Bioscience Co., Ltd. (Okayama, Japan) [4] is a derivative of the B-domain of native protein A and has a cross-linked polyvinyl backbone with a mean particle size of 70 μm. The domain number of Byzen Pro has not been disclosed by the manufacturer. The ligand of the MabSelect SuRe affinity medium (GE Healthcare, Uppsala, Sweden) is a tetramer of the modified B-domain of native protein A and has an agarose backbone. It is alkali-tolerant and has a mean particle size of 85 μm. TOYOPEARL AF-rProtein A HC-650F (Tosoh Bioscience, Griessheim, Germany) is stable under alkaline conditions as well and has a hexameric structure. The ligand is based on the C-domain of native protein A and coupled to a polymethacrylate matrix and the mean particle size is 45 μm. Ligand densities are not disclosed for any of the materials.

Most recombinant antibodies are derived from a primary sequence of the immunoglobulin class G (IgG) which plays a major role in the humoral immune response. The IgG class can further be divided in four subclasses: IgG1, IgG2, IgG3 and IgG4 (in order of decreasing abundance in human serum). From a biopharmaceutical point of view, IgG1 and IgG2 are of special interest as the majority of approved antibody products is accounted to one of those two classes. Antibodies of the IgG1 and IgG2 type show a high sequence homology of ~90%, but the slight variations in the constant region, particularly in the hinge regions and upper CH2 domains that are responsible for major differences in antigen responses and receptor affinities [9,10]. IgG2 has a shorter hinge region than IgG1 and is therefore considered less flexible. Antibodies of the IgG2 subclass also show a lower capacity on staphylococcal protein A chromatography media [11]. It has been shown by crystallographic refinement that SpA binds the antibody in the Fc region between the CH2 and CH3 domains [12] and that the E- and D- domains can bind antibodies of the V_H3 subclass in their variable region [13].

To elucidate the effect of temperature on antibody binding we performed equilibrium and dynamic binding capacity measurements and column experiments with loading and elution. Structural alterations upon binding of the antibody were tested by size exclusion chromatography, circular dichroism and differential scanning calorimetry. The raw heat of adsorption was determined by isothermal titration calorimetry.

2. Material and methods

All chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

2.1. Antibodies

Experiments were carried out with recombinant, human antibodies of the subclasses IgG1 and IgG2. For the EBC studies, we used both subclasses as model proteins. Due to material shortage, breakthrough studies, differential scanning calorimetry, circular dichroism and isothermal titration calorimetry were performed with the IgG2 molecule only.

The antibody concentration was measured by UV absorption at 280 nm and concentration was calculated using the molar extinction coefficient of 1.40 for IgG1 and 1.38 for IgG2. Purity was determined by analytical size exclusion chromatography and was above 99% for both antibodies

2.2. Equilibrium binding capacity

Equilibrium binding capacity (EBC) studies were performed in 96-deep well plates with a total volume of 2 ml (Thermo Fisher, MA, USA) and a reaction volume of 440 μl. Each well contained 20 μl of chromatography medium which was added as a slurry solution of 20%. The chromatography medium was equilibrated in 20 mM sodium phosphate, pH 6.9. Initial protein concentrations were between 0.16–4.90 g/l for IgG1 and 0.17–5.90 g/l for IgG2. Plates were incubated at temperatures ranging from 4 to 40 °C in Eppendorf ® ThermoMixers (model: R) and equilibrated overnight. For the experiments at 4 and 12 °C, the devices were placed in the cold room and set to the respective temperature. Experiments at 22, 30, and 40 °C were performed in a lab at room temperature with the device set accordingly. After incubation, the plates were centrifuged at 500 x *g* for 5 min. Aliquots (100 μl) of supernatant of each well were transferred to UV-Star® UV-Transparent Microplates (Greiner Bio-One, Kremsmünster, Austria) and absorbance at 280 nm was measured at a TECAN Infinte 200 PRO. Protein concentration was determined based on a standard. The isotherms of the adsorbed proteins were obtained from the mass balance. The data was fitted to Langmuir isotherm [14] given in Eq. (1).

$$q = q_{max} \frac{K_L * C}{1 + K_L * C} \quad (1)$$

The capacity at a given concentration C in mg/ml is denoted by q, q_{max} is the maximum binding capacity in mg/ml and K_L is the affinity constant in ml/mg. These parameters were fitted for each temperature and material.

2.3. Breakthrough curves

An ÄKTA pure (GE Healthcare, Uppsala, Sweden) was used for breakthrough studies. For each material, a column with a volume of 1.05 ± 0.05 ml was packed according to the manufacturer's protocols. As hardware, Tricorn 5/50 columns (GE Healthcare) were used. Column performance was tested by salt injections using 20% ethanol, 0.4 M NaCl as running buffer, and 20 μl of 20% ethanol, 2 M NaCl as a pulse. Columns with asymmetries between 1.0 and 1.2 were used for the experiments. To perform the experiments at different temperatures, a pre-heating loop with a volume of 5 ml was placed in front of the column. Loop and column were equilibrated in water baths of the respective temperature before loading. The temperature of the water bath was monitored by two temperature sensors that were located in the water bath at the column in- and outlet. The column was equilibrated with 10 CV of 20 mM sodium phosphate, pH 6.9. For the load, mAb was dialyzed against the equilibration buffer and diluted to a concentration of 2.0 g/l. The sample was kept at 4 °C during loading and was preheated to the necessary temperature in the pre-heating loop in the water bath. Columns were loaded to a breakthrough of 80%, then washed with 20 CV of 20 mM sodium phosphate, 2 M NaCl (pH: 6.9), eluted with 15 CV step elution of 0.1 M glycine-HCl (pH 4.0 for Byzen Pro, pH 3.0 for Mab Select SuRe and TOYOPEARL AF-rProtein A HC-650F) and re-equilibrated with 10 CV of 20 mM sodium phosphate, pH 6.9.

2.4. Elution by acid, salt and temperature

For the elution experiments, we used the same columns as for the breakthrough curves. Conditions were identical for all three

chromatography media. For acid and salt elution, the equilibration buffer was 20 mM sodium phosphate buffer at pH 6.9. For the elution by heat, we used a buffer with 20 mM sodium phosphate, 50 mM NaCl at pH 6.9.

Columns were loaded to $DBC_{10\%}$ at room temperature with mAb at 1 g/l in equilibration buffer. For acid and salt elution, the loading was performed at room temperature. For heat elution, the loading was performed in a water bath at 4 °C. Loading was followed by 10 CV of washing with the equilibration buffer, for heat elution this was also performed at 4 °C. For acid elution, we worked at two pH values. The recommended pH range for Byzen Pro is 4.0–8.0, therefore we used a 0.1 M glycine-HCl buffer at pH 4.0. For MabSelect SuRe and TOYOPEARL AF-rProtein A HC-650F, the experiments were also performed with 0.1 M glycine-HCl buffer at pH 3.0. Salt elution was achieved with a buffer containing 20 mM sodium phosphate, 1.5 M NaCl, and pH 6.9. For heat elution, the buffer was kept constant during the entire run. After washing, the pump was stopped and the loop and the column were placed in a 40 °C water bath. After 5 min of equilibration, the pump was started again. For comparability, the loop was utilized for all experiments, although not necessary for salt and acid elutions. Eluted peaks were collected in 96-deep-well plates. Samples eluted in acidic buffer were neutralized by addition of 5% (v/v) of 500 mM sodium phosphate, pH 8. Samples were dialyzed against 20 mM sodium phosphate buffer at pH 6.9 and stored at 4 °C until further analysis.

2.5. Nano-differential scanning calorimetry (DSC)

For measurements of the free antibody, samples were diluted to a concentration of 1 mg/ml and dialyzed against 20 mM sodium phosphate buffer at pH 6.9 overnight. This solution was loaded into the sample cell of a TA-Instruments (New Castle, DE, USA) Nano DSC instrument (model: 602000). The reference cell was filled with 20 mM sodium phosphate buffer at pH 6.9 and a thermoscan from 4 °C to 100 °C with a scan rate of 1 °C/min was performed. In between sample runs, the instrument was cleaned by flushing with water and buffer. Before starting measurements with a different material, additionally a cleaning solution containing 0.5 M NaCl, 0.1 M acetic acid, and 1 mg/ml pepsin followed by flushing with water was used. The cell was incubated with the pepsin-containing solution for 3 h at 37 °C and then flushed with 2 l of water.

The obtained thermogram data were analyzed using the TA Instruments NanoAnalyse software. Blank runs were performed with the buffer (20 mM sodium phosphate, pH 6.9) and the three chromatography media in buffer. The buffer blank was subtracted from all samples where the antibody was in solution (IgG2 unbound and the eluted fractions from the different elution strategies). The blank runs of the chromatography media were used for the samples where IgG2 was adsorbed on the respective medium. The signals of these blank runs contributed less than 5% to the antibody signal.

2.6. Size exclusion chromatography (SEC)

Size exclusion chromatography was used to determine antibody yield, purity, and the amount of high molecular mass impurities. We performed high-performance liquid chromatography by isocratic elution on a Dionex UltiMate 3000 HPLC system equipped with a diode array detector (Thermo Scientific, Waltham, MA, USA). The running buffer was 50 mM sodium phosphate buffer with 150 mM NaCl (Sigma-Aldrich) at a pH of 7.0, prepared with 0.22 μm filtration (GSWP04700, Merck KGaA). We applied 100 μl of a 0.2 μm vacuum-filtered sample (0.2-μm GHP AcroPrepTM 96 filter plate; Pall Life Sciences, Ann Arbor, MI, USA) to a TSKgel® G3000SWXL HPLC Column (5 μm, 7.8 × 300 mm) with a TSKgel SWXL Guard Column (7 μm, 6.0 × 40 mm; Tosoh, Tokyo, Japan). We used ChromeleonTM 7 software (Thermo Scientific) to monitor the signals at 280 nm (for aggregate content and yield).

2.7. Circular dichroism (CD)

Circular Dichroism spectra were obtained on a Chirascan CD Spectrometer from AppliedPhotophysics (Surrey, UK). The dialyzed samples were diluted to concentrations of 0.3 g/l and measured in cells with a width of 0.1 cm. Spectra were obtained in the range of 180–260 nm. The bandwidth was set to 1 nm and the signal was averaged over 10 s. The detector reached saturation at 195 nm, therefore, we show data in the range from 195 to 260 nm.

2.8. Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry experiments were carried out on a VP-ITC microcalorimeter (Malvern Instruments, UK). The reference cell was filled with 20 mM sodium phosphate buffer, pH 6.9. As sample we used the IgG2 molecule. The antibody was dialysed against 20 mM sodium phosphate buffer, pH 6.9 overnight. All buffers and samples were degassed before usage. After each run, the sample cell and the syringe were cleaned with a solution of 1% Decon 90 and flushed with water. The cell temperature was set to 25.0 °C. Each experiment consisted of 20 injections with 10 μl injected over 20 s in intervals of 13 min. The stirring speed was set to 915 rpm and a reference number of 25 μcal/sec was used. For TOYOPEARL AF-rProtein A HC-650F and Byzen Pro, we used a slurry of 25% in the sample cell and the protein concentration was 5.8 g/l. For MabSelect SuRe we could not obtain a signal under these conditions, therefore we conducted the experiment with a slurry of 50% and a protein concentration of 11.6 g/l. To account for the heat of dilution, we performed blank experiments under the same conditions with protein being titrated into buffer and buffer being added to the cell with chromatographic media. Peak integration was done in the software Origin 7.0 (Originlab, USA).

For thermodynamic analysis, we followed the approach used by Ueberbacher et al. [15]. The change in Gibbs energy associated with the adsorption of a protein to a stationary phase is calculated according to Eq. (2).

$$\Delta G_{ads} = -R * T * \ln(K) \tag{2}$$

R denotes the universal gas constant, T is the temperature and K is the equilibrium constant. K is calculated by extrapolation of q/c to an infinite dilution of the protein according to Eq. (3).

$$K = \lim_{C \to 0} \frac{q}{C} \tag{3}$$

The capacity q is defined by the parameters determined in the EBC studies, according to the Langmuir adsorption isotherm described by Eq. (1) in chapter 2.2.

Isothermal titration calorimetry enables to measure the heat Q_{ads} that is released upon protein adsorption on the stationary phase. The cumulative amount of released heat is given by integration of the power P over time as shown in Eq. (4).

$$Q_{ads} = \int_{t_2}^{t_1} P * \Delta t \tag{4}$$

The heat Q_{ads} relates to ΔH_{ads}, the enthalpy change associated with the adsorption of the protein on the stationary phase, as follows in Eq. (5).

$$\Delta H_{ads} = \frac{Q_{ads}}{V * q}$$

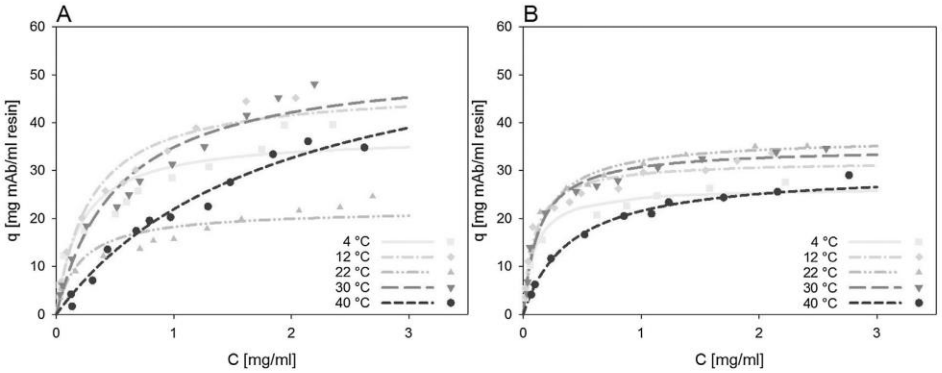

Fig. 1. Adsorption Isotherms of Antibody on Byzen Pro at Different Temperatures, Subclass IgG1 (A) and Subclass IgG2 (B).

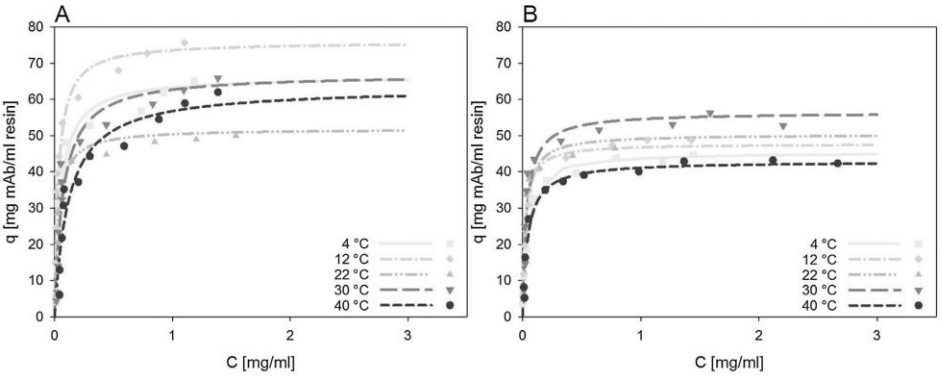

Fig. 2. Adsorption Isotherms of Antibody on MabSelect SuRe at Different Temperatures, Subclass IgG1 (A) and Subclass IgG2 (B).

V is the volume of the stationary phase and q is the capacity from the Langmuir isotherm.

Q_{ads} also contains the contributions from the heat of dilution of the protein ($\Delta H_{dil}{}^{prot}$), the stationary phase ($\Delta H_{dil}{}^{sp}$) and the adsorbed ions ($\Delta H_{ads}{}^{ion}$). This is taken into account by subtracting the respective blank signals.

The change in entropy caused by the adsorption of the protein (ΔS_{ads}) is calculated from ΔH_{ads} and ΔG_{ads} (Eq. (6)).

$$\Delta G_{ads} = \Delta H_{ads} - T * \Delta S_{ads}$$

3. Results and discussion

3.1. Equilibrium binding capacity at different temperatures

We determined the equilibrium binding capacity at five temperatures, from 4° to 40 °C, with finite bath adsorption. Antibodies of the IgG1 and IgG2 subtype were adsorbed onto three protein A chromatography media, Byzen Pro, MabSelect SuRe, and TOYOPEARL AF-rProtein A HC-650F. From the finite bath adsorption measurements, we constructed isotherms and the data was fitted with the Langmuir adsorption isotherm (Figs. 1–3). Maximum adsorption (q_{max}) and affinity constants (K_L) were determined by the Langmuir fit [14]. All chromatography media showed temperature-dependent adsorption behavior, but for Byzen Pro we observed the greatest dependence in adsorption capacities based on temperature (Table 1). For all three chromatography media, we observed a higher temperature sensitivity for the IgG1 subtype than for the IgG2 subtype. Standard deviations are not listed in the following paragraphs but can be found in the corresponding table (Table 1).

For Byzen Pro, the lowest detected EBC for IgG1 was found at 22 °C with 22.0 g/l, the maximum was reached at 40 °C with a capacity of 63.5 g/l (Table 1, Fig. 1). For IgG2, the EBC ranged from 26.5 g/l (4 °C) to 36.9 g/l (22 °C). The shape of the isotherm curves at 40 °C deviated from the shape observed at other temperatures, with the curves being less steep, indicating a lower affinity of the material although the equilibrium binding capacity are not at a minimum at this temperature. Typically, isotherms of protein A material will take on an almost rectangular form. This change in the shape of the isotherms was observed for both subclasses, IgG1 and IgG2 and is also reflected by minima in the affinity constants (Table 2).

For MabSelect SuRe, we also observed a temperature dependence of the adsorption and that the adsorption of antibody with subclass IgG1 was more thermosensitive than the adsorption of antibody with subclass IgG2. For the antibody with subclass IgG1, the EBC ranged from a minimum of 51.9 g/l at 22 °C to 75.9 g/l at 12 °C (Table 1, Fig. 2). For IgG2, the EBC ranged from 42.9 g/l (40 °C) to 56.4 g/l (30 °C). The binding capacities were significantly higher than those we observed for Byzen Pro. We did not observe a shal-

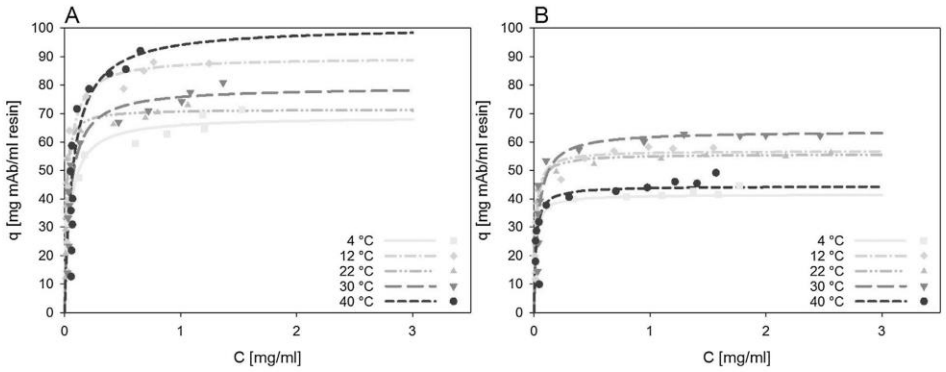

Fig. 3. Adsorption Isotherms of Antibody on TOYOPEARL AF-rProtein A HC-650F at Different Temperatures, Subclass IgG1 (A) and Subclass IgG2 (B).

Table 1
Equilibrium Binding Capacities (q_{max}) with Standard Deviations of the Three Protein A Chromatography Media.

	Byzen Pro (mg/ml)		MabSelect SuRe (mg/ml)		TOYOPEARL AF-rProtein A HC-650F (mg/ml)	
	IgG1	IgG2	IgG1	IgG2	IgG1	IgG2
4 °C	37.3 ± 3.8	26.5 ± 0.9	66.5 ± 4.1	45.5 ± 1.1	68.9 ± 2.1	41.6 ± 3.0
12 °C	47.5 ± 3.3	32.0 ± 1.2	75.9 ± 4.9	47.8 ± 1.7	89.7 ± 6.0	57.0 ± 2.1
22 °C	22.0 ± 2.2	36.9 ± 1.2	51.9 ± 2.8	50.4 ± 3.0	71.5 ± 3.6	55.8 ± 1.9
30 °C	53.3 ± 5.3	34.8 ± 1.1	67.1 ± 4.1	56.4 ± 2.8	79.3 ± 3.9	63.9 ± 3.3
40 °C	63.5 ± 8.5	30.1 ± 1.1	63.3 ± 4.3	42.9 ± 1.3	100.6 ± 2.5	44.6 ± 3.2

Table 2
Affinity constants (K_L) based on Langmuir Fit for the Three Protein A Chromatography Media.

	Byzen Pro (ml/mg)		MabSelect SuRe (ml/mg)		TOYOPEARL AF-rProtein A HC-650F (ml/mg)	
	IgG1	IgG2	IgG1	IgG2	IgG1	IgG2
4 °C	4.9	10.6	20.0	23.3	21.9	63.0
12 °C	3.5	10.8	31.4	45.9	31.3	67.3
22 °C	4.9	6.7	32.6	37.5	37.1	68.3
30 °C	4.9	7.6	14.3	29.1	21.5	68.8
40 °C	0.5	2.5	8.7	23.0	14.3	60.0

lowed adsorption isotherm at 40 °C as was present in the isotherms of Byzen Pro, which means that the affinity does not change for MabSelect SuRe even at drastically different temperatures, but, rather, the maximum binding capacity changes.

TOYOPEARL AF-rProtein A HC-650F showed the highest binding capacities for both antibodies. Again, the temperature sensitivity was higher for the antibody with subclass IgG1 than for IgG2. For antibody subclass IgG1, the lowest EBC of 68.9 g/l was observed at 4 °C and the highest at 40 °C with 100.6 g/l. The IgG2 antibody showed the lowest capacity at 4 °C (41.6 g/l) and the highest at 30 °C (63.9 g/l) (Table 1, Fig. 3).

The equilibrium binding capacity was dependent on IgG subclass, which has been also reported by others [11,16]. All tested chromatography media reacted sensitively to temperature changes, but to different extents, with the highest temperature dependence observed for the thermosensitive mutant Byzen Pro, which was expected. There are no clear trends regarding the maximum or minimum binding capacities. For MabSelect SuRe and Byzen Pro, the lowest capacities of IgG1 were observed at 22 °C, but for TOYOPEARL AF-rProtein A HC-650F, the lowest EBC was found at 4 °C. For IgG2, Byzen Pro and TOYOPEARL AF-rProtein A HC-650F showed the lowest EBCs at 4 °C, whereas MabSelect SuRe had its minimum at 22 °C. Although our data do not permit us to extract a general rule, we certainly showed that operating at the wrong temperature, even for chromatography media not marketed as thermosensitive, can mean capacity losses of up to 30%.

Experimental studies of protein adsorption on surfaces show that conformational changes take place upon surface adsorption [17]. Due to structural differences between the subclasses, IgG1 is considered more flexible than IgG2. We assume that this flexibility accounts for the greater deviations in equilibrium binding capacities for IgG1 upon temperature changes.

For Byzen Pro, we observed that the isotherms at 40 °C do not show the typical rectangular shape that is normally observed in protein A isotherms. This variation is a clear indication that the modifications introduced into the ligand have decreased the affinity of the material at elevated temperature. At 40 °C which is the recommended elution temperature, the ligand is however not fully denaturated and still able to bind antibodies.

3.2. Dynamic binding capacities

The dynamic breakthrough analysis of any adsorption system is a combination of equilibrium binding capacity, adsorption kinetics, and system dispersion [18]. The performance was evaluated via breakthrough curve analysis as a function of the residence

Table 3
Dynamic Binding Capacities at 10% Breakthrough ($DBC_{10\%}$) of the Three Protein A Chromatography Media (Data for IgG2).

	Byzen Pro (mg/ml)		MabSelect SuRe (mg/ml)		TOYOPEARL AF-rProtein A HC-650F (mg/ml)	
	125 cm/h	250 cm/h	125 cm/h	250 cm/h	125 cm/h	250 cm/h
4 °C	22.6	14.9	18.6	11.0	33.2	27.8
22 °C	20.9	16.5	25.6	17.9	40.8	33.2
40 °C	18.3	15.8	30.8	24.7	40.2	34.2

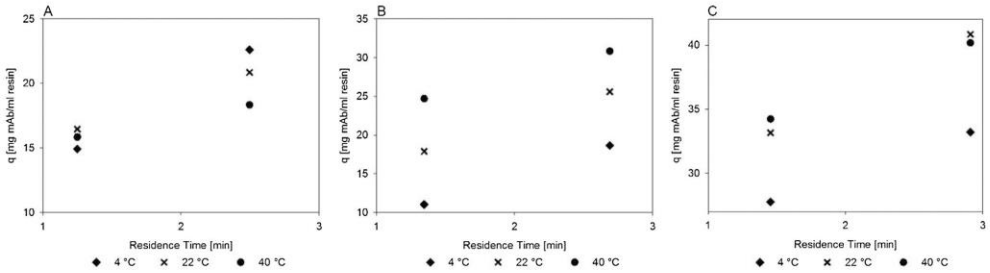

Fig. 4. Dynamic Binding Capacities at 10% Breakthrough ($DBC_{10\%}$) of Byzen Pro (A), MabSelect Sure (B) and TOYOPEARL AF-rProtein A HC-650F (C).

time. The dynamic binding capacity (DBC) decreases as the flow rate is increased because there is less time for diffusion in a pore diffusion limited process. To elucidate temperature sensitivity in column experiments, we packed 1 ml columns and placed a loop with a volume of 5 ml in front of the column. Loop and column were equilibrated and loaded in water baths set to the temperature we wanted to test. The temperature of the water bath was monitored by two temperature sensors that were located in the water bath at the column in- and outlet. The dynamic binding capacity at 10% breakthrough ($DBC_{10\%}$) was determined at two flow rates, 125 cm/h and 250 cm/h, which correspond to residence times of ~1.4 and ~2.7 min, respectively. (The maximum recommended flow rate for Byzen Pro is 250 cm/h.) Based on the manufacturer's protocol, we expected to see high binding capacity at low temperature and low binding capacity at high temperatures for Byzen Pro. This trend could, however, only be verified at the slow flow rate while at high flowrates, the capacity was similar for all three temperatures: 14.9 g/l at 4 °C, 16.5 g/l at 22 °C and 15.9 g/l at 40 °C (Table 3, Fig. 4A). The invariability of binding capacities at faster flow rates can be explained by mass transfer limitations which govern the process under these conditions. At low flow rates, we would expect that the binding capacity increases with temperature due to the increased diffusivity which is described by the Stokes-Einstein equation. However, in the case of Byzen Pro, the diffusivity appears to have a subordinate role as the binding capacities decrease with increasing temperatures. This confirms the observations made in the EBC studies where the isotherm curves of Byzen Pro at 40 °C had a non-rectangular shape, showing that the affinity of the material is reduced at this temperature.

MabSelect SuRe showed the same trend at both flow rates, the $DBC_{10\%}$ increased with temperature (Table 3, Fig. 4B) which shows that the process is not entirely governed by mass transfer limitations under these conditions. Rather, this is a diffusion-limited process, where the dynamic binding capacity is determined by the equilibrium binding capacity and the diffusivity.

For TOYOPEARL AF-rProtein A HC-650F, the lowest $DBC_{10\%}$ was observed at 4 °C whereas the $DBC_{10\%}$ values at 22 °C and 40 °C were similar (Table 3, Fig. 4C). The finite batch experiments showed that the EBC at 40 °C is lower than that at 22 °C. In the $DBC_{10\%}$, the decreased capacity is most likely compensated by the enhanced diffusivity, resulting in similar capacities for both temperatures.

For MabSelect SuRe and TOYOPEARL AF-rProtein A HC-650F, the $DBC_{10\%}$ is mostly governed by the temperature dependence of pore diffusion. For Byzen Pro, there is only a temperature trend recognizable at the higher residence time, where the diffusion limitation is less of an issue while the mass transfer resistance dominates the process at short residence times. In absolute numbers, TOYOPEARL AF-rProtein A HC-650F has the highest capacity, followed by MabSelect SuRe and Byzen Pro.

3.3. Elution profiles (Elution by salt pulse, acid and heat)

We tested three different elution strategies on the chromatography media. The first was a conventional acidic elution. The recommended pH range for the use of Byzen Pro is 4.0–8.0, therefore the acid elution was performed at 4.0. For MabSelect SuRe and TOYOPEARL AF-rProtein A HC-650F, we performed acid elution at pH 3.0 and 4.0. In EBC and DBC studies, we observed that the affinity of the antibody to Byzen Pro was weaker compared to the conventional chromatography media. Therefore, we also tested elution by a step elution with a high salt buffer. The third elution type was based on heat and followed the protocol provided by Nomadic Bioscience Co., Ltd, the manufacturer of the thermosensitive Byzen Pro material. Originally, we wanted to use the same buffer that had already been used for EBC and DBC studies (20 mM sodium phosphate, pH 6.9) for all three elution protocols but under these conditions, the heat elution of the Byzen Pro material was not possible. Therefore, we added 50 mM NaCl to the equilibration buffer for the heat elution experiments and this allowed the elution of antibodies from Byzen Pro.

At pH 4.0, elution was possible from all three chromatography media, with yields of 99% for Byzen Pro, 77% for MabSelect SuRe, and 83% for TOYOPEARL AF-rProtein A HC-650F (Table 4). MabSelect SuRe and TOYOPEARL AF-rProtein A HC-650F can however be used at the lower pH of 3.0 which leads to optimized elution behavior and yields >99% (Table 4) [19,20]. Byzen Pro can however not be operated in this pH range, therefore elution at pH 3.0 could not be

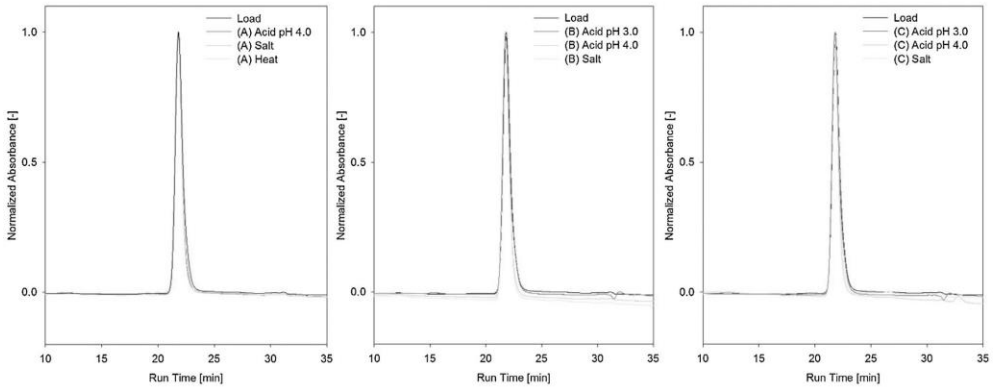

Fig. 5. SEC Data of IgG2 before (Load) and after Adsorption on Protein A Chromatography Material. (A) Byzen Pro, (B) Mab Select SuRe, (C) TOYOPEARL AF-rProtein A HC-650F.

Table 4
Yield (%) for Different Elution Types for all Chromatography Media. Acid Elution with 0.1 M Glycine-HCl at pH 3.0 and 4.0, Salt Elution with 20 mM Sodium Phosphate, 1.5 M NaCl and pH 6.9. For Heat Elution the Buffer (20 mM Sodium Phosphate, 50 mM NaCl, pH 6.9) is Kept Constant and Column is Heated from 4 °C to 40 °C (Data for IgG2).

	Byzen Pro	MabSelect SuRe	TOYOPEARL AF-rProtein A HC-650F
Acid [pH 4.0]	99%	77%	83%
Acid [pH 3.0]	–	>99%	>99%
Salt	98%	55%	11%
Heat	96%	No Elution	No Elution

tested with this material. Especially for acid-sensitive antibodies, the weaker binding of the antibody to the medium is advantageous.

Byzen Pro is also salt sensitive. Elution of the antibody with a salt pulse of 1 M NaCl was possible with a yield of 98%. Consequently, a high salt wash as is frequently used in protein A chromatography was not possible with this chromatography medium. On the other hand, elution can be achieved by salt, if a low pH elution is not practicable. It is also important to mention that elution with salt can be more easily scaled up compared to temperature elution. The milder elution behavior of Byzen Pro occurs at the expense of binding capacity; about 80% of Mab Select SuRe and 50% of TOYOPEARL AF-rProtein A HC-650F under optimal temperature conditions ($DBC_{10\%}$ at 22 °C, 125 cm/h).

For temperature elution, the column was equilibrated in a water bath at 4 °C, then the pump was stopped for 5 min to ensure that the column had achieved the desired temperature before being loaded to $DBC_{10\%}$. For the entire loading and washing, the column was kept at 4 °C. Then the column was transferred to a 40 °C water bath and the pump was stopped for another 5 min. After equilibration at the elevated temperature, the pump was started and elution started. When using this protocol, the buffer stays constant during the entire run. Heat elution was successful only for the Byzen Pro material with a yield of 96%. There was no elution detectable for TOYOPEARL AF-rProtein A HC-650F and Mab Select SuRe (Table 4). For Byzen Pro, the elution worked only when we used a buffer containing small quantities of sodium chloride (20 mM sodium phosphate, 50 mM NaCl, pH 6.9). When using a salt-free running buffer (20 mM sodium phosphate at pH 6.9), the elution from Byzen Pro was not possible. Heat elution of Mab Select SuRe and TOYOPEARL AF-rProtein A HC-650F was tested with both buffers but neither of them allowed any elution.

Table 5
Transition Temperature of Antibodies T_m Observed in DSC.

Sample	T_m1	T_m2
Antibody subclass IgG2 on TOYOPEARL AF-rProtein A HC-650F	78.5	–
Antibody subclass IgG2 on Byzen Pro	70.3	77.7
Antibody subclass IgG2 on MabSelect SuRe	78.4	–
Antibody subclass IgG2 in Solution	73.6	78.4
Load/Pooled IgG2 Fractions after Elution	73.6	78.4

When looking at the results of the EBC and DBC experiments, it is surprising that heat elution does not take place since Mab Select SuRe and TOYOPEARL AF-rProtein A HC-650F both have higher binding capacities at 40 °C than at 4 °C. We suspect that the ligands undergo different temperature dependent changes depending on whether there is already protein adsorbed or not. In other words, antibodies that are already adsorbed to Mab Select SuRe and TOYOPEARL AF-rProtein A HC-650F will remain on the material under unfavorable conditions even if the initial adsorption process would not take place to the same extend if these conditions were already present before adsorption. Therefore, it is not possible to predict process performance from the EBC data solely.

SEC analysis showed that the elution type had no influence on the formation of aggregates for this molecule (Fig. 5). There are, however, antibodies which show salt and acid-sensitivity in regard to aggregation propensity [21].

3.4. Probing of structural changes by differential scanning calorimetry and circular dichroism

To gain insight into structural changes of the antibody upon binding to the protein A ligand, the melting curves of the free antibody and antibody bound on the chromatography medium were determined by differential scanning calorimetry. Two peaks were observed in the thermogram of antibodies. The thermal unfolding of an IgG consists of a two phase transition (Fig. 6). The first peak is associated with the unfolding of the CH2 and the Fab domain while the second peak corresponds to the transition temperature (Tm) of the CH3 domain [22]. The antibody of the subclass IgG2 molecule used for our study has its maxima at 73.6 and 78.4 °C (Table 5).

When the antibody was bound to Byzen Pro, it still showed a two phase unfolding process with transition temperatures at 70.4 and 77.7 °C (Fig. 6). We conclude that the antibody is slightly destabilized when it is bound to the protein A ligand. When the antibody is adsorbed on MabSelect SuRe or TOYOPEARL AF-rProtein

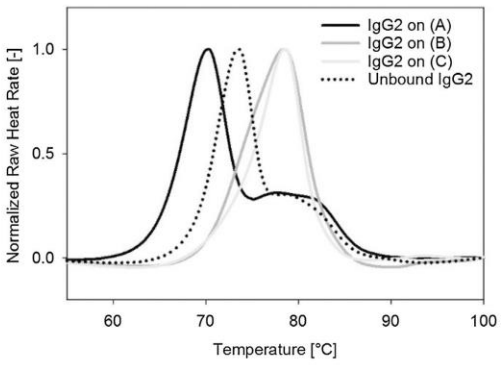

Fig. 6. Nano DSC Thermograms of IgG2 in Solution (Dotted Line) and IgG2 after Immobilization on (A) Byzen Pro, (B) Mab Select SuRe, (C) TOYOPEARL AF-rProtein A HC-650F.

A HC-650F, only a single-phase transition can be observed. The unfolding occurs at a Tm of 78.4 °C for MabSelect SuRe and 78.5 °C for TOYOPEARL AF-rProtein A HC-650F. The decrease in unfolding temperature observed on Byzen Pro demonstrates that the antibody undergoes structural changes while binding leads to lower melting temperatures for both the CH2 and CH3 domains, whereas the adsorption onto MabSelect SuRe and TOYOPEARL AF-rProtein A HC-650F stabilizes the antibody causing a shift to a higher melting temperature. The change from a biphasic unfolding reaction to a single phase transition indicates that the individual domains are stabilized by varying extents which results in overlapping peaks.

To determine if these structural changes are permanent, we also performed DSC and CD measurements of the eluted antibody from the column experiments with different elution protocols. After elution, the samples were dialyzed against equilibration buffer and DSC experiments were performed. In regard of the main unfolding event, the eluted antibodies were identical to the thermograms of antibodies before binding to protein A (Fig. 7A–C). There are slight variations in the unfolding temperatures of the more stable species in the range of 78–88 °C. However, the CD measurements indicate that the structures of the antibody were identical before (Load) and after protein A adsorption (Fig. 8A–C). Based on that, we conclude that adsorption on protein A materials causes only transient, structural changes of the IgG molecules which are fully reversible. We can thereby confirm the findings made by Gagnon et al. [23,24] who showed that the hydrodynamic radii of the antibodies is altered when they have undergone an acid elution from protein A media. Additionally, our results show that these structural changes can differ depending on the choice of protein A material.

The DSC measurements showed that the antibody undergoes structural changes during the adsorption. These changes are dependent on the materal and also the use of Byzen Pro caused a structural change in the antibody that was associated with a decreased stability and was substantially different from conventional chromatography media.

3.5. Isothermal titration calorimetry

Isothermal titration calorimetry experiments were performed to determine the raw heat of adsorption of the IgG2 subclass

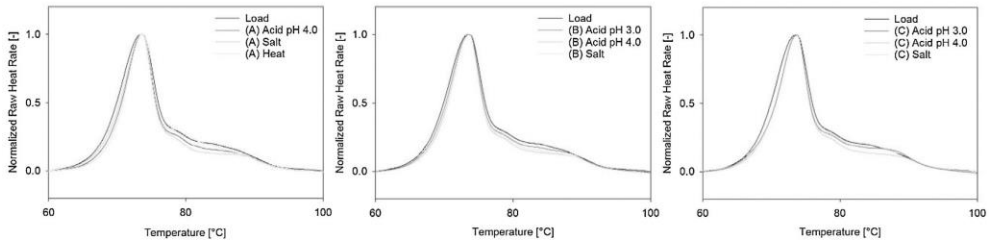

Fig. 7. Nano DSC Thermograms of IgG2 before and after Protein A Chromatography. (A) Byzen Pro, (B) Mab Select SuRe, (C) TOYOPEARL AF-rProtein A HC-650F.

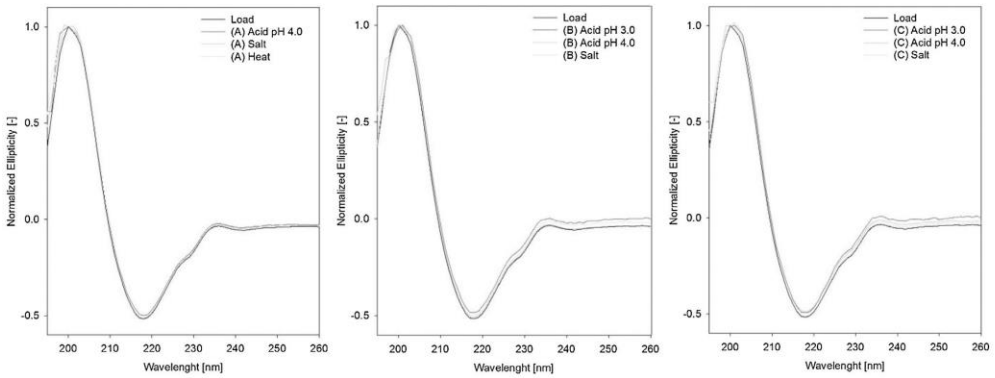

Fig. 8. CD Spectra of IgG2 before and after Adsorption on Protein A Chromatography Material. (A) Byzen Pro, (B) Mab Select SuRe, (C) TOYOPEARL AF-rProtein A HC-650F.

Table 6
Thermodynamic Parameters of the Three Protein A Chromatography Media for IgG2.

	Byzen Pro	MabSelect SuRe	TOYOPEARL AF-rProtein A HC-650F
ΔG_{ads} [kJ/mol]	−13.7	−18.7	−20.4
$-T*\Delta S_{ads}$ [kJ/mol]	151.8	3.5	−14.5
ΔH_{ads} [kJ/mol]	−165.5	−22.2	−5.9

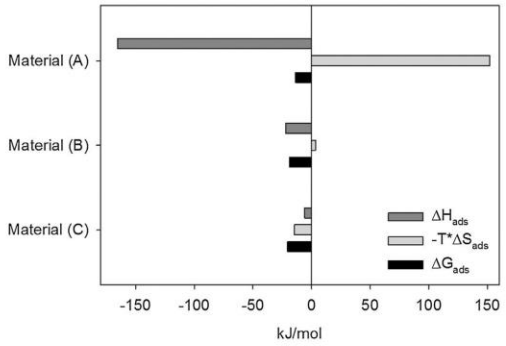

Fig. 9. Thermodynamic Parameters of Byzen Pro (A), Mab Select SuRe (B), TOYOPEARL AF-rProtein A HC-650F.

(ΔH_{ads}) for the three materials at 25.0 °C. The experimental data for Byzen Pro (Fig. 9, Table 6) show that the adsorption of the antibody is driven by enthalpy suggesting that hydrogen and other non-covalent bonds are formed. The adsorption also causes the system to undergo a loss of conformational freedom which is shown by the unfavorable entropy contribution. The change in Gibbs free energy accounts for −13.7 kJ/mol.

The adsorption on Mab Select SuRe is also driven by enthalpy but in contrast to Byzen Pro, the entropy has only a weakly opposed contribution (Fig. 9, Table 6). The change in Gibbs free energy is −18.7 kJ/mol. The decrease in entropy could be an indicator for a partial unfolding of the protein which creates access to side chains that are otherwise buried in the interior of the protein. This increases the number of water accessible sites on the surface. From this viewpoint, it would also be interesting to see if the alterations of the hydrodynamic radii of the eluted antibodies can be affected by the choice of protein A material [23,24]. Byzen Pro is an interesting candidate for these types of experiments as it allows elution by salt and the low pH elution can be avoided.

TOYOPEARL AF-rProtein A HC-650F shows the strongest exothermic adsorption behavior with a change in Gibbs free energy of −20.4 kJ/mol (Fig. 9, Table 6). Interestingly, the adsorption is not only driven by an increase in enthalpy but also by an increase of entropy. This indicates that the molecules have a considerable mobility after adsorption and that translation on the surface takes place to a greater account. Similarly, it would be possible that the hydrophobic effect causes the antibody molecules to adopt a more tightly packed conformation which decreases the surface area and consequently also the water accessible sites. On the other hand TOYOPEARL AF-rProtein A HC-650F consists 6 domains. This compared to four for Mab Select SuRe and less then six for Byzen Pro. This might also contribute to this exothermic effect.

The changes in ΔG_{ads} are in good agreement with the EBC and DBC studies as, where TOYOPEARL AF-rProtein A HC-650F yields the highest capacities, followed by MabSelect SuRe and Byzen Pro.

4. Conclusion

The thermosensitive chromatography medium Byzen Pro displayed shallow isotherms at higher temperature, whereas the other protein A chromatography media had typical rectangular adsorption isotherms. Our study shows that antibodies undergo transient structural changes during protein A chromatography and that these changes differ from medium to medium. The conformation of the antibody is stabilized when it is bound to MabSelect SuRe and TOYOPEARL AF-rProtein A HC-650F while it is slightly destabilized upon binding to the thermosensitive ligand. This cannot be explained by the different number of domains only by the domain structure. The adsorption of the IgG2 subclass is most exothermic for TOYOPEARL AF-rProtein A HC-650F, followed by Mab Select SuRe and Byzen Pro. Due to the increased thermosensitivity of Byzen Pro, elution can be carried out by increasing the temperature in the presence of sodium chloride. The temperature elution was enabled at the expense of lower dynamic binding capacity and yield. Temperature elution is difficult to scale up and working at elevated temperature carries the risk of increased protease activity and microbial growth. Due to weakened interaction between antibody and ligand, elution can also be carried out at a pH of 4.0 or with a pulse of concentrated sodium chloride buffer. This may be useful for pH sensitive antibodies, but high salt washes cannot be conducted. The thermosensitivity of conventional protein A chromatography media is not sufficient to elute the proteins by raising the temperature. The thermosensitive protein A is a contribution to antibody manufacturing especially for pH sensitive antibodies. While a temperature elution is possible on the lab scale, the better option for production is a high salt elution as it is easier to scale up, and does not need any additional equipment for heating or cooling.

Acknowledgments

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11.2 Publication 2

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RESEARCH ARTICLE

BIOTECHNOLOGY PROGRESS

Mid-manufacturing storage: Antibody stability after chromatography and precipitation based capture steps

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Abstract

Antibodies of the IgG2 subclass were captured from the clarified cell culture fluid either by protein A chromatography or by polyethylene glycol precipitation. The captured intermediates were stored as neutralized eluates (protein A chromatography) or in solid form as polyethylene glycol precipitates over a period of 13 months at three temperatures, −20°C, 5°C, and room temperature to compare the capture technologies in regard of the resulting product storability. Monomer content, high molecular mass impurities product loss and changes in the composition of the charge variants were determined at six time points during the storage. At the beginning and end of the study, samples were additionally tested by differential scanning calorimetry, differential scanning fluorimetry, and circular dichroism to determine structural alterations occurring during storage. Protein A purified material was highly stable at all tested temperatures in regard of monomer content and product losses. A transient, acidic isoform was formed during the chromatography step which re-converted to the main charged variant upon storage within a matter of days. Precipitated antibodies could be stored at −20 or 5°C for 3 months without product losses but afterwards recovery yields dropped to 65%. At room temperature, the precipitated antibody was not stable and degraded within 3 months.

KEYWORDS
chromatography, immunoglobulin, precipitation, storability, storage

1 | INTRODUCTION

Stability and shelf life of formulated antibodies have been studied extensively but there is limited data available for process intermediates. Research is classically based on ideal storage conditions for final drug formulations[1-3] but nowadays biopharmaceutical operations are often conducted on a global scale and the materials might be shipped mid-manufacturing. In 2016, the European Medicine Agency (EMA) published a guideline on process validation for the manufacturing of biotechnology-derived products[4] where they recommend to evaluate the impact of hold steps, mid-manufacturing storage, and transportation on process *intermediates*. To determine the influence of unexpected process interruptions on the intermediate, they suggest to perform studies under worst-case and non-standard conditions. Furthermore, intermediate characterization is crucial for process development, design of hold steps, and development of in-process control methods. Therefore, protein alterations and stability at all stages during processing are important.

Stability studies are time and cost consuming, since the samples must be stored under the respective temperature and conditions for the projected time period to get good quality data. Accelerated shelf-life studies have been suggested in which the sample is stored at higher temperature and then possible storage time at lower temperature is extrapolated using the Arrhenius equation.[5] For elaboration of

shelf-life some quality parameters reflecting the degradation of the protein must be determined.

All proteins, including recombinant antibodies, are susceptible to alterations during production, processing, and storage. Common modifications of antibodies are sialylation, glycosylation, deamidation, oxidation, N-terminal pyroglutamine cyclization, C-terminal lysine cleavage, aspartate isomerization, and disulfide bond shuffling.[6] These modifications give rise to a high degree of microheterogeneity and can affect antibody functionality and stability.[7] It is not possible to capture all these variants therefore surrogate parameters are used during product and process development. Importance has however to be put on the identification of critical protein modifications in order to limit degradation pathways and define optimum storage conditions in regard of temperature, humidity, and light irradiation.

Rapid methods to investigate antibody destruction during storage are usually HPLC based with the most widely used techniques being size exclusion chromatography, cation-exchange chromatography or content analysis by affinity chromatography. Such methods capture the aggregate formation, degradation/clipping of the antibody to a smaller molecular variant and formation of charge variants.

Nowadays, the majority of antibody products is produced in mammalian host cell lines and after primary recovery, the purification is based on protein A chromatography which typically results in yields around 95%[8] and purities >98%.[9] Due to the high affinity of protein A towards antibodies and the high capacities achieved by modern protein A materials, the protein solution is rather concentrated and pure at this stage. The high antibody concentration in combination with the harsh, acidic elution conditions can promote the formation of aggregates and therefore potentially to a loss in product.[10] Also, protein A chromatography material is rather expensive, adding significantly to the overall process costs, especially in early development phases when the material is not utilized to its maximum lifetime.[11] Therefore, several groups – including our own – have presented alternative approaches for antibody capture steps in recent years, for example, by mixed-mode chromatography,[12] aqueous two-phase extraction[13] or precipitation.[14-16] Precipitation offers a viable, non-chromatographic alternative which can be easily customized for continuous manufacturing. It scales only with volume and is able to deal with varying titers in the upstream feed. Burgstaller et al. recently demonstrated that polyethylene glycol (PEG) precipitation in combination with tangential microfiltration results in similar purity and process yields as protein A chromatography.[17] In a PEG precipitation capture step, the solubility of the antibody in the clarified culture supernatant is lowered by addition of PEG leading to precipitation of the product. The solid precipitate is harvested by microfiltration and resolubilized in a dissolution buffer. The antibody is thereby concentrated and host cell proteins (HCPs) are largely depleted. To meet the requirements in regard of purity for drug products, the protein has to be further processed by at least one more polishing step before it will be transferred into a formulation buffer by an ultra-/diafiltration step.

In this work, we compared two antibody capture technologies in regard of effects on the intermediate storability. Antibodies were captured either by protein A chromatography or by precipitation from the clarified cell culture fluid. The protein A eluate was neutralized and stored in liquid phase. The precipitated material was stored in its solid form after decantation of the supernatant. Samples were then stored under identical conditions at three temperatures (−20°C, 5°C, and room temperature) for 13 months. Storage at −20 and 5°C are typical storage conditions for biopharmaceutical intermediates awaiting further processing. Storage at room temperature (ambient, non-monitored) was included as a worst-case scenario. The EMA guideline for process validation[4] recommends tests at higher temperatures and for elongated times to substantiate the recommendations for ideal intermediate handling during manufacturing. In both cases, the material was stored mid-manufacturing without performing any additional processing steps before storage. It should be noted that this results in different high molecular weight impurities (HMWI) and low molecular weight impurities (LMWI) concentrations for the two pools since protein A chromatography is more efficient in HCP clearance. The precipitated material is stored under precipitation conditions, that is, 13.2% (w/w) of polyethylene glycol are present. Storage of the material in its precipitated form poses the advantages that less volume is being stored which leads to a decrease in storage costs.

The reconstituted material was tested by HPLC based methods (size exclusion and cation exchange pH gradient) at six time points during storage (starting material and after 1, 3, 6, 10, and 13 months at the respective temperature). Starting and end material were additionally probed by differential scanning calorimetry (DSC), differential scanning fluorimetry (DSF), and circular dichroism (CD) to identify structural changes during storage.

2 | MATERIAL AND METHODS

All chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise.

2.1 | Antibodies

Experiments were carried out with recombinant, human antibodies of the subclass IgG2. We used clarified cell culture fluid (CCCF) of a CHO run with an antibody monomer concentration of 3.3 g/L. The antibodies were then either captured by protein A chromatography (chapter 2.1.1) or precipitation (chapter 2.1.2) using CCCF from the same CHO run.

2.1.1 | Protein A chromatography

Protein A chromatography was carried out on Mab Select SuRe resin (GE Healthcare, Sweden). Column was equilibrated with 50 mM Na-phosphate at pH 7.0 and washing was performed with 100 mM Na-citrate at pH 5.5. Antibodies were eluted by a single step gradient using 100 mM Na-acetate at pH 3.6. They were then incubated at pH 3.6 for 1 hr before the pH was readjusted to 6.0.

2.1.2 | Precipitation

Polyethylene glycol with an average molecular weight of 6,000 Da was used for antibody precipitation as described in previous publications.[14] In short, a stock solution of 40% PEG6000 (w/w) was prepared in a 100 mM Tris buffer (pH 7.5). The CCCF was mixed in a polypropylene tube with the stock solution to a final PEG concentration of 13.2% (w/w). Then, the solution was mixed on a mechanical end-over-end shaker (Cole-Parmer, IL) for 10 min. The precipitate was centrifuged for 5 min at 2,000 rcf and the liquid phase decanted. The precipitate was washed with a solution of 13.2% PEG in 100 mM Tris (pH 7.5) (gravimetrically determined to be the same amount as previously removed) and again incubated on the shaker for 10 min. After another centrifugation (5 min at 2,000 rcf), the CCCF was decanted and the precipitate was put into storage. Before analysis, the precipitated antibody was reconstituted in the same volume previously removed by decantation. As reconstitution buffer, 100 mM Tris–HCl buffer (pH 7.5) was used. The protocol and screening for precipitation conditions was already presented in more detail by Burgstaller et al.[17]

2.2 | Storage conditions

In both cases, protein A purification and precipitation, the material was stored without performing any additional process steps. The protein A purified material was stored in liquid form after neutralization. The precipitated material was stored in the precipitated form after removal of the supernatant. Conical, lightproof polypropylene tubes were used for storage. Storage at −20°C was done in a standard lab freezer from Electrolux (Stockholm, Sweden) and storage at 5 ± 3°C was done in a refrigerator by KBS Kältetechnik (Wiesbaden, Germany). Room temperature storage was performed in one of our labs with room temperature ranging from 20.0 to 25.0°C. This scenario was included to mimic worst-case conditions like an unexpected process interruption where fast degradation kinetics can be expected.

2.3 | Nano differential scanning calorimetry (nanoDSC)

Samples were dialyzed in Slide-A-Lyzer cassettes (Thermo Fisher Scientific, Waltham, USA) with a molecular weight cut-off (MWCO) of 10.000 Da to a 20 mM sodium phosphate buffer, pH 6.9 and then diluted to a concentration of 3 mg/ml. The solution was loaded into the sample cell of a TA-Instruments (New Castle, DE) Nano DSC instrument (model: 602000). The reference cell was filled with a 20 mM sodium phosphate buffer pH 6.9 and a thermoscan from 20 to 100°C with a scan rate of 1°C/min was performed. In between sample runs, the instrument was cleaned by flushing the cells with water and buffer. At the end of each set of experiments, the instrument was cleaned with a solution containing 0.5 M NaCl, 0.1 M acetic acid, and 1 mg/mL pepsin which was incubated for 3 hr at 37°C and then flushed with 2 L of water. The obtained thermogram data were analyzed using the TA Instruments NanoAnalyse software. Buffer blanks were subtracted from all samples.

2.4 | Circular dichroism (CD)

Circular dichroism spectra were obtained on a Chirascan CD Spectrometer from Applied Photophysics (Surrey, UK). The samples were diluted to concentrations of 0.3 g/L and measured in cells with a path length of 0.1 cm. Spectra were obtained in the range of 180 to 260 nm. The bandwidth was set to 1 nm and the signal was averaged over 10 s. The detector reached saturation at 195 nm, therefore, data in the range from 195 to 260 nm is shown.

2.5 | Size exclusion chromatography (SEC)

SEC was used to assess the monomer and HMWI content and product losses. Experiments were carried out on a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, Waltham). Isocratic elution was carried out with a 50 mM sodium phosphate buffer with 150 mM NaCl at pH 7.0. 10 µl of 0.22 µm filtered sample were applied to a TSKgel® G3000SWXL HPLC column (5 µm; 7.8 mm × 300 mm) with a TSKgel® SWXL guard column (7 µm; 6.0 × 40 mm) (both Tosoh, Japan). Chromeleon™ 7 software (Thermo Scientific) was used to monitor the signals at 215 nm (for HMWI) and 280 nm (for monomer content and product loss). The starting material is referred to as T_0 material. Monomer content is based on the ratio of product peak area (monomer, detected at 280 nm) to the sum of all peak areas. HMWI are calculated by taking the sum of all peaks eluting before the main antibody peak (at 215 nm) and dividing it by the sum of main peak and earlier eluting species. Concentration in relation to T_0 is the ratio between the monomer peak at 280 nm of the respective sample with the T_0 sample of the respective capture method (protein A or precipitation). Average values and standard deviations are based on triplicate measurements. Injection volume and dilutions were kept constant for all samples.

2.6 | Isoform characterization by pH gradient chromatography

Antibody isoforms were monitored by a cation exchange (CIEX) HPLC method based on a pH gradient method developed by Lingg et al.[18] In short, measurements were performed on a Dionex UltiMate 3000 HPLC system (Thermo Scientific). A ProPac™ WCX-10G Guard Column (10 µm, 4 × 50 mm) and a ProPac™ WCX-10 column (10 µm, 4 × 250 mm) (both Thermo Scientific) were used as stationary phase. Samples were diluted to 1 g/L and the injection volume was 100 µl. The flow rate of the method was set to 1 ml/min. The column was equilibrated for 1 CV at 100% A, before a 12 CV gradient from 0 to 100% B was performed. The method ended with a 1 CV wash step at 100% B. Mobile phase A was 5.5 mM HEPES, 4.2 mM Bicine, 9.5 mM CAPSO, 0.8 mM CAPS and 6.3 mM NaCl (pH 8.0). Mobile phase B was 10.5 mM Bicine, 2.5 mM CAPSO, 7.0 mM CAPS (pH 10.5). The outlet was monitored at 280 nm. The most abundant isoform is defined as main charge variant (MCV). Isoforms eluting earlier than the MCV are called acidic variants, later eluting ones are called basic variants. Accuracy, precision and the lower limit of quantification of

the method can be found at Lingg et al.[19] Injection volume and dilutions were kept constant for all samples.

2.7 | Nano differential scanning fluorimetry (nanoDSF)

Nano differential scanning fluorimetry measurements were performed on a Tycho NT.6 (Nanotemper, Germany). Samples were diluted with 25 mM Tris (pH 7.5) to a concentration of 1 g/L and taken up in glass capillaries which were placed in the nano differential scanning fluorimeter. Initial temperature was set to 35°C and the ramp-up rate was set to 30°C/min. Spectra were taken between 35 and 95°C.

3 | RESULTS

3.1 | Monomer content, HMWI, and product loss

To determine the stability of the antibody intermediate, we used clarified cell culture supernatant and purified it either by a conventional protein A chromatography step or by precipitation of the antibody with PEG at a concentration of 13.2% (w/w). The protein A eluate was kept at the elution pH of 3.6 for 1 hr and then readjusted to the storage pH of 6.0. The PEG precipitated sample was stored in its solid form and in the presence of 13.2% polyethylene glycol. To simulate different intermediate storage conditions, samples were stored at three different temperatures. Storage at −20 and 5°C corresponds to typical storage conditions mid-manufacturing while storage at room temperature was included as a worst-case condition where rapid degradation kinetics are expected. The material was stored for a total of 13 months. Multiple aliquots for both protein A purified and precipitated material were stored at these temperatures and for each analysis an independent aliquot was used and discarded after analysis to avoid repeated freeze–thaw cycles of samples. Samples were tested at the start and after 1, 3, 6, 10, and 13 months in storage with different methods for monomer content, aggregates and product losses. Changes in protein structure were determined by CD, DSC, and DSF. Figure 1 shows the composition of the samples before and after capture step. HMWI as well as LMWI present in the CCCF are more efficiently removed by protein A chromatography than by precipitation. For further information regarding the efficiency of precipitation methods in comparison to affinity chromatography, see Sommer et al.[14,20] and Hammerschmidt et al.[15]

Figure 2 gives the monomer content for both purification techniques at the three tested temperatures. Over the testing period of 13 months, the monomer content of the protein A purified samples decreases approximately 2% from 99.8% at T_0 to ~98% at all tested temperatures. This decrease is mirrored by a rise in HMWI (Figure 3).

For the precipitated antibody, the monomer content (purity) stays constant at −20°C, decreases slightly at 5°C and strongly when stored at room temperature. For the material stored at 5°C, the monomer loss is counteracted by an increase in HMWI. The monomer content of the precipitated material decreases at a constant rate over the observation period. However, HMWI stay constant for the first

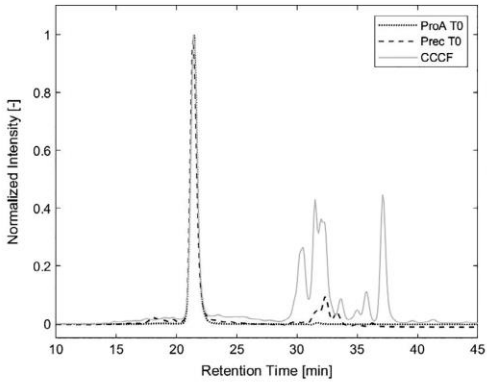

FIGURE 1 Size exclusion chromatogram of the antibodies before (CCCF) and after capture step (either protein A chromatography or precipitation). Due to different purification efficiencies, the amount of HMWI and LMWI are lower in the protein A purified pool compared to the precipitated material. CCCF, clarified cell culture fluid; HMWI, high molecular weight impurities; LMWI, low molecular weight impurities

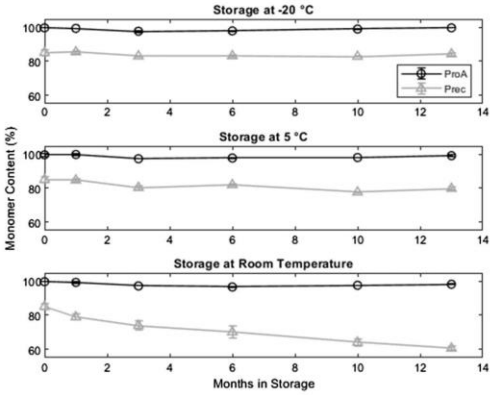

FIGURE 2 Monomer content of protein A purified and precipitated material at three storage temperatures. For protein A purified material, the monomer content is stable at all tested temperatures. For the precipitate, the monomer content is stable at −20 and 5°C but decreases when stored at room temperature. Error bars are based on triplicate measurements

6 months in storage and start rising only afterwards. Therefore, the monomer loss of the first 6 months has to be the result of degradation processes leading to the formation of LMWI. Generally, the HMWI content in the precipitated samples is around 5% at the start of the storage, while it is less than 1% for the protein A purified samples.

We do not observe any product losses for the protein A purified materials at any temperature (Figure 4). For the precipitated material,

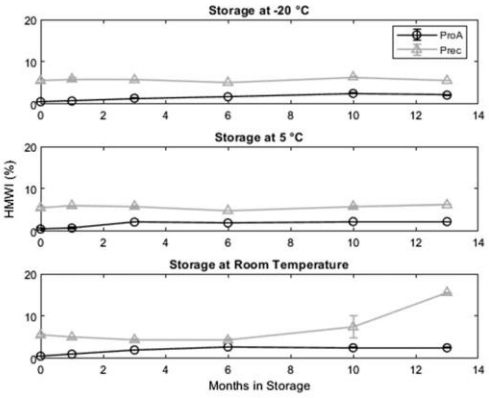

FIGURE 3 HMWI content in protein A purified and precipitated material at three storage temperatures. For protein A purified material, the HMWI stay constant at all tested temperatures. In the precipitated material, the HMWI concentration is significantly higher compared to the protein A purified material. When stored at −20 or 5°C, the HMWI content in the precipitated sample remains stable. At room temperature, the HMWI of the precipitated material accumulate over time. Error bars are based on triplicate measurements. HMWI, high molecular weight impurities

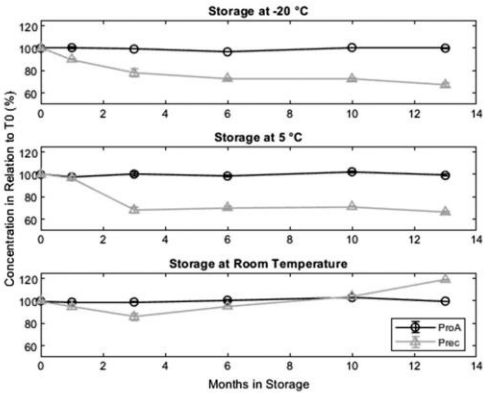

FIGURE 4 Concentration of protein A purified material and precipitated material in relation to the concentration at T_0. Yields for the protein A purified material are stable at all tested temperatures. The precipitated material stored at −20 or 5°C needs to be reconstituted within the first 3 months of storage, otherwise yields drop to 65% where they stay constant. Precipitated material stored at RT is degraded as shown by CIEX-HPLC (Figure 5b). Error bars are based on triplicate measurements

the picture looks quite different. The product loss of the material stored at −20 and 5°C is less than 10% if reconstituted within 1 month but if the precipitate is stored longer than that, the losses increase to 30 to 35%. To keep recovery yields high, it is therefore necessary to reconstitute the precipitated material within the first month of storage.

For the precipitated material stored at room temperature, we observe a small product loss during the first 3 months of storage, afterwards the SEC HPLC data indicate no product losses resulting in resolubilization yields above 100% [sic] after 13 months. While the overall yield is stable at 100%, the product is unstable at room temperature. We assume degradation into product related impurities (degradation products) as the yield is stable, but the monomer content of the sample drops down to 60% during storage at room temperature. Due to the results regarding monomer content (Figure 2), HMWI (Figure 3), and charged variants (Figure 5), we assume that the antibody is degraded after 13 months of room temperature storage and that the degraded antibody has a higher UV absorbance than the native form explaining a yield above 100%. Since obtaining product losses less than 0% is virtually impossible, we assume that tryptophan residues which are buried in the hydrophobic core of the native protein are becoming exposed to the hydrophilic solvent due to the loss of conformation and therefore increase the UV signal. The data shown in Figures 2 and 3 is presented in tabular form in as well (Table S1).

3.2 | Acidic variants formed during protein a chromatography

3.2.1 | Variant alterations during storage

Isoform composition of the stored material was determined at six time points during storage of 13 months for the materials stored at −20°C, 5°C, and room temperature to catch any changes in the isoform composition indicating possible degradation of the antibody during storage. The T_0 sample of the protein A purified material was additionally tested for isoform pattern changes in the first 120 hr of storage at temperatures 5°C, room temperature, and 40°C since we detected an acidic variant in the starting material that vanished in storage within the first month. We hypothesized that the formation of the acidic variant could be a result of the low pH hold step after protein A elution, therefore we prepared a sample of precipitated material resolubilized and stored for 1 hr at low pH to capture any changes in isoform composition which are not due to the protein A purification itself, but due to the following low pH viral inactivation. Figure 6 shows a pH gradient HPLC chromatogram of the antibody before capture of clarified cell culture fluid (CCCF), the samples of the protein A purified material and the precipitated material after low pH incubation before storage.

Protein A chromatography leads to the formation of a new isoform in the sample which is more acidic than the one present in the CCCF while precipitation or viral inactivation at low pH does not result in formation of any new variants. In this chapter, we will however focus on the alterations during long-term storage while the formation of the acidic variant during protein A chromatography is dealt with in more detail in chapter 3.2.2. Figure 7a shows the protein A purified material during long term storage at −20°C. As we can see, the acidic variant is converted into the MCV within the first month of

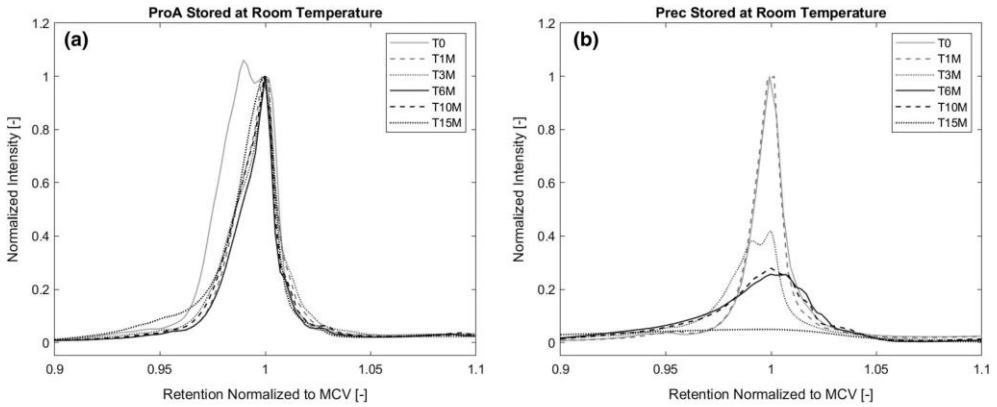

FIGURE 5 (a) Isoform pattern of protein A purified material and (b) precipitated material stored at room temperature. Acidic variant of protein A purified material reconverts to MCV within first month of storage. Precipitated material is not stable at room temperature, the formation of various new isoforms can be observed during storage. MCV, main charge variant

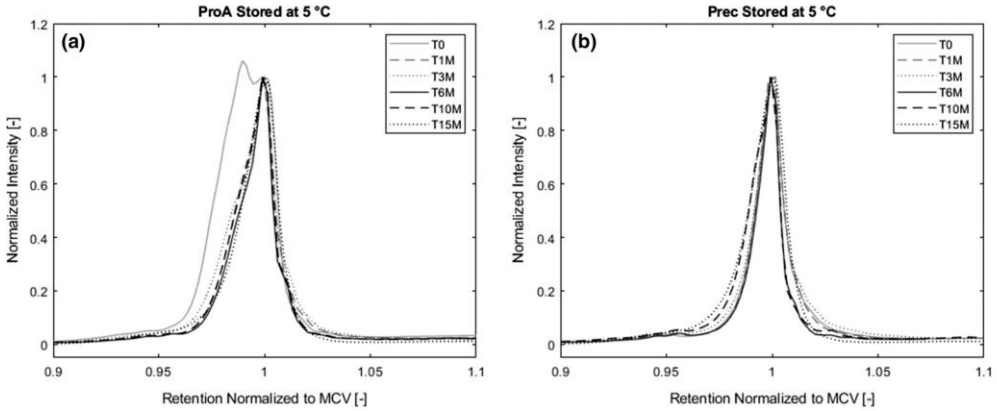

FIGURE 6 (a) Isoform pattern of protein A purified material and (b) precipitated material stored at 5°C. Acidic variant of protein A purified material reconverts to MCV within first month of storage. Precipitated material has a stable isoform pattern. MCV, main charge variant

storage. Afterwards, the isoform pattern is stable again. The precipitated antibody stored at −20°C depicts a stable isoform pattern over the monitoring period (Figure 7b).

At 5°C (Figure 8), we observe the same trend as at −20°C, the acidic variant in the protein A purified sample turns into the MCV while the precipitated antibody shows a stable isoform pattern over the whole time. For the protein A purified material stored at room temperature, we see the same course that was already described for the storage at −20 and 5°C (Figure 5a), meaning a generation of an additional isoform during protein A chromatography that is not visible anymore after 1 month in storage. After the initial conversion of the acidic isoform to the main charge variant, the pattern stays stable.

For the precipitated material stored at room temperature, the isoform pattern is only stable for 1 month (Figure 5b). After storage of 3 months, we see the appearance of new, acidic isoform and after 6 months, the sample seems to be completely degenerated with new acidic and basic variants present.

3.2.2 | Formation of acidic isoform during protein a chromatography

In recent years, multiple groups, including our own, have observed conformational plasticity of antibodies during protein A chromatography.[21-23] In this work we could again observe the formation of a

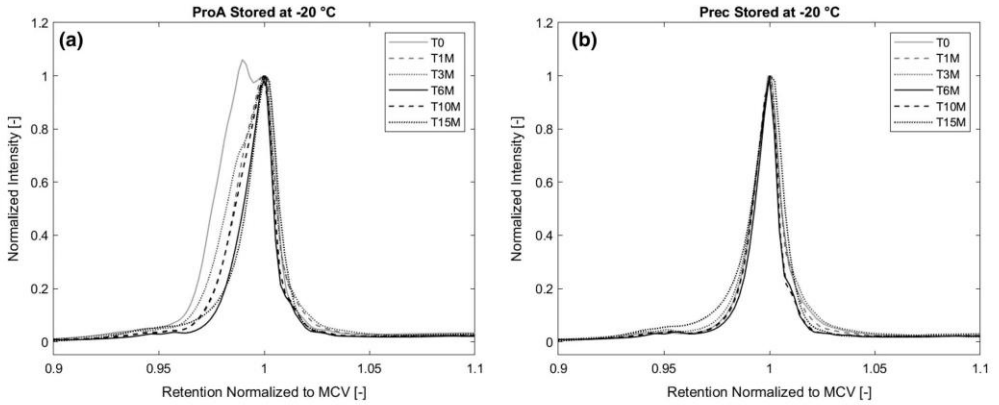

FIGURE 7 (a) Isoform pattern of protein A purified material and (b) precipitated material stored at −20°C. Acidic variant of protein A purified material reconverts to MCV within first month of storage. Precipitated material has a stable isoform pattern. MCV, main charge variant

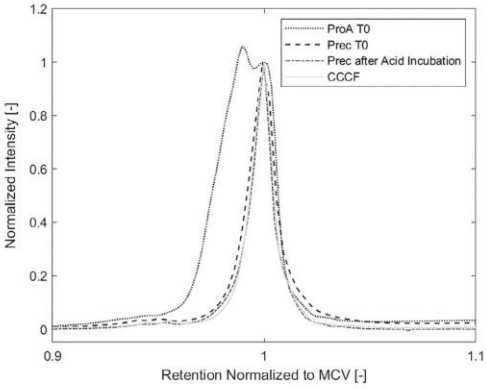

FIGURE 8 Isoform pattern for protein A purified, precipitated material, precipitated material after acid incubation and CCCF. During protein A chromatography, a transient, acidic variant is formed. CCCF, clarified cell culture fluid

transient antibody isoform. We incubated the T_0 material after protein A chromatography at three different temperatures (5°C, room temperature and 40°C) for a total of 120 hr and tested the charged variant composition at T_0, 24, 48, and 120 hr.

We were able to show that re-conversion of the acidic isoform to the MCV is temperature dependent. Figure 9a–c shows chromatograms of the pH gradient HPLC of the T_0 material after protein A purification.

When incubated at 5°C or room temperature, the acidic isoform remains visible for the first 48 hr. Between 48 and 120 hr, it is almost entirely converted to the MCV and just a small shoulder remains visible. Since the yields are stable (see chapter 3.1), we know that the acidic isoforms reforms into the MCV and is not just degraded. At an incubation temperature of 40°C, the acidic isoform is almost entirely gone after 48 hr and not detectable anymore after 120 hr showing that the kinetics are temperature dependent.

To see whether the formation of the acidic isoform results from the low pH that the antibodies are exposed to during elution in protein A chromatography or the subsequent incubation at low pH for viral inactivation. Therefore, we incubated the precipitated antibody after resolubilization in pH 3.6 for 1 hr and performed pH gradient analysis again. As seen in Figure 6, the low pH incubation does not lead to change in isoform pattern, meaning that the structure change seen for protein A purified samples is due to the chromatography itself and not the shift to low pH.

3.3 | Structural alterations during long-term storage

In addition to monomer content, product loss, HMWI, and isoform composition, we wanted to check for possible changes in the structure of the stored antibodies. For this we used DSC, DSF as well as CD. In DSC, unfolding of antibodies follows a biphasic transition where the unfolding of the Fab and CH2 domain occur approximately at the same temperature and overlap in the thermogram. The CH3 domain endures higher temperatures and unfolds a bit later.[24] On average, the main unfolding event (Fab and CH2) of the protein A purified material lies at 73.7°C while the precipitated material unfolds at 73.3°C (Figure 10a, Table S2).

This indicates that the precipitated material is slightly less stable than the protein A purified one. At higher temperatures, we observe slight differences in the unfolding of the CH3 domains of the antibodies. We noticed that the presence of residual polyethylene glycol influences the DSC signal strongly but this was taken into account by respective blank subtractions. Also the co-presence of different host cell proteins could impact the thermal stability of the samples. In the

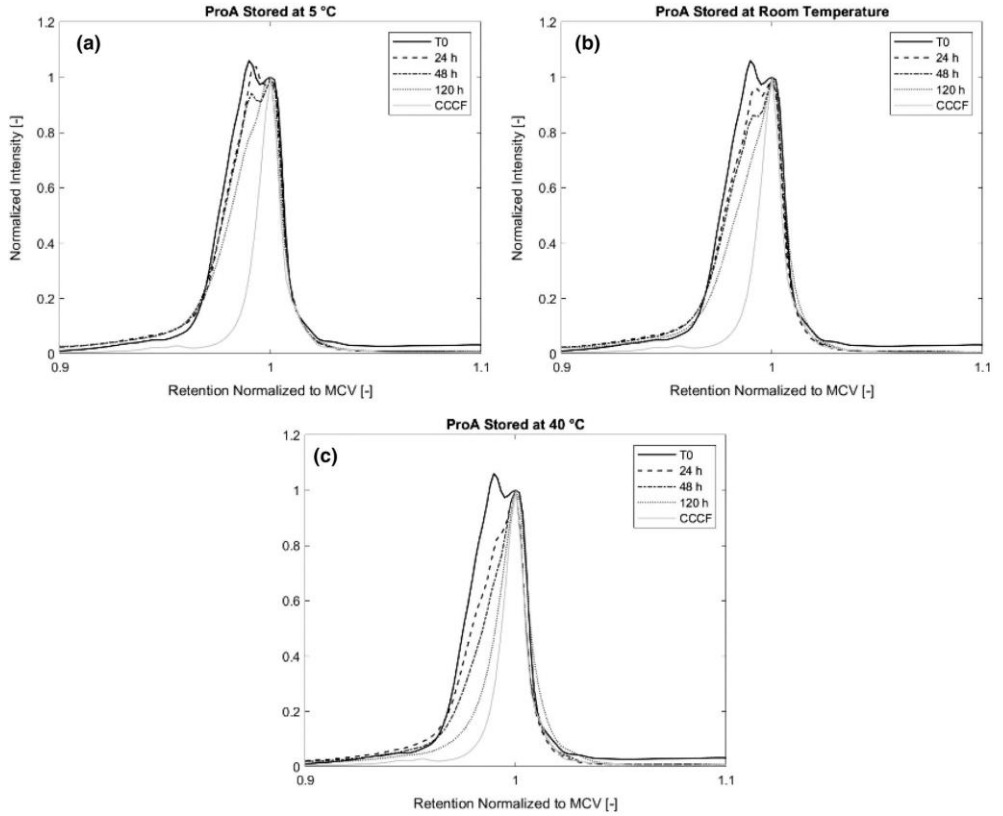

FIGURE 9 (a–c) Isoform pattern of protein A purified material stored at 5°C, room temperature and 40°C for 120 hr compared to CCCF. Incubation of the protein A purified material with the acidic isoform shows temperature dependent reconversion of the acidic isoform the MCV within a matter of days. CCCF, clarified cell culture fluid

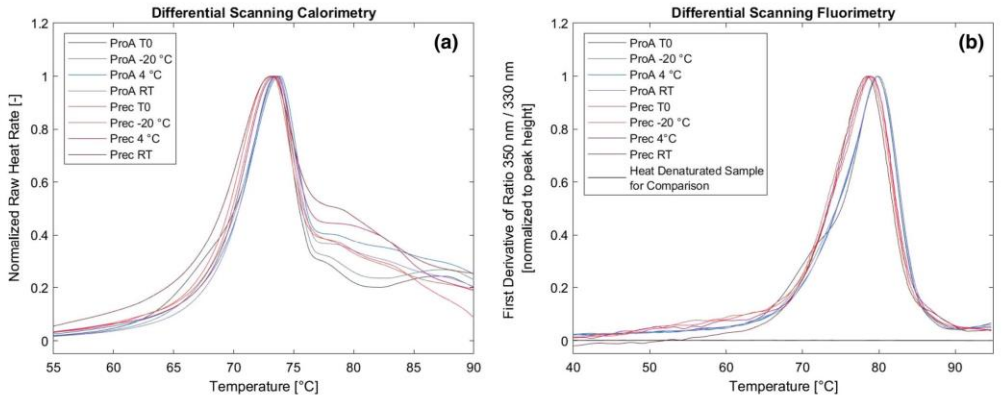

FIGURE 10 (a) Differential scanning calorimetry and (b) differential scanning fluorimetry spectra for protein A purified and precipitated material at start and end points of storage. Both techniques, DSC and DSF, indicate a slight decrease in stability of the precipitated material

differential scanning fluorimetry the inflection temperature of the protein A purified samples is on average at 79.8°C and is therefore about 1°C higher than the one of the precipitated antibodies which is at 78.7°C (Figure 10b, Table S2). Due to the fast heat ramping in the DSF (30°C/min), the unfolding of the antibodies occurs in a single transition rather than a biphasic one as is usually observed in DSC experiments. In both cases (DSC and DSF), the protein A purified sample shows a slightly higher unfolding temperature than the precipitated material. This can be either due to impurities in the precipitation sample adding to the signal, or due to differences in the stability of the antibody itself. To get more insight into the structure and possible structure differences, we measured the CD spectra of precipitated and resolubilized antibody in comparison to protein A purified samples.

In CD measurements, the precipitated antibodies have an observed maximum at 198 nm while the protein A purified material has its maximum 202 nm (Figure 11). All samples have a minimum at 218 nm with the precipitated antibodies giving a stronger signal than the protein A purified material.

Deconvolution of the spectra shows that the precipitated antibody contains a higher amount of random coil. This could be the reason for the reduced stability observed in DSC and DSF experiments. We performed a series of experiments to rule out the possibility that the changes between the protein A purified and the precipitated material are caused by residual PEG presence or the co-presence of host cell proteins. Spiking the HCP pool of the precipitated material with protein A purified antibody did not cause a shift in the CD signal as it was observed for the precipitated material. Neither did the spiking of the protein A purified material with PEG concentration between 0.5 to 5%. The resulting data is presented in Figure S1.

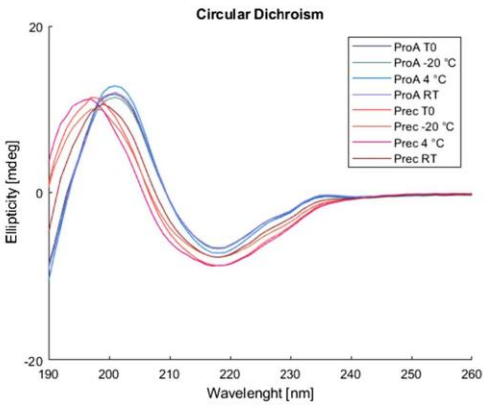

FIGURE 11 Circular dichroism spectra for protein A purified and precipitated material at start and end points of storage. Deconvolution of the spectra shows that a higher amount of random coil is present

4 | DISCUSSION

In our study we compared protein A purified antibody to precipitated antibody in terms of intermediate stability at different temperatures. For the protein A purified material, the storage temperature had no significant impact on monomer content, HMWI or product loss compared to the starting material. HMWI increased only by 2% during the 13 month storage period which could be easily removed in a polishing step later in the downstream process. The protein A purified material can therefore be stored at −20°C, 5°C, and even room temperature with similar stability.

Precipitated material can be stored at −20 and 5°C but product losses have to be expected if the material is stored for more than 1 month. To obtain yields >90%, it is necessary to reconstitute the antibody within the first month of storage, making precipitate storage an option for intermediate storage before the next process step, but not feasible for long term storage. For the protein A purified material stored at −20°C, 5°C, and room temperature as well as the precipitated material stored at −20 and 5°C, the product losses are counteracted by increases in HMWI. For the precipitated material stored at room temperature, the HMWI start rising only after 6 months but product losses are observed gradually during the whole storage period. Therefore, the initial product losses have to be a result of degradation processes which lead to the formation of LMWI. The decrease in resolubilization yields observed for the precipitated material stored at −20 and 5°C could be due to the formation of compact precipitates which are harder to solubilize. Performing a buffer resolubilization screening for the precipitates stored for longer periods (>3 months) could improve resolubilization yields. After this initial drop, the resolubilization yields remain stable for the residual storage period. Storage at room temperature is not recommended at all as it leads to formation of new isoforms and degradation starts visibly after 1 month.

DSF, DSC, and CD spectra show differences between the protein A purified material and the PEG precipitates. All three methods indicate that the stability of the precipitated material is slightly decreased compared to the protein A purified material. This is in contrast to observations that our group has made earlier with antibodies of the subclass IgG1 that showed no differences based on the capture conditions. Our study shows that antibodies of the IgG2 have a higher susceptibility of structural changes during manufacturing. This could be due to the fact that IgG2 is less flexible than IgG1 as it has a shorter hinge region and contains more inter-heavy chain disulfide bonds which make it more rigid[25] and potentially leads to difficulties in the resolubilization process. An additional complication for IgG2 could be more possibilities for disulfide bridge shuffling. An interesting fact is that the degradation was only visible in isoform analysis while the SEC chromatograms lead to believe structural integrity.

It would be very interesting to determine the exact structural nature of precipitates by for example, high-resolution microscopy in the future to explain these differences in IgG1 and IgG2 behavior. Product alterations could also be followed in unit operations further

downstream. Thereby it becomes possible to see if the alterations detected after the capture step have an impact on the final drug product. Due to our analysis, we recommend a broad pallet of analytical tools to track product variations and product alterations. While one method, for instance size exclusion, could lead to the assumption of a stable protein, another method, for instance CD, could show significant changes in the structure of the product. We at least recommend three complementary methods, while one tracks product degradations in size (SEC), one tracks product alterations in structure (CD) and one tracks product alterations in charge (ion exchange). In our case, following the product through the complete process was not possible since the process was not yet fully developed when we evaluated the stability of the capture intermediates. For production, the product alterations have to be tracked through the complete process chain.

Changes in the charged isoform pattern were detected by HPLC-CIEX with a pH gradient. We observed the formation of an acidic variant during protein A chromatography that slowly converts back into the main charged variant in the first days of storage. The kinetics of these conversions depend on the storage temperature, with a faster conversion for higher temperatures where for storage at 5°C the conversion takes a week, while at elevated temperatures of 40°C it takes only 48 hr. We were also able to show that the formation of this acidic isoform is connected to the chromatography itself. Incubation of the precipitated material at a pH of 3.6 for 1 hr does not lead to a change in the isoform pattern and a formation of this isoform due to the low pH at elution can therefore be ruled out. This confirms the findings of our previous work[21] where it was shown that the conformational changes occur while the antibodies are adsorbed to the material. Several protein modifications are known to shift the isoelectric point of a protein to the acidic side, most commonly deamidation reactions but also C-terminal lysine cleavages, sialylation, and oxidation/reduction,[6] but none of them are known to be completely reversible in the timeframes observed in our experiments. Others have shown that protein A chromatography can lead to significant structural changes in the antibody resulting in reduced hydrodynamic radii.[23] Such structural changes could be the cause of the phenomenon we see in our experiments, but we observed no change of hydrodynamic radius of the antibody in our samples (data not shown).

In summary, the antibodies had better storage abilities when they were captured by protein A chromatography than by precipitation. The protein A purified material was found to be well storable at all tested temperatures. With the analytics used in this study we could not detect any structural changes within the protein A purified samples over time. Monomer content and product loss were constant over a period of 13 months. The precipitated antibodies stored at room temperature started to degrade after 3 months resulting in an increase of LMWI impurities first which are later followed by the formation of HMWI too. The reduced storability of the precipitated proteins could be due to the higher presence of HCP in the precipitated samples which could include proteases leading to degradation of the product. Especially at room temperature, the precipitated material is prone to aggregate formation as well. At −20 and 5°C, the precipitated material can be stored without any product losses but reconstitution yields suffer if the material is stored for more than 1 month. The working window is however large enough to ship the material to a different production site and conduct experiments on the material mid-manufacturing or continue processing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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