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Characterization of excipients for their application during purification of monoclonal antibodies and other therapeutic proteins

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LIST OF ABBREVIATIONS

Abbreviation	Explanation
Λ^-	Concentration of deprotonated form of ligand
$S_{Acetate^-,OH^-}$	Selectivity coefficients of anions
S_{Na^+,H^+}	Selectivity coefficients of cations
$c^B_{Acetate^-}$	Concentration of dissociated acetate species in the bulk volume
$c^B_{H^+}$	Concentration of protons in the bulk volume
$c^B_{Na^+}$	Concentration of sodium ions in the bulk volume phase
$c^R_{Na^+}$	Concentration of sodium ions in the resin pore phase
pH_B	pH value in bulk phase
pH_R	pH value in resin pore phase
r_D	Donnan ratio
ADC	Antibody-drug conjugate
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
ANS	Anilinonaphthalene-sulfonic acid
APC	Antigen presenting cells
C1q	C1 complex
CDC	Complement-dependent cytotoxicity
CEX	Cation exchange chromatography
CHES	N-Cyclohexyl-2-aminoethanesulfonic acid
CHO	Chinese Hamster Ovary
CIP	Cleaning-In-Place
COVID-19	Coronavirus disease 2019
CV	Column Volume
DIX	Donnan Ion Exchange Model
DNA	Deoxyribonucleic acid
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FcRn	Neonatal Fc receptor
FcγR	Fc gamma receptor
FDA	Food and Drug Administration (USA)
Hc	Heavy chain
HCP	Host cell protein
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonic acid
HMW	High molecular weight (aggregates)
IEF	Isoelectric Focusing
IgG, IgA, IgM, IgE, IgD	Natural occuring immunoglobulins
kDa	Kilodalton
Lc	Light chain
LGE	Linear gradient elution
LMW	Low molecular weight (fragments)
mAb	Monoclonal Antibody
MES	2-(N-morpholino)ethanesulfonic acid
mM, M	mmol/L, mol/L

MOPSO	β-Hydroxy-4-morpholinepropanesulfonic acid
Nano-DSF	Proprietary name for system to measure differential scanning fluorimetry
N-state	Native-like state of protein
pg	picogram
P-state	Perturbed state of protein
RSA	Reversible Self-Association
SE-HPLC, SEC	Size exclusion (high performance liquid) chromatography
TAPS	[tris(hydroxymethyl)methylamino]propanesulfonic acid
UV	Ultra-Violet
Λ	Ligand density

1 INTRODUCTION

1.1 Antibodies – the protein class

The immune system is a network of cells, molecules, and organs to protect the human body against foreign invaders, also known as pathogens, and keep it healthy. The immune system can be categorized into two parts. The innate immune system is the first line of defense against pathogens and is composed of barriers (skin), small molecules (complement) and cells (macrophages or dendritic cells). This part of the immune system works without the need for preconditioning from the environment and kills or removes the pathogens from the infected area (Megha and Mohanan 2021).
Immunoglobulins and antibodies are a part of the adaptive and humoral immune system. It depends on the ability of cells like B-lymphocytes to learn and adapt to repeated exposure of antigens. Antigens are proteins or carbohydrate chains of glycoproteins, that pose a certain risk against the health of tissues, cells, and the human body in total (Megha and Mohanan 2021). Macrophages and others are specific cell types of the immune system that are responsible in part for presenting and transporting the antigens to the lymph nodes (e.g. as antigen presenting cells (APC's)), where B- and T-cells are working on destroying the antigen or antigen-infected cells and learning about the antigen structure to enhance immune response with repeated exposure of the certain antigen. Especially B-cells differentiate into plasma cells to be able to produce antibodies against that specific antigen. The main features of acquired immune response are: specificity and diversity of recognition, immunological memory, specialized response, and recognition towards the self and non-self components of the organism itself (Megha and Mohanan 2021).
Antibodies make up a specific class of glycoproteins and are an integral part of the human body and the immune system. There are 5 different isotypes of antibodies or immunoglobulins, IgG, IgA, IgM, IgE, and IgD, and each with a specific role in the immune system. The basic structure of a monomeric antibody is Y-shaped (see Figure 1). Human immunoglobulins have two identical light chains (L_C) and two identical heavy chains (H_C), which are connected through disulfide bonds (Chiu et al. 2019). The two small polypeptide light chains are connected with the larger polypeptide heavy chains through disulfide bonds and non-covalent interactions like hydrogen bonds, salt linkages, or hydrophobic bonds (Megha and Mohanan 2021). Furthermore, the bottom part of the Y shape makes up the Fragment crystallizable (Fc) and the two identical arms of the antibody are the Fragments antigen binding (Fab). Both Fab arms can each bind an antigen of the same structure. The Fc part is responsible for binding to FcγR (Fc-gamma-receptors) and the first subcomponent of the C1-complex (C1q) to mediate antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), trogocytosis, induction of secretion of mediators, and endocytosis of opsonized particles, as well as modulation of tissue and serum half-life through interaction with the FcRn (Chiu et al. 2019; Daëron 1997; Rouard et al. 1997; Taylor and Lindorfer 2015). The Fc has been the focus of significant engineering to modulate effector function activities found on monocytes, macrophages, dendritic cells, neutrophils, T and B lymphocytes, and natural killer cells (Chiu et al. 2019; Mimoto et al. 2016). The hinge region contains disulfide bridges that connect the two heavy chains together and make the antibody a very flexible molecule.

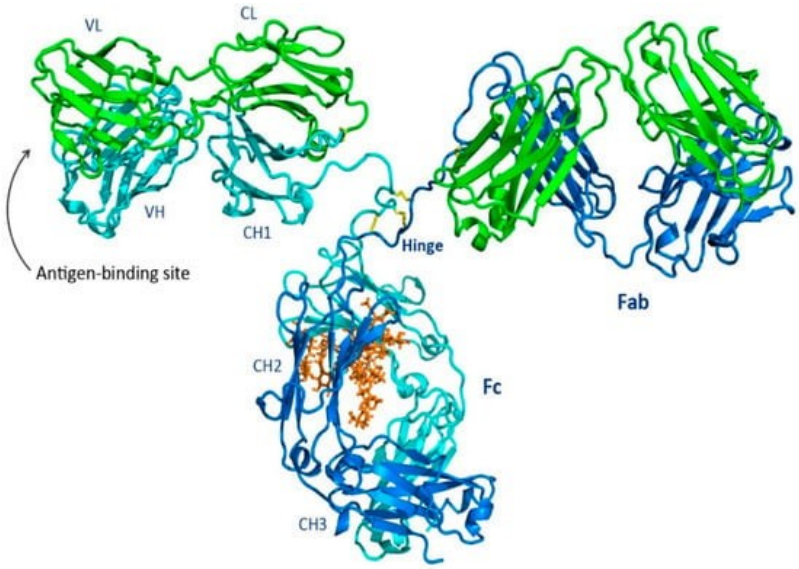

Figure 1: A ribbon representation of an intact IgG, Protein Data Bank (PDB) id: 1igt (Harris et al. 1997), which is a mouse IgG2a isotype. The light chains are green, the heavy chains are cyan and blue, the glycan is orange sticks, and the interchain disulfides are yellow sticks. Copied from (Chiu et al. 2019).

The five types of antibodies are distinguished mainly by their concentration in the plasma serum. IgD only contributes to about 0.2% of the total immunoglobulin in the serum and it mostly functions as an antigen receptor for B-cells which have not been exposed to antigens (Megha and Mohanan 2021; Schroeder and Cavacini 2010) and it can activate basophils and mast cells to produce antimicrobial factors (Chen et al. 2009; Megha and Mohanan 2021).
IgE antibodies are also present in a very low concentration in the serum, but they play an important role in the hypersensitivity reactions that are responsible for the symptoms of e.g. hay fever, asthma, or hives. They trigger a histamine release from mast cells and basophils due to binding to the allergens and also protect against parasitic worms (Megha and Mohanan 2021; Pier et al. 2004).
IgM, different from IgD, IgE, and IgG which are monomeric antibodies with a Y-shape, is expressed and secreted by the plasma cell as a pentamer. All five monomeric subunits are linked together through the Fc regions in the center of the pentamer and ten antigen-binding sites on the outside of the molecule. Its main purpose is the primary response to an antigen and therefore has the ability to eliminate pathogens in the early stages of B cell-mediated humoral immunity before there is sufficient IgG. The concentration of IgM in plasma is about 5-10% (Geisberger et al. 2006; Megha and Mohanan 2021; Pier et al. 2004).
IgA is found with a concentration of 10-15% in the serum. It is part of the mucosa in the respiratory tract, gut and urogenital tract and can also be found in saliva, sweat, tears and breast milk. Therefore, it is part of the immune system in the most common entryways of pathogens. This immunoglobulin can be found as a monomer but also in multimeric forms, like dimers, trimers and some tetramers (Megha and Mohanan 2021; Underdown and Schiff 1986).
The most abundant by far under the class of immunoglobulins is IgG, with its different subtypes IgG1, IgG2, IgG3, and IgG4. They can be distinguished by differences in γ-chain sequence, the size of the hinge region and the number and position of the interchain disulfide bonds between the heavy chains and their concentration in the serum and overall make up about 80% of the immunoglobulins in the serum (Megha and Mohanan 2021; Punt et al. 2013; Wang et al. 2007). This is also the most produced antibody for protein therapeutics and the focus in this project and dissertation.

1.2 Antibodies as drug substance

Since antibodies are involved in many different cascades in the immune system and their structure is mostly conserved, even though they are specific for each antigen, it is not surprising, that a lot of research over the last decades has been dedicated towards using antibodies as drug substances. Protein therapeutics, to which antibodies as a drug substance belong to, are a growing field of interest for the pharmaceutical industry. Small chemical molecules still play a significant role in therapeutics, but protein therapeutics opened up a whole new set of molecular targets in the human body (Johnson-Léger et al. 2006). For example, disorders such as chronic renal failure, dwarfism and infertility, once untreatable, are now successfully managed using protein therapeutics (Johnson-Léger et al. 2006).
Monoclonal antibodies, due to their high specificity is a part of a new era of personalized treatment strategy and therefore show less adverse therapeutic outcomes (Megha and Mohanan 2021). Their indications are wide-spread and range from cancer therapeutics over immunoregulation, transplantation and pulmonary disorders (Leader et al. 2008). As of 2020, the FDA and European Medical agency has approved around 90 antibodies for the therapy of autoimmunity, cancer, infectious and inflammatory diseases, reported by Kaplon et al. (Kaplon et al. 2020). And of course the COVID-19 pandemic was a significant driver for monoclonal antibody therapies since the start in 2020 (Kaplon and Reichert 2021).
Most of the approved antibody treatments are IgGs with the 4 subgroups, but there is an increase in novel antibody formats, which are not naturally occurring in the human body. Through the possibility of protein engineering these novel formats play a more and more significant role in protein therapeutics. These formats include Fc-Fusion proteins, multivalent antibodies, bispecific antibodies, Fab-fragments, antibody-drug conjugates (ADC's) and so-called nano-bodies (Deonarain et al. 2015; Fischer and Léger 2007; Hendriks et al. 2017; Li and Zhu 2010; Miller et al. 2020). The aim is to create antibodies and antibody-related biologics with the appropriate functional and biophysical properties to address specific therapeutic needs. The engineering approaches applied to antibodies, antibody fragments, and antibody fusion products include effector function engineering, antibody humanization, affinity modulation, and stability enhancement to emprove efficacy and manufacturability (Chiu et al. 2019).
One example for the multiple mechanisms that mAb-based therapeutics can be used for is cancer immunotherapy indications. It is based on three main mechanisms summarized by Kimiz-Gebologlu et al.: (1) inhibition of the factors and receptors that activate the signal pathways used by the cancer cells in division and angiogenesis by antibody binding; (2) the antibody-dependent cellular cytotoxicity (ADCC) which is composed of target monoclonal antibodies formed from either chimeric or full human antibody components that bind to specific tumor associated antigens and (3) complement-dependent cytotoxicity (CDC) by complement activation (Harris and Drake 2013; Kimiz-Gebologlu et al. 2018; Mayor et al. 2016).

1.3 Production and purification of antibodies

Ever since the first hybridoma technology for pure antibody production developed by Köhler and Milstein in 1975 (Köhler and Milstein 1975), great advances have been made to enhance productivity while reducing immunogenicity towards the patient. The first developed therapeutic mAb, muromonab-CD3 (OrthokloneOKT3) was approved by the FDA in 1986 (Ecker et al. 2015), and was a murine mAb against T-cell-expressed CD3 that functions as an immunosuppressant for the treatment of acute

transplant rejection (Megha and Mohanan 2021). Due to the fact that this antibody was originally coming from a mouse, the immunogenicity, i.e. the immune reaction in the human body was quite high and production of this drug substance was eventually stopped. Since then technologies to reduce the mouse part of the protein sequence (chimeric antibody) (Foster and Wiseman 1998; Maloney et al. 1997b; Maloney et al. 1997a; Morrison et al. 1984) or eventually be able to produce fully human antibodies have been employed (Kempeni 1999; Megha and Mohanan 2021; Watier and Reichert 2017).

Antibodies are mostly produced in mammalian cell lines, e.g. Chinese hamster ovary (CHO) cells. And since antibody therapies currently require large doses either in volume or concentration, manufacturing capacity becomes an issue because the drug substance must be produced in large quantities with cost and time efficiency to meet clinical requirements and commerzialization (Li et al. 2010). One way to significantly reduce process development timelines for upstream as well as downstream activities, is to develop a platform process. This is one way for companies to deliver their pipelines efficiently by utilizing streamlined platform processes that include standardized process conditions and procedures (Li et al. 2010). Use of a platform process for upstream allows acceleration of early stage process development activities, e.g. clone selection, process lock and technology transfer to clinical manufacturing (Li et al. 2010). For downstream activities, the use of a platform process has similar advantages in terms of selection of chromatography and filtration steps. The similarity of molecular characteristics and properties among different mAbs make the platform approach feasible, although the processes may not be fully optimized for every molecule (Li et al. 2010).

For upstream production of antibodies, a mammalian cell is likely chosen, due to the similar glycosylation pattern compared to human proteins. CHO (Chinese hamster ovary) cells are the predominant host used to produce therapeutic proteins. About 70% of all recombinant proteins produced are made in CHO cells (Dr. Wei-Shou Hu 2007; Li et al. 2010). Genetical engineering is focused on improving the host cells to improve or modify the product quality or improve the host cell robustness (Li et al. 2010). Through upstream production, the cell culture gets scaled up to multiple ~10,000 liter at manufacturing scale. Advancements in the fields of cell line, media and bioreactor conditions, cell specific productivity of over 20 pg/cell/day can be routinely achieved (Li et al. 2010; Wurm 2004), and product titers of about 10 g/L have been reported.

Mammalian cells are secreting the antibody after production inside the cell and therefore no cell disruption is needed for capturing the product. Nevertheless, the first step of downstream activities after cell culture is the protein harvest, where the cell culture broth with the target protein is separated from cells and cell debris. At commercial manufacturing scale this is achieved through a combination of centrifugation and depth filtration (Maybury et al. 2000; Olson 1995).

After depth filtration the target protein is still surrounded by many other process- and product related impurities, like proteins or DNA from the cell host and media components. These are separated in a first capture affinity chromatography step, Protein A chromatography. The main advantage for Protein A chromatography over other chromatography resins is the high affinity for the Fc part of antibodies to bind to immobilized Protein A, a protein derived from *Staphylococcus aureus* (Hjelm et al. 1972; Kronvall 1973). Protein A chromatography is able to purify the target up to 95% (Pete Gagnon 1995; Shukla et al. 2007) with a yield of over 90%. Elution of the bound antibody is done with a gradient or step to low pH values (~3.5). After Protein A chromatography, one of the two orthogonal virus inactivation or virus removal steps required by the FDA (FDA 1998), is a low pH virus inactivation step. The elution pool from the capture step

is set to a low pH value (~3.6-3.4), then incubated over a certain time and then neutralized again. Low pH treatment has been shown to successfully inactivate retroviruses for a variety of biotechnological products (G. Sofer 2003; Shukla et al. 2007). The next chromatography steps are employed as polishing steps to remove any residual HCPs, DNA, aggregates or other impurities. These chromatographies can be selected based on which impurities need to be removed and which mode of action is therefore required. One to two chromatography polishing steps are usually done. The most popular chromatography modes in platform applications are cation- and anion exchange chromatography, hydrophobic interaction chromatography or mixed-mode chromatography (Shukla et al. 2007). Ion exchange chromatography is based on electrostatic interactions of the charged protein surface with the oppositely charged immobilized ligand. Hydrophobic interaction chromatography is based on hydrophobic interactions of the protein to the resin matrix. And mixed-mode chromatography is most often a mixture of ion exchange and hydrophobic ligands immobilized on the resin matrix and can therefore offer a whole different separation compared to the interactions alone. Cation exchange chromatography is used in bind-and-elute mode, where the target antibody is bound at pH ~5.0 to the resin. The impurities either flow through the column during load step or, when the target is eluted mostly in a salt gradient, the impurities either get separated in the gradient, i.e. aggregates elute later than monomer, or do not get eluted and have to be removed during a strip/CIP step after the gradient with sodium hydroxide solution. Following the polishing chromatography steps is usually the second orthogonal step of virus removal, a virus filtration (Shukla et al. 2007). And the last part of the platform process is ultrafiltration and diafiltration to concentrate the antibody solution and to change the buffer into the formulation buffer (van Reis et al. 1997). This is the step where the antibody is stabilized with excipients to increase shelf-life before final filtration and filling into primary containers.

1.4 Challenges for protein therapeutics

As described above, the last step of the purification train is designed to exchange the buffers from the last purification unit operation and mix the target antibody with a formulation buffer. The overall goal of protein formulation development is to transform a highly-purified, recombinant protein solution (drug substance) into a stable, efficacious biopharmaceutical drug or vaccine (dosage form) for administration to patients (Kamerzell et al. 2011). Pharmaceutical excipients may be added to a formulation to stabilize the protein, facilitate in manufacturing the dosage form, control or target delivery in the body, or provide tonicity to minimize pain upon injection (Kamerzell et al. 2011). Examples include buffers controlling solution pH value, carbohydrates as bulking agents for lyophilization, polymers as viscosity agents for topical applications, and salts or sugars adjusting solution osmolality into a physiological range (Kamerzell et al. 2011).
Product- and process-related impurities, like HCP’s, host cell DNA, HMW and LMW species, leached Protein A, and charge variants, should mostly be removed at the point of formulation. However, an adverse immune response from the patient is always possible and can have a lot of reasons. Johnson-Léger et al. formulated it this way: “The assumption is that the closer a biopharmaceutical is to “self”, the less likely it is to induce an antibody response. However, to date it is true to say that almost every protein therapeutic, human and non-human, has elicited some kind of antibody response. The immunogenicity of therapeutic proteins can be influenced by many factors, including the genetic background of the patient; the presence of mutations in the protein; the formulation; the presence of conjugates or aggregates; the route of administration,

dose frequency and duration of treatment; and the presence of contaminants introduced during manufacturing, handling and storage" (Johnson-Léger et al. 2006). Specifically aggregates or HMW's pose a risk for the drug substance and the final formulation (Moussa et al. 2016). Differences in biological activity of the aggregates compared to the activity of the monomeric protein can significantly impair the potency of a protein-based drug (Vázquez-Rey and Lang 2011). Aggregation is a complex cascade with different origins, mechanisms and pathways, e.g. colloidal and conformational stability of proteins (Oyama et al. 2020). Several different interactions play a role in aggregation and it always depends on the surroundings of the monomeric antibody. Protein aggregation during manufacturing and purification is a common effect. Aggregation can occur during cell culture, exposure to low pH value conditions (e.g. virus inactivation after capture chromatography), or pH values near the isoelectric point of the protein, agitation, buffer characteristics, equipment contact materials, ultrafiltration, pumping, final filling and freeze-thaw and storage (Vázquez-Rey and Lang 2011). Therefore, the process needs to be mild and robust at the same time to be able to remove aggregates without introducing new ones.

1.5 Objective for this project

This chapter has partially been published in first author papers (Stange et al. 2022b; Stange et al. 2022a).

Monoclonal antibodies are subjected to chemical, physical and mechanical stresses during manufacturing, transportation, and storage, which can cause deamidation, oxidation, fragmentation, and unfolding (Guo et al. 2014; Oyama et al. 2020). Especially unfolding is a concern since it is often thought of as a starting point for aggregation (Guo et al. 2014; Rosenberg 2006). Cation exchange chromatography is part of the platform process in a monoclonal antibody purification train (Man et al. 2019; Shukla et al. 2007). It is based on electrostatic interactions between the protein and the ligands on the stationary phase. Overall, it is known as a relatively mild technique to separate the target protein from process- and product-related impurities, like host-cell proteins, aggregates, and charge variants. Other polishing techniques, like hydrophobic interaction chromatography, are known to induce conformational changes and aggregation of the target protein (Fogle et al. 2006; Muca et al. 2010). However, over the last couple of years, more and more reports of surface-induced conformational changes or even aggregation of monoclonal antibodies in cation exchange chromatography have been published (Farys et al. 2018; Gillespie et al. 2012; Guo et al. 2014; Guo et al. 2016; Guo and Carta 2014; Huang et al. 2020; Kimerer et al. 2019a, 2019b; Luo et al. 2014; Luo et al. 2015; Man et al. 2019). The origin of this effect is not yet fully understood and seems to differ between proteins. Kimerer et al. have seen multiple peak elution behaviors for a bispecific antibody with no hold time and no measurable aggregation and attributed this to an interconversion of the flexible protein from one species to another (Kimerer et al. 2019a, 2019b). Voitl et al. have proposed two different binding conformations or sites, with the second being rate limiting, for their elution behavior of human serum albumin (Voitl et al. 2010a, 2010b). This hypothesis was tested by Luo et al., because their monoclonal antibody showed a double-peak elution behavior without unfolding or aggregation and found that their observation was due to histidine protonation-based charge variants (Luo et al. 2015). In some cases, the observation of multiple peak elution was correlated with a structurally unstable aglycosylated monoclonal antibody (Gillespie et al. 2012) or Fc-fusion protein (Chen et al. 2016). For some proteins the predominant effect was reversible self-association (RSA) or reversible

aggregation (Chen et al. 2016; Farys et al. 2018; Gillespie et al. 2012; Guo et al. 2014; Guo et al. 2016; Luo et al. 2014; Man et al. 2019; Poplewska et al. 2021; Stańczak et al. 2020), while for others permanent unfolding and aggregation was present (Farys et al. 2018; Guo et al. 2014; Guo and Carta 2015; Huang et al. 2020; Man et al. 2019). Guo et al. and other authors attributed this effect to the ligand structure and polymer grafted extenders of the cation exchange resins (Farys et al. 2018; Guo et al. 2014; Guo and Carta 2015). Huang et al. and other authors meanwhile, have hypothesized that their observations were likely due to the hydrophobicity of either the base matrix or the polymer grafted extenders (Huang et al. 2020; Luo et al. 2014; Poplewska et al. 2021; Stańczak et al. 2020). All the authors, who have published about this phenomenon, however, seem to agree, that this effect is predominantly affected by the overall stability of the protein. And while some have done solution experiments outside the resin to prove this hypothesis, none have accounted for the significant decrease of pH-value inside the resin pore during the load and hold times. The Donnan effect (F. G. Donnan 1911) was originally described for ion exchange resins in the 1950s by Gregor et al. and further developed by other authors (Gregor 1951; Helfferich 1995; Jansen et al. 1996; Jansen et al. 1997; Ståhlberg 1999). It incorporates the ion distribution in ion exchange resins not only for counterion species, but also co-ions, neutral, and zwitterionic species. For the mathematical description of the Donnan effect, Jansen et al. developed the Donnan ion exchange (DIX) model for the linear region of the adsorption isotherm (Jansen et al. 1996). Using this model, the differences in charged ions between the resin and bulk phase can be calculated, which is dependent on the salt concentration and the bulk pH-value (Wittkopp et al. 2018). This results in a significantly lower pH-value inside the resin pore, which has an influence on the conformational stability of the monoclonal antibody. In addition, the resin phase pH depends on the ligand density. Earlier investigations into the role of ligand density in ion-exchange chromatography focused on the retention differences of model proteins (Wu and Walters 1992), impurity removal like host-cell proteins, aggregates, and charge variants (Fogle et al. 2012; Fogle and Persson 2012), static binding capacity and yield (Fogle and Persson 2012; Wrzosek et al. 2009). For tentacular ion-exchangers effects of ligand density variation on structural characteristics of the polymer layer like pore size changes, conformation of the polyelectrolyte chains, grafting density per surface area, ligand density per polymer chain, and length of the polymer chains further increase complexity of the phenomena (Bhambure et al. 2016; Bhambure et al. 2017; Franke et al. 2010; Thomas et al. 2013; Wrzosek et al. 2009). The ligand density influence on the pH in the Donnan volume was mainly investigated for weak ion exchangers (Sanchez-Reyes et al. 2021; Wittkopp et al. 2018).

To ensure a monoclonal antibody product's long shelf-life (up to 3 years), stabilizing excipients are added at the final stage of production before filling and packaging. Their effect on the stability of the antibody is tested using different biophysical methods, e.g., spectroscopy in various applications, microscopy, and calorimetry (Kamerzell et al. 2011). Most of these biophysical methods are used to study the protein-protein or protein-excipient interactions in solution during the application of various stresses, like mechanical stress from stirring or physical stress, like freeze-thaw cycles (Hawe et al. 2009; Mahler et al. 2005). One of the major causes of protein unfolding and aggregation is also the interaction with surfaces, e.g., stainless steel tanks (Vázquez-Rey and Lang 2011) or chromatographic resin material (Beyer and Jungbauer 2018; Guo et al. 2014). Potential influential parameters during the on-column unfolding, like temperature and hold time are investigated in this project. Other groups have shown that these parameters could affect the multiple peak elution behavior to a great extent (Chen et

al. 2016; Farys et al. 2018; Gillespie et al. 2012; Guo et al. 2014; Kimerer et al. 2019a; Luo et al. 2015; Voitl et al. 2010a, 2010b). However, my main focus is the influence of excipients on the two peak elution behavior and their use as a potential screening tool for surface-induced unfolding and aggregation. First, five excipients commonly used in pharmaceutical formulations or otherwise interesting were chosen for the first set of experiments: Sucrose, Trehalose, Urea, a charged Excipient A, and the combination of Excipient A and Sucrose. Sucrose and Trehalose are common stabilizing excipients (Akers 2002; Barnett et al. 2016; Kim et al. 2003; Le Basle et al. 2020; Sudrik et al. 2017), which are preferentially excluded from the protein surface (Sudrik et al. 2019). Urea at low concentration is commonly used as a refolding agent (Hamada et al. 2009) and usually lowers protein stability at higher concentration (Hou et al. 2010; Man et al. 2019). Ionic Excipient A and the combination with Sucrose were used to get more insight into the stabilizing potential for the proprietary excipient from Merck KGaA (Darmstadt, Germany). Further studies with a minimized parameter set were then conducted using other potentially influential excipients, Sorbitol and Mannitol, Taurine, Arginine, Glycine, and PEG4000. While Sorbitol and Mannitol have similar properties regarding protein stabilization in pharmaceutical formulations and are used widely as bulking agents (Akers 2002; Chang et al. 2005b; Chang et al. 2005a; Cleland et al. 2001; Kadoya et al. 2010; Le Basle et al. 2020; Meyer et al. 2009; Sudrik et al. 2019), Taurine has not been applied in formulation development and is mostly used for food and feed applications and studied for its effect on the human body (Chesney et al. 1998; Schaffer et al. 1998; Stohs and Miller 2014). Arginine and Glycine are both amino acids but with different abilities to interact with the protein, which can impact their stability (Arakawa et al. 2006; Cloutier et al. 2020; Kamerzell et al. 2011; Le Basle et al. 2020; Li and Nail 2005; Maity et al. 2009; Meyer et al. 2009; Shukla and Trout 2010; Sudrik et al. 2017; Svilenov et al. 2020; Thakkar et al. 2012; Zhang et al. 2016). PEG4000 is commonly used as a co-solvent in parenteral formulations (Akers 2002), for refolding of recombinant proteins (Lee and Lee 1987), but slightly decreases the thermal stability of proteins, likely due to interactions with the protein surface upon unfolding (Barnett et al. 2016; Zielenkiewicz et al. 2006). Sucrose, Arginine, and Glycine have been used by other groups to influence the multiple peak elution behavior but have published mixed results (Chen et al. 2016; Farys et al. 2018; Gillespie et al. 2012; Guo et al. 2016; Guo and Carta 2015; Luo et al. 2014; Luo et al. 2015; Man et al. 2019; Thakkar et al. 2012). For example, while in two studies, the addition or exchange of Arginine showed no effect (Chen et al. 2016; Gillespie et al. 2012), in others, it led to a reduction of second peak (Gillespie et al. 2012; Guo et al. 2016; Guo and Carta 2015; Luo et al. 2014), and yet in another study led to an enlarged second peak (Luo et al. 2015). This shows that the influence of different types of excipients is yet to be clarified and compared with other orthogonal techniques to elucidate their influence on the conformational stability of the protein overall. Therefore, I used nanoDSF to compare their influence on the stability of the monoclonal antibody during low pH conditions. I show that, while in some instances, their effects are similar, in others, they differ significantly, which could be due to the fact, that different unfolding and aggregation pathways are triggered, and it further proves that such a screening tool for surface-induced unfolding and aggregation could be helpful in formulation development.

In this project I used a stable, glycosylated IgG1 antibody and followed the practical approach of Guo et al. (Guo and Carta 2015), as I used a sodium acetate buffer system, three strong cation exchange resin prototypes with tentacle technology and different ligand densities or a commercially available Fractogel® SO_3 (M) resin, a hold time before elution and a 20 CV elution gradient to 1 M NaCl. I investigated the overall

effect with potential several influencing factors, including ligand density variation, buffer composition, temperature, hold times, and excipients to elucidate the effects of these parameters on the two-peak elution behavior of a monoclonal antibody in cation exchange chromatography.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and buffer substances

Sodium acetate trihydrate (Emprove® Expert), Sucrose (Emprove® Expert, low in nanoparticles), PEG4000 (Emprove® Essential), Mannitol (Emprove® Expert), Trehalose dihydrate (Emprove® Expert) and Sorbitol (Emprove® Essential), Excipient A, and Glycine (cryst. Emprove® Expert) was supplied by Merck KGaA, Darmstadt, Germany. Na2HPO4*2H2O (Emprove® Expert, #1.37036), NaOH (Emprove® bio, #1.37020), Urea, Taurine, Arginine, and NaCl (Emprove® Expert, #1.37017) were purchased at Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Acetic acid (glacial, min. 99.0%, Chemsolute) was purchased at Th. Geyer GmbH & Co. KG (Renningen, Germany). CHES and MOPSO (both ≥99.0%) were both purchased at Sigma-Aldrich (Merck KgaA, Darmstadt, Germany). MES*H_2O, TAPS, and HEPES (all for buffer solutions) were purchased at AppliChem GmbH (Darmstadt, Germany).

2.1.2 Protein preparation

The model protein used in this study is a chimeric monoclonal antibody (mAb) provided by Merck KgaA (Darmstadt, Germany). The mAb is a glycosylated IgG1 antibody with molecular mass of ~ 150 kDa and the determined isoelectric point values of the glycosylated forms varied between 8.15 – 8.60 by IEF. The protein sample was provided as a Protein A purified sample. It was diluted with the appropriate equilibration buffer to 5 mg/mL. SE-HPLC analysis of the protein solution showed an aggregate content of 1.5-2%.

2.1.3 Columns

The cation exchange resin Fractogel® SO_3 (M) used in this work was commercially obtained from Merck KGaA (Darmstadt, Germany). The resin was packed in OPUS® MiniChrom columns by Repligen (Weingarten, Germany). A commercially available Fractogel® SO_3 (M) with an inner diameter of 5 mm and a length of 50 mm (V_c = 1.0 mL) as well as with an inner diameter of 8 mm and a length of 50 mm (V_c = 2.5 mL) were used.
The strong cation exchange resin prototypes used in this work, are based on the same base matrix used for Fractogel® and were synthesized with different ligand densities (here denoted as low P1, middle P2, high P3, see Table 1) and were provided by Merck KGaA (Darmstadt, Germany). According to the manufacturer, the resin is based on a cross-linked polymethacrylate support and has sulfoisobutyl groups as ligands. All resins were packed in OPUS® MiniChrom columns by Repligen (Weingarten, Germany). The prototype resins were packed with an inner diameter of 8 mm and a length of 50 mm (Vc = 2.5 mL). These resins have been further characterized by Bhambure et al. for their functional and structural properties (Bhambure et al. 2016; Bhambure et al. 2017).

Table 1: Ligand density of the cation exchange prototype resins applied

Resin ID	$\Lambda_{Dry\ gel}$ /(µeq·g^{-1})	Λ_{packed} /(µeq·mL^{-1})	Λ_{pore} /(mmol·L^{-1})[1)]
Prototype resin P1	395	82.94	0.196
Prototype resin P2	483	113.65	0.256
Prototype resin P3	645	151.76	0.338

[1)]calculated from Λ_{packed}

Furthermore, a strong cation exchange column with smaller bead size Fractogel® SO3 (S) (~35 µm) was used to compare the influence of the bead size. Also, a weak cation exchange column Fractogel® COO was used in this project. Both were commercially obtained from Merck KgaA (Darmstadt, Germany). The resins were packed in OPUS® MiniChrom columns by Repligen (Weingarten, Germany). Both have an inner diameter of 8 mm and a length of 50 mm (V_c = 2.5 mL).
To evaluate if the tentacle-structure of Fractogel® resins had an influence on the two-peak elution behavior, a resin with similar ligand and properties but without the tentacle-structure was used, Toyopearl® SP-650 M. The column was commercially obtained from Tosoh Bioscience LLC (Tosoh Bioscience GmbH, Griesheim, Germany). The resin was packed in OPUS® MiniChrom columns by Repligen (Weingarten, Germany). It has an inner diameter of 8 mm and a length of 50 mm (V_c = 2.5 mL).

2.2 Methods

2.2.1 Chromatography experiments

The buffer used for equilibration, loading, and hold step was 40 mM sodium acetate trihydrate adjusted with acetic acid to pH 4.25, 4.50, 4.70, or 5.00, unless otherwise indicated. Elution buffer was the same as the equilibration buffer plus 1 M NaCl. The excipients were added as 500 mM, 5% in case of PEG4000 or, instead of sodium acetate trihydrate for the ionic excipients (Arginine and Excipient A), as 40 mM concentration either in just the equilibration buffer, just the elution buffer system, or in both. Strip solution was 20 mM Na_2HPO_4*$2H_2O$ plus 1 M NaCl adjusted to pH 7.00. 0.5 M NaOH was used as a CIP-solution after every 1000 min hold experiment.
The pH values of the buffers were adjusted offline using a WTW inoLab_IDS Multi 9420 pH meter equipped with a SenTix® 940 pH electrode (both Xylem Analytics, Weilheim, Germany). All buffers were filtered using a 0.45 µm Cellulose Nitrate Filter (Sartorius, Göttingen, Germany) and degassed in an ultrasonic bath.
The linear gradient elution chromatography runs were performed on an Äkta purifier 10 or an Äkta Explorer 100, equipped with a UV detector measuring at 280 nm, a conductivity cell, and a pH electrode. If the runs were performed with temperature control, the column was placed in an HPLC column oven (column thermostat, Jetstream 2 plus, Jasco Inc., Easton, USA) set at 25°C, unless otherwise indicated.
The column was equilibrated at 180 cm/h with the specific equilibration buffer. For the experiments at low loadings, 1 mg/mL$_{CV}$ (e.g. 200 µL of 5 mg/mL solution, diluted with equilibration buffer) of the mAb solution was injected onto the column, unless otherwise stated. Following the injection of the samples, the columns were washed with equilibration buffer. Then the flow was stopped for 1000 min, unless otherwise indicated. Afterward, another wash step of 5 CV with equilibration buffer was done before elution of the antibody with a salt gradient to 1 M NaCl over 20 CV. Following the elution was a Strip and CIP step (after 1000 min experiment) to remove any residual protein bound to the resin. Second peak percentages were evaluated using the software PeakFit v4.12 (Seasolve Software Inc., Framingham, USA) and a deconvolution algorithm. The

mathematical software TableCurve 2D v.5.01 (Systat Software Inc., Richmond, USA) was used for the fitting of the first-order formation kinetics in 3.3.6.
For the experiments with the reinjection of the first and second peak fractions, the original protein load was 5 mg/mL$_{CV}$. The resulting fractions of first and second peak were buffer exchanged into the equilibration buffer using PD-10 columns (Cytiva, Uppsala, Sweden) before reinjection.

2.2.2 Aggregate determination by SE-HPLC

Size exclusion chromatography was performed with a BioSep s3000 column with a diameter of 7.8 mm and a length of 300 mm (Phenomenex, Torrance, USA). The analytical SEC was performed on an ÄKTAmicro™ system coupled to an autosampler A-905 (GE Healthcare, Uppsala, Sweden). The running buffer consisted of 0.05 mol/L sodium phosphate and 0.3 mol/L sodium chloride at pH 7.00. Fractions from the CEX chromatography runs were injected into the SEC column and eluted at a flow rate of 1 mL/min. Determination of the aggregate content was done by manual integration of the chromatograms using the Unicorn® software (GE Healthcare, Uppsala, Sweden).

2.2.3 Analytical cation exchange chromatography

The analytical cation-exchange experiments were performed on an Äkta micro (GE Healthcare, Uppsala, Sweden) with built-in 10 mL/min pumps, UV and conductivity sensors. The evaluation has been done using the Unicorn® 5.31 software package (GE Healthcare, Uppsala, Sweden). Chromatography column was a YMC BioPro® SP-F with a column length of 50 mm, an inner diameter of 4.6 mm and a column volume of 0.83 mL. The experiments were done using the fractions 1-3 of the second peak and the original mAb solution and elution in a gradient. Buffer A of the buffer system contained 8.8 mM Acetic acid, 10.0 mM MES*H_2O, 15.5 mM MOPSO, 22.5 mM NaCl, 6.5 mM NaOH with a resulting buffer pH value of 5.0. Buffer B of the buffer system contained 7.7 mM CHES, 18.4 mM HEPES, 7.2 mM TAPS and 29.0 mM NaOH with a resulting buffer pH value of 9.5. The flow was set to 0.8 mL/min and after loading and 2 CV wash and 5 CV at 25% buffer B, a gradient over 45 CV to 100% buffer B led to the elution of the antibody charge variants. To remove any still bound species, 4 CV of 100% B was done after the gradient.

2.2.4 Calculation of the resin pH value

Using the model originally described by Jansen et al. (Jansen et al. 1996), we can calculate the Donnan ratio with the following equation (1) (Wittkopp et al. 2018),

$$r_D = \frac{c^R_{Na^+}}{c^B_{Na^+}} = \frac{\Lambda^-}{2c^B_{Na^+}} + \sqrt{S_{Acetate^-,OH^-}S_{Na^+,H^+} + \left(\frac{\Lambda^-}{2c^B_{Na^+}}\right)^2} \quad (1)$$

with r_D symbolizing the Donnan ratio, $c^R_{Na^+}$ is the concentration of Na^+ ions in the resin phase, $c^B_{Na^+}$ is the concentration of Na^+ ions in the bulk phase, Λ^- is the concentration of deprotonated form of the ligand. However, since the resin prototypes are strong cation exchange resins, the ligands are fully deprotonated, therefore $\Lambda^- equals \Lambda$. $S_{Acetate^-,OH^-}$ and S_{Na^+,H^+} are the selectivity coefficients of the involved anions and

cations, respectively. The selectivity coefficients, in this case, are set to 1 for simplicity, which was also done in a previous publication (Kawakita and Matsuishi 1991).
The derivation of equation 1 neglects the H^+ and OH^- concentration. For not too low pH values $c^B_{H^+} << c^B_{Na^+} = c^B_{Acetate^-}$.
Now the pH-value in the resin pore can be calculated with

$$pH_R = pH_B - log_{10}(r_D) \quad (2)$$

It is important to note, that the ion concentrations and the ligand densities are defined with respect to the pore volume (Pedersen et al. 2003; Wittkopp et al. 2018) and the exchange reaction is between the bulk phase and the immobile phase of the pore volume, which is called Donnan volume (Wittkopp et al. 2018).

2.2.5 Aggregation propensity of mAb in solution

For the determination of aggregation of the mAb in solution, the antibody was diluted in 40 mM sodium acetate buffer at pH 3.3, 3.5, and 3.7 to a concentration of 1 mg/mL. The aggregate content was measured at different timepoints over an incubation period of 1000 min with SE-HPLC. After the 1000 min period, 2 M NaCl solution was added to the antibody to reach a final concentration of 500 mM NaCl and a protein concentration of 0.75 mg/mL. Then the monomer/aggregate ratio was again measured with SE-HPLC over timepoints of 0 min (directly after dilution), 60 min, 120 min and 240 min. SE-HPLC measurements were done according to the method described in 2.2.2..

2.2.6 Differential scanning fluorimetry using nano-DSF

The differential scanning fluorimetry (DSF) measurements were conducted on a Prometheus® NT.48 (NanoTemper Technologies GmbH, Munich, Germany). For each experiment, the capillaries were filled with quadruplicates of ~10 µL of sample with a concentration of 2 mg/mL. For the experiments with excipients, the buffers were prepared similar to the chromatography experiments and the antibody diluted to 2 mg/mL in the buffers. The capillaries were filled in triplicates. After initial temperature equilibration, the temperature gradient was done from 20°C to 95°C with a slope of 1°C/min with an excitation power at 10%. Fluorescence signals were recorded at 330 nm and 350 nm. The thermal unfolding experiments of the monoclonal antibody were carried out using the nanoDSF methodology. This method does not need the addition of an external dye but rather measures the changes in the intrinsic fluorescence intensity of the aromatic amino acid residues (e.g., tryptophan).

3 RESULTS

3.1 Elution behavior of the monoclonal antibody

As described in the introduction, a monoclonal antibody immediately eluting at pH <5.00 shows most likely a single peak. However, if the mAb was held on the column without flow for a certain time, the resulting chromatogram shows likely more than one peak. In this case, I followed the approach of Guo et al. (Guo and Carta 2015) and held the mAb on the strong cation exchange resin Fractogel® SO_3 (M) for 1000 min before eluting it in a 20 CV salt gradient to 1 M NaCl.

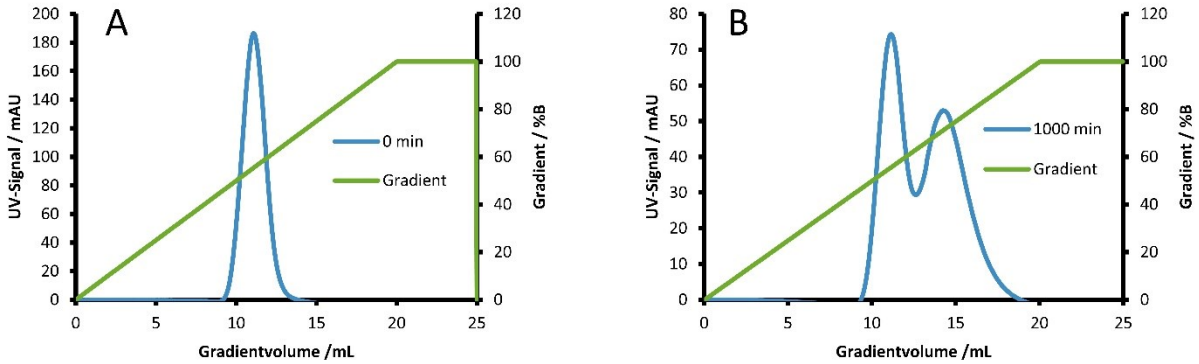

Figure 2: Elution of mAb from Fractogel® SO_3 (M) in salt gradient to 1 M NaCl in 20 CV at pH 4.70 (A) elution after 0 min hold time (B) elution after 1000 min hold time.

The resulting chromatograms with 0 min hold time (A) and 1000 min hold time (B) are shown in Figure 2. In this case a second peak is clearly visible after the 1000 min hold time.

As will be described throughout this chapter, this two-peak elution behavior has several influencing factors, including, but not limited to, bead size, excipients, pH value of the buffer system and temperature. An explanation of this phenomenon will be given by calculating the Donnan effect and its subsequent internal resin pH value, which is much lower than the buffer pH value. Furthermore, known stabilizing excipients were added at different stages during the experiment to compare effects on this unfolding and aggregation pathway to excipient effects in in-solution experiments. Since temperature control proved to be a very crucial factor for reproducibility of results, it is important to mention here, that not all of the following investigations were done under temperature control. The following chapters have experiments with temperature control: 3.3.5, 3.3.6, 3.3.7, 3.3.9, and 3.5. Nevertheless, since the other experiments were done during a relatively short period of time, they should be reproducible and comparable within each other, and the overall trend should be confirmed.

3.2 SE-HPLC analysis of 2nd peak after elution

This chapter has been published in a first author paper (Stange et al. 2022b).

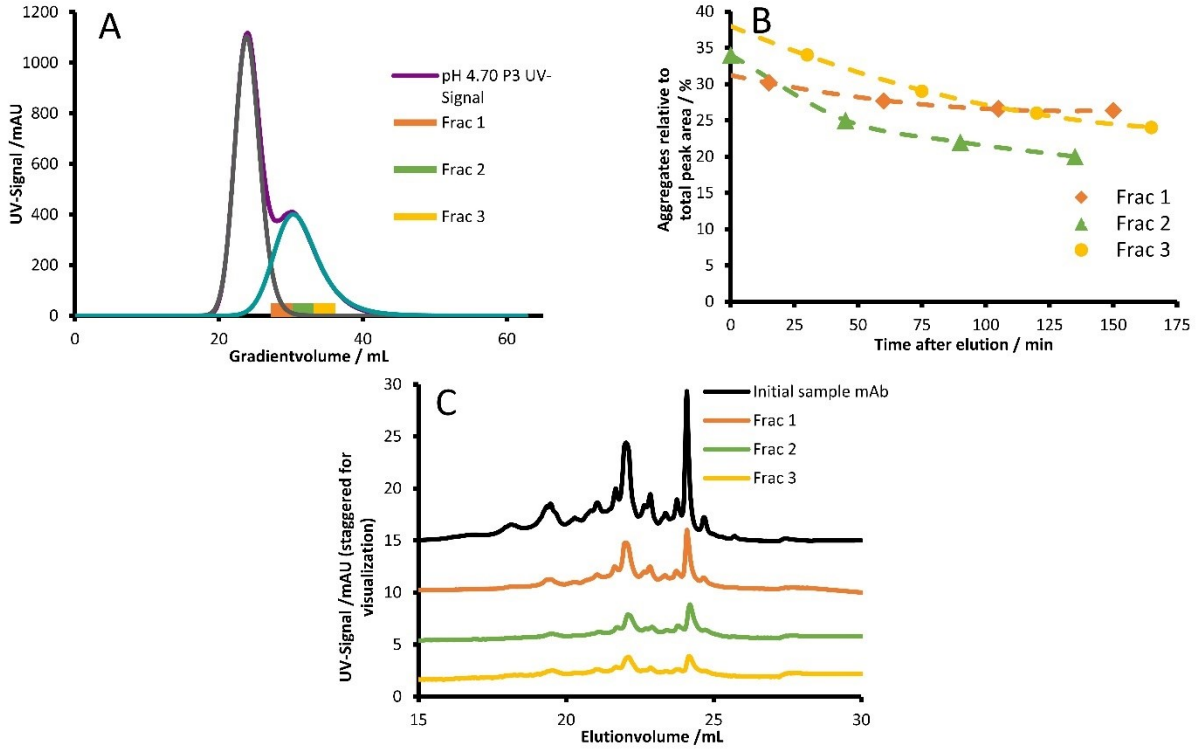

Figure 3: (A) Elution of mAb from P3 in salt gradient at buffer pH value 4.70 after 1000 min hold time. Elution was done in a 20 CV gradient to 1 M NaCl. Protein load was 10 mg/mL$_{CV}$. UV signal was recorded at 280 nm. The second peak was collected in three fractions and analyzed with SE-HPLC and analytical CEX. First and second peak were deconvoluted using PeakFit® software. (B) Aggregate content measured with SE-HPLC after elution for the three collected fractions of the second peak depicted in (A). (C) CEX analysis after elution for the three collected fractions of the second peak depicted in (A).

To characterize the second peak, the mAb was loaded onto the high ligand density prototype column (P3) and eluted after a hold step of 1000 min. This was done due to the high influence of ligand density and load density on the amount of second peak created (see 3.3.7 and 3.3.8), so that enough second peak pool could be generated for the analysis. The second peak was fractionated (see Figure 3 (A)) and analyzed after elution with SE-HPLC (in Figure 3 (B)). Fraction 2 was analyzed immediately after elution. The different times for the first data points of fractions 1 and 3 are due to the analytical delay time of about 20 min. As can be seen in Figure 3 (B) the multimer content is similar for all three fractions independent of their elution volume. Extrapolation back towards 0 min for fraction 1 and 3, shows that the first fraction contains the least multimers directly after elution, and the third fraction, which elutes the latest, contains the most multimers directly after elution.

The dominating species in the fractions 1 to 3 is a monomeric form of the mAb co-eluting with the multimers in the second peak. Deconvolution of the double peak function using the multiple peak-fit tool of the software PeakFit® (Figure 3 (A)) indicates that fraction 3 shows no peak overlap by the first peak. The monomeric species found in fraction 3 show a different elution behavior in CEX compared to the mAb monomer in the first peak. Analytical cation exchange chromatography of the fractions throughout first and second peak did not reveal different charge variant species (see Figure 3 (C)). The second peak is composed of a mixture of multimeric species (see Figure 4)

and monomeric forms. The low resolution of the LGE run does not allow a more complex deconvolution process.

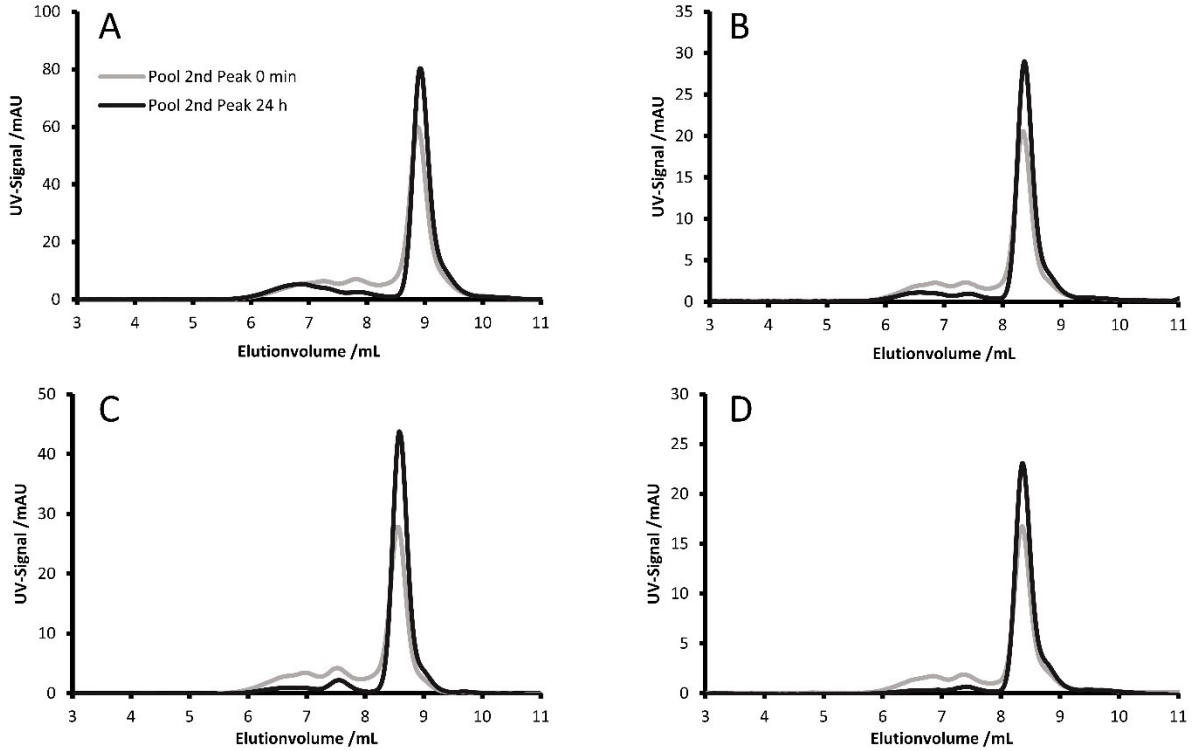

Figure 4: SE-HPLC analysis of 2nd peak pool directly after elution (grey) and after 24 hour incubation time at room temperature (black). Elution of mAb from P3 in salt gradient at different buffer pH values ((A) at pH 4.25; (B) at pH 4.50; (C) at pH 4.70; (D) at pH 5.00) after 1000 min hold time. UV-Signals were recorded at 280 nm.

The multimeric species in fraction 1 to 3 show a slow conversion into monomers over time (Figure 3 (B)). To further analyze the kinetics of multimer dissolution, the mAb was loaded onto the high ligand density prototype column (P3) for four pH buffer values 4.25, 4.50, 4.70, and 5.00 and eluted in a salt gradient after 1000 min hold time. The elution was fractionated and pooled this time according to the first peak and second peak (combined fractions 1 to 3). Measuring the aggregate content of the first peak-pool in SE-HPLC showed almost 100% monomer. The second peak-pool (Figure 4) showed 25-33% of aggregates, mostly dimers and oligomers independent of the mobile phase pH value.

Measuring the aggregate content over time after elution showed a reversal back to monomer for all four buffer pH values (Figure 5). However, the extent of reversibility of aggregates is dependent on the buffer pH values used in the experiments. For the run at pH 4.25, only dimers reverse back to monomer and most of the multimer content stays constant after 24 h (Figure 4 (A)). For the experiments done at pH 4.50, 4.70 and 5.00, both multimer and dimer species dissociate into monomers over 24 h (Figure 4 (B-D)). Experiments at pH 4.70 (see Figure 5 (B)) showed, that over the time span of a few days, the antibody solution reaches the pre-experiment aggregate content.

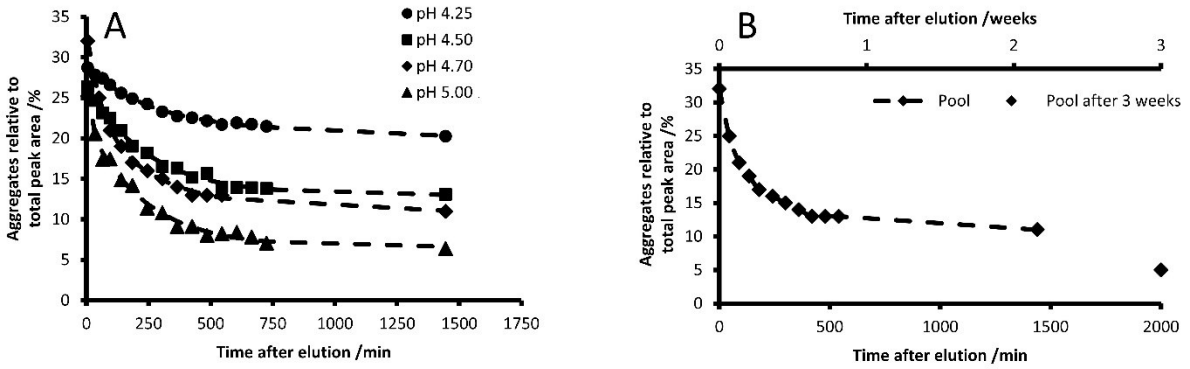

Figure 5: (A) Aggregate level of 2nd peak elution pool at different buffer pH values (● pH 4.25; ■ pH 4.50; ◆ pH 4.70; ▲ pH 5.00) in relation to time after elution. (B) Aggregate level of pH 4.70 2nd peak elution pool in relation to time after elution. The single point corresponds to the upper x-axis and represents the aggregate level after 3 weeks.

Figure 5 correlates the multimer level directly after elution and its reversal back to monomer over time. While the overall multimer level directly after elution is similar for all 4 pH values, the reversibility is clearly pH dependent. All curves show an almost biphasic behavior, as in the first 300 min after elution the multimer content decreases quickly and then more slowly after that. Especially in the case of pH 4.25, more stable aggregates are produced. For the run at pH 4.00 with the commercial Fractogel® SO3 (M) column, the aggregates appear to be even more stable, following the trend, and their content did not change significantly over 24 h (see Figure 6). It is likely that the adsorbed protein is a mixture of different conformational states that changes with pH value.

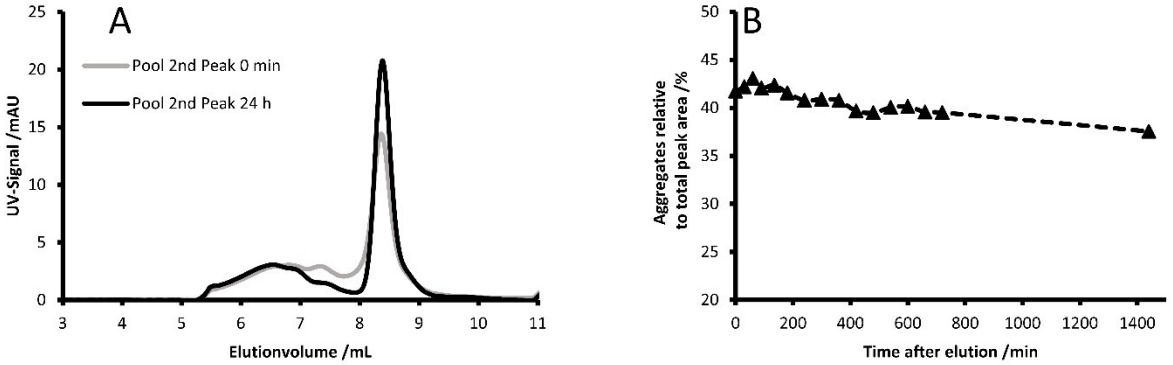

Figure 6: (A) SE-HPLC analysis of 2nd peak pool directly after elution (grey) and after 24 hour incubation time at room temperature (black). Elution of mAb from commercial Fractogel® SO3 (M) in salt gradient at buffer pH value 4.00 after 1000 min hold time. UV-Signals were recorded at 280 nm. (B) Aggregate level of pH 4.00 2nd peak elution pool in relation to time after elution.

It also should be mentioned that the antibody in the 2nd peak elution pool was injected directly onto the SEC column without dilution or buffer exchange, which means that most of the aggregates are converted to monomers even though they are incubated after elution at relatively low pH, high salt (~500 mM NaCl) concentration and at room temperature. Experiments with reinjection of the first and second peak have been done as well (Figure 7).

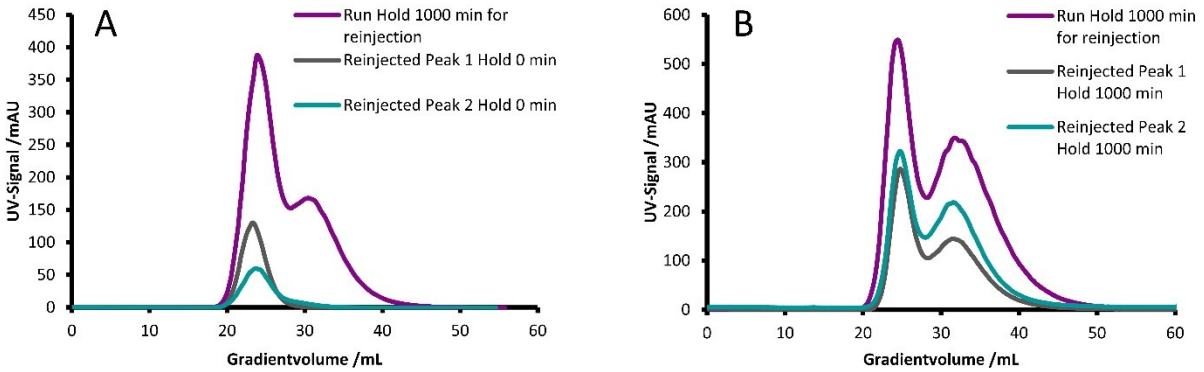

Figure 7: Elution of mAb from P3 in salt gradient at buffer pH value 4.50 after 1000 min hold time. Elution was done in a 20 CV gradient to 1 M NaCl. Protein load was 5 mg/mL$_{CV}$. UV signal was recorded at 280 nm (pink line in both graphs) (A) Blue and green lines: The fractions from first and second peak were collected after elution, buffer exchanged and then injected again to the column and eluted with no hold time in the same gradient conditions. Green line: Elution of fraction of first peak directly after buffer exchange. Blue line: Elution of fraction of second peak directly after buffer exchange. (B) Same conditions as for (A), but the reinjected fractions of first and second peak were again held on the resin for 1000 min before elution.

For these experiments, the collected fractions had to be buffer exchanged to bind to the column for re-chromatography. Both reinjections (former first and second peak separately), led to the same result, one peak at the position of the first peak. In Figure 7 (B) the mAb from the first and second peak was again held for 1000 min on the column after buffer exchange and reinjection and leads to the same elution pattern as in the other 1000 min experiments.

3.3 Influencing factors on two-peak elution behavior

As already mentioned, the two-peak elution behavior can be influenced by many different factors. This chapter is a summary of the influencing factors I investigated.

3.3.1 Bead size

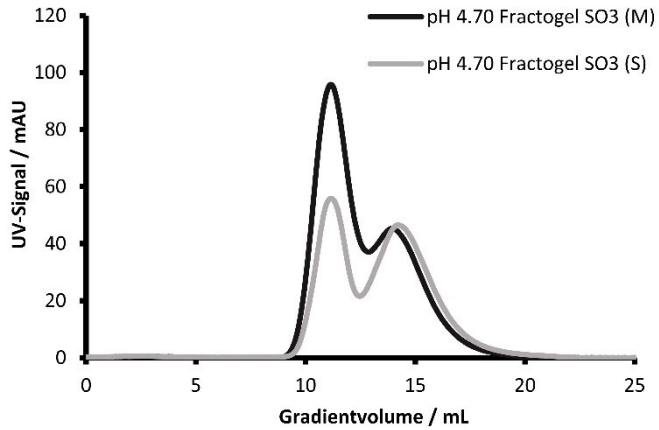

Figure 8: Elution of mAb from Fractogel® SO$_3$ (M) (black) and Fractogel® SO$_3$ (S) (grey) in salt gradient to 1 M NaCl in 20 CV at pH 4.70 after 1000 min hold time.

Figure 8 shows the difference in the two peak elution behavior for two different bead sizes but same base matrix, ligands, and ligand density. As can be seen, the smaller bead size Fractogel® SO$_3$ (S) results in a significant increase in second peak percentage and a decrease of first peak. The elution conductivities for both peaks are similar to the larger bead size. The smaller bead size seems to promote surface induced unfolding and aggregation of the mAb during the 1000 min hold time.

3.3.2 Strong/weak cation exchange resins

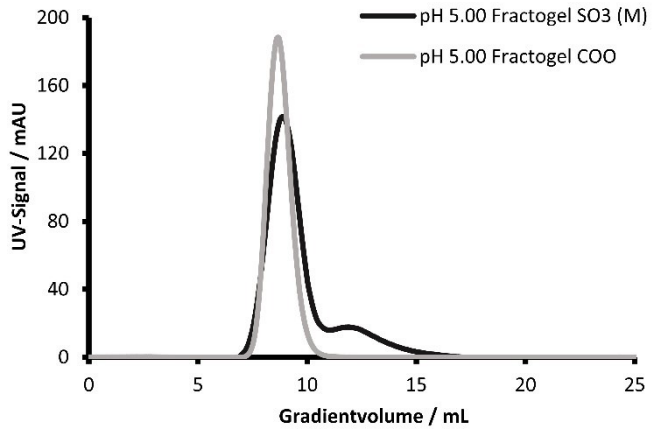

Figure 9: Elution of mAb from Fractogel® SO_3 (M) (black) and Fractogel® COO (grey) in salt gradient to 1 M NaCl in 20 CV at pH 5.00 after 1000 min hold time.

In Figure 9, a comparison between a strong cation exchange resin Fractogel® SO_3 (M) and a weak cation exchange resin Fractogel® COO is shown. A weak cation exchange ligand is negatively charged only over a relatively small pH value range and therefore can bind proteins only in this range. In this case, to ensure a fully deprotonated ligand on the COO resin, a buffer pH value of 5.00 was chosen, even though that also decreases the two peak elution phenomenon for the strong cation exchange resin. However, as can be seen in Figure 9, the weak CEX resin clearly shows a gaussian peak shape of one single peak eluting at the same conductivity as the first peak of the strong CEX resin. This indicates that the surface induced unfolding and aggregation is not promoted with the weak CEX ligand over the 1000 min hold time.

3.3.3 Ligand structure

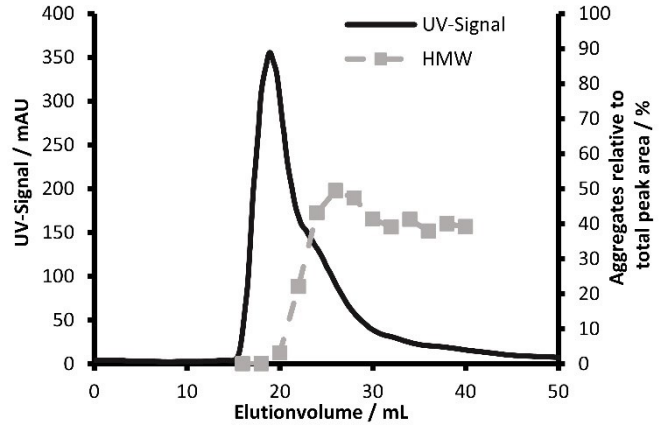

Figure 10: Elution of mAb from Toyopearl® SP-650M in salt gradient to 1 M NaCl in 20 CV at pH 5.00 after 96h hold time. The elution volume of the peak was fractionated and the aggregate content measured with SE-HPLC. The measurements were not done directly after elution but after a certain time.

Experiments were done with Toyopearl® SP-650M, which is a strong cation exchange resin and has the same ligand (SO_3) and similar properties than the Fractogel® SO_3 (M), but the main difference between the resins is the missing tentacle-structure of the ligand in the Toyopearl® resin. In Figure 10 the mAb had to be held on the resin for 96 hours before a peak shoulder was visible. This elution was fractionated and measured with SE-HPLC after some days. It can be seen from this overlay, that the first fractions

contain only monomer, comparable to the first peak elution pools sampled from the Fractogel® resin. In the apparent shoulder of the peak, the HMW content increases significantly to over 40-50%. Since the measurements were not done directly after the elution, it can be estimated that the aggregate content is most likely stable and not partially reversible, like for the Fractogel® aggregates.

3.3.4 Buffer and Counterion concentration

The original setup for these experiments was based on the parameters described by Guo et al. (Guo and Carta 2015), in that a 40 mM Sodiumacetate buffer system at a specific pH value is used. In this chapter, it was investigated how the second peak percentage is influenced by a different buffer concentration and by different counterion concentrations.

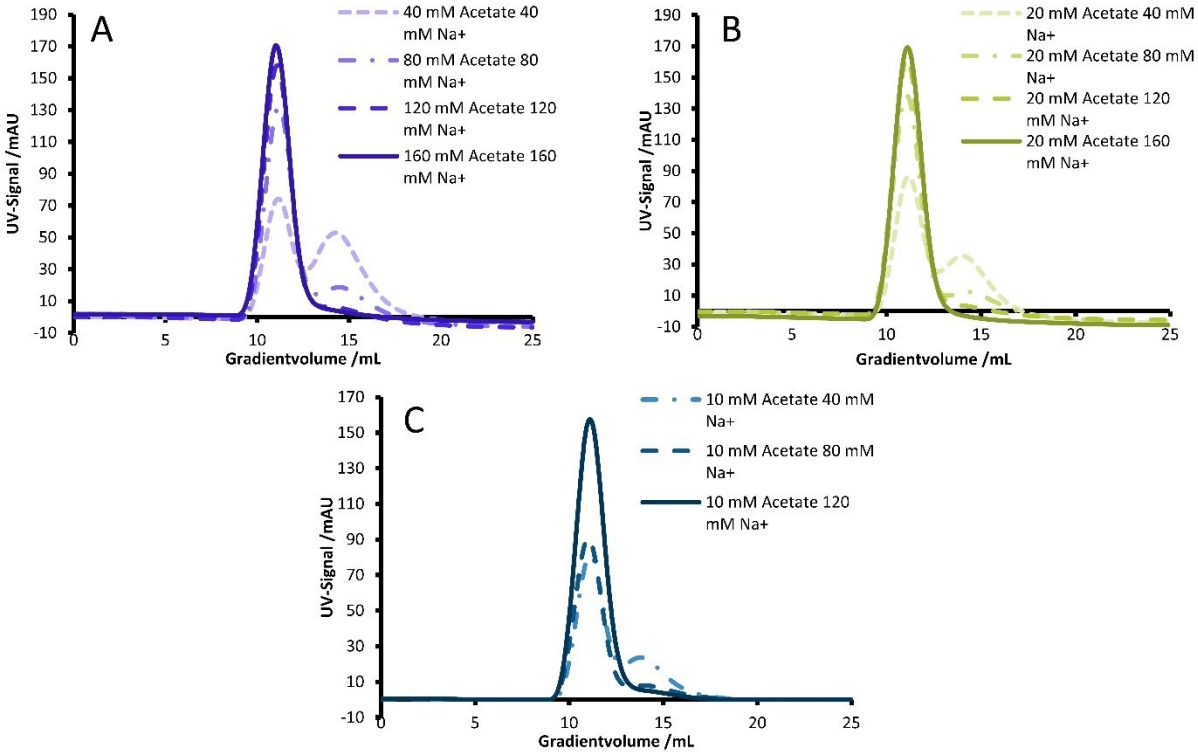

Figure 11: Influence of different buffer concentrations and different counterion concentrations during the load phase. (A) Overlay of chromatograms with increase of buffer (Acetate) and counterion (Na+) concentration. (B) Overlay of chromatograms with constant buffer (Acetate) and increased counterion (Na+) concentration. The concentration of Acetate is decreased (20 mM) compared to the reference run at 40 mM Na-Acetate. (C) Overlay of chromatograms with constant buffer (Acetate) and increased counterion (Na+) concentration. The concentration of Acetate is decreased (10 mM) compared to the reference run at 40 mM Na-Acetate and to the runs in (B). All experiments were performed on a Fractoge®I SO3 (M) column at pH 4.70.

Figure 11 shows the influence of buffer species concentration, i.e. dissociated Acetate concentration and the influence of counterion concentration, i.e. Sodium ion concentration. All runs were performed at pH 4.70 and the difference in buffer and counterion species was only used in the respective loading buffer system. For the elution buffer, the “standard” 40 mM Sodiumacetate + 1 M NaCl buffer was used. In Figure 11 the other species present in the buffers according to the buffercalculating software Buffermaker® are: Undissociated acetic acid and Chloride ions, however they are not suspected to be involved in the binding and unfolding of the antibody during the 1000 min hold time. When the buffer concentration and the counterion concentration is increased, the amount of second peak is reduced (see Figure 11 (A)). The same trend

is visible when the buffer concentration is held constant at a lower than standard concentration and just the counterion concentration is increased (see Figure 11 (B) and (C)).

3.3.5 Temperature

This chapter has been published in a first author paper (Stange et al. 2022a).

Conformational stability and aggregation of proteins are highly dependent on factors like temperature. Therefore, the column was placed into a column oven to control the temperature during the experiments.

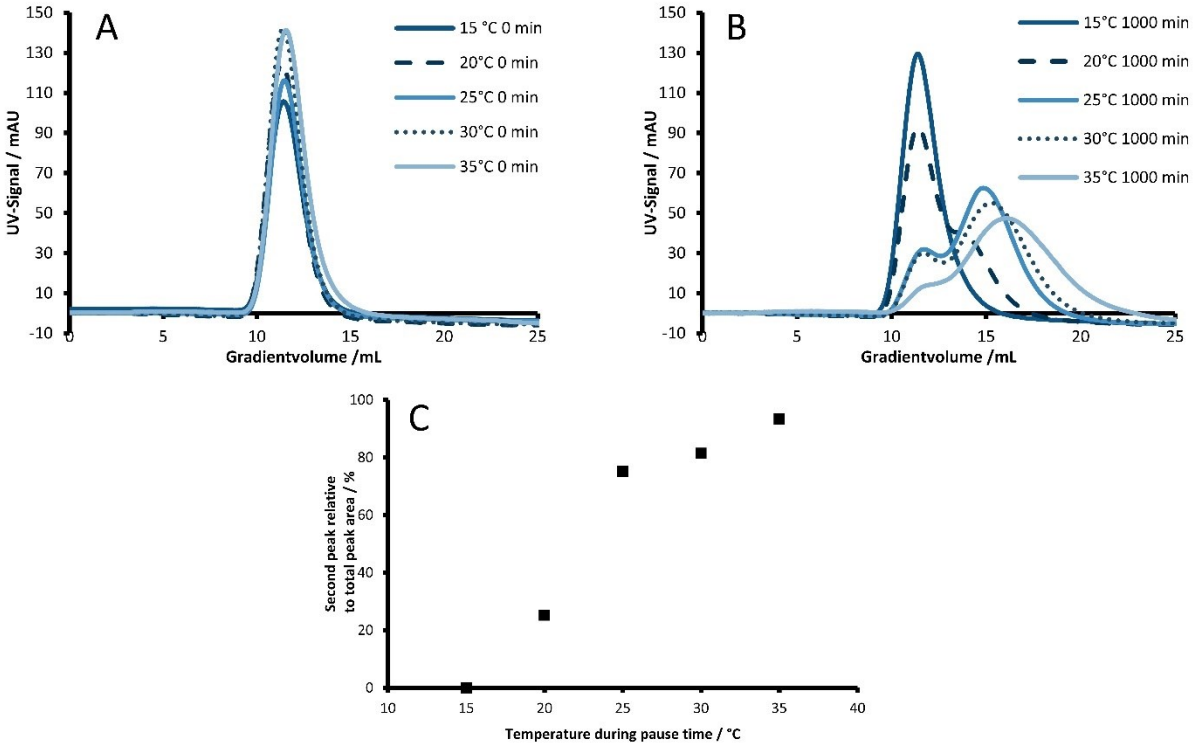

Figure 12: Elution of mAb from Fractogel® SO3 (M) in salt gradient at pH 4.70 after 1000 min hold time. (A) Elution chromatograms for mAb after 0 min hold time at different temperatures (B) Elution chromatograms for mAb after 1000 min hold time at different temperatures (C) Second peak area relative to total peak area for different temperatures.

Figure 12 shows the resulting chromatograms for 0 min (A) and 1000 min (B) hold time and the second peak percentage (C) for the different temperatures between 15°C and 35°C. Figure 12 (A) shows an overlay of the peaks resulting after 0 min hold time. All investigated temperatures show a single elution peak, with no shift of the peak maximum towards higher elution volume. For the runs at 1000 min hold time, Figure 12 (B) shows a significant difference for the different temperatures. At 15°C, a slight tailing of the first peak is visible, but no second peak is observed. Increasing the temperature up to 25°C increases the second peak percentage to 80%. In particular, the "jump" in the percentage of the second peak in the experiments between 20°C and 25°C is essential for the reproducibility of the results obtained at "room temperature". For even higher temperatures of 30°C and 35°C, the peak maximum of the second peak shifts to higher elution volumes/conductivities and exhibits more tailing. The setup with the temperature-controlled column oven resulted in a smaller deviation and higher reproducibility.

3.3.6 Hold time

This chapter has been published in a first author paper (Stange et al. 2022a).

Since mAb unfolding, refolding and aggregation and hence the second peak percentages are clearly dependent on kinetic factors like temperature, the hold time between injection and elution should be a contributing factor, too. Therefore, the two most interesting pH values were selected to determine the second peak percentages for different hold times. Figure 13 shows the experimental results for pH 4.25 (A) and pH 4.70 (B) as dots and the fitted first-order formation curve as the line.

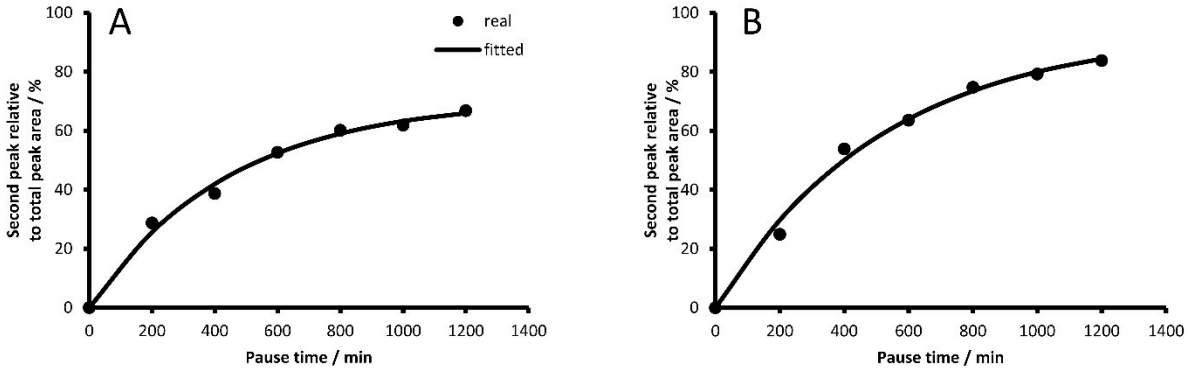

Figure 13: Kinetic development of second peak percentage (A) at pH value 4.25 (B) at pH value 4.70 at 25 °C.

Both graphs show the same trend, i.e., the longer the hold time, the larger the second peak, and even after 1200 min, the elution peak is still split into two peaks. The increase of second peak percentage is more substantial for pH 4.70, and after 1200 min, more second peak is produced.

3.3.7 Load density

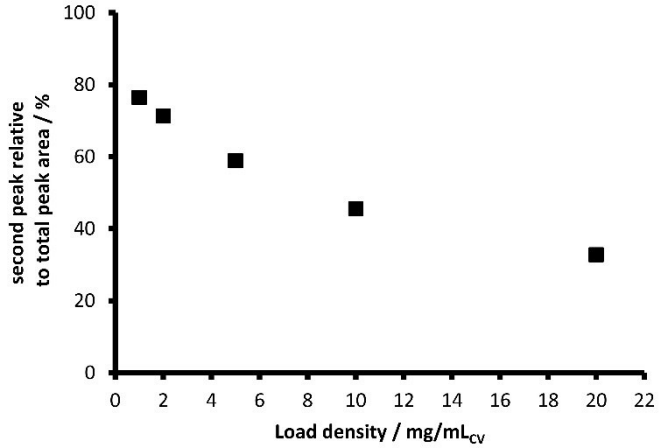

Figure 14: Elution of mAb from Fractogel® SO3 (M) in salt gradient at pH 4.70 after 1000 min hold time at different load densities (1, 2, 5, 10, and 20 mg/mL$_{CV}$).

To determine if the observed effects are only due to the low loading of protein onto the CEX column, or if they could be visible during medium to higher loadings, more mAb was loaded on the Fractogel® SO3 (M) column. After 1000 min hold time, the protein was eluted analogous to the low loading experiments. Second peak percentages were evaluated as described in 2.2.1 and graphically displayed in Figure 14. Up to a medium load challenge of 20 mg/mL$_{CV}$ second peak percentage is decreased but still visible.

3.3.8 Ligand density variations

This chapter has been published in a first author paper (Stange et al. 2022b). These experiments were done by Dr. Gabriela Sanchez-Reyes.

To determine how the ligand density of the resin influences the two-peak elution of a monoclonal antibody on a strong cation exchanger, linear salt gradient elution experiments at 5 different pH values (5.00, 4.85, 4.70, 4.50 and 4.25) and on three different prototype resins with low ligand density (Prototype 1, P1), middle ligand density (Prototype 2, P2), and high ligand density (Prototype 3, P3) were performed.

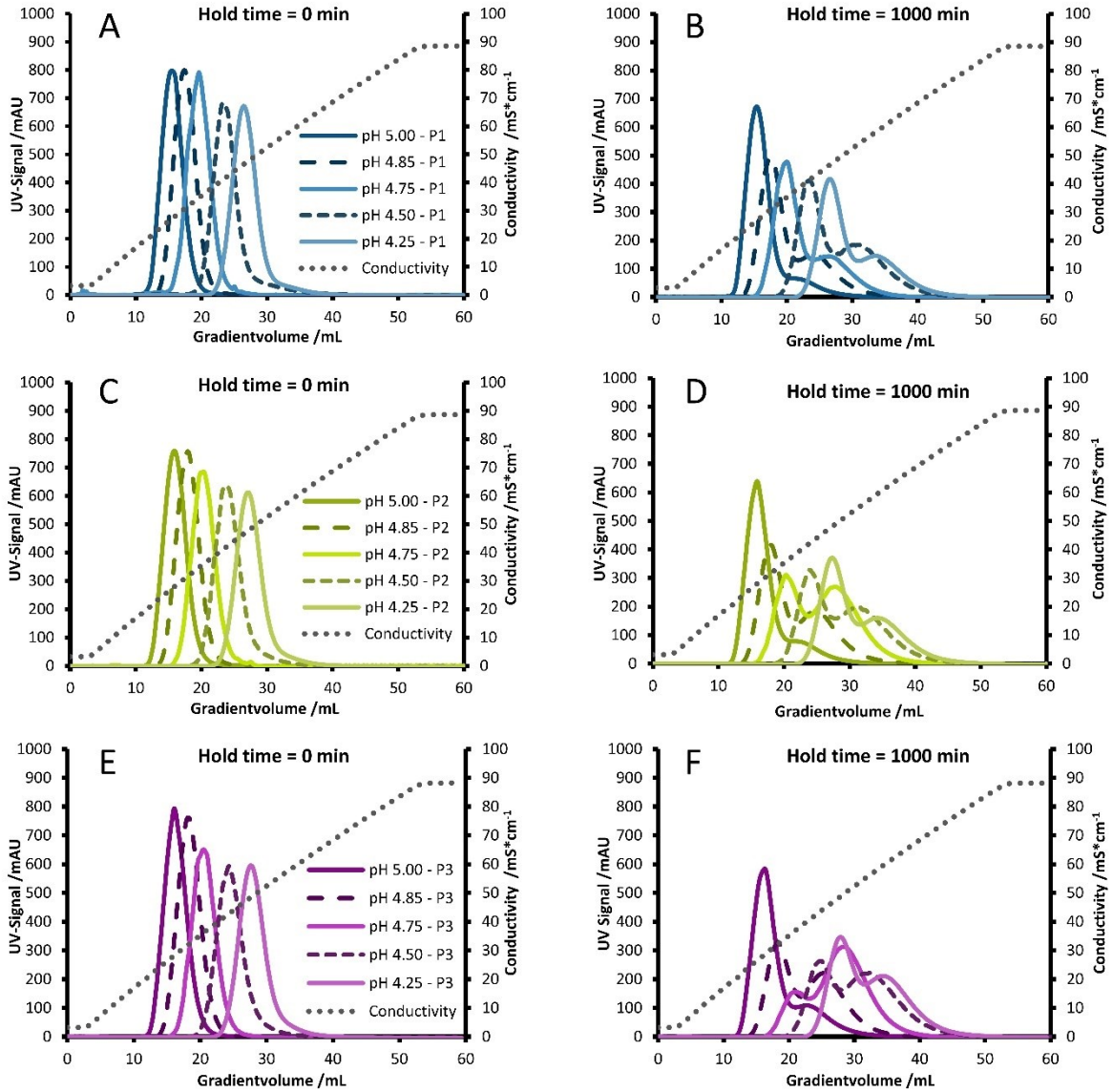

Figure 15: Elution of mAb from Fractogel® (A/B) low P1, (C/D) middle P2, and (E/F) high P3 ligand densities with 20 CV salt gradient at 5 different buffer pH-values (pH 4.25, 4.50, 4.70, 4.85, 5.00). UV-signals were monitored at 280 nm. Protein load was 1 mg/mLCV. (A/C/E) elution after 0 min hold time and (B/D/F) elution after 1000 min hold time

Figure 15 shows the chromatograms of the antibody elution after 0 min hold time (A/C/E) and 1000 min hold time (B/D/F) on the resin with the three ligand density prototypes at different buffer pH values. When the protein is eluted directly after the load/wash step, only a single peak within the elution salt concentration of 270-440 mM is visible. With decreasing pH of the mobile phase buffer, a higher salt concentration is needed to elute the protein off the column. For the 1000 min hold time

chromatograms a second peak is visible for all pH values and ligand density prototypes but the area of second peak differs. It can be observed that the higher the ligand density, the more second peak is produced after 1000 min.

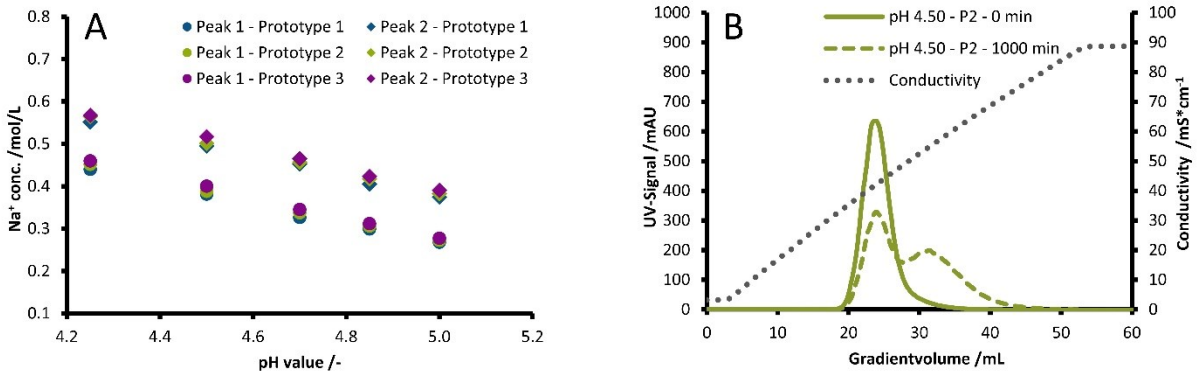

Figure 16: (A) Elution Na+ -ion concentration of the first and second peak for the 5 different pH values 4.25, 4.50, 4.70, 4.85 and 5.00 with the three prototypes P1, P2, and P3. (B) Elution behavior of mAb after 0 min and 1000 min hold time from prototype P2 at pH 4.50. UV-signal was recorded at 280 nm. This graph is representative for the other pH values and the other prototypes.

As expected for cation exchange chromatography, the lower the mobile phase pH value, the higher the counterion concentration that is needed for elution of the first and second peak species (Figure 16 (A)). The elution of both peaks slightly depends on ligand densities (Figure 16 (A)) with the highest ligand density leading to the strongest increase in the eluting salt concentration. No influence of the hold time on the elution Na^+ -concentration of the first peak is observed (Figure 16 (B)).

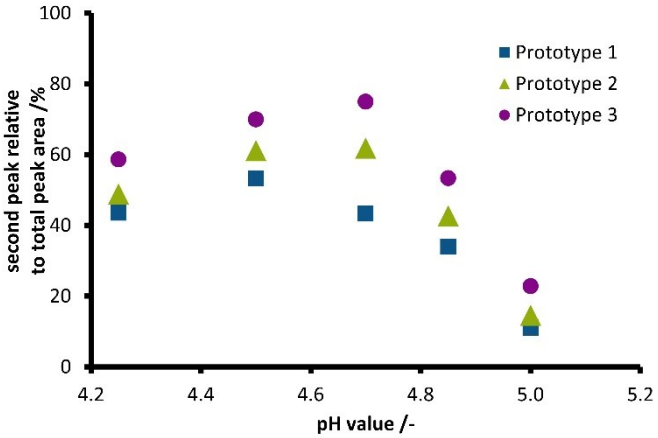

Figure 17: Second peak area relative to total peak area for the three different ligand density prototypes at 5 different buffer pH values

Figure 17 displays the dependency of second peak percentage and buffer pH value for the three prototypes. The highest ligand density creates overall the largest second peak, whereas the lowest ligand density is responsible for the least second peak percentage. The largest second peak area is created at pH 4.70 for the middle and high ligand density resins and at pH 4.5 for the lowest ligand density. However, the bell-shaped correlation is similar for all three prototypes. At pH values above pH 5 the two-peak elution behavior is no longer observed for the low and middle ligand density and to a marginal extend for prototype 3.

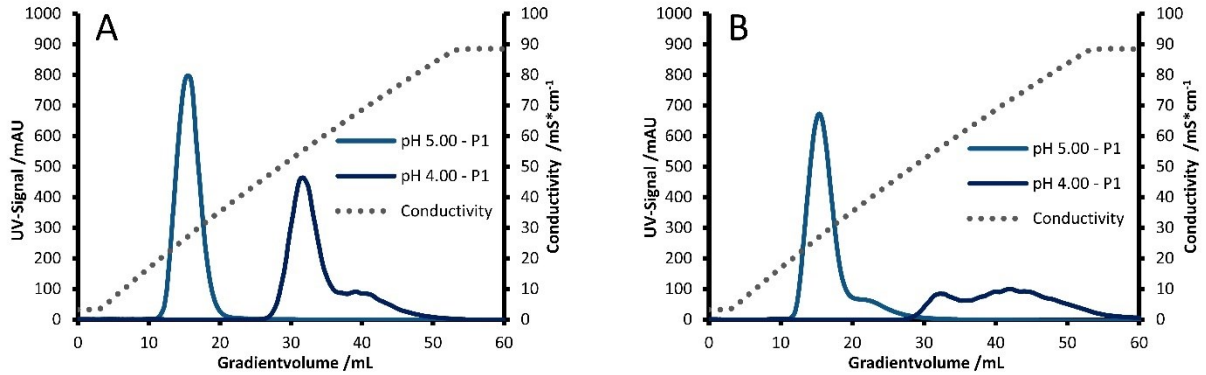

Figure 18: Elution of mAb from prototype low P1 ligand density with salt gradient at different buffer pH-values. (A) elution after 0 min hold time and (B) elution after 1000 min hold time. The UV-Signal was recorded at 280 nm.

As can be seen in Figure 18 (A), a lower pH value than 4.25 produces a second peak already at 0 min hold time with the lowest ligand density resin. After 1000 min hold time at pH 4.00 the mAb eluted with a strongly tailing second peak reaching the end of the gradient. The yield of antibody, which could be eluted with the salt gradient, is reduced to 60%. A sodium hydroxide CIP step is required to wash the presumably aggregated mAbs from the column. For the middle ligand density, this trend was confirmed, as even less antibody could be recovered from the column after the hold step (15%) (Figure 18 (B) dark blue). For the highest ligand density prototype, the run at 0 min hold time showed no recovery of the antibody during the gradient. This clearly indicates that buffer pH value of 4.25 represents another point of interest, as the resulting on-column unfolding, multimerization and aggregation shifts to irreversibility (see Figure 5). Full mAb recovery during elution is possible at pH above 4.25 only.

3.3.8.1 Donnan effect

This chapter has been published in a first author paper (Stange et al. 2022b). These experiments and the calculations were done by Dr. Gabriela Sanchez-Reyes.

As the mAb shows no aggregation in solution at pH values > 4 (see Figure 25), the two peak elution and aggregation is related to the conditions during the bind/elute steps in the chromatography setup. Most publications attribute the two peak elution behavior to the interaction of the proteins with the functional groups on the surface of the stationary phase (Chen et al. 2016; Gillespie et al. 2012; Guo et al. 2016). The conditions in the pore network and near the surface are assumed to be identical to the mobile phase conditions.
The Donnan ion exchange (DIX) model does not assume complete exclusion of co-ions, which results in concentration differences of charged ions between the resin and the bulk phase. It can be used to calculate the internal resin pH value, which is also dependent on the ligand density of the resin (F. G. Donnan 1911; Gregor 1951; Helfferich 1995; Jansen et al. 1996; Jansen et al. 1997; Lendero et al. 2008; Ståhlberg 1999; Wittkopp et al. 2018). For our antibody and chromatographic setup, the model predicts a significantly lower pH value in the pore phase compared to the bulk phase, see Figure 19 (A).

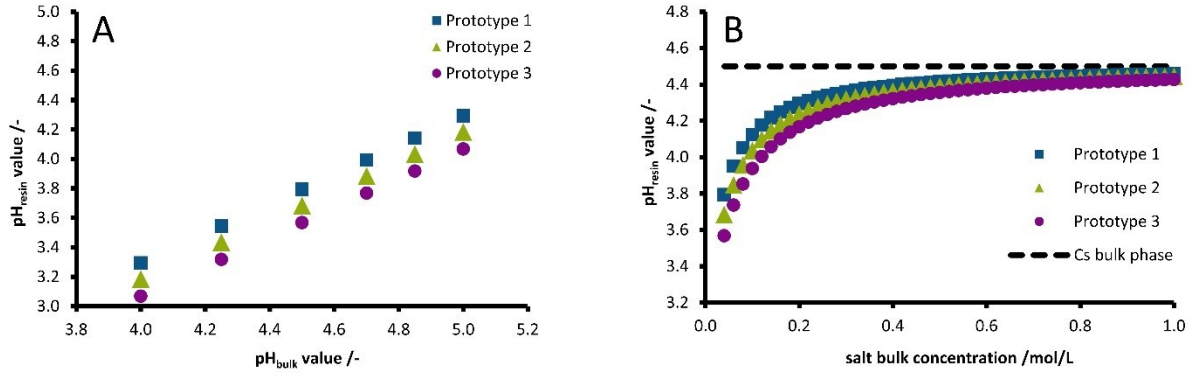

Figure 19: (A) Calculation of the resin pore pH value in relation to the bulk pH value for the three prototypes. (B) Calculation of the resin pore pH value in relation to the bulk salt concentration at bulk pH value 4.50 for the three prototypes.

The high ligand density prototype results in the largest difference between bulk pH value and resin pore pH value and vice versa for the low ligand density resin prototype (Figure 19 (A)). And since the effect is salt-dependent, it is more significant at low salt concentrations, which is the case in the load buffer during the 1000 min hold time (Figure 19 (B)). For the lowest pH-value tested on the column it means, that if the buffer was adjusted to a pH value at 4.25, the pore pH value is almost 1 pH-unit lower at around 3.3.

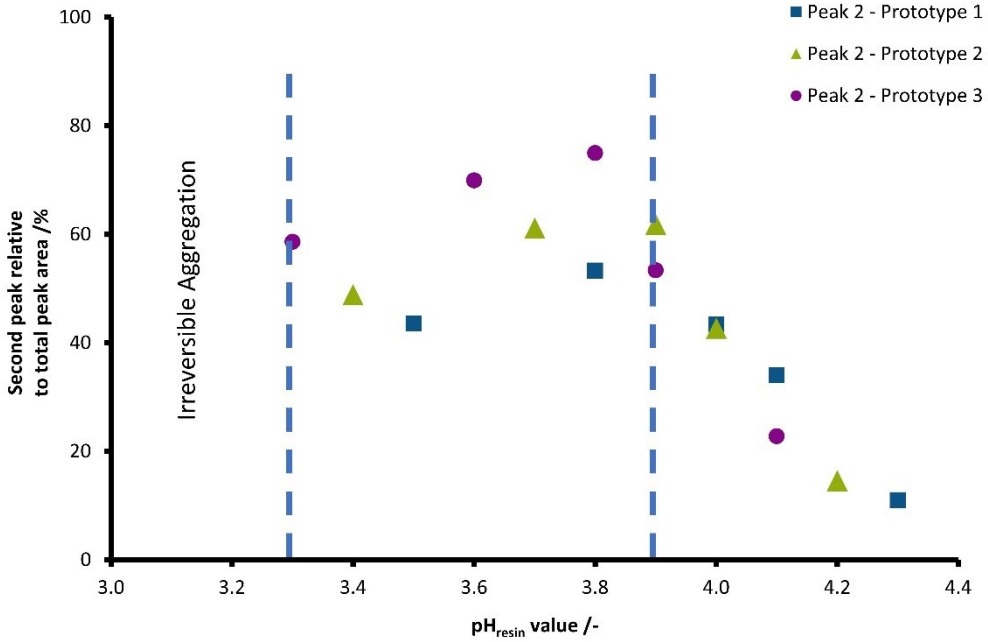

Figure 20: Second peak area relative to total peak area for the three different ligand density prototypes in relation to the pH value in the resin pores

Figure 20 shows the correlation between resin pore pH-value and the percentage of second peak area. An almost linear relationship is visible for the experiments with a pore pH-value above 3.9 and the extent of second peak formation primarily depend on the internal pH during the hold time. Between pH 3.3 and pH 3.9 the percentage of second peak is ligand density dependent and the highest ligand density produces the most second peak.
Around pH 3.3, which corresponds to a buffer pH value of ~4.25, the antibody shows irreversible on-column aggregation with ligand density dependent decrease of mAb recovery during elution. Even after 0 min hold times, a second peak can be observed and the resulting yields are decreased, as can be seen in Figure 18.

3.3.9 Excipients

This chapter has been published in a first author paper (Stange et al. 2022a).

As can be seen in the previous chapters, the two peak elution behavior is a complex phenomenon with several crucial influencing factors. To investigate excipients on this phenomenon, all the aforementioned factors were kept constant, including temperature.

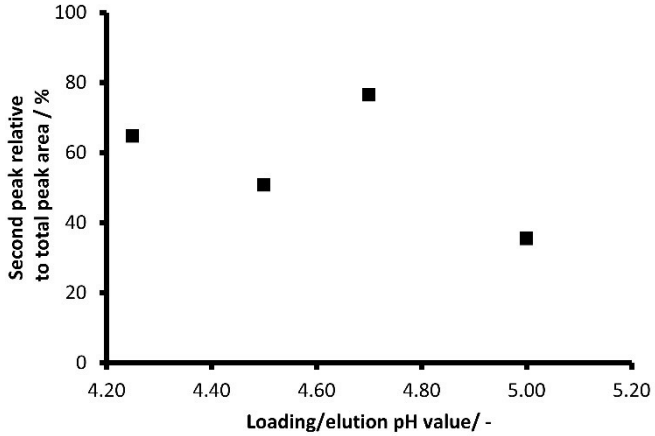

Figure 21: Second peak area relative to total peak area for 4 main buffer pH values (pH 5.00, 4.70, 4.50, and 4.25)

Figure 21 shows the percentage of second peak created when the pH value during the load and elution phase without excipients is changed. The most amount is created at pH 4.70, but at pH 4.50 and 5.00, the second peak is decreased, and at pH 4.25, second peak percentages increased again. It shows that the underlying unfolding and aggregation events are very dependent on the pH value (external and internal) and are very complex in nature. Reproducibility of the second peak percentage for pH 4.70 was recorded over several months as repeat experiments between the regular screening experiments. The experiments shown with temperature control are very reproducible, and the overall standard deviation is less than 5% (based on 15 experiments conducted over several months).

Since the 1000 min hold time results in unfolding/aggregation of the mAb due to the low internal resin pH value, excipients should influence this phenomenon, comparable to conventional in-solution stability and aggregation studies. Therefore, excipients were added at three stages during the experiment. Either they were added only during the load and hold phase, and elution was done without them at the same buffer pH value. Alternatively, they were washed into the column after the hold time, and the antibody was eluted in the presence of the excipients. Or the excipients were present during load, hold, and elution.

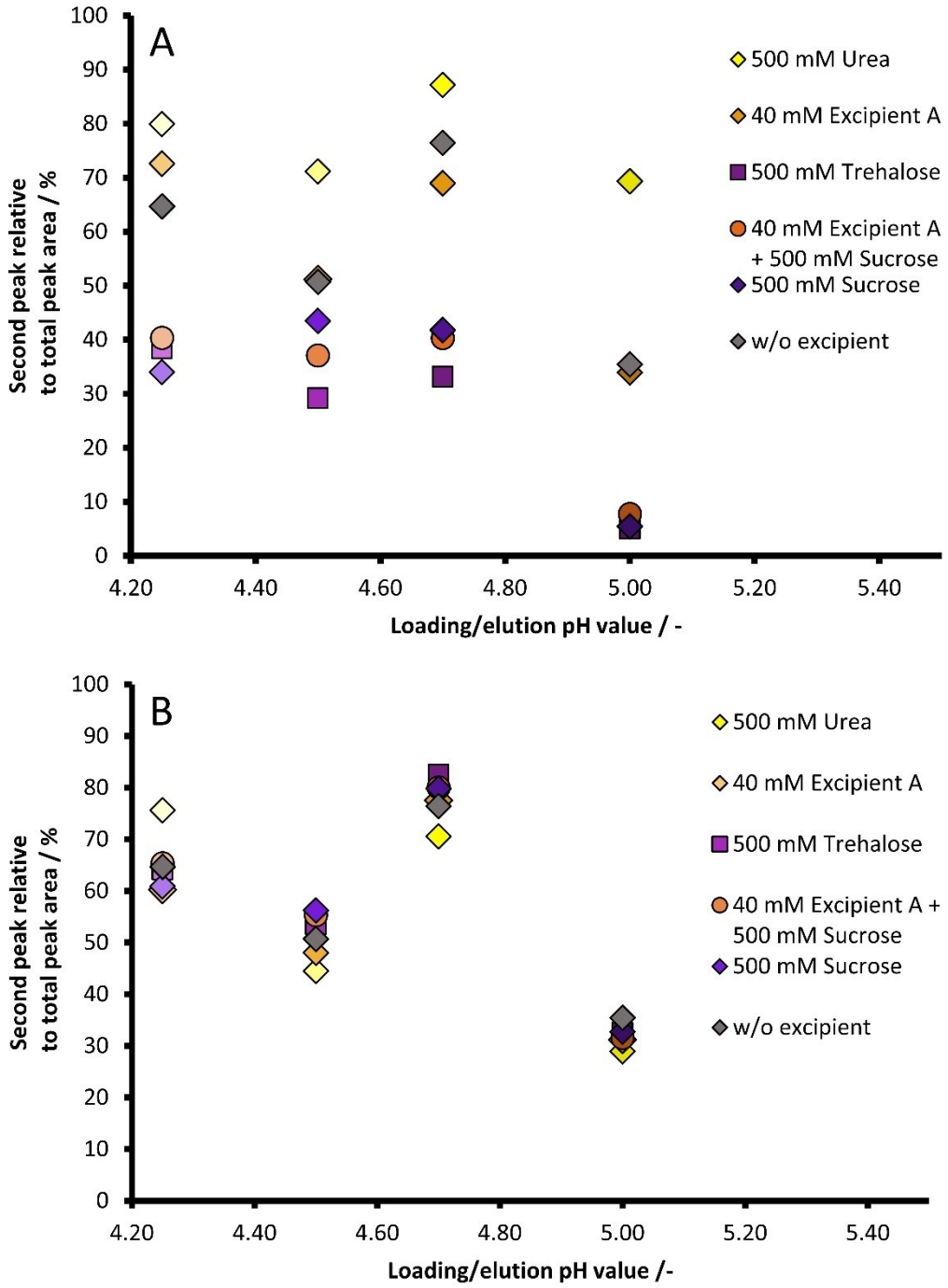

Figure 22: Influence of excipients at different pH values when (A) added only into the load buffer and (B) added only into the elution buffers. Some data points are similar to each other and may therefore not be clearly visible.

Figure 22 shows the resulting second peak percentages for five excipients or combinations if they were either present only (A) during the load and hold phase or (B) only during the elution. It shows that the presence of excipients during the load and hold phase has a stronger influence on the amount of second peak percentage than their presence in the elution buffer system. This has also been observed in other studies (Guo et al. 2016). However, the overall trend between the four buffer pH values stays the same with the addition of the excipients. Clearly, this is another indication that the conditions the mAb is subjected to during the hold time are the major contributors to the second peak occurring.

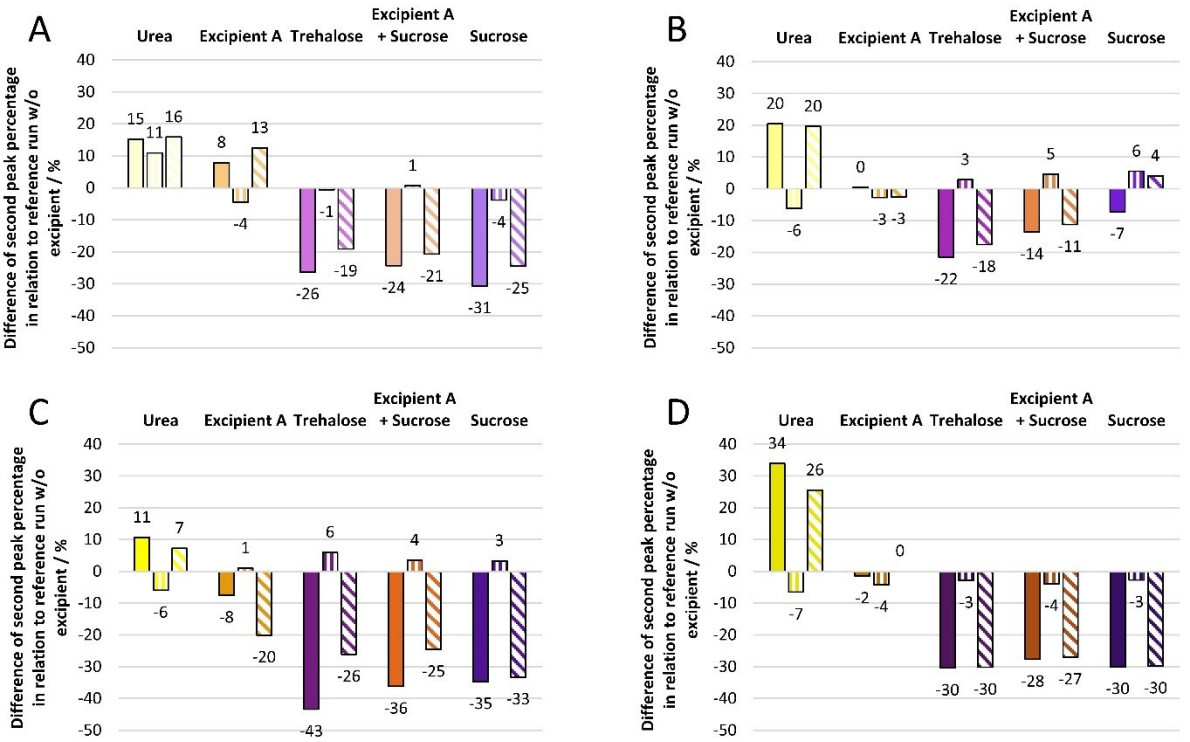

Figure 23: Influence of excipients when added only into the load buffer (full color), only into the elution buffers (striped color), or in both load and elution buffer (cross striped color). The reference (0) is the run without any excipients in load and elution buffers. Negative values indicate less second peak percentage compared to the reference run. If the bar shows a positive value, there is more second peak percentage than the reference run. (A) runs were done at buffer pH value 4.25, (B) pH 4.50, (C) pH 4.70, and (D) pH 5.00.

Looking more closely into the effects of the excipients when added during either of the three possibilities, Figure 23 shows the resulting second peak percentages compared to the runs without excipients. In this case, negative values show a reduction of second peak percentage and, therefore, a stabilization of the antibody, and a positive value shows an increase in second peak percentage, which is equal to further destabilization of the mAb. The full-color bars show the impact of the excipient when it is added only during the load/hold phase; the striped color bars represent the impact when the excipient is added only during elution, and the cross-striped color bars stand for the impact when the excipient was present during the whole experiment (from left to right for each excipient). The most significant effect can be achieved when the excipient is either present only during the load and hold phase or if it is present during all stages of the chromatography run. Furthermore, the pH values 4.25 (A) and 4.70 (C) show overall the biggest impact of the excipients on the amount of second peak created.
As a known destabilizing agent, Urea results in a larger second peak at all pH values when present during the hold step. Excipient A has a small impact overall, which could be because it is only used as a counterion in a concentration of 40 mM to replace sodium in the sodium acetate buffer system. The sugars Trehalose and Sucrose and the combination of Excipient A and Sucrose, most probably due to the impact of Sucrose in this case, show stabilizing abilities for all pH values and almost all conditions tested in these experiments. Especially at pH 4.70, Trehalose can reduce the amount of second peak by over 40% when added only during the load and hold phase. However, none of the stabilizing excipients could completely inhibit the unfolding and aggregation during the low-pH incubation on the column.
The effect of the excipients during the elution step only is small and shows a different pH dependency compared to the presence during the hold step. At intermediate pH

values of 4.5 and 4.7, the excipients (except Urea) increase the second peak, while a lower percentage of the second peak is observed at the other pH values.

Since the excipients were used at a specific concentration for the screening experiments, it was also worth looking into the effect of different concentrations. Figure 24 shows the influence of different Sucrose concentrations on the second peak percentage for the two most influential pH values, 4.25 and 4.70.

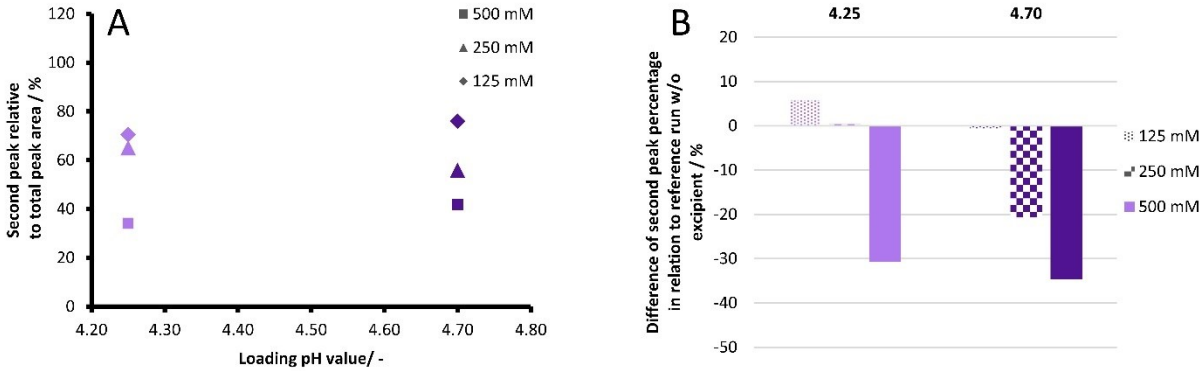

Figure 24: Influence of Sucrose when added only into the load buffer at different concentrations (125 mM, 250 mM, and 500 mM). (A) second peak area relative to the total peak area. (B) Bar chart of second peak relation. The reference (0) is the run without any excipients in load and elution buffers. If the bar shows a negative value, there is less second peak percentage than the reference run. If the bar shows a positive value, there is more second peak percentage than the reference run. The runs were done at buffer pH values 4.25 and 4.70.

There is a clear correlation between second peak percentage and therefore stabilizing effect and concentration. The chosen concentration of 500 mM for Sucrose has the biggest impact for both pH values and the tested concentrations. Interestingly, the concentration effect is strongly pH dependent. At pH 4.70, 250 mM Sucrose reduces second peak formation, while at pH 4.25, it shows no effect, and 125 mM Sucrose even promotes second peak formation at pH 4.25.

3.4 Stability and reversibility studies of the monoclonal antibody in solution

So far, it was shown that the calculated low internal resin pH values have a significant effect on the unfolding and aggregation in cation exchange chromatography in combination with several other influential factors. However, it is important to understand if this is actually a surface-induced unfolding phenomenon or if these effects can be replicated by the protein in solution. This chapter will describe the effects of the calculated low internal resin pH value on the conformational stability of the protein.

3.4.1 Aggregation of mAb in solution at low pH

This chapter has been published in a first author paper (Stange et al. 2022b).

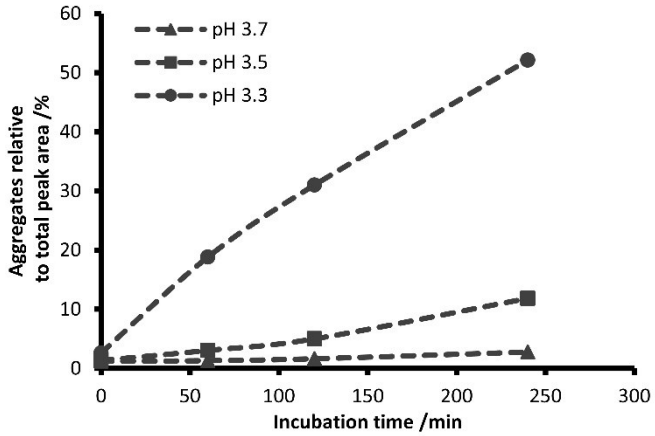

Figure 25: SE-HPLC measurements of mAb in solution at different pH values. Incubation of mAb for 1000 min with buffer A at pH 3.7, pH 3.5 and pH 3.3 and then addition of 500 mM NaCl to incubated solution and SE-HPLC measurements of mAb solutions at different timepoints. Protein concentration after dilution with 2 M NaCl solution is 0.75 mg/mL.

Size exclusion measurements were used as a tool to investigate if the observed reversible aggregation is only due to the low internal resin pH value or if the binding of the mAb to the resin ligands plays a significant role. When the antibody is incubated at low pH (pH 3.3, pH 3.5, pH 3.7) and a comparable salt concentration of the CEX during the 1000 min hold step, aggregation is minimal. Afterwards 500 mM NaCl buffer (comparable salt concentration of elution in CEX) was added to the mAb solutions. In a timeframe close to the column experiments for elution, the mAb showed pH dependent aggregation kinetics (Figure 25). The lower the pH value, the higher the aggregation rate. No aggregation of the mAb at pH > 4 in the absence or presence of NaCl was observed.

3.4.2 Nano-DSF measurements at different pH values

This chapter has been published in a first author paper (Stange et al. 2022b).

Thermal unfolding experiments were carried out using a label-free differential scanning fluorimetry approach (nanoDSF). The antibody showed multiple unfolding transitions in the plot of the fluorescence versus temperature.

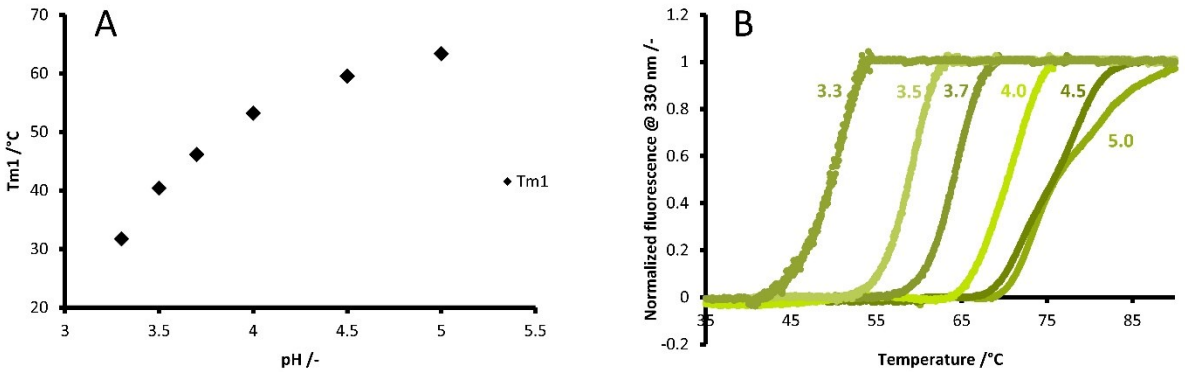

Figure 26: (A) dependence of Tm1 unfolding transition midpoints on pH value (B) Temperature-induced normalized fluorescence measurements (330 nm) in regard to temperature at different pH values

Figure 26 (A) shows the relationship between the temperature of the first transition (Tm1) and the corresponding pH-value of the solution. The relationship is non-linear, with a strong decrease of Tm1 starting at pH value smaller than 4. At higher pH values the Tm1 values seems to reach a plateau value and display constant thermal stability. The Prometheus® system and the accompanying evaluation software uses the fluorescence ratio to determine Tm values, since especially the first transition is not always visible for IgGs. The normalized fluorescence signals in Figure 26 (B) shows further transition(s) of the partially folded antibody to the unfolded protein. For pH 5.00 there are two clear transitions visible in the curve and the transitions occur over a wide temperature range of about 20 °C. If the solution pH is decreased these transitions start to overlap and at pH < 4 only a single transition is visible. Additionally, the transitions have different slopes, indicating that the antibody unfolds in a more cooperative manner with less populated unfolding intermediates. At pH 3.3 the temperature range for unfolding is 5 °C and the antibody is fully unfolded above 55 °C.

3.4.3 Influence of excipients on the reversibility of aggregates

So far, excipients were only added in the buffer systems for load or elution or both and the amount of second peak created was compared. However, these excipients could also potentially influence the amount of aggregates and their reversibility after elution. Therefore, a series of experiments have been performed at different pH values and at different addition points for the excipients.

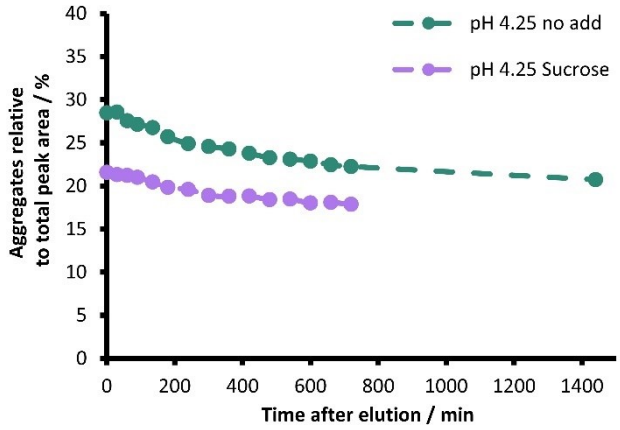

Figure 27: Aggregate level of 2nd peak elution pool at pH 4.25 in relation to time after elution. Green: No addition of excipients in elution buffer; Violet: Addition of 500 mM Sucrose in elution buffer system.

In Figure 27, pH 4.25 was chosen as the buffer pH value, since it has shown in the experiments without excipients, that the resulting aggregates are only reversible to a limited extent. In this case the result without excipient at this pH value is shown and compared to the addition of 500 mM Sucrose in the elution buffer system. Sucrose was added at this point, because in the on-column experiments (see 3.3.9), the addition of excipients did not result in a change of second peak percentage. As can be seen from this figure, the amount of aggregates created in the second peak is reduced with the addition of Sucrose. However, the reversibility is similar to the experiment without excipient. It shows, that for this buffer pH value, the effects on the amount of aggregates is small and the effect on the reversibility is negligible.

The second influential buffer pH value was determined to be at pH 4.70. In this case, the Sucrose was added after the elution in the resulting second peak elution pool. Furthermore, it was added as a solid powder to a resulting concentration of 500 mM and the falcon tube was inverted carefully to dissolve the Sucrose before measuring the aggregate content with SE-HPLC.

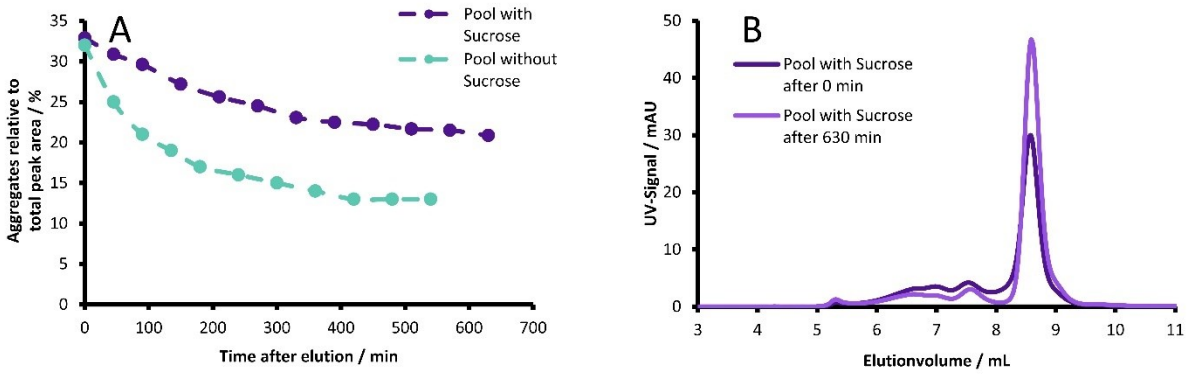

Figure 28: (A) Aggregate level of 2nd peak elution pool at pH 4.70 in relation to time after elution from Fractogel® SO3 high ligand density P3 prototype. Green: No addition of excipients in elution pool; Dark Violet: Addition of 500 mM Sucrose after elution in second peak elution pool. (B) SE-HPLC chromatogram overlay of pool after addition of Sucrose in powder form after 0 min and after 630 min.

As can be seen in Figure 28, the initial aggregate content of both experiments is similar, but the reversibility of these aggregates is significantly slowed down with the addition of Sucrose. In the SE-HPLC chromatograms, a small peak at high molecular weights was seen for the experiment with Sucrose. So far, it was unclear, if the stabilization of aggregates was due to the mixing of Sucrose powder with the elution pool or if it was due to the overall stabilizing activity of Sucrose.
Therefore, Sucrose, and other influential excipients from the on-column experiments (Arginine and Glycine), were added or substituted, in the case of Arginine, into the elution buffer systems at pH 4.70 for the same reasons stated at the beginning of this chapter.

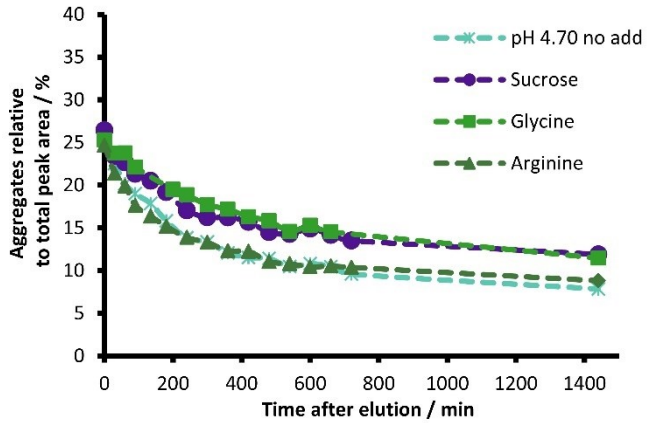

Figure 29: Aggregate level of 2nd peak elution pool at pH 4.70 in relation to time after elution. Mintgreen: No addition of excipients in elution buffer; Dark Violet: Addition of 500 mM Sucrose in elution buffer system; Green: Addition of 500 mM Glycine in elution buffer system; Dark green: Substitution of 40 mM Arginine for Sodium in elution buffer system.

Figure 29 shows the comparison of reversibility for the three excipients and without excipients. For all conditions tested, the amount of aggregate created during the 1000 min hold time on the column is similar. While the addition of 500 mM Sucrose and 500 mM Glycine seems to slow the reversibility of the aggregates down to some extent, the substitution of 40 mM Arginine in the elution buffer system has no visual effect. This somewhat confirms the observations made in the previous figures, that Sucrose, and apparently Glycine do not just have a stabilizing effect on the monomer during the 1000 min hold time, but also have a similar effect on the resulting aggregates.

3.4.4 Influence of excipients on the stability of mAb in solution at low pH

Results for this specific topic can be found in chapter 3.5.

3.5 Comparison of excipient effects on mAb in solution and during on-column unfolding/aggregation

This chapter has been published in a first author paper (Stange et al. 2022a).

In the previous chapter, it was shown that the monoclonal antibody is significantly destabilized at low pH values. Since excipients can stabilize the protein to some extent during the on-column experiments, they should have an influence on the stability of the mAb at low pH values in solution. This was tested again with nano-DSF. A comparison of all the excipients tested during the on-column experiments and complementary in-solution experiments is shown in this chapter.

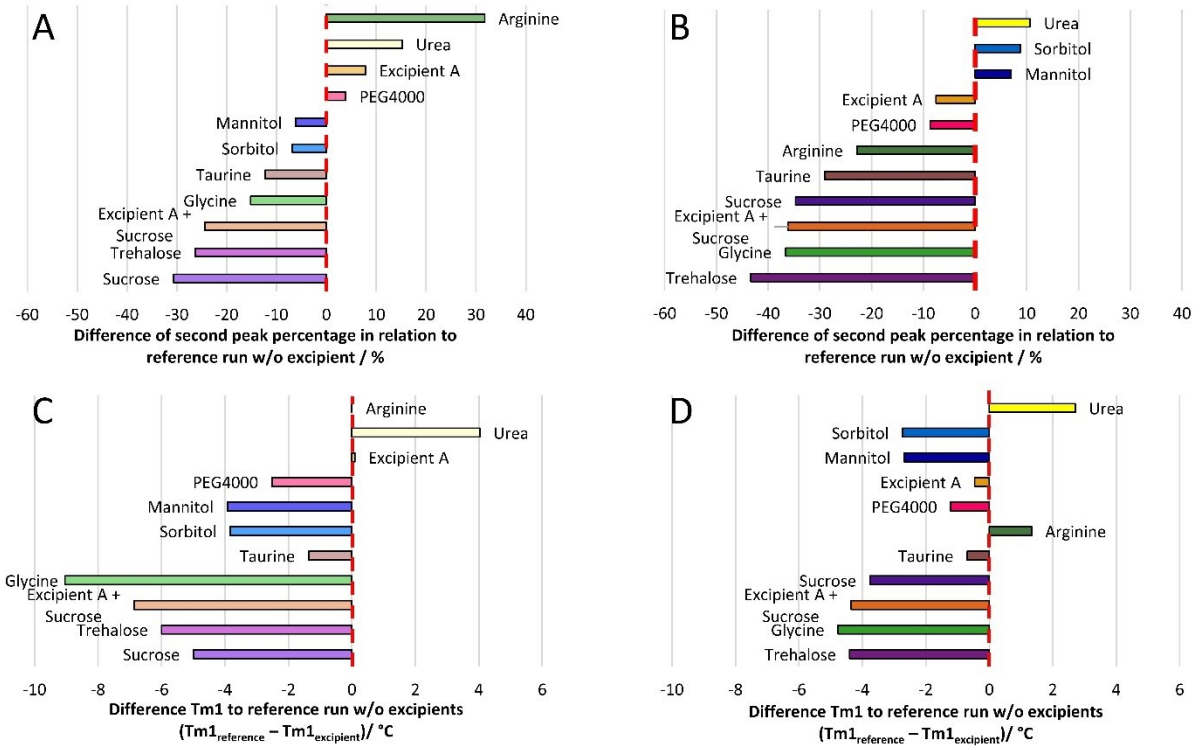

Figure 30: Influence of excipients on the two peak elution behavior and nanoDSF measurements. (A) and (B) show the amount of second peak created in comparison with the reference run without excipients at (A) pH 4.25 (internal resin pH value of ~3.3) and (B) pH 4.70 (internal resin pH value of ~3.7). The excipients were only added to the load buffer. The reference (0, red line) is the run without any excipients in load and elution buffers. If the bar shows a positive value (facing right), there is more second peak percentage than the reference run. If the bar shows a negative value (facing left), there is less second peak percentage compared to the reference run. (C) and (D) shows the Tm1 values in comparison with the reference experiment without excipients at (C) pH 3.3 and (D) pH 3.7. The reference (0, red line) is the experiment without excipients. If the bar shows positive values (facing right), the Tm1 values are decreased compared to the Tm1 reference, and therefore the mAb is destabilized. If the bar shows negative values (facing left), the Tm1 values are increased and therefore indicate a stabilization of the mAb.

Figure 30 compares the excipients between the on-column experiments and the nanoDSF measurements. (A) and (B) are the results from the on-column experiments performed at buffer pH-values of (A) pH 4.70 and (B) pH 4.25, which correspond, according to the Donnan effect, to internal resin pH-values of pH 3.70 and 3.30, respectively. Therefore, the nanoDSF measurements were done at the calculated low pH values to directly compare the effects seen on-column for conformational stability and the effects of the excipients during thermal unfolding. For (A) and (B), if the bars are facing right, it means more second peak was produced during the 1000 min hold time compared to the run without excipient. This should match the results from the nanoDSF measurements so that the first unfolding transition temperature (Tm1) is lower than

without excipient. This holds only partially true. While the excipient Urea has a destabilizing effect at both pH values under all conditions tested, Arginine and Excipient A show no significant change in Tm1 at pH 4.25 (3.3 for nanoDSF), and the addition of PEG4000 leads to a slightly higher Tm1. For pH 4.70 (3.70 for nanoDSF), Sorbitol and Mannitol should be slightly destabilizing and have a smaller Tm1 value; however, the opposite is the case. The Tm1 values are slightly elevated. Looking at the excipients, which led to a smaller amount of second peak, therefore bars are facing left of the red line in (A) and (B), they should show an increase in thermal unfolding temperature Tm1 in (C) and (D). That conclusion is true, with a few amendments. Since the excipients are ordered in the way that the most stabilizing excipient in the on-column experiments is at the bottom of the graph in (A) and (B), this should be mirrored by the highest increase in Tm1 value for the same excipient on the far right in the graphs (C) and (D) for the nanoDSF measurements. Therefore, the same trends as seen in the chromatography experiments cannot be fully confirmed by nanoDSF. Furthermore, there is one outlier in the nanoDSF measurements for pH 3.70 (4.70 for chromatography). While Arginine can be used as a stabilizing excipient in the column experiments, in nanoDSF, it results in a slightly decreased stability and, therefore, Tm1 value.

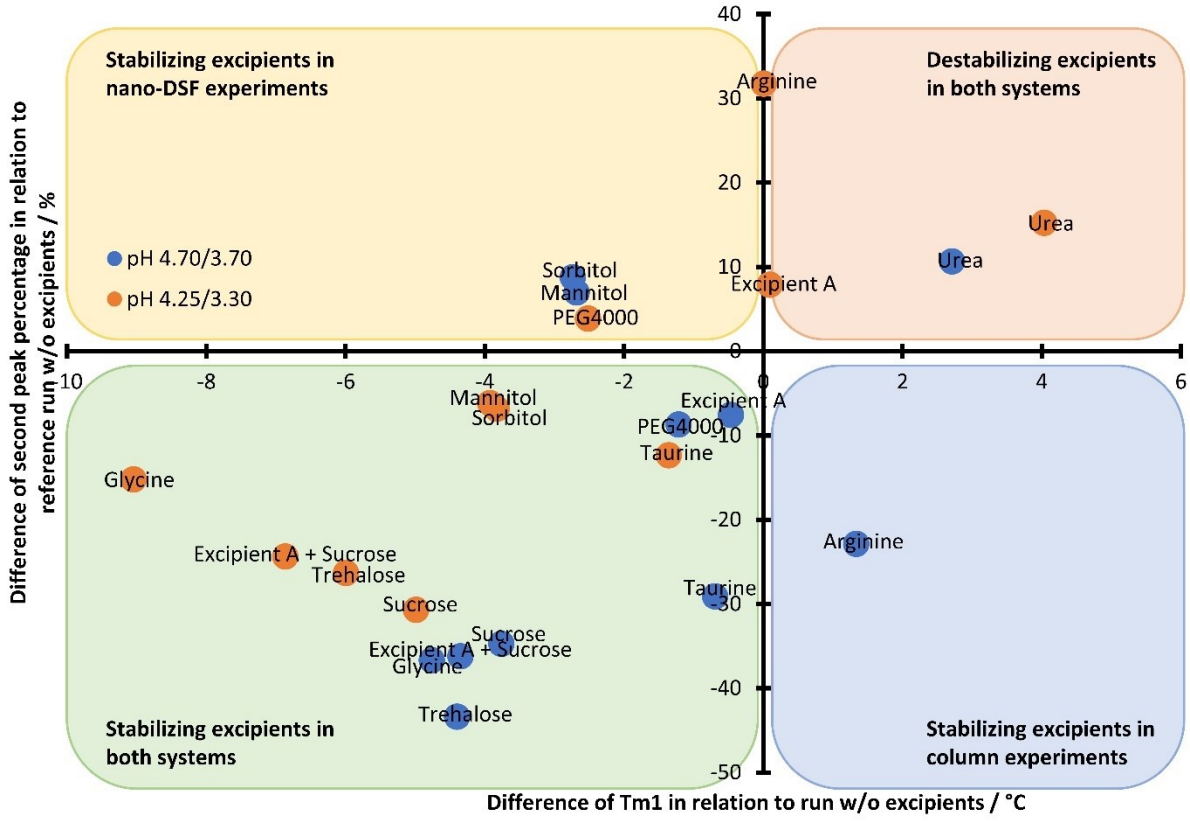

Figure 31: Overview of the effects from nanoDSF and on-column studies combined.

In Figure 31, the effects are shown combined for both pH values (pH 4.25/3.30 and pH 4.70/3.70) and nanoDSF and on-column studies. As can be seen in Figure 31, most of the excipients have stabilizing abilities in both methods and for both pH values tested (down left/green). There is a visible trend between the two pH values for some of the excipients. While the lower pH value (4.25/3.3) results in better stabilization during nanoDSF measurements compared to the higher pH value (4.70/3.70), i.e. lower Tm1 values, their stabilizing abilities during the on-column experiments are not as good. This can be seen as a shift of the corresponding dots towards the bottom right when the pH value is increased. The opposite holds true for Mannitol, Sorbitol, Excipient A,

Arginine, and Urea. This shows that the underlying mechanisms between the different screening tools and the pH values are diverse and more complex.

4 DISCUSSION

In the following chapters, I will discuss the previously shown results of my experiments and put them into context of other publications.

4.1 Composition of the 2nd peak

This chapter has partially been published in a first author paper (Stange et al. 2022b).

As a first step to investigate two-peak elution behavior and its influential parameters, it is important to understand, what exactly the second peak represents. Comparing all publications made about this phenomenon, it is clear that the multiple peak elution behavior is protein dependent. In some cases, no aggregates were found (Luo et al. 2015; Voitl et al. 2010a), but the question there remains, if the elution fractions were analyzed directly after elution. In the beginning stages of this research, almost no aggregates were found in SE-HPLC, but it was later realized, that the fractions were measured days after elution and therefore most of the aggregates had already refolded back to monomer (see Figure 5 (B)). As shown in chapter 3.2, when the second peak was collected and analyzed directly with SE-HPLC, the three fractions contained a similar amount of aggregates throughout the second peak, with the third fraction containing the highest amount (see Figure 3).
The protein load for the linear gradient elution (LGE) runs is well below the dynamic binding capacity of the prototype resins and protein-protein interactions in the adsorbed phase are of minor importance. Instantaneous multimerization is expected to occur predominantly in the liquid phase during desorption of the different conformational states of the protein.

Figure 6 shows the reversibility for pH 4.00 over 24 hours after elution. Only a fraction of the dimer refolds back into monomer and the rest stays constant over this time frame. It further establishes that this phenomenon is pH-dependent.

Since for all three fractions, the predominant variant is ~70% monomer and charge variant separation is unlikely to be the cause of this peak splitting (see Figure 3 (C)), the second peak is most likely assigned to the mAb monomer which at first unfolded upon adsorption into different conformational states. During elution they desorb and refold partially into the mAb monomer and partially aggregate into multimeric forms. Deconvolution of the elution peaks into two gaussian peaks is a strong simplification of the heterogeneous nature of the 2nd elution peak. And the LGE experiments do not allow for a more complex deconvolution.

With respect to the complex composition of the second peak, regarding most probably multiple monomeric variants and multimeric variants, and their pH dependent reversal over time, these binding-induced conformational changes do “not proceed gradually but rather in distinct steps” (Norde and Giacomelli 1999) and “the protein population in flatland appears to be heterogeneous with respect to the conformational state of the molecules: a fraction being in a native-like (N) state and the remainder in one or more perturbed (P) states” (Norde 2008). Poplewska et al. used 3 different P states in their proposed mechanism to simulate unfolding and aggregation of a mAb in CEX (Poplewska et al. 2021).

Both reinjections (former first and second peak separately), led to the same result after 0 min hold time, one peak at the position of the first peak (see Figure 7). This supports the statement, that a decomposition of multimers is observable even under high salt conditions but could be accelerated when the salt is removed during buffer exchange. In addition, the different conformational monomeric states are fully reversible and fold back into the species eluting in peak 1. On the other hand, after 1000 min hold time, both reinjections show a two peak elution behavior again. Other groups have reported similar effects (Gillespie et al. 2012; Kimerer et al. 2019a; Voitl et al. 2010a).

4.2 Influencing factors on two peak elution behavior

4.2.1 General influencing factors

This chapter has partially been published in a first author paper (Stange et al. 2022b; Stange et al. 2022a).

It was shown that two-peak elution behavior of a monoclonal antibody in cation exchange chromatography is influenced by the resin and resin structure, the buffer systems and kinetic parameters like temperature and hold time.

Bead size (see chapter 3.3.1 and Figure 8) was tested in the way, that elution behavior was compared between Fractogel® SO_3 (M) (~60 µm) and Fractogel® SO_3 (S) (~35 µm). The smaller bead size (Fractogel® SO_3 (S)) seems to have a promoting effect towards unfolding and aggregation. The second peak percentage is increased for the smaller bead size. Bead size is also an influential factor in resolution, e.g. compare resolution improvement for HPLC columns against preparative chromatography columns. Since no SE-HPLC data was measured for these experiments, it is unclear, if the smaller bead size really promoted the unfolding and aggregation pathway or if the resolution was just improved in a way that it increases second peak percentage. In other publications regarding the multiple peak elution behavior, bead size was not investigated as a potential influential factor.

Another potential influencing parameter towards the two-peak elution behavior is the comparison between strong and weak cation exchange resins. Strong cation exchange resins have a ligand that is electrostatically charged over a broad pH value range, whereas the weak cation exchange ligand is only negatively charged over a small range. In this case, the strong resin and baseline experiment was performed with Fractogel® SO_3 (M) and compared to a run with Fractogel® COO (M) (see chapter 3.3.2 and Figure 9). The runs were performed at pH 5.00 to ensure that the weak cation exchange ligand is fully charged. It could be seen that the weak CEX resin did not show a shoulder or distinguishable second peak after 1000 min hold time. Weak cation exchange resin did not show two peak elution behavior for human serum albumin (Voitl et al. 2010a)

In chapter 3.3.3, it was shown that a cation exchange resin without a tentacle structure between bead and resin, leads only to unfolding and aggregation after a significant longer hold time (96 hours compared to 1000 min or less). Furthermore, the resulting aggregates from the elution seem to be stable and do not refold after some time. Luo et al. also investigated a double peak elution profile and included different resins and resin structure in their work to separate, what they concluded are protonated/deprotonated histidine charge variants. They found a similar elution profile for Toyopearl® SP-

650 M, as in only one elution peak occurs. Other resins showed at least a shoulder, but the main difference between the resins showing a shoulder or nothing and the ones with a distinguishable second peak is the tentacle-structure or a bi-modal pore size distribution. Except for Nuvia S resin, which has grafted polymers. In the case of Luo et al. this resin only showed a shoulder instead of a second peak (Luo et al. 2015). Guo et al. also did an extensive investigation of different resin structures on the two-peak elution behavior. They also found a clear correlation of second peak percentage between resins with grafted polymer extenders or bi-modal pore size distribution (as in the case of Poros 50 HS). They concluded that the open pore structure and the lack of this tentacle structure is the main reason for the difference for the appearance of the second peak. They, however, found a clear second peak with Nuvia S, instead of just a shoulder, but they had to reduce the flow rate significantly to increase the resolution of the second peak (Guo et al. 2014; Guo and Carta 2015).

Chapter 3.3.4 showed the effect of different buffer and counterion concentrations on the two-peak elution behavior. The runs were all performed at pH 4.70, so the external buffer and internal pore pH value do not influence this effect. Furthermore, it is not expected that the other species present in some of the loading buffers (undissociated acetic acid and Chloride ions) have a direct influence on this phenomenon. It was shown, that the lower the buffer concentration (dissociated Acetate species), the less second peak is produced (compare runs with 40 mM Na^+ in Figure 11 (A), (B), and (C)). And when the counterion concentration is increased, but the buffer concentration is held constant, again the second peak percentage is significantly decreased. This can be explained by the Donnan effect. The Donnan effect is clearly dependent on the concentration of ions in the buffer system. The higher the ion concentration, the less influence on the internal resin pH value (see Figure 19 (B)). Guo et al. showed some results in this regard as well (Guo and Carta 2015), but they attributed the effect on the second peak to the overall increased or decreased binding strength and not on the internal resin conditions. Gillespie et al. increased the buffer concentration on a Fractogel® SO3 (M) during a two-peak elution. It resulted in a decrease in aggregate formation in the second peak, but not to the degree expected, since there was still a 6-fold increase in HMW's (Gillespie et al. 2012).

As can be seen in chapter 3.3.5, temperature is a crucial parameter, that should be kept constant over the 1000 min hold time to achieve reproducible results. Other authors have included temperature in their studies of the two peak elution behavior and found mixed results. Voitl et al. have also reported that for HSA on a strong cation exchange column, as the temperature influences the two peak elution in a similar but not so strong manner, suggesting a kinetic process in the adsorption/desorption as well as the aggregation (Voitl et al. 2010a). Similar observations were made by Gillespie et al. for their aglycosylated IgG1 (Gillespie et al. 2012). In Kimerer et al., increased temperature led to the merging of multiple peaks into one, which eluted earlier (Kimerer et al. 2019a), suggesting a faster interconversion of the eluting subspecies. In our case, however, the retention of the first peak stays constant with increasing temperature, while the second peak becomes increasingly broader with the peak maximum shifted to higher retention times. As the temperature effect on peak width is small (Figure 12 (A)), the change in the peak shape and position of the second peak indicates a temperature-dependent change in the composition of the second peak.

The results for the increased hold time are shown in chapter 3.3.6. The experimental data points were fitted with a mathematical CurveFit software, called TableCurve®. The

fitted curve of first-order formation fits the experimental results well, even though the underlying mechanism of surface-induced unfolding and then partial refolding and aggregation upon desorption from the column is probably more complex (Poplewska et al. 2021). Unfolding and refolding/aggregation are kinetic processes that follow different pathways. A more comprehensive study of the aggregation mechanism and pathway was outside the scope of this study. Philo et al. gave an overview of different unfolding and aggregation mechanisms (Philo and Arakawa 2009). In this case, we hypothesize that the binding to the resin surface and the low pH value are causing a surface-induced unfolding. Upon elution, the protein desorbs and partially aggregates into multimeric forms and partially refolds into the mAb monomer. The second peak is composed of a mixture of multimeric species and monomeric forms (Stange et al. 2022b). Several other authors who have investigated this multiple-peak elution behavior have termed it similarly (Farys et al. 2018; Gillespie et al. 2012; Guo et al. 2016).

In chapter 3.3.7 it can be seen that, the higher the load density, the smaller the second peak area relative to total peak area. However, up to a medium load challenge, a second peak is still clearly distinguishable. Since the protein immediately adsorbs to the resin surface due to the high positive charge at this low pH value, most of the protein will be adsorbed directly at the column entrance. Therefore, the increase in column loading likely reduces the degree of conformational changes due to steric hindrances and repulsive protein-protein interactions (“crowding effect”) (Chen et al. 2016; Guo et al. 2014; Stańczak et al. 2020).

Lot-to-lot ligand density variations of the same resin are common and can, in some cases, have a significant influence on the elution behavior of the protein. In this case, we chose three prototype resins with significantly different ligand densities to investigate this resin structure difference for the two peak elution behavior. It was shown in chapter 3.3.8, that the three different prototypes have a significant influence on the amount of second peak produced. The higher the ligand density, the more second peak is produced, and the elution conductivity is increased. Similar results have been observed in IEX, showing stronger retention of the protein in the resins with a higher ionic capacity (Fogle et al. 2012; Fogle and Persson 2012; Sanchez-Reyes et al. 2021; Wu and Walters 1992).

It was also shown that if the buffer pH value is further decreased as pH 4.25, only a fraction of the protein load can be recovered from the column in the gradient (see Figure 18). This indicates a significant unfolding during the 1000 min hold time and therefore a much stronger interaction between the resin and the ligand.

Irreversible, partial irreversible and reversible desorption of proteins from surfaces is frequently described (Latour 2020; Talbot 1996). The extent of irreversibility is often strongly “binding history” dependent owing to the slow interconversion of nonequilibrium states (Tie et al. 2003). Several publications demonstrated surface-binding induced slow conformational changes of the bound proteins during the hold step in ion exchange chromatography (Chen et al. 2016; Farys et al. 2018; Gillespie et al. 2012; Guo et al. 2016; Guo and Carta 2014, 2015).

The extend of the conformational changes depend on the resin/surface chemistry and the protein properties. Huang et al. hypothesized that in cation exchange chromatography the base matrix and its inherent hydrophobicity is also likely to play a role in the on-column aggregation of a mAb (Huang et al. 2020). It is well known that hydrophobic

interactions between resin/ligand and protein can lead to unfolding and aggregation (Fogle et al. 2006; Muca et al. 2010). Investigations with Toyopearl® SP-650 M, which has the same functional group and base matrix as the Fractogel® prototypes, but no grafted polymer extenders, revealed a single monomeric peak after 1000 min hold time and a second peak only after 96 h incubation, which contained mostly non-reversible aggregates (Figure 10). This result points to a more important role of the physicochemical properties of the polymer surface layer than the base matrix. Similar conclusions were drawn by other groups comparing different polymer-modified and non-polymer CEX resins (Farys et al. 2018; Guo and Carta 2015).

Several authors have published a change in second peak, when the concentration of NaCl, the buffer concentration or the buffer pH-value is changed (Chen et al. 2016; Gillespie et al. 2012; Guo et al. 2014; Guo and Carta 2015), but none correlated this with the effective conditions inside the polymer pore network. During the load and hold time, the monoclonal antibody is therefore subjected to a far lower pH-value than was originally set in the buffer.

The extent of second peak formation correlates with the decrease of the resin pH down to pH 3.9. The major effect of the ligand density variation is on the drop of the internal resin pH value. At a resin pH value between pH 3.3 and 3.9 the second peak formation shows a ligand density dependency. mAb-ligand interaction is likely to play an additional role in production of reversible conformational species. A recent detailed characterization of the three prototypes describes differences in the structural properties of the resins (Bhambure et al. 2016). A 2-fold reduction in average pore size and a stronger tendency of the polymer chains to form ionic aggregates with increasing ligand density was reported. The local microenvironment of the bound mAb might be different, effecting the extent and strength of the protein-tentacle interactions. A similar argument was used to explain the functional differences of the prototypes for three model proteins including a monoclonal antibody (Bhambure et al. 2017).
At a resin pH < 3.3 the mAb recovery during the salt gradient starts to decrease. Irreversible on-column aggregation is observed, presumably caused by more severe unfolding of the mAb. Philo et al. described this mechanism as surface-induced aggregation (Philo and Arakawa 2009), which means that the binding of the protein to the ligand leads to conformational changes and then to an aggregation-prone non-native monomer state. Low pH induced mAb aggregation is also reported during the elution in Protein A chromatography (Mazzer et al. 2015).

4.3 Stability and reversibility studies of the monoclonal antibody in solution

To test the hypothesis that the significantly lower internal resin pH value is the main factor in the extend of the two-peak elution behavior, some studies in solution were done at the calculated internal resin pH value.

4.3.1 Aggregation of mAb in solution at low pH

This chapter has been published in a first author paper (Stange et al. 2022b).

In chapter 3.4.1 the mAb was subjected to the calculated low internal resin pH value over 1000 min and then the NaCl concentration was increased to roughly the elution concentration of 500 mM. During the 1000 min hold time in solution without salt, the antibody did not show any aggregation in SE-HPLC. But after the salt was increased, it aggregated depending on the set pH value. Bickel et al. studied the effect of NaCl-

induced aggregation at low pH level and found that in the absence of salt, an increase in fluorescence intensity is an indicator for a conformational change of the proteins tertiary structure and when salt is added these exposed hydrophobic patches interact, leading to aggregation (Bickel et al. 2016). The relationship between unfolding, aggregation and solution pH-value has been studied extensively by other groups (Arosio et al. 2013; Chen et al. 2016; Mahler et al. 2005; Mahler et al. 2009).
However, the percentage of aggregates formed cannot completely explain the observed on-column aggregation. First, only the percentage of aggregates at pH 3.3, which corresponds to a buffer pH value of ~4.25, is close to the observed on-column aggregation level after 120 min, which is comparable to the timeframe for elution in the on-column experiments. For pH 3.5 and 3.7, aggregation levels remained lower than in CEX experiments. Second, the aggregates that were created using this solution assay are non-reversible aggregates. Therefore, there seems to be an influence of the resin structure and the binding conformation to this two-peak elution behavior.

4.3.2 Nano-DSF measurements at different pH values

This chapter has been published in a first author paper (Stange et al. 2022b).

One other possibility to study the conformational stability of the antibody is through intrinsic or extrinsic fluorescence measurements. In extrinsic fluorescence measurements a dye-molecule (e.g. ANS) is added to the solution and when the protein unfolds, it binds to the antibody and the fluorescence signal can be detected. For intrinsic fluorescence measurements no dye is necessary. As the protein unfolds, tryptophans and tyrosines, that are usually located more in the hydrophobic center of the folded protein, emerge, and can be detected through a fluorescence signal. In a temperature gradient the protein gets stressed and starts to unfold, which can be in stages or all at once. Therefore several “melting temperatures” or Tm’s can be detected in the fluorescence signal. In this case, the antibody was subjected to a wide range of pH values, to determine the conformational stability in relation to solution pH value. The nano-DSF measurements clearly show that this IgG1 antibody is significantly destabilized at low pH values. Several publications have demonstrated similar observations and the different unfolding transitions were assigned to domain unfolding. The lowest Tm usually corresponds to the CH2 domain followed by the Fab then the CH3 domain at highest temperature (He et al. 2010; Temel et al. 2016). Depending on the pH, the unfolding of the antibody follows different pathways. Interestingly, the stronger influence of pH on stability and unfolding of the mAb starts around pH 4, which coincides with the pH of 3.9 seen in the ligand density dependency of second peak formation (see Figure 20).
Latour pointed out that the unfolding/folding pathway of protein on surfaces is not necessarily identical to a pathway seen in solution (Latour 2020). This might explain the differences seen in the aggregation tendency of mAb species produced at low pH in solution and those released from surfaces after prolonged incubation.

4.4 Excipients as a main influencing factor

This chapter has been partially published in a first author paper (Stange et al. 2022a).

The two peak elution behavior can be influenced by many different parameters and I have shown that the main driving factor is the conformational stability of the antibody subjected to the binding to the resin and the low internal pore pH value in the resin pore. In formulation development for drug products, excipients are added at least in the final stages of production to ensure a stable product over the designated shelf-life.

Some publications already added excipients to their buffer systems to investigate the effect on their multiple peak elution behavior, but with mixed results (Chen et al. 2016; Farys et al. 2018; Gillespie et al. 2012; Guo et al. 2016; Guo and Carta 2015; Luo et al. 2015). Therefore, it was important to first establish a system with which excipients could be reproducibly screened. This was mainly achieved through a constant temperature during the hold time using a column oven (see Figure 21). It resulted in a slightly different percent second peak in relation to total peak area for the four main pH values than previously shown. Nevertheless, since all experiments for one influencing parameter without temperature control, were done in relatively quick succession then the overall trends and conclusions should hold true. Furthermore, it was important to investigate, when excipients have the biggest effect on the two peak elution behavior. 5 excipients from different classes were selected to be added either only in the load buffer system (and then washed out after 1000 min hold time) or only in the elution buffer system (washed in after 1000 min hold time) or in both stages. As could be seen from the graphs (see Figure 22 and Figure 23), the addition (or substitution) during the load phase or during both phases had the biggest influence on the second peak. When the excipients were added only during the elution phase, the effects were negligible. This shows again that the conditions during the load and hold phase are crucial for the conformational stability of the antibody and can only be influenced at that stage. Another crucial parameter is the concentration of excipient. Figure 24 shows this exemplary for Sucrose concentrations added only in the load and hold phase. The effect is clearly concentration-dependent.

Since the aggregates are partially reversible, depending on the original buffer pH value during load and hold phase, it was suspected that excipients could also influence the reversibility. Therefore, excipients were added either during just the elution phase, because then the influence on the second peak is low and the excipient is already in the elution pool buffer, or they were added afterwards as solid powder or dissolved solution. The addition of Sucrose as a solid powder resulted in the problem, that the powder had to be dissolved in the elution pool solution. This meant that mechanical stress was performed on the protein in the solution by slightly shaking, stirring or inverting it. And even though the stress was kept minimal, the aggregate level could be influenced by it. Therefore, it was concluded that adding excipients in the elution buffer system would be the less stressful setup for the protein and likely not influence the outcome. It could be seen in Figure 29, that the addition of 500 mM Sucrose or Glycine slightly slow the conversion or refolding down and stabilize some of the aggregates. Even though this effect is in the realm of possibilities for these excipients, to the author’s knowledge, it has not been published.

After 5 excipients have been tested for their effect during the different stages of the on-column experiment, several more were added to the screening. And to be able to compare the effects between this new on-column screening method and a more conservative screening approach, nano-DSF was again used. The pH values for the nano-DSF experiments were set to the calculated low internal pH values to improve comparability. Figure 30 and Figure 31 show the results from both screenings. In the following part of the discussion, I will discuss the used excipients and their effects in more detail.

Arginine is one of those excipients that showed mixed effects. While in some studies, the addition or substitution of Arginine resulted in a reduction or removal of second peak and reversible self-association was mitigated by it (Gillespie et al. 2012; Guo et al. 2016; Guo and Carta 2015; Luo et al. 2014; Man et al. 2019), in others it

exacerbated aggregation at low pH or showed no stabilization during on-column experiment, when it was added in the load and hold buffer (Chen et al. 2016; Gillespie et al. 2012; Luo et al. 2015; Svilenov et al. 2020). Arginine increases protein solubility and stabilizes the mAb against thermal unfolding and aggregation (Arakawa et al. 2006; Le Basle et al. 2020; Maity et al. 2009). It is preferentially excluded from the mAb surface at higher concentrations (Sudrik et al. 2017). Furthermore, it can electrostatically interact with the protein and through cation-π interactions with the aromatic side chains on the protein surface (Cloutier et al. 2020; Le Basle et al. 2020; Sudrik et al. 2017). Its effect on thermal stability might be related to the type of anions present in the formulation, with acetate arginine salt, which was present in this experiment, improving the stability of an IgG1 and reducing aggregation kinetics (Le Basle et al. 2020; Zhang et al. 2016). Arginine has the potential to form clusters, due to different charges throughout the molecule (C-terminus is negatively charged, N-terminus and R-group are positively charged). Therefore it can interact with antibodies and other proteins through the negatively charged residue and the free end can be involved in clustering (Cloutier et al. 2020; Shukla and Trout 2010). The effect of Arginine on the global thermal stability and tertiary structure is dependent on the mAb (Thakkar et al. 2012). Arginine reduces repulsive protein interactions, which lowers the colloidal stability and leads to a lower aggregation onset temperature (Svilenov et al. 2020).

Glycine, on the other hand, is another amino acid used as a stabilizing pharmaceutical excipient. In this setup, it revealed stabilizing abilities for the on-column experiment, as well as the thermal unfolding screening with nano-DSF. It was also already used in other studies concerning the multiple peak elution behavior with various results. Either it showed no effect, when it was added during load/hold phase (Chen et al. 2016; Gillespie et al. 2012), or as an addition in the elution buffer showed little effect (Farys et al. 2018), or Glycine in the buffer system reduced second peak but did not erase it (Gillespie et al. 2012). Normally, Glycine is used as a bulking agent in freeze-dried formulations comparable to Mannitol (Le Basle et al. 2020; Li and Nail 2005; Meyer et al. 2009).

Mannitol and Sorbitol were used in this study, because, similar to Glycine, they are often employed as bulking agents in freeze-dried formulations for their stabilizing abilities (Akers 2002; Le Basle et al. 2020; Meyer et al. 2009). Mannitol is probably the most widely used bulking agent, because of its many positive properties with respect to crystallinity, high eutectic temperature, and matrix properties (Akers 2002). It provided less protection during storage of a model recombinant humanized monoclonal antibody, compared to Sucrose and Trehalose (Akers 2002; Cleland et al. 2001). Both Mannitol and Sorbitol are preferentially excluded from the protein surface, but Sorbitol is less excluded compared to Sucrose, Trehalose and Mannitol (Sudrik et al. 2019). Their effects on monomer loss rates, as well as potency for reversible self-association in aggregation studies showed a dependency on the mAb and its attributes (Sudrik et al. 2019). Sorbitol is also commonly used as a co-solvent in parenteral formulations (Akers 2002). However, it can stabilize proteins in solution and in the lyophilized state (Chang et al. 2005b; Chang et al. 2005a; Kadoya et al. 2010). Sorbitol is also able to stabilize monoclonal antibodies during thermal unfolding transitions (Barnett et al. 2016).

Sucrose, as a commonly used stabilizing excipient for different protein stress conditions, has also been used in a few multiple peak elution behavior studies. In these cases the addition in the load/hold buffer did not help (Chen et al. 2016; Gillespie et al.

2012). In others, Sucrose stabilized the protein at low pH values (Man et al. 2019; Svilenov et al. 2020; Thakkar et al. 2012) or reversible self-association was mitigated by it (Man et al. 2019). Sucrose can increase the thermodynamic stability of the native state mAb in proportion to its concentration, but not necessarily in a linear relationship (Barnett et al. 2016; Kim et al. 2003). This is also the case for the results at different and increasing concentrations. Sucrose shifts the conformational equilibria within the native-state ensemble toward more compact, structurally ordered species with lower surface area, which is due to its preferential exclusion from the protein surface (Kim et al. 2003; Sudrik et al. 2019). Its effect on monomer loss rates, as well as potency for reversible self-association in aggregation studies showed a dependency on the mAb and its attributes, similar to Sorbitol, Mannitol and Trehalose (Sudrik et al. 2019; Thakkar et al. 2012). The nature of the interaction between Sucrose and protein seems to be isotropic and unspecific (Svilenov et al. 2020).

And while Sucrose can be hydrolyzed into Glucose and Fructose, which then can lead to glycation of proteins (Fischer et al. 2008; Le Basle et al. 2020), Trehalose is a non-reducing sugar (Le Basle et al. 2020), but with similar stabilizing abilities, as can be seen in this study. Trehalose is used as a bulking agent in freeze-dried formulations (Akers 2002). It is also preferentially excluded from the protein surface (Sudrik et al. 2019) and its effect on monomer loss rates, as well as potency for reversible self-association in aggregation studies showed a dependency on the mAb and its attributes (Sudrik et al. 2019). As seen in this study as well, Trehalose stabilizes the protein during thermal unfolding transitions (Barnett et al. 2016).

Excipient A was used a potential excipient with stabilizing abilities. It can be categorized as an osmolyte, like the polyol and sugar excipients. In this study however, either the concentration of the ionic/electrostatically charged excipient is too low at 40 mM, or it simply only has minor influences on the conformational stability of antibodies. It is important to note, however, that the effect on-column, despite being smaller, are comparable to Arginine, the other ionic/electrostatically charged excipient used in this study. For the combination of Excipient A and Sucrose, it did not yield an additional advantage over using Sucrose as excipient alone.

Urea is a known destabilizing excipient. In this context, the concentration was smaller than it is usually applied, however, the destabilizing effects can be seen in the resin experiments and the nano-DSF measurements. In other studies, Urea exacerbated aggregation at low pH and it was ineffective in controlling reversible self-association (Man et al. 2019). Urea led to changes in secondary and tertiary structure, which resulted in changes of their characteristic charge for several proteins in anion exchange chromatography (Hou et al. 2010). However, it is commonly used as a refolding agent (Hamada et al. 2009).

Taurine is not used as a pharmaceutical excipient in protein formulation, but can be categorized under osmolytes (Kamerzell et al. 2011). So far, investigations into the effect of Taurine has been limited to food and feed application and its effects on the human body (Chesney et al. 1998; Schaffer et al. 1998; Stohs and Miller 2014). In this study, Taurine showed good stabilizing abilities for the on-column experiments, as well as the nano-DSF studies.

PEG4000 is commonly used as a co-solvent in parenteral formulations, as a solubilizing agent (Akers 2002) and for refolding of recombinant proteins (Lee and Lee 1987).

In some cases it was shown, that it decreases the thermal stability as well as overall stability of proteins (Akers 2002; Barnett et al. 2016). This is likely due to interactions with hydrophobic side chains of the protein at high PEG concentrations, which get exposed during unfolding (Barnett et al. 2016; Zielenkiewicz et al. 2006). In our study it shows only minimal effects on the stability of the protein on-column, as well as during thermal unfolding in nano-DSF.

4.5 Conclusion

This chapter has partially been published in first author papers (Stange et al. 2022b; Stange et al. 2022a).

In this publication, I showed important influencing factors and parameters on the two-peak elution behavior of the mAb. The observed double peak elution behavior on the CEX column Fractogel® prototypes is due to reversible self-association and aggregation during the 1000 min hold time. Bead size, strong vs. weak cation exchange, ligand structure, buffer and counterion concentration, temperature, hold time, load density, ligand density, excipients, and pH value all play a major role in this phenomenon. Furthermore, it is clear from other publications that this effect is also protein-dependent. As temperature is known to be very impactful in unfolding and aggregation kinetics, it was shown that in the case of surface-induced unfolding and aggregation, it is a major influencing factor. The setting of a specific temperature is crucial for the reproducibility of results. Furthermore, the hold time seems to follow a kinetic progress, as was shown for the two most influential pH values (pH 4.70 and pH 4.25). I also found that, the amount of protein that dissociates after elution is strongly dependent on the mobile phase pH value and the ligand density of the resin. The ligand density variation influences the Donnan effect, which is responsible for significantly lower pH values inside the resin pores. The extent of the double peak formation correlates with the internal resin phase pH down to pH ≥ 3.9. At lower pore pH values the effect is ligand density dependent. The mAb is considerably destabilized at pH values <pH 4.00 but shows no aggregation in solution experiments. The addition of salt during the solution experiments leads to aggregation of the protein, but the level of aggregation and its reversibility is different from the column experiments. Therefore, I conclude, that the interaction with the ligand, ligand structure or base matrix plays a key role in this phenomenon.

As the main focus of this study, I have shown various excipients from different classes and their effects on the two-peak elution behavior, as well as compared these effects to studies from thermal unfolding with nanoDSF. Overall trends can be confirmed between the two orthogonal techniques as common stabilizing excipients, like Sucrose and Trehalose, also show stabilizing abilities here. Furthermore, common destabilizing excipients, like Urea, are also destabilizing the mAb here. In between these two extremes, the effects are mostly minimal or sometimes differ between the techniques, possibly due to different interactions in the unfolding and aggregation pathways. Especially interesting is the effect of Arginine between the two different pH values. An explanation or description of this effect has yet to be made and was outside of the scope of this investigation. However, it shows why different groups have reported different results on the multiple-peak elution behavior when they also applied Arginine as an excipient.
Nonetheless, the results shown here give clearer insight into the phenomenon and the possible management in a production process for less stable mAbs and other proteins. Furthermore, comparing the two orthogonal techniques shows that the method can be

applied to screen excipients regarding surface-induced unfolding and aggregation. I have shown that by keeping a few influential factors in mind, e.g., temperature, buffer pH value, ligand density, and hold time, this method could give insight into stabilizing abilities of excipients for a different unfolding/aggregation mechanism.
A future investigation could include a deeper investigation into the effects of excipients during the reversal of aggregates or include more biophysical methods to investigate the root of the unfolding/aggregation. Other groups have established e.g. HX-MS to get better insights into the mass change of protein fragments during binding and elution. To optimize the screening tool for the excipients, a semi-high-throughput method could be established using e.g. a Tecan Robocolumn system with a different detection system (UV shift for aggregates or Fluorescence probes).

5 SUMMARY

Antibodies are an integral part of the human immune system. They are mostly Y-shaped monomeric molecules with different roles in the destruction of specifically targeted cells or molecules. Therefore, their specific targeting and binding ability has been explored by researchers and pharmaceutical industry for their application as drug substance. Ever since 1975, when the first hybridoma technology was invented by Köhler and Milstein, to produce pure mouse antibody, technologies involving antibody production has increased significantly.
Today, mostly IgG monoclonal antibodies with their subtypes are used as drug substance and their production is done through genetically engineered CHO cells in a cell culture, followed by a multi-step purification train. As part of this purification platform, cation exchange is often used to mildly separate aggregates and other product- and process-related impurities, that could potentially elicit an immune response from the patient. However, in the recent years, more and more publications showed a phenomenon during cation exchange chromatography, the two-peak elution behavior. In mostly a salt gradient elution, and sometimes after a certain hold time on the column, the protein elutes in two distinct peaks, instead of just one, as expected. These publications also show that this phenomenon is mostly protein-dependent and has several different explanations.
In my project, I used a standard approach to elicit this two-peak elution behavior with a monoclonal antibody held on a cation exchange resin for 1000 min before elution in a salt gradient to 1 M NaCl. To elucidate what exactly the second peak is, I used size exclusion chromatography. It was found that the second peak is mostly comprised of monomer, which are probably conformationally altered, but also ~30% aggregates, which are reversible, depending on the buffer pH value.
I further investigated several influential factors on this phenomenon. Bead size, tentacle technology, buffer composition, weak vs. strong cation exchange ligands, and load density were found to have an effect on the two-peak elution behavior. Interestingly, especially temperature and hold time had a strong influence on the amount of second peak created. This is a clear indication that the unfolding and aggregation on the resin surface and in the resin pores is a kinetic process.
Since SE-HPLC analysis showed a correlation between pH value of the buffer system and stabilization of the resulting aggregates, I wanted to test different ligand densities on the cation exchange resin. I found that, when the Donnan effect is calculated for the specific buffer pH value, counterion concentration, and the ligand density of the resin, the monoclonal antibody is subjected to a significantly lower pH value during the 1000 min hold time than originally thought of. The lower pH value inside the resin pore has a significant impact on the conformational stability of the antibody, which was shown with nano-DSF (intrinsic fluorescence) measurements.
Another big impact on the second peak had the addition or substitution of excipients into the buffer systems. Excipients are mostly used at the end of the purification train to stabilize the protein, increase shelf-life, and facilitate easier application to the patient. In this case, I wanted to study the effects of excipients on the surface-induced unfolding and aggregation. In a first smaller study I used 5 common excipients added or substituted during the three phases of the chromatography (only during load and hold phase, only during elution or present throughout the chromatography). It showed that the biggest influence of excipients can be achieved during either only the load and hold phase or when present during the entire chromatography step. I then added several more excipients to further study the effects on this phenomenon and again, I used

nano-DSF measurements to compare these effects with a more “traditional” in-solution screening approach. Some excipients showed similar stabilizing/destabilizing effects in both systems, while others had completely opposite effects. Nevertheless, this approach, following a guideline to avoid other influential parameters, can deliver more diverse insight into protein-excipient interactions, while the protein is surface-bound.

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7 CURRICULUM VITAE

PERSONAL

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SCHOOL EDUCATION

2005 – 2008	Berufliche Schulen Gelnhausen, Ernährungsgymnasium
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UNIVERSITY EDUCATION

WS2008-SS2010	University education (B.Sc. Food chemistry) at the Justus-Liebig University Gießen – not finished
SS2013-WS2016	University education (B.Sc. Biotechnology) at the University of Applied Sciences Mannheim
28.10.2016	B.Sc. Biotechnology (Final thesis topic: Optimization of charge variant purification using mathematical modelling), Final mark: 2.2
WS2016-SS2018	University education (M.Sc. Biotechnology – Bioprocess development) at the University of Applied Sciences Mannheim
19.07.2018	M.Sc. Biotechnology – Bioprocess Development (Final thesis topic: Influence of excipients on Protein A chromatography), Final mark: 1.5
Since 11.12.2019	Doctoral student with goal Dr.sc.hum at the Medicinal Faculty of Ruprecht-Karls University Heidelberg

EXPERIENCE/EDUCATION OUTSIDE OF UNIVERSITY

September 2010 – January 2013	Apprenticeship as chemical laboratory technician at Sanofi-Aventis Deutschland GmbH, appointed to the following departments: - Process development Biotechnology (DSP), - Pharmaceutical sciences (Liquid formulations) - Analytical sciences (HPLC-Analytics)
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