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Antibody-Fragments: Protein Engineering, Process Development, and their Application as Affinity Ligands

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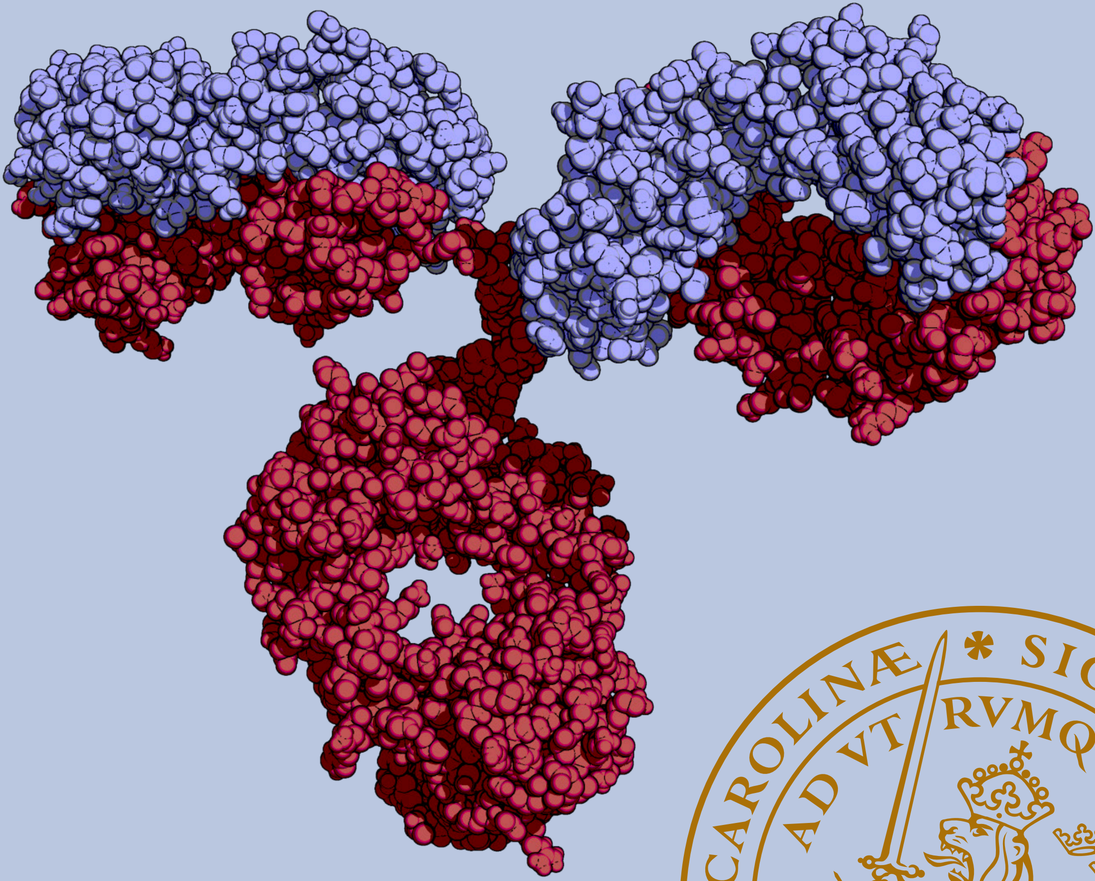

Antibody-Fragments: Protein Engineering, Process Development, and their Application as Affinity Ligands

Laila Sakhnini

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Antibody-fragments: Protein Engineering, Process Development and their Application as Affinity Ligands

Abstract

Monoclonal antibody (mAb) fragments have become the formats of choice for both biopharmaceuticals and ligands in analytical and preparative applications. Their smaller size enables additional advantages over full-length mAbs, such as improved tissue penetration, faster blood clearance for diagnostic applications, improved capacity, and enablement of multivalent formatting. However, limitations in their biophysics and downstream processing can become a challenge in their development. In this thesis, three different aspects of these limitations have been investigated.

First, a strategy to improve the developability potential of a model antigen binding fragment (Fab) was investigated. 393 Fab variants with single and double/triple mutations in the frameworks and complementarity determining regions were screened by *in silico* computational models. Out of this library, 26 variants were selected for *in vitro* developability assessment. A majority of the variants exhibited same or improved thermodynamic stability relative to wild-type. In addition, they were improved with regards to non-specificity. Variants containing [28D]-FR1-H, [31D]-CDR1-H and [53D]-CDR2-L showed to be improved in regard to aggregation propensity. Remarkably, the affinity to the target protein was fully retained in all variants.

Second, a downstream process was developed for a single-chain variable fragment (scFv) with kappa light chain II, which cannot be purified by conventional methods. Design of experiments was employed together with a proteomics approach for identification and relative quantitation of host cell protein (HCP). Capture and polishing were performed by hydrophobic-charge induction multimodal chromatography (MMC) and anion hydrophobic MMC, respectively. The polish step was developed and optimised by screening and optimisation DoE. Operational parameters were successfully screened, and optimal purity, yield, and HCP reduction factor were estimated to >98 %, >98 %, and 14, respectively.

Third, a systematic study for the development of high-capacity immunoaffinity adsorbents was conducted. MAb, Fab, scFv and multimeric scFv fusions were investigated as affinity ligands. The accessibility of the binding sites had substantial effect on the binding capacity. It turned out to be largely impacted by the ligand format, ligand density, and orientation of ligands. Altogether, highest binding capacity was achieved for adsorbents with site-directed monomeric and multimeric scFv ligands; up to 20 mg/mL for a target protein of 50 kDa.

Key words

Monoclonal Antibodies, Aggregation, Biophysical Characterisation, Process Development, Affinity Adsorbents

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Dedicated to my dear mother and all the people who believed in me and supported me.

Contents

Preface ix

Acknowledgements xi

Populärvetenskaplig Sammanfattning xv

List of Papers xvii

Abbreviations xix

1. Introduction 1
    - 1.1 Research Objectives 1
2. Monoclonal Antibodies and Their fragments 3
    - 2.1 Structure and Function 3
    - 2.2 Past and Present Developments 6
    - 2.3 Biophysical Challenges 11
        - 2.3.1 Thermodynamic Stability 11
        - 2.3.2 Colloidal Stability 14
        - 2.3.3 Protein Aggregation 17
        - 2.3.4 Engineering Antibodies for Aggregation Resistance 18
3. Downstream Processing of Antibody Fragments 21
    - 3.1 Overview 21
    - 3.2 Process Development 23
    - 3.3 Antibody Processes 26
4. Immunoaffinity Adsorbents 31
    - 4.1 Overview 31
    - 4.2 Important Factors 31
    - 4.3 Characterisation of Affinity Adsorbents 34
    - 4.4 Past and Present Developments 35
    - 4.5 Limitation in Regeneration Procedure 37

5. Concluding Remarks and Future Directions.. 39
References.. 41

Preface

This doctoral dissertation is submitted in partial fulfilment of the degree of Doctor of Engineering in Biochemistry at Lund University, Sweden. The research was conducted in Global Research Technology at Novo Nordisk A/S, Måløv, Denmark, under guidance of Prof. Leif Bülow, Dr. Anja K. Pedersen and Dr. Maria Dainiak between March 2016 and May 2019. The work resulted in the generation of this doctoral thesis, four research papers and several oral/poster presentations at different conferences (e.g. PEGS 2017 in Boston, US; BPP 2017 in Copenhagen, Denmark; and ISPPP 2018 in Berlin, Germany).

Laila Sakhnini
October 2019

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Laila Sakhnini
October 2019

Populärvetenskaplig Sammanfattning

Antikroppar är protein delaktiga i kroppens immunförsvar mot främmande ämnen. De har en unik funktion som består av att de binder till specifika molekyler. I läkemedelsindustrin har man utnyttjat denna funktion för att utveckla nya antikroppsbaserade läkemedel och teknologier. En antikropp är en rätt så stor molekyl. Storleken har visat sig vara ett problem i vissa avseenden. För att undvika problem, har man med hjälp av biokemi kunnat producera mindre fragment av antikroppar med bibehållen funktion. Dock har det visat sig att dessa fragment kan vara svåra att producera. Det gör att de blir dyra och inte lika tillgängliga för patienter runt om i världen. Dessutom har det visat sig att en del av dem inte är så stabila. Deras instabilitet kan ha en negativ effekt på deras funktion, bland annat att de kan börja aggregera och bilda stora proteinkomplex. Detta är ett stort problem för proteinbaserade läkemedel eftersom det kan bland annat initiera kroppens immunförsvar.

I det här projektet har tre olika aspekter undersökts. Den första aspekten var att undersöka ny strategi för att göra ett antikroppsfragment mer stabilt. Metoden bestod av att införa negativa laddningar på ytan av fragmentet. Det visade sig fungera och de bibehöll sin funktion. Den andra aspekten var att utveckla en reningsprocess för ett annat svår-producerat antikroppsfragment. Processen bestod av två olika kromatografisteg. Det sista steget optimerades med hjälp av statistiska modeller. Resultatet visade att det var möjligt att få ett bra utbyte med en tillräcklig bra renhet. Den tredje aspekten var att utveckla antikroppsbaserade material för affinitetskromatografi som binder till ett specifikt protein. Problemet bestod av att det ursprungliga materialet hade en låg bindningskapacitet. Hypotesen var att antikroppen var för stor. Olika antikroppsfragment undersöktes som alternativ, vilket resulterade i nya material med förbättrad bindningskapacitet.

List of Papers

Papers included in this thesis

This thesis is based on the following papers, which will be referred to in the text by Roman numbers. The papers are appended at the end of this thesis.

Paper I	**L.I. Sakhnini**, P.J. Greisen, Z. Bozoky, C. Wiberg, S. Lund, A.M. Wolf Pérez, H.S. Karkov, J-J. Hansen, K. Huus, L. Bülow, N. Lorenzen, M.B. Dainiak, A.K. Pedersen, Improving the developability of an antigen binding fragment by aspartate substitutions. Biochemistry 58 (2019) 2750-2759.
Paper II	**L.I. Sakhnini**, A.K. Pedersen, I. Rodríguez Léon, P.J. Greisen, J-J. Hansen, M.B. Vester-Christensen, L. Bülow, M.B. Dainiak, Optimizing selectivity of anion hydrophobic multimodal chromatography for purification of a single-chain variable fragment. Eng. Life Sci. 19 (2019) 490-501.
Paper III	**L.I. Sakhnini**, A.K. Pedersen, H. Ahmadian, J-J. Hansen, L. Bülow, M.B. Dainiak, Designing monoclonal antibody fragment-based affinity resins with high binding capacity by thiol-directed immobilisation and optimisation of pore/ligand size ratio. J. Chromatogr. A 1468 (2016) 143-153.
Paper IV	**L.I. Sakhnini**, A.K. Pedersen, M.B. Dainiak, L. Bülow, Multimeric fusion single-chain variable fragments as novel high-capacity ligands (2019, manuscript in-preparation).

Papers not included in the thesis

A.M. Wolf Pérez, P. Sormanni, J. Sonne Andersen, **L.I. Sakhnini**, I. Rodriguez-Leon, J. Rose Bjelke, A. Juhl Gajhede, L. De Maria, D. Otzen, M. Vendruscolo, N. Lorenzen, In vitro and in silico assessment of the developability of a designed monoclonal antibody library. mAbs 11 (2019) 388-400.

My contribution to the papers

Paper I	I planned and designed the study. Dr. Per Jr. Greisen performed all the *in silico* experiments in Rosetta. I performed all the *in vitro* experiments, of which the Biacore SPR and the aggregation studies were performed inclusively with Dr. Charlotte Wiberg and Dr. Nikolai Lorenzen, respectively. All plasmids were constructed by Dr. Søren Lund. Furthermore, I drafted the paper closely together with Dr. Nikolai Lorenzen, thereafter refined it under guidance of all co-authors.
Paper II	I participated in the planning of the study and performed all the experiments, with an exception to the nano LC-MS/MS analyses which were performed by Dr. Ileana Rodríguez Léon. I drafted the paper, thereafter refined it under guidance of all co-authors.
Paper III	I participated in the planning of the study, performed all the experiments and drafted the paper, thereafter refined it under guidance of all co-authors.
Paper IV	I designed the multimeric fusion scFv ligands, planned the study, performed all the experiments and wrote the paper.

Abbreviations

AC	Affinity chromatography
ADC	Antibody-drug conjugate
AIEC	Anion exchange chromatography
BET	Brunauer-Emmett-Teller isotherm
BiAb	Bispecific antibody
BiTE	Bispecific T-cell engager
CD spectroscopy	Circular dichroism spectroscopy
CDR	Complementarity determining region
CHO	Chinese hamster ovary cells
CIC	Cross-interaction chromatography
CIEC	Cation exchange chromatography
CIP	Cleaning-in-place
C_H	Constant domain of heavy chain
C_L	Constant domain of light chain
C-terminal	Carboxyl-terminal
CV	Column volume
DBC	Dynamic binding capacity
DoE	Design of experiments
DSC	Differential scanning calorimetry
DSF	Differential scanning fluorometry
ELISA	Enzyme-linked immunosorbent assay
Fab	Antigen binding fragment
Fc	Constant/crystallisable fragment
FDA	Food and Drug Administration
FR	Framework
FT	Flow-through

Fuc	Fucose
ΔG	Gibb’s free energy
Gal	Galactose
GlcNAc	N-acetylglucosamine
GRM	General rate model
HCP	Host cell protein
HEK293	Human embryonic kidney 293 cells
HIC	Hydrophobic interaction chromatography
HTE	High-throughput experimentation
Ig	Immunoglobulin
LC-MS	Liquid chromatography mass spectrometry
mAb	Monoclonal antibody
Man	Mannose
MMC	Mixed-mode or multimodal chromatography
MS	Mass spectrometry
MWCO	Molecular weight cut-off
NPC	Normal phase chromatography
ND	Not determined
N-terminal	Amino-terminal
OFAT	One-factor-at-a-time
PAT	Process analytical technologies
QbD	Quality by design
RPC	Reverse phase chromatography
SAA	Solvent accessible area
SAP	Spatial aggregation propensity
SEC	Size-exclusion chromatography
scFv	Single-chain variable fragment
SE-HPLC	Size-exclusion high-performance chromatography
SE-UPLC	Size-exclusion ultra-performance chromatography
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMA	Steric mass-action
SMAC	Standup-monolayer adsorption chromatography
SPR	Surface plasmon resonance

T_m	Temperature of midpoint of heat denaturation
TNF	Tumour necrosis factor
UF/DF	Ultrafiltration and diafiltration
V_H	Variable domain of heavy chain
V_L	Variable domain of light chain
V-NAR	Variable new antigen receptor
wt	Wild-type

1. Introduction

Antibody-fragments have become indispensable tools in the biopharmaceutical industry. Their smaller size has made them the format of choice relative to full-length antibodies in applications ranging from therapeutic drugs, probes in diagnostic applications to affinity ligands in preparative downstream processes and analytical enzyme-linked immunosorbent assays (ELISA) [1,2,3,4,5,6,7]. However, there are challenges in their biophysical stability and downstream processing that can affect their production and long-term functionality. This thesis aims to review past and present developments of antibody-fragments and their application as affinity ligands in affinity chromatography.

1.1 Research Objectives

In this doctoral thesis, antibody-fragments, such as the antigen binding fragment (Fab) and the single-chain variable fragment (scFv), were investigated in terms of biophysical stability, downstream processing and their application as ligands in affinity adsorbents for purification of biopharmaceuticals. The aims were to:

I. Gain insight into the effect of different biophysical properties on the aggregation propensity of a Fab,

II. Explore multimodal chromatography for the development of non-conventional purification processes for antibody-fragments,

III. Investigate the importance of ligand size, immobilisation chemistry and pore size in the development of high-capacity immunoaffinity adsorbents.

2. Monoclonal Antibodies and Their fragments

2.1 Structure and Function

Antibodies, also known as immunoglobulins (Igs), are bivalent glycoproteins originally part of the humoral immune response of the adaptive immune system, which is one of the lines of defence against pathogens in the human body. The biological function of antibodies is to signal the presence of foreign invasion by recognising and binding to specific elements, defined as epitopes, of foreign molecules known as antigens. There are five different classes of antibodies, IgA, IgD, IgE, IgG and IgM, differing in their structure and biological function. The IgG is further split into four subclasses; IgG1, IgG2, IgG3 and IgG4. Furthermore, these proteins are produced by plasma cells (B lymphocytes) in the blood and they are all found in different concentrations, with IgG being present in the highest concentration [8].

An IgG antibody consists of two heavy chains and two light chains (kappa or lambda class) connected via disulphide bridges. The heavy chain consists of a variable domain (V_H) followed by three constant regions, C_H1, C_H2 and C_H3, whereas the light chain consists of a variable domain (V_L) followed by a constant domain (C_L) (see Figure 1) [9].

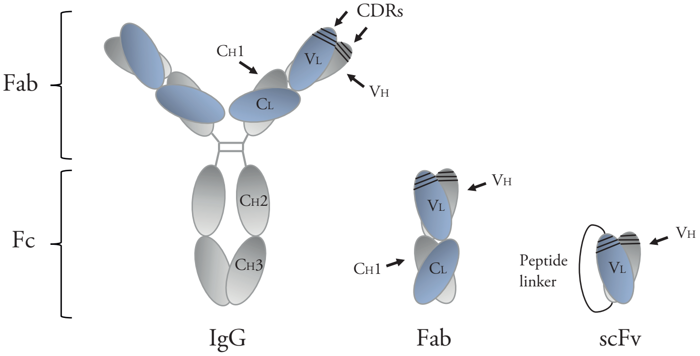
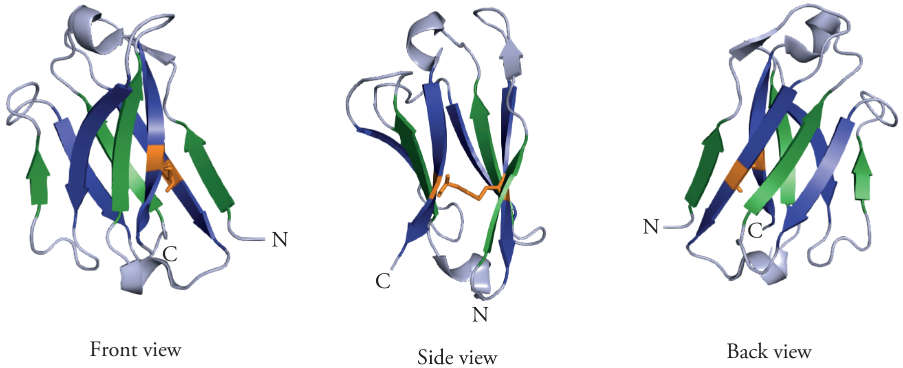


Figure 1. A schematic presentation of IgG, Fab and scFv. The figure was inspired from reference [9].

Furthermore, an IgG consists of 12 domains, each adopting the immunoglobin fold, i.e. two β-sheets of anti-parallel configuration, bridged together via a single intrachain disulphide bond, with a hydrophobic core (see IgG fold of V_H domain of Adalimumab in Figure 2) [8]. The variable regions, V_H and V_L, consists of four framework (FR) regions and three hypervariable peptide loops, respectively, known as the complementarity determining regions (CDRs) containing paratopes. The CDRs are solvent-exposed loops that are often partially disordered and contain both hydrophobic and charged amino acid residues to provide specific binding to antigens. They are structurally supported by the FRs [26]. The strength of the interaction between a paratope of an antibody and an epitope of an antigen is defined as affinity, which can be measured by e.g. surface plasmon resonance (SPR) [10].



Front view

Side view

Back view

Figure 2. The structure of the IgG fold of the V_H domain of Adalimumab (based on PDB 4NYL). The intrachain disulphide bridge is indicated by the orange coloured Cys residues.

Variable regions from different antibodies can be studied and compared to each other with help of numbering systems used for annotation of the variable regions. The most common ones are the Kabat [11] and the Chothia [12] numbering systems. The Kabat numbering is based on primary sequence data, whereas the Chothia numbering is based on structurally correct positions. An example of a Kabat numbering assignment can be seen in Figure 3.

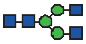
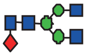
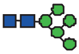
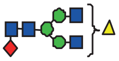
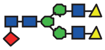
```
         1         2         3         4         5
1234567890123456789012345678901234567890123456789012A3456789
                              11111                22222222222
EVQLVESGGGLVQPGRSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSAITWNSGHIDY

6         7         8            9         10            11
01234567890123456789012ABC34567890123456789 0ABCD123456789012
222222                                333333333333
ADSVEGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAKVSYLSTASSLDYWGQGTLVTVS
```

Figure 3. Kabat numbering assignment of the V_H domain of Adalimumab (based on PDB 4NYL). The CDRs are designated the numbers 1, 2 and 3.

Smaller fragments containing the variable regions can be generated by protein engineering. Such fragments are the Fab consisting of V$_H$-C$_H$1 and V$_L$-C$_L$ connected via an interchain disulphide bridge, and the scFv consisting of V$_H$ and V$_L$ connected via a peptide linker [9]. A schematic presentation of these molecular formats can be seen in Figure 1.

Glycosylation is an important post-translational modification of an antibody. It can affect conformation, stability, bioactivity and immunogenicity [13,14]. All IgG molecules contain N-glycans, which are polysaccharide molecules covalently attached to asparagine (Asn) residues of the Fc region via a N-glycosidic bond [14,15]. Humans have 36 different types of N-glycans [15], of which all have a common core consisting of three mannoses (Man) and two N-acetylglucosamines (GlcNAc) (see Table 1) [14].

Table 1. Common N-glycans of human mAbs; Fuc = fucose, Gal = galactose, Glc = glucose, GlcNAc = N-acetylglucosamine, Man = mannose.

N-glycan	**Full name**	**Average mass [g·mol^{-1}]**	**Structure**
G0	GlcNAc$_2$Man$_3$ GlcNAc$_2$	1299.21	
G0F	GlcNAc$_2$Man$_3$ GlcNAc$_2$Fuc	1445.35	
Man5	GlcNAc$_2$Man$_5$	1217.10	
G1F	GalGlcNAc$_2$Man$_3$ GlcNAc$_2$Fuc	1607.49	
G2F	Gal$_2$GlcNAc$_2$Man$_3$ GlcNAc$_2$Fuc	1769.63	

2.2 Past and Present Developments

In 1975, Köhler and Milstein from the MRC Laboratory of Molecular Biology in Cambridge (UK) discovered a way to generate monoclonal IgG antibodies (mAbs), which are identical proteins produced by a single cell line, using the hybridoma technology [16]. They fused immortal mouse myeloma cells with spleen cells from mouse immunized with sheep red blood cells. This resulted in antibody-producing hybridoma cells that could be cultured [16]. Using this technology, the first therapeutic mouse mAb, OKT3, was developed and later on approved in 1986 by the Food and Drug Administration (FDA) for clinical use during kidney transplants to prevent rejection of the new organ. However, the mouse OKT3 mAb was later reported to be immunogenic due to high response of human anti-mouse antibodies in the human body [17]. Humanisation strategies to minimize the immunogenicity included making chimeric mAbs [18,19], which were a hybrid of human constant regions and mouse variable regions, and variable region resurfacing [20,21], which aimed to replace surface residues of a mouse variable region with a human set of surface residues.

Advancements in recombinant technology enabled production of mAbs in mammalian cells instead of less-defined hybridoma cells [22,23]. Mammalian cells, such as Human Embryonic Kidney 293 (HEK293) and Chinese Hamster Ovary (CHO) cells, were preferred over bacterial expression systems, such as Escherichia coli (*E. coli*), due to the larger size of the mAb (approximately 150 kDa) relative to the host cell. However, it was found that mAb fragments, such as Fab and scFv, could successfully be expressed in *E. coli* due to their smaller size [22,23,24]. Particularly, their smaller size has made them more attractive than full-length antibodies due to several advantages such as improved tissue penetration, faster blood clearance for diagnostic applications, lower immunogenicity due to the absence of the constant/crystallisable fragment (Fc) region, improved capacity and enablement of multivalent formatting [25,26,27,28]. Despite their shorter half-life in the human body due to the lack of the Fc region, which enables circulation via the neonatal Fc receptor, chemical modifications such as PEGylation and PASylation have recently shown to be successful for increasing their half-life [29,30,31,32,33,34].

Following the Hybridoma technology and the immunization methodology for discovery of antibodies, a breakthrough in science was made in 1985 when Smith [35] discovered that foreign DNA could be inserted into the filamentous phage gene III, resulting in the display of antigens on the phage surface. Based on this finding, McCafferty *et al.* [36] showed that it was possible to display and isolate a rare scFv on the surface of a filamentous phage. Ultimately, Winter *et al.* [37] later reported on the screening of combinatorial antibody phage libraries, thereby revolutionizing antibody discovery by evading immunization of animals.

M13 is the most commonly used bacteriophage in the antibody phage display technology and it is approximately 1 µm in length. It consists of single stranded DNA (ssDNA), found inside the phage, displaying five copies of the pIII coat protein. The methodology is iterative and consists of the following steps; (1) a phage library of size 10^9-10^{11}, displaying e.g. scFvs with randomized paratopes, is screened against a desired target protein immobilized on an ELISA plate, (2) excess, non-bound phages are washed off from the ELISA plate, (3) bound phages are eluted by e.g. changing the pH or adding solutions with different salt concentrations, (4) eluted phages are amplified by infecting *E. coli* host cells, and (5) antibody phages are purified and sequenced for identification. The whole process is usually repeated 3-4 rounds to enrich higher affinity antibodies [38,39]. An overview of the process can be seen in Figure 4.

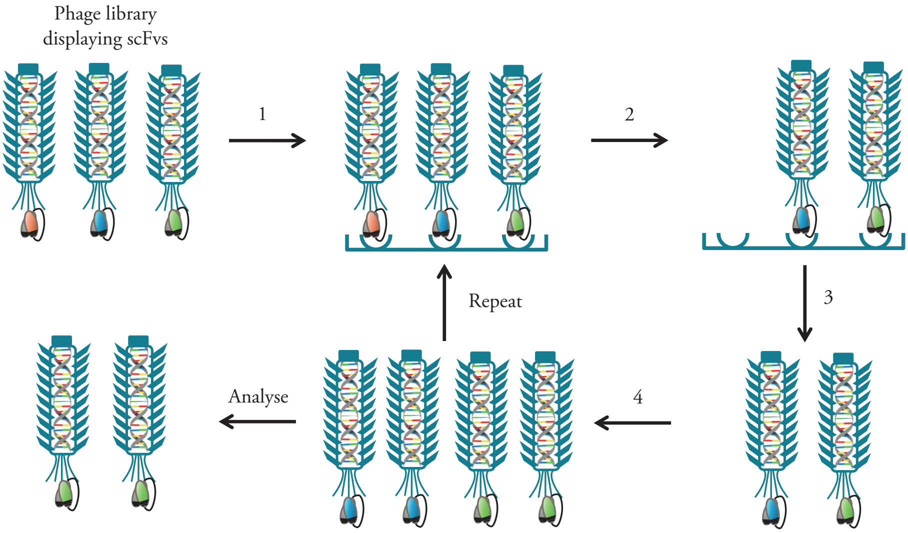


Figure 4. An overview of the phage display process; 1) phages bind to the target molecule, 2) excess phages are washed away, 3) bound phages are eluted, 4) eluted phages are amplified for further analysis. The figure was inspired from reference [38].

Adalimumab was the first phage display-derived antibody, developed by Cambridge Antibody Technology, that got approved by the FDA in 2002 [40,41]. It acts by neutralising the tumour necrosis factor (TNF) alpha, which is a proinflammatory cytokine involved in apoptosis, cell survival, immunity and inflammation [42]. Today, Adalimumab is sold by AbbVie under the commercial name Humira® for treatment of several diseases such as plaque psoriasis, psoriatic arthritis, rheumatoid arthritis, Crohn's disease, polyarticular juvenile idiopathic and active enthesitis-related arthritis and non-infectious uveitis [43]. Humira® is the world's most sold pharmaceutical drug with global sales of 19.9 billion US dollars in 2018 [44]. The therapeutic mAb market has been expanding ever since the first approved mAb, OKT3, in 1986; around 70 mAbs will be on the market by 2020 and the total global sales are estimated to 125 billion US dollars [45].

Furthermore, the molecular format of antibodies has evolved beyond just mAb, Fab and scFv. The demands for multi-specific targeting, higher affinity, shorter half-life and cytotoxic properties have led to the development of exotic formats such as bispecific antibodies (BiAb), antibody-drug conjugates (ADC), single-domain V antibodies, minibodies, diabodies and recombinant fusions (see Figure 5) [28,46,47,48,49].

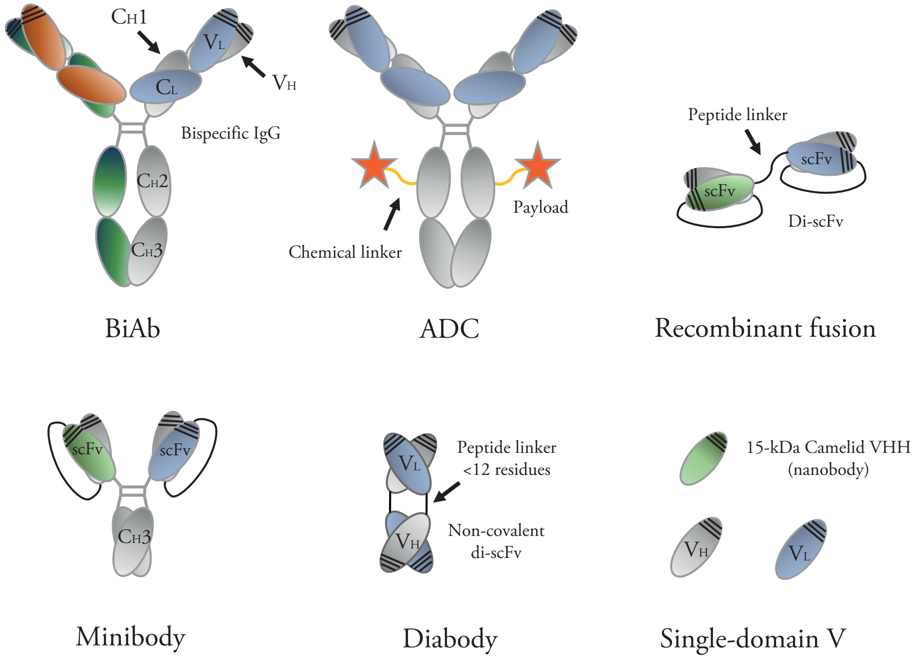

C_H1
V_L
V_H
C_L
Bispecific IgG
C_H2
C_H3
BiAb
Payload
Chemical linker
ADC
Peptide linker
scFv
scFv
Di-scFv
Recombinant fusion
scFv
scFv
C_H3
Minibody
V_L
Peptide linker
<12 residues
V_H
Non-covalent
di-scFv
Diabody
15-kDa Camelid VHH
(nanobody)
V_H
V_L
Single-domain V

Figure 5. A schematic presentation of a selection of emerging molecular formats of antibodies within therapeutic and diagnostic applications. The figure was inspired from references [28,48,49].

BiAbs are bispecific bivalent antibodies that can bind to two different antigens. There are different types of bispecific antibodies; bispecific IgGs, bispecific antibody fragments, bispecific recombinant fusions and bispecific antibody conjugates [47]. In particular, bispecific IgGs can be challenging to produce. Co-expression of the heavy and light chains can lead to homodimerisation of the heavy chains and mispairing of the light chains. In addition, separation of such mixture with closely related antibodies can be a major challenge [47,50]. Nevertheless, several strategies have been developed to overcome these issues, e.g. knobs-in-holes (introduction of mutations in C_H3 to avoid homodimerisation) [51], DuoBody® technology (introduction of mutations in C_H3 to facilitate Fab-arm exchange) [52], CrossMAb® technology (domain crossover of V_H-C_H1 and V_L-C_L of one of the Fab-arms to avoid mispairing of the light chains, and knobs-in-holes strategy to avoid homodimerisation) [53], and charge-pair (introduction of mutations in C_H3 to favour heterodimerisation of the Fc via attractive charge interactions) [54]. Furthermore, a common approach in the preparation of bispecific IgGs has been to express and purify the two different IgG molecules separately; then perform the assembly [47]. Until now, two BiAbs have been approved by the FDA for therapeutic use; Blinatumomab [55] and Emicizumab [56] (see Table 2). Blinatumomab is a bispecific T-cell engager (BiTE), developed by Amgen, for treatment of patients with B-cell precursor acute lymphoblastic leukaemia. It is a recombinant fusion di-scFv, with the two scFvs being separated by a Gly/Ser peptide linker [57]. Emicizumab is a bispecific IgG, discovered by Chugai Pharmaceuticals and further developed by Genentech (subsidiary of Roche), for treatment of patients with Haemophilia A. The bispecific IgG binds to the coagulation factors IXa and X [58].

ADCs are monoclonal antibodies that are chemically conjugated to a drug, i.e. a payload. They have become of great interest for selective targeting of cancer cells for delivery of cytotoxic molecules [59]. Today, there are four ADCs approved by the FDA for the treatment of cancer [60] (see Table 2).

Minibodies and diabodies are scFv-based formats that have emerged as superior alternatives to scFvs. A minibody is a scFv-C_H3 dimer, whereas a diabody is a scFv dimer that self-assemble when the peptide linker between V_H and V_L is shorter than 12 residues (see Figure 5). They have attracted a great deal of interest in tumour localisation and therapy as they have shown to accumulate in tumours at higher levels relative to IgGs and scFvs [28].

Single-domain V antibodies are the smallest antigen binding fragments of antibodies. There are different types of single-domain V antibodies; domain antibodies (V_H and V_L), Camelid-derived heavy-chain variable domains (nanobodies) and shark-derived variable new antigen receptor (V-NAR) domains. In particular, nanobodies and V-NAR domains have shown to be more attractive due to higher affinity, solubility and stability under stringent conditions relative to mouse and human domains [28,61]. The world's first nanobody, Caplacizumab, was approved by the FDA in 2019 for the treatment of thrombotic thrombocytopenic purpura, which is a rare blood clotting disease (see Table 2) [62].

Table 2. A list of BiAbs, ADCs and single-domain V antibodies with regulatory approval.

Drug	Molecular format	Regulatory approval	Targets	Ref.
Catumaxomab (Removab®, Neovii Biotech)	BiAb (trifuctional rat/mouse IgG)	EMA approval* (2009)	Tumour cell protein EpCAM and T-cell surface protein CD3	[63]
Blinatumomab (Blincyto®, Amgen)	BiAb (recomb. fusion di-scFv)	FDA approval (2014)	B-cell antigen CD19 and T-cell surface protein CD3	[55]
Emicizumab-kxh (Hemlibra®, Genentech)	BiAb (humanised IgG)	FDA approval (2017)	Factor IXa and X	[56]
Brentuximab vedotin (Adcetris®, Seattle Genetics)	ADC (Chimeric IgG and toxin)	FDA approval (2011)	Tumour cell antigen CD30	[64]
Ado-trastuzumab emtansine (Kadcyla®, Genentech)	ADC (humanised IgG and toxin)	FDA approval (2013)	Human epidermal growth factor receptor 2 (HER2)	[65]
Inotuzumab ozogamicin (Besponsa®, Wyeth)	ADC (humanised IgG and toxin)	FDA approval (2017)	B-cell antigen CD22	[66]
Gemtuzumab Ozogamicin (Mylotarg®, Wyeth)	ADC (humanised IgG and toxin)	FDA approval (2017)	Tumour cell antigen CD33	[67]
Caplacizumab (Cablivi®, Ablynx/Sanofi)	Single-domain V (bivalent nanobody)	FDA approval (2019)	Von Willebrand factor	[68]

*The market authorisation was withdrawn by the European Commission in 2017 at the request by Neovii Biotech [69].

2.3 Biophysical Challenges

There are several important biophysical properties, in addition to desired antigen binding (equilibrium dissociation constant ranging from nanomolar to picomolar [70]), low immunogenicity and high expression level, that needs to be met during early-stage development of antibodies;

I. high solubility,
II. low non-specificity,
III. high thermodynamic and colloidal stability [71].

The measurement of these properties is often referred to as developability assessment [72,73]. Primarily, there are two types of protein stabilities; (1) *chemical* such as deamidation, oxidation and hydrolysis, and (2) *physical* such as thermodynamic changes, adsorption to surfaces and aggregation. Particularly, protein aggregation has a great impact on manufacturing, stability and safety, and is therefore considered as a major challenge during the drug development [74,75]. It has shown to be associated with cytotoxicity and immunogenicity in patients [76]. Furthermore, a variety of different factors, such as amino acid sequence, biophysical stability (e.g. structural and colloidal), concentration and formulation, can affect the protein aggregation process [77,78,79,80].

2.3.1 Thermodynamic Stability

The thermodynamic stability of a protein is fundamentally described by Gibbs free energy of unfolding ($\Delta G_{\text{unfolding}}$), which is the difference in free energy between the native state and the denatured state. Thermodynamic stability is referred to the reversibility of protein denaturation. Protein folding is a complex, spontaneous process with the hydrophobic effect being the main driving force. In addition, it is a *cooperative* process as the conformation of one amino acid affects the conformation of another amino acid in a protein. To study thermodynamic stability and cooperativity, which are equilibrium properties unlike folding kinetics, proteins should be studied at thermodynamic *equilibrium*. For instance, in order to study unfolded proteins at equilibrium, the folded state should be destabilised, e.g. by changing the pH of the protein solution, to get the temperature of denaturation closer to more practical conditions [81] (see example in Figure 6).

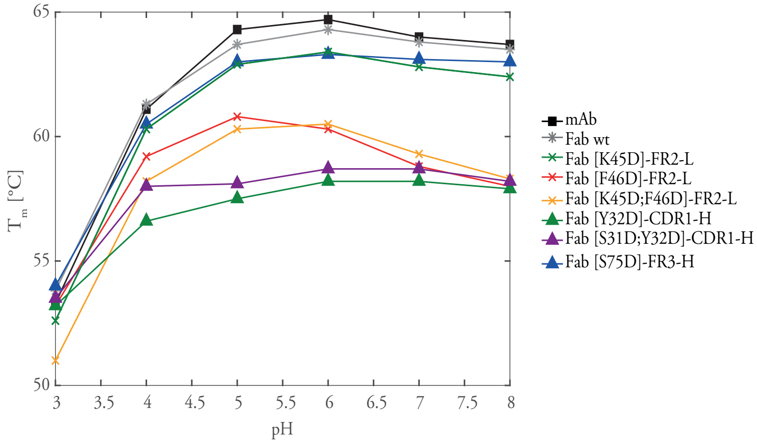


Figure 6. An illustration of the pH dependence of the temperature of midpoint of heat denaturation (T_m) for a mAb and seven Fab variants (unpublished data). T_m is shown for the first unfolding transition.

Protein unfolding can be followed by a variety of different variables, e.g. molar ellipticity (circular dichroism, CD, spectroscopy), heat capacity (differential scanning calorimetry, DSC) and fluorescence (differential scanning fluorometry, DSF), during denaturation by increasing the temperature. If at least two of the measured variables result in same normalised denaturation curve, the denaturation process can be approximated to a *two-state model.* A protein that follows a two-state model is either in the native or denatured state, i.e. the population of intermediates is negligible at any given temperature. For higher unfolding states, intermediates can exist as detectable unfolding transitions [81].

For large proteins that contain multiple domains, several unfolding transitions can be observed in case the different domains unfold independently, i.e. have different intrinsic stability. A full-length mAb, which is a 12-domain protein, usually exhibit three unfolding transitions corresponding to C_H2, C_H3 and Fab, respectively. The order in which the domains unfold has shown to depend on the antibody subclass [82]. Furthermore, the reason to why three transitions are normally observed is due to same intrinsic stability of the domains within the C_H2, C_H3 and Fab, respectively. An example of a Fab with multiple unfolding transitions can be seen in Figure 7. The Fab containing the unstable mutation [F46D]-FR2-L exhibit two unfolding transitions, indicating altered intrinsic stability of one or several of the domains relative to wild-type (wt).

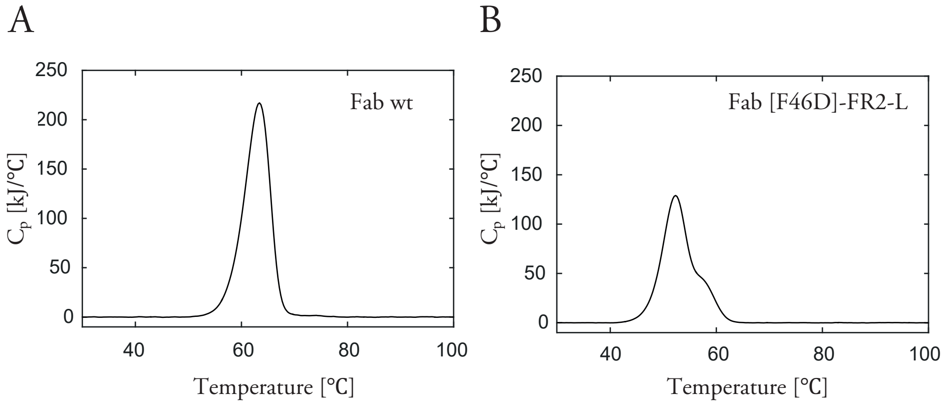


Figure 7. DSC thermal unfolding curves for (A) Fab wt and (B) [F46D]-FR2-L at pH 8.3 (unpublished data).

For a protein with a two-state model behaviour and a temperature independent heat capacity change upon unfolding (ΔC_p), the thermodynamic stability ($\Delta G_{unfolding}$) can be expressed as Eq. 2.1 [83].

$$\Delta G_{unfolding} = \Delta H_m \cdot \left(\frac{T_m - T}{T_m}\right) + \Delta C_p \cdot \left(T_m - T \cdot \left(1 - ln\left[\frac{T}{T_m}\right]\right)\right) \tag{2.1}$$

Where T_m is the temperature of midpoint of heat denaturation at which the $\Delta G_{unfolding}(T_m) = 0$, ΔH_m is the enthalpy of unfolding at T_m, and T is the temperature. ΔC_p can be estimated by DSC experiments. At $\Delta G_{unfolding}(T) > 0$, the native state is stabilised, of which the maximum stability is achieved at $T = T^*$.

2.3.2 Colloidal Stability

The colloidal stability of a protein depends on all attractive and repulsive short-range interactions such as exchange repulsion (i.e. hard-sphere), van der Waals interaction (i.e. all distance dependent intermolecular interactions), and electrostatic and hydrophobic interactions [80]. Protein-protein interactions are highly dependent on molecular shape, i.e. shape complementarity [84]. For antibodies, the colloidal stability can be measured in terms of protein self-association. There are several ways for measuring protein self-association:

- second virial coefficient (B22) by static light scattering or membrane osmometry,
- diffusion interaction parameter (k_d) by dynamic light scattering,
- plasmon wavelength (λ_p) by affinity-capture self-association nanoparticle spectroscopy [85].

As the success of therapeutic proteins is greatly impacted by surface interactions [85], efforts to predict non-specific protein-protein interactions have been taken. Chennamsetty *et al.* [86] developed the spatial aggregation propensity (SAP) tool, which is an *in silico* predictor for identification of potential hydrophobic regions on surfaces of proteins that are prone to non-specific protein-protein interactions. Based on a three-dimensional structure of a protein, SAP scores are calculated for each of the side-chain atoms (see Eq. 2.2). The SAP score of one amino acid is then calculated by averaging the SAP scores for its constituent atoms within a certain radius (SAP radius). A SAP radius of 10 Å has previously been shown useful for identification of potential hydrophobic patches [87]. The SAP scale is normalized to the Gly residue, e.g. positive SAP scores are on the hydrophobic scale whereas negative SAP scores are on the less hydrophobic scale [86].

$$SAP_{atom\ i} = \sum\nolimits_{\substack{simulation\\ average}} \left\{ \sum\nolimits_{\substack{Residues\ with\ at\ least\\ one\ side-chain\ atom\\ within\ R\ from\ atom\ i}} \left(\frac{\substack{SAA\ of\ side-chain\\ atoms\ within\ R}}{\substack{SAA\ of\ side-chain\ atoms\\ of\ fully\ exposed\ residue}} \cdot Residue\ hydrophobicity \right) \right\} \tag{2.2}$$

Where SAA is the solvent accessible area and R is the SAP radius. SAA is commonly used to find the exposure of amino acids in proteins. For illustration, a SAP map for a scFv can be seen in Figure 8.

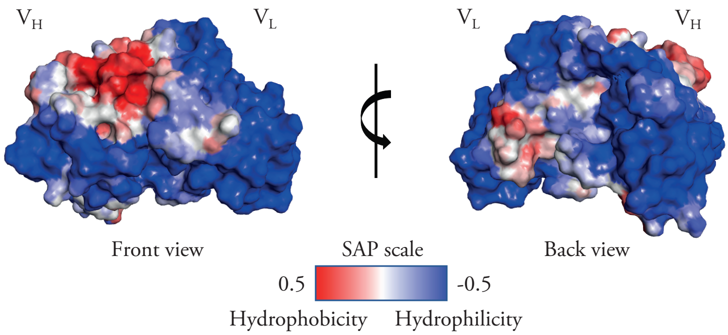


Figure 8. An illustration of a SAP map of a scFv against target protein X.

Although SAP has been shown to be a useful tool for some antibodies [86,87,88], some structural scientists argue that the SAP tool cannot be generally applied for proteins as it does not consider shape complementarity, which is the fundamental basis for protein-protein interactions.

Other approaches for prediction of non-specific protein-protein interactions are standup-monolayer adsorption chromatography (SMAC) and cross-interaction chromatography (CIC). In SMAC, proteins are passed through a size-exclusion chromatography column (i.e. Zenix HPLC column, Sepax Technologies Inc., USA) [89], whereas in CIC they are passed through a column containing immobilised polyclonal IgGs [90]. The retention times of the proteins in SMAC and CIC are then related to their colloidal stability. For antibodies, they have been shown to correlate well with protein self-association [91].

Colloidal properties have important implications on the behaviour of proteins. An example is the behaviour of the scFv against target protein X in solution at physiological conditions. Native mass spectrometry (MS) indicated the presence of non-covalent dimers. As the concentration increased from 0.6 to 1 mg/mL, the population of dimers increased, indicating concentration-dependent monomer-dimer equilibrium (see Figure 9A-B). The population of dimers could also be observed by size-exclusion ultra-performance liquid chromatography (SE-UPLC) in Figure 9C-D. When the same scFv sample was analysed by SE-UPLC with running buffer containing 10 % isopropanol, one population of monomers could be observed (see Figure 9E). This suggests that the monomer-dimer interface, that was disrupted by the isopropanol, could potentially have involved hydrophobic residues. The monomer-dimer equilibrium may then have been a way to stabilise the scFvs by shielding solvent-exposed hydrophobic residues from surrounding water molecules.

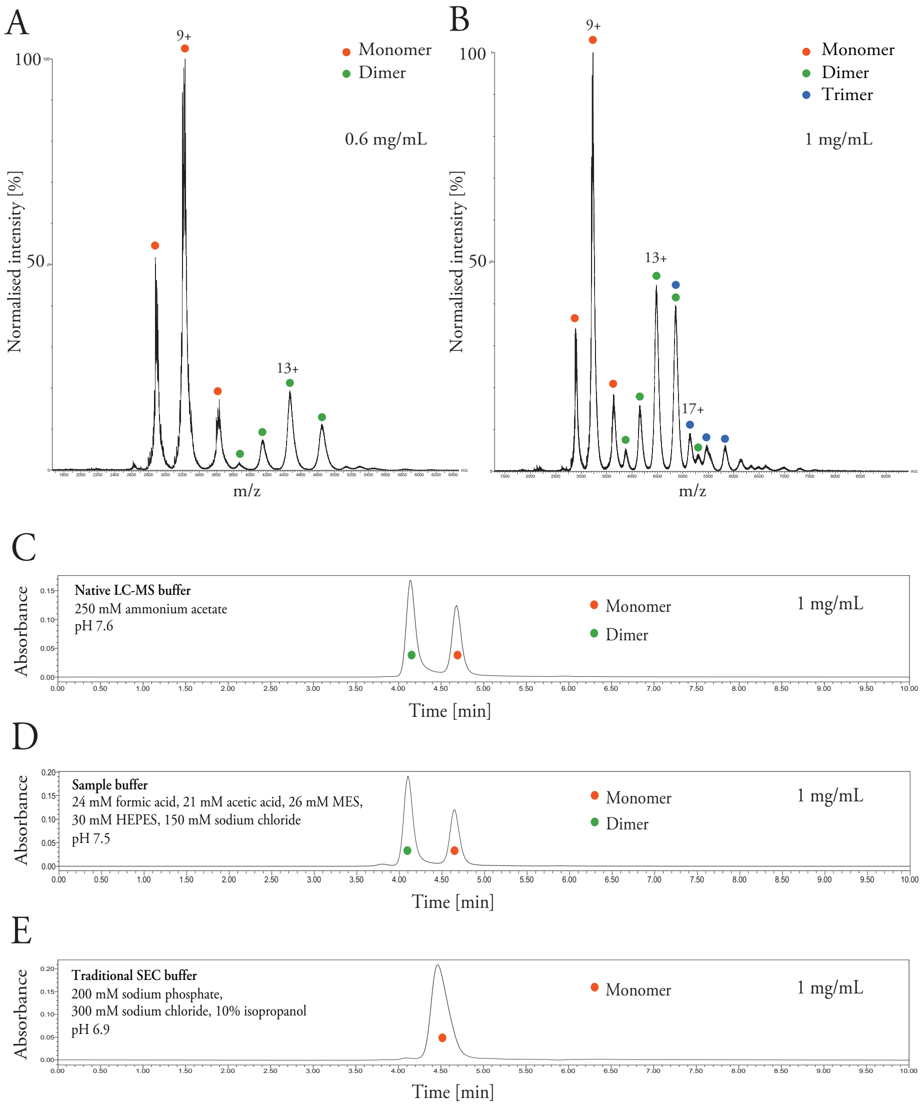


Figure 9. Analyses of a scFv against protein X (unpublished data). Native MS spectra are shown for scFv at (A) 0.6 mg/mL, and (B) 1 mg/mL, repsectively. SE-UPLC chromatograms are shown for scFv (1 mg/mL) analysed with (C) native LC-MS buffer, (D) sample buffer and (E) buffer with 10 % isopropanol, respectively. The native MS analyses were performed by Dr. Jun Frederiksen (Novo Nordisk A/S, Denmark).

2.3.3 Protein Aggregation

There are two types of aggregation pathways; non-native and native. In the non-native process, proteins undergo a thermodynamic change by full or partial unfolding, whereafter stable aggregates are formed [92]. In the native process, proteins self-associate or associate to other proteins (polyspecific interactions) at native conditions via interactions of aggregation-prone hotspots on the surface, whereafter stable aggregates are formed (see Figure 10) [93,94]. Previous studies have shown that protein aggregation can be mediated by protein/protein interfaces such as those between CDRs and antigens [85,95,96,97,98,99].

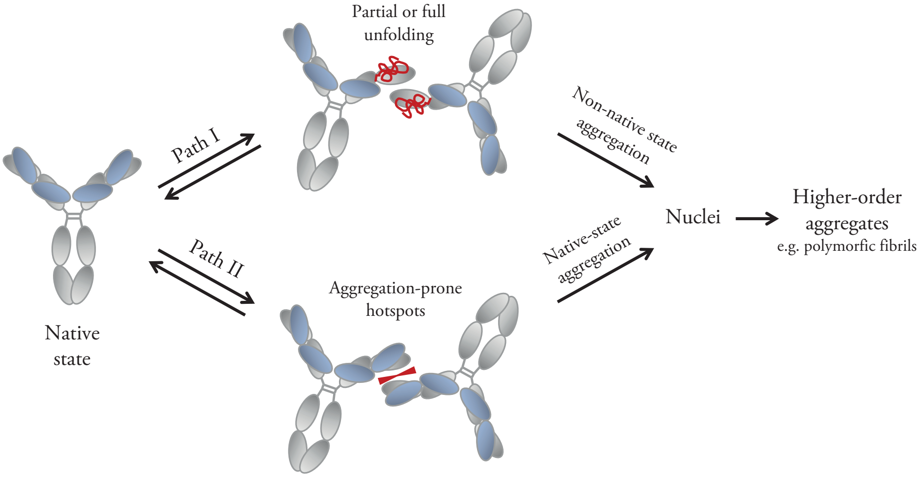


Figure 10. Common aggregation pathways; (I) native antibody becomes partially or fully unfolded, and (II) native antibody self-associate via aggregation-prone hotspots. All paths leading to the formation of a nuclei, which is defined as the smallest stable irreversibly formed aggregate such as a dimer, are often reversible. Following nucleation, higher order aggregates are formed. The figure was inspired from reference [94].

In the pharmaceutical industry, assessment of aggregation is crucial for the estimation of product shelf-life, which is defined as the time in which the product remains within the approved specifications while stored under defined conditions. Real-time stability studies can take 6 months to 2 years to conduct. This may not be feasible during early-stage screening [94,100]. Accelerated stability studies at higher temperatures have become more common for extrapolation of real-time stability studies at lower temperatures. However, due to the complexity of the aggregation process and temperature dependence of thermodynamic stability, such type of aggregation has shown to follow a non-Arrhenius behaviour, i.e. the natural logarithm of the reaction rate coefficient is not proportional to the inverse of the temperature (see Arrhenius equations in Eq. 2.3-2.4) [94]. This suggests that stability studies at higher temperatures may not extrapolate real-time stability studies well at lower temperatures.

$$k = A \cdot e^{-\frac{E_a}{R \cdot T}} \qquad \text{Arrhenius equation} \qquad (2.3)$$

$$\ln k = \ln A - \frac{E_a}{R \cdot T} \qquad \text{Linear form of the Arrhenius equation} \qquad (2.4)$$

Where k is the reaction rate coefficient, A is the pre-exponential factor, E_a is the energy of activation for the reaction, R is the universal gas constant and T is the temperature.

2.3.4 Engineering Antibodies for Aggregation Resistance

There are three different ways to engineer proteins; (i) rational design, (ii) directed evolution and (iii) semi-rational design. Rational design is based on detailed understanding of the structure, function and biophysical properties of the protein, whereas directed evolution is an iterative process based on random mutagenesis, screening and selection, such as the phage, mammalian and yeast display technologies [101,102,103]. Semi-rational design is a combination of rational design and directed evolution, i.e. screening of a library of multiple mutations assembled on the basis of structural, functional or biophysical information [104]. A selection of these design studies for antibody stability engineering is reviewed below.

Rational and semi-rational design studies have been widely reported for the generation of stable antibodies. Ewert *et al.* [105] analysed the biophysical properties of consensus variable domains from the human germline family. They found that the consensus domain from the V_H3 family was the most stable one, followed by the V_H1 and V_H5, in terms of aggregation propensity relative to the V_H2, V_H4 and V_H6. Based on this, Ewert *et al.* described two different methods for generation of stable, aggregation-resistant variable domains; grafting of CDR loops into stable FRs and rational stability engineering by insertion of point mutations in the FRs [105]. These methods have shown to be successful in several studies by the same research group [106,107,108]. In another rational design study, Pepinsky *et al.* [109] investigated the effect of hydrophobic CDRs and isotype switch on solubility and aggregation. The study showed that mutagenesis into less hydrophobic CDRs of an IgG1 Fab resulted in substantially lower aggregation at physiological conditions. In addition, an isotype switch of the IgG1 mAb to IgG2 resulted in improved solubility and decreased aggregation. Pepinsky *et al.* suggest that factors outside the Fab, such as position of interchain disulphide bonds, may also influence the aggregation propensity of when reformatting Fabs to mAbs [109].

Courtois *et al.* [110] showed that insertion of glycosylation sites close to hydrophobic patches, mapped by the *in silico* SAP tool, can affect the aggregation propensity of a Fab domain. An up to 3-fold decrease in soluble aggregation was reported for variants with N-glycans near hydrophobic patches relative to wt after incubation at 52 °C for 48 h [110].

Perchiacca *et al.* [111] investigated homologous V_H domains differing in aggregation propensity. They wanted to understand whether the difference in aggregation propensity was due to a specific property of a single of CDR loop or a combined property of multiple CDR loops. Positively and negatively charged V_H domains were generated by site-directed mutagenesis of the most solvent-exposed residues in the CDRs. It was found that aggregation was more than dependence of surface net charge. Instead, highly positional residues, 29 and 31-33 of CDR1 in V_H, were shown to play a substantial role in aggregation measured after incubation at 70 °C for 20 min [111]. Similarly, but in a directed evolution approach, Dudgeon *et al.* [112] identified highly specific positions in the CDRs and reported on a general approach for decreasing the aggregation propensity of human V_H domains from the V_H3 and $V_\kappa1$ germline families. Positions in the CDR1 of heavy chain (CDR1-H) and CDR2 of light chain (CDR2-L) were identified and targeted for substitutions into aspartate (Asp) residues by high-throughput phage display. The variants possessed decreased aggregation propensity after heating to 80 °C. According to Dudgeon *et al.* [112], the stabilising effects of the identified Asp-substitutions were due to act on aggregation-prone unfolded or partially unfolded states.

The effect of these highly positional CDR mutations on the aggregation propensity has not been examined at physiological conditions. Therefore, this was investigated in paper I; an *in silico* library of highly positional Asp mutations (single, double and triple) in the FRs and CDRs of a Fab was screened by computational models in Rosetta. 26 different mutants were selected, produced and characterized in terms of antigen binding and biophysical stability. All variants retained the antigenic binding affinity to the target protein, and the thermodynamic stability in terms of T_m was retained or enhanced relative to Fab wt. Thirteen of the variants resulted in decreased aggregation propensity after a six-day incubation at 45 °C. [28D]-FR1-H, [31D]-CDR1-H and [53D]-CDR2-L were identified as stabilizing both as single mutations and when combined in both variable domains. With the increasing number of Asp-substitutions, it was possible to reduce the non-specificity, which together with decreased aggregation propensity are important biophysical properties for biopharmaceuticals (paper I). The reduction of non-specificity by the introduction of Asp residues aligns with a recent report by Rabia *et al.* [113].

3. Downstream Processing of Antibody Fragments

3.1 Overview

Liquid chromatography is one of the most common methods for analytical and preparative separation of proteins, peptides and smaller molecules. The experimental setup consists of a *column* packed with a *stationary phase*, e.g. porous, spherical adsorbent with certain chemical properties. The molecules of interest are dissolved in the *mobile phase*, e.g. aqueous buffer, and passed through the column. As the molecules move through the column, they will each interact differently with the stationary phase, thereby get a different *retention time*, which is the time taken for a molecule to pass through the column. The ability of the chromatographic system to distinguish between the molecules is defined as *selectivity* and it is modulated by both the properties of the stationary phase and conditions of the mobile phase.

There are two types of chromatographic modes; bind/elute and flow-through (FT). In the bind/elute-mode, the chromatographic process consists of the following steps; (i) equilibration of column, (ii) application of sample, (iii) wash of excess, unbound molecules, (iv) elution of adsorbed molecules by changing the conditions of the mobile phase, (v) Cleaning-in-Place (CIP) and (vi) storage. The elution can be performed either in isocratic mode, i.e. when the conditions of the mobile phase are kept constant, or gradient mode, i.e. when the conditions of the mobile phase are varied. In the FT-mode, the conditions of the mobile phase are selected so that the molecule of interest passes through the column without adsorbing to the stationary phase, while the other molecules adsorb to the stationary phase. The molecule of interest is collected in the FT, whereas the other molecules can be recovered following the steps described for the bind/elute-mode.

An overview of the different chromatographic techniques can be seen in Table 3. The type of technique is typically chosen based on the properties of the molecules to be separated. The selectivity can be further optimized by changing the operational parameters, e.g. pH, conductivity and composition of the mobile phase during application, wash and elution.

Table 3. An overview of different chromatographic techniques used in preparative and analytical scales.

Technique	Abbreviation	Basis of separation	Examples	Ref.
Affinity	AC	Specificity	Preparative purification of affinity tagged proteins with specificity towards the affinity tag, e.g. polyhistidine, FLAG™, Protein C and calmodulin binding peptide (CBP).	[114]
Size-exclusion	SEC	Size	Separation of protein biotherapeutics from high-molecular weight (HMWP) species, e.g. aggregates.	[115]
Reverse phase	RPC	Polarity	Analytical separation of mAb product-related variants. The stationary phase is more hydrophobic (e.g. silica bonded with C_{18}) than the mobile phase (e.g. aqueous/organic solvent mixture).	[116]
Normal phase	NPC	Polarity	Analytical separation of highly hydophilic molecules that cannot be retained on RPC, e.g. separation of N-glycans from mAb for investigation of glycoforms. The stationary phase is more hydrophilic (e.g. silico bonded with carbamoyl, diol or amino groups) than the mobile phase (e.g. organic solvents).	[117,118]
Hydrophobic interaction	HIC	Polarity	Preparative purification of mAbs for removal of aggregates and host cell protein (HCP) in flow-through (FT)-mode. The stationary phase is more hydrophobic (by definition less hydrophobic than RPC) than the mobile phase (e.g. aqueous buffer). This technique can separate highly hydrophobic species as compared to RPC.	[119,120]
Ion-exchange	CIEC (cation) AIEC (anion)	Charge	Preparative purification of mAb at pH below its isoelectric point (pI) for removal of aggregates, DNA and basic HCP by CIEC.	[121]
Mixed-mode	MMC	Hydrophobicity and charge	Preparative purification of mAbs by cation hydrophobic MMC. The stationary phase has both hydrophobic and charged properties.	[122]

3.2 Process Development

The regulatory initiatives Quality by Design (QbD) and Process Analytical Technologies (PAT) have shaped the way downstream processes are developed in the biopharmaceutical industry. These initiatives were introduced into the chemical manufacturing control review process in 2004 [123] and they are defined in the International Conference on Harmonisation guidelines as:

> **QbD:** A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management [124].
>
> **PAT:** A system for designing, analysing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality [124].

The aim of QbD is to implement quality into the product and the process. To achieve this, knowledge and understanding of the product and the process are required. This can be achieved by establishing a design space, which is the range of product attributes and process parameters that comply with the quality requirements, as well as applying PAT as a tool [124,125]. There are several approaches for establishing a design space; trial-and-error approach, expert knowledge-based approach, high-throughput experimentation (HTE) approach, model-based approaches and hybrid methods [126,127]. An overview of the different approaches can be seen in Table 4.

Table 4. Approaches for establishment of design space during process development.

Approach	Methods	Principle	Drawbacks	Ref.
Trial-and-error	One-factor-at-a-time (OFAT)	One factor at a time is changed, while keeping the other factors constant. A conclusion is then drawn from the resulting effect.	- Suboptimal results - Many experiments - Time-consuming - Material-consuming - No extrapolation	[126]
	Design of Experiment (DoE)	Statistical approach where different factors are simultaneously changed. It is used to identify significant factors, whereafter optimising a response with respect to them.	- Depending on number of factors included in the design, it can be many experiments, and time- and material-consuming - No extrapolation	[126,128]
Expert knowledge-based	Rational method based on expert knowledge in large-scale production in a system of rules	Based on proteomics data on each protein impurity, an algorithm selects the most efficient purification method. Then, purity, number and concentration of each protein after separation is predicted by a second algorithm.	- Requires initial proteomics characterisation of the starting material - Data from each protein impurity has to be available in a database	[126,129]
HTE	- Miniature robot columns - 96-well plate format	HTE is a fast investigation of many factors in miniaturized and automated format requiring only small amount of material. It accelerates screenening of factors that are otherwise time-consuming and allows determination of kinetic and isothermic parameters.	- HTE does not guarantee optimal results, therefore it should just be used as a screening tool - Requires high-throughput analytical technologies	[126]
Model-based	Mechanistic models, e.g. the general rate and the steric mass-action (SMA) models	Instead of real-time experiments, *in silico* simulation of different condictions are performed, allowing accurate extrapolation. It is based on fundamental understanding of the process.	- Requires parameter estimation - The approach can be less accurate for complex starting materials	[126,127]
Hybrid methods	HTE in combination with mechanistic model-approach	Combines experimental knowledge generation and model-based process design models. Allows scalability to be implemented in the mechanistic model.	- Requires high-throughput analytical technologies - The approach can be less accurate for complex starting materials	[126,127]

Design of experiments (DoE) is a widely used approach for establishing a design space. It is a systematic approach based on multivariate models correlating process parameters to main process responses. The aim of DoE is to perform a minimum number of experiments for screening and optimization of process parameters for a given process. Several studies have been reported on the successful use of DoE for process development [130,131,132,133]. However, for a high number of process parameters, it can require many experiments for correlating interactions between process parameters and main responses [134]. In addition, the multivariate models are often not reliable for extrapolation of process responses [126,127].

On the contrary, mechanistic models allow accurate extrapolation of process responses. They are useful for the understanding of chromatographic processes. The general rate model (GRM) is a commonly used mechanistic model for simulation of packed-bed chromatography (see Eq. 3.1). It considers convection (mass transfer by flow), axial dispersion (back mixing and diffusion in the axial direction), mass transfer through film from the bulk to the outer surface of the stationary phase bead (see Eq. 3.2), and intraparticle diffusion (e.g. Fick's diffusion, see Eq. 3.3) [135].

$$\frac{\partial c_i}{\partial t}(t,z) = \overbrace{-u\frac{\partial c_i}{\partial z}(t,z)}^{\text{Convection}} + \overbrace{D_{ax}\frac{\partial^2 c_i}{\partial z^2}(t,z)}^{\text{Axial dispersion}} - \overbrace{\frac{1-\varepsilon_c}{\varepsilon_c}\frac{3}{r_p}j_{film,i}(t,z)}^{\text{Mass transfer}} \qquad \text{The GRM} \tag{3.1}$$

$$j_{film,i}(t,z) = k_{film,i}\left(c_i(t,z) - c_{p,i}(t,z,r_p)\right) \qquad \text{Film diffusion} \tag{3.2}$$

$$\frac{\partial c_{p,i}}{\partial t}(t,z,r) = D_{p,i}\left(\frac{\partial^2 c_{p,i}}{\partial r^2}(t,z,r) + \frac{2}{r}\frac{\partial c_{p,i}}{\partial r}(t,z,r)\right) - \frac{1-\varepsilon_p}{\varepsilon_p}\frac{\partial q_i}{\partial t}(t,z) \qquad \text{Fick's diffusion} \tag{3.3}$$

Where c_i is the concentration of component i at the axial position z $\in [0, \mathrm{L}]$, L is the length of the column, t is the time, u is the interstitial velocity, D_{ax} is the axial dispersion coefficient, ε_c is the column void, r_p is the radius of the stationary phase bead, $j_{film,i}$ is the flux through the film from the bulk to the stationary phase bead for component i, $k_{film,i}$ is the film mass transport coefficient, $c_{p,i}$ is the concentration of component i in the stationary phase, $D_{p,i}$ is the pore diffusion coefficient for component i, ε_p is the porosity of the stationary phase bead, and $\partial q_i/\partial t$ is the adsorption rate for component i.

The adsorption rate can be described by different adsorption isotherm models in the kinetic form, e.g. Langmuir [136], Freundlich [137], Henry's law [138], and steric mass action (SMA) [139]. A commonly used one is the SMA model, which was proposed by Brooks and Cramer in 1992 for protein adsorption at equilibrium in non-linear ion-exchange chromatography [139]. The SMA model considers (i) displacement of salt counter ions when the protein with higher affinity binds to the stationary phase, and (ii) steric hindrance of salt counterions due the protein occupying binding sites when bound to the stationary phase. This steric hindrance makes the bound salt counterions unavailable for ion exchange with other molecules.

The application of mechanistic models for the development and understanding of chromatographic processes has been reported in several studies. Iyer *et al.* [140] developed an AIEC step for separation of a mAb from its dimers using the SMA model. Huuk *et al.* [141] showed that it was possible to optimize two consecutive ion-exchange steps (CIEC and AIEC). Wang *et al.* [142] combined the GRM and the stochiometric displacement isotherm model with machine learning based on artificial neural network to simulate experimental errors in ionic capacity and salt elution gradients during CIEC.

3.3 Antibody Processes

In the biopharmaceutical industry, recombinant mAbs are produced in cells that are engineered to secret the protein. Commonly used expression host cells are murine myeloma (e.g. NS0 and Sp2/1) and mammalian cells (e.g. CHO and HEK293). During the recent years, protein titers have increased considerably due to optimized cell lines and shifts from fed-batch (8 g/L) to perfusion (27 g/L). The demand for higher titers due to economic reasons has moved the bottlenecks from the upstream to the downstream section of the production [143,144]. For mAbs, the cost of the downstream section corresponds to around 45-80% of the total production costs [145].

A typical downstream process for a mAb is illustrated in Figure 11. The aim of downstream processing is to achieve high purity of the protein of interest while maintaining high yield. The process consists of clarification, capture, virus inactivation, intermediate purification and polishing. First, the expressed mAb is harvested by centrifugation and filtration for removal of host cells and cell debris. Then, the mAbs are captured by Protein A-based affinity chromatography, in which host cell protein (HCP) and nucleic acids are removed to a large extent [146,147,148]. Protein A binds to the Fc region with high affinity and it is originally found in the cell wall of Staphylococcus aureus [149,150]. Following the capture step, viruses are inactivated by a low pH hold. Intermediate purification is then performed for removal of aggregates and leached Protein A ligand, as well as further removal of basic HCP and nucleic acids. Traditionally, CIEC in bind/elute-mode has been used as an intermediate purification step. After this step, polishing is performed by AIEC in FT-mode for removal acidic HCP and residual DNA, followed by filtration for removal of viruses [146,147,148]. An alternative technique to IEC is the non-conventional MMC, which separates proteins on the basis of two or more molecular interactions such as hydrophobicity and charge [151]. It has been shown to remove aggregates and viruses, as well as decrease the level of insoluble aggregation during the chromatographic process [132,152,153,154,155, 156,157,158]. Examples of frequently used MM adsorbents are the anion hydrophobic Capto Adhere adsorbent [153,154,155], the cation hydrophobic Capto MMC adsorbent [152], the ceramic hydroxyapatite adsorbent [153,156], as well as the hydrophobic charge induction MEP HyperCel adsorbent [132,153,155,157,158,159]. For downstream processing of mAbs, both Capto Adhere™ and MEP HyperCel have been shown to decrease the level of HCP by up to 98% [154,153]. The chemical properties of three common MM adsorbents can be seen in Figure 12.

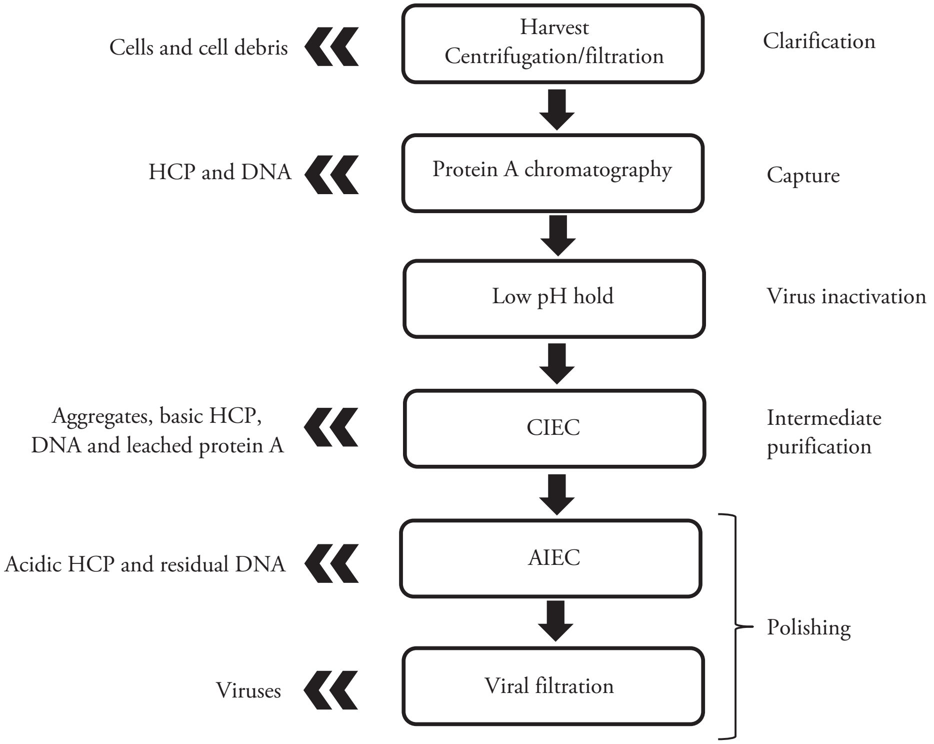

Cells and cell debris
Harvest
Centrifugation/filtration
Clarification
HCP and DNA
Protein A chromatography
Capture
Low pH hold
Virus inactivation
Aggregates, basic HCP,
DNA and leached protein A
CIEC
Intermediate
purification
Acidic HCP and residual DNA
AIEC
Polishing
Viruses
Viral filtration

Figure 11. A typical process for a mAb consisting of clarification, capture, intermediate purification and polishing. The figure was inspired from references [146,147,148].

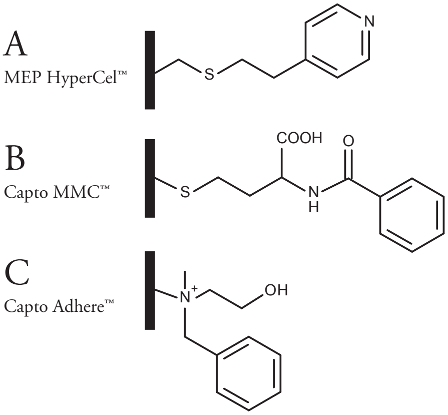


Figure 12. Different MM ligands. (A) MEP HyperCel (Pall, USA), (B) Capto MMC (GE Healthcare, Sweden) and (C) Capto Adhere (GE Healthcare, Sweden). The molecular structures were drawn in Accelrys draw 4.1 software (Accelrys, USA).

Antibody fragments, such as Fab and scFv, can be challenging to purify due to the absence of the Fc region required for binding to Protein A. However, Protein G and Protein L, as well as nanobody-based KappaSelect™, LambdaSelect™ and CaptureSelect™ can be used as alternatives to Protein A for some fragments (see Table 5). Apart from Protein A- and Protein L-based adsorbents, there are no other commercial affinity adsorbents for the purification of scFvs. However, they are currently under the development at the biotech company Thermo Fisher Scientific [160].

Table 5. Affinity resins against human mAbs and their fragments.

Affinity adsorbent	Ligand size	Targets	Ref.
Protein A, e.g. MabSelect Sure™ (GE Healthcare)	42 kDa (Protein A) 6.7 kDa (Z domain)	Fc and Fab (V_H3 family only)	[161,162]
Protein G, e.g. Protein G Sepharose™ (GE Healthcare)	30 kDa	Fc and some interactions with C_H1 of Fab	[163,164]
Protein L, e.g. Capto L™ (GE Healthcare)	34 to 106 kDa	Kappa LC I, III and IV of Fab and scFv	[165]
KappaSelect™ (GE Healthcare)	13.2 kDa	Kappa C_L of Fab	[166]
LambdaSelect™ (GE Healthcare)	13.1 kDa	Lambda C_L of Fab	[166]
CaptureSelect™ (Thermo Fisher Scentific)	13-14 kDa	Available for Fc, C_H1 of Fab, Kappa C_L of Fab, Lambda C_L of Fab and isotype specific (IgA, IgM, IgE) and subclass specific (IgG1,3,4).	[167]

Nevertheless, Muzard *et al.* [168] showed that it can be possible to purify scFvs by grafting Protein L-binding activity. However, this requires protein engineering in the early-stage development of the molecule. The use of affinity tags may serve as an alternative, however they can be an issue for biopharmaceuticals in terms of immunogenicity [169,170,171]. To avoid this, enzymatic cleavage of the affinity tag after purification is required, demanding an additional purification step for removal of the enzyme and the cleaved tag.

Currently, there is a lack of studies reporting on alternative approaches for purification of scFvs. Gagnon *et al.* [172] conducted a screening study for the purification of an anti-prostate stem-cell antigen mini-body, produced in murine myeloma NS0 cells, and demonstrated the potential use of MMC when traditional methods are lacking. Lindner *et al.* [173] reported on a successful non-conventional purification process for a scFv produced periplasmatically in *E. coli* cells. The process consisted of four purification steps; capture by Capto MMC, intermediate purification and polishing by AIEC and CIEC, respectively, and further polishing by ceramic hydroxyapatite MMC [173].

Furthermore, MMC was investigated in paper II for purification of a scFv produced in mammalian HEK293 cells. A two-step purification process was developed by statistical design of experiments (DoE) approach. A proteomics approach using nano LC-MS/MS was applied for identification and relative quantitation of HCP. Capture was successfully performed by MMC with MEP HyperCel. Polishing was performed with Capto Adhere and resulted in an improvement in purity from 86 % to > 98 %. In addition, the level of HCP was reduced by a factor of 14. Altogether, MMC was shown to be an excellent technique for purification of the scFv (paper II).

4. Immunoaffinity Adsorbents

4.1 Overview

Affinity chromatography is a widely used technique for purification of specific molecules. Due to the specificity to one target molecule only, the technique has been shown to be excellent for direct capture from crude starting materials [161,162,163,164,165]. Affinity adsorbents can be developed when there is an antibody, i.e. *affinity ligand*, available against a molecule of interest and such adsorbents are known as *immunoaffinity adsorbents*. The process of developing immunoaffinity adsorbents consists of four steps; (i) chemical activation of support material, (ii) immobilisation of affinity ligands to activated support material, (iii) wash of excess affinity ligands, and (iv) blocking of unreacted active-groups of support material [174,175].

4.2 Important Factors

Several important factors should be considered when developing affinity adsorbents. However, before the start of any development, an affinity ligand with suitable properties should be available; it should have a high affinity to the desired target protein and the binding should be reversible to enable desorption of the target protein during elution. In addition, it should be stable enough to endure CIP. When such ligand exists, the following factors should be considered. The support material should preferably be spherical and uniform with a porous network. A high level of porosity is important to enable good flow and allow intraparticle diffusion of the target protein [176]. The porous support material, i.e. resin, should be hydrophilic and inert to maintain specificity to the target protein only. Several types of support materials exist, such as cellulose- and methacrylate-based, as well as cross-linked agarose, which is the most commonly used one. Cross-linked agarose is commercially called Sepharose and it is classified according to its agarose content; typically around 2-6% [175].

The support material should have the capability to be functionalized with different chemical groups for immobilization of the affinity ligand. A wide range of pre-activated resins are available commercially (see Table 6). The choice of the activation chemistry depends on the immobilisation approach. Immobilisation via amino- or carboxyl-groups of the mAb-based affinity ligand will typically result in random, multipoint attachment to the support material due to the high predominance of these reactive groups on the protein surface. However, some lysine (Lys) residues in proteins can be more reactive than others. Therefore, site-direction can be achieved by immobilisation of amino-groups of reactive Lys in some special cases [177,178]. Moreover, site-directed immobilisation can be achieved by immobilisation to one reactive group such a thiol-group of a Cys residue [175]. Other reactive groups, such as glycan and azide groups, have also been reported for site-direction [179,180,181].

The pore/ligand size ratio is an important factor that should also be considered when designing immunoaffinity adsorbents for a certain target protein of interest. The resin has a certain pore space that can potentially be a limiting factor for larger affinity ligands and target proteins. It has been suggested that the pore size should be five times larger than the average size of the ligand. Nevertheless, a consequence of a larger pore size is a smaller surface area for immobilisation [174].

The physical and chemical stability of the support material is important to consider. It should withstand the conditions of the immobilization as well as the chromatographic process (e.g. elution and CIP). The developed affinity adsorbent should then in turn be stable over a wide pH and temperature range, as well as re-useable for a long period of time. It should also be resistant to bacterial degradation and have a low level of leached ligands [176].

The affinity adsorbent should have a binding capacity that aligns with the specifications of the production process. It should be selected based on the biochemical understanding of the target protein, the adsorption isotherm and the desired productivity. The binding capacity will affect both the productivity and the manufacturing costs. For a low-capacity adsorbent, a larger column is usually needed to achieve higher productivity, i.e. columns with diameters of up to 2 m at manufacturing scale. This will in turn lead to extra costs due to the need of larger quantities of adsorbent and consumables, as well as potential issues with column pressure [182].

Table 6. An overview of different commercially available pre-activated resins.

Commercial activated resin	Material	Reactive group of affinity ligand	Resin-ligand linkage
CNBr-activated Sepharose 4FF (GE Healthcare)	4% cross-linked agarose	-NH_3	Isourea linkage
NHS-activated Sepharose 4FF (GE Healthcare)	4% cross-linked agarose	-NH_3	Amide bond
Actigel ALD Superflow (Sterogene)	Cross-linked agarose	-NH_3	Secondary amine linkage
Cellufine Formyl (JNC Corporation)	Cellulose	-NH_3	Secondary amine linkage
Toyopearl AF-tresyl 650 M (TOSOH)	Methacrylate polymer	-NH_3, -SH	Secondary amine linkage, thioether bond
Toyopearl AF-epoxy 650 M (TOSOH)	Methacrylate polymer	-NH_3, -SH	Secondary amine linkage, thioether bond
Epoxy-activated Sepharose 6B (GE Healthcare)	6% cross-linked agarose	-NH_3, -SH, -OH	Secondary amine linkage, thioether bond, ether bind
Sulfhydryl-reactive resin (DivBio Science)	6% cross-linked agarose	-SH	Thioether bond
Carboxyl-reactive resin (DivBio Science)	6% cross-linked agarose	-COOH	Amide bond
CarboxyLink (Pierce)	4% cross-linked agarose	-COOH	Amide bond
Toyopearl AF-amino 650 M (TOSOH)	Methacrylate polymer	-COOH	Amide bond

4.3 Characterisation of Affinity Adsorbents

Immunoaffinity adsorbents can be characterised by their dynamic binding capacity (DBC), chromatographic breakthrough curve, utilisation yield and adsorption isotherm at a certain ligand density, ρ. The DBC is defined as Eq. 4.1 and describes the capacity at a certain ligand density and column residence time.

$$DBC = \frac{m_{Target}}{V_c} \tag{4.1}$$

Where m_{Target} is the amount of target molecules recovered during elution, and V_c is the column volume.

The utilisation yield is defined as Eq. 4.2 and describes the capacity of the immunoaffinity adsorbent relative to the theoretical capacity. It is normalised to the number of antigenic binding sites.

$$Utilisation\,yield = \frac{DBC}{\sum_{i=1}^{n} M_{Target} \cdot \rho_i \cdot N_i} \tag{4.2}$$

Where M_{Target} is the molecular weight of the target molecule, and N is the number of antigenic binding sites of affinity ligand.

The breakthrough curve describes the concentration profile of the target molecule through the column and it has a typical sigmoid shape. The steepness of the curve describes the width of the mass transfer zone, which is the segment of the column where mass transfer from the mobile phase to the binding sites of the affinity adsorbents occur [184]. A broad breakthrough curve indicates a wide mass transfer zone, which is a result of resistance due to mass transfer in mobile phase, film diffusion, pore diffusion or adsorption kinetics [183].

The adsorption isotherm describes the relationship between the binding capacity and the concentration of the target molecule in the mobile phase at equilibrium. The simplest model is the Langmuir adsorption isotherm described by Langmuir in 1916 (see Eq. 4.3) [136,184]. Langmuir was awarded the Nobel Prize in Chemistry in 1932 for his discoveries and investigations in surface chemistry [185].

$$q = \frac{q_m \cdot c}{K_d + c} \qquad \text{Langmuir model} \tag{4.3}$$

Where q is the equilibrium binding capacity, c is the concentration of target molecule in the mobile phase at equilibrium, q_m is the maximum equilibrium binding capacity, and K_d is the equilibrium desorption constant. It is based on three assumptions; (i) the target proteins in the mobile phase is in contact with the surface of the adsorbent, (ii) the adsorbent has a limited number of binding sites, and (iii) monolayer adsorption.

Another adsorption isotherm model is the Brunauer-Emmett-Teller (BET), which was described by Brunauer *et al.* [186] in 1938. It was originally developed for multilayer adsorption of gas molecules. For a finite number of adsorption layers, the BET model can be written for adsorption in liquid phase according to Eq. 4.4.

$$q = q_m \frac{K_S \cdot c\,[1-(n+1)(K_L \cdot c)^n + n(K_L \cdot c)^{n+1}]}{(1-K_L \cdot c)\left[1+\left(\frac{K_S}{K_L}-1\right)K_L \cdot c - \left(\frac{K_S}{K_L}\right)(K_L \cdot c)^{n+1}\right]} \qquad \text{BET model} \qquad (4.4)$$

Where n is the number of adsorption layers, K_L is the equilibrium constant of adsorption for the first layer and K_S is the equilibrium constant of adsorption for the upper layer.

4.4 Past and Present Developments

One of the earliest developments of affinity adsorbents was reported in 1951 by Campbell *et al.* [187]; bovine albumin was coupled to diazotized amino-cellulose. The intended use of the developed bovine albumin cellulose adsorbent was to isolate rabbit antibodies against bovine serum albumin from antisera. However, it was not until the mid-1960s when it was reported that rabbit antibodies had been immobilised to various materials for the development of immunoaffinity adsorbents [188,189]. Today, there are commercial mAb-based affinity adsorbents available. Examples include the anti-FLAG M1 and M2 affinity adsorbents [190,191]. However, a drawback of full-length mAb-based affinity adsorbents is low binding capacity, e.g. anti-FLAG M2 affinity adsorbent has a capacity of 0.6 mg/mL adsorbent for a FLAG-BAP target protein (49 kDa) [191].

Nevertheless, the development of immunoaffinity adsorbents has moved to immobilisation of smaller fragments, rather than full-length mAbs, to achieve higher binding capacity, e.g. camelid-derived nanobodies (15 kDa) (see Figure 5) [192]. Examples include KappaSelect, LambdaSelect and VIIISelect with a binding capacity around 8-20 mg/mL [166,193].

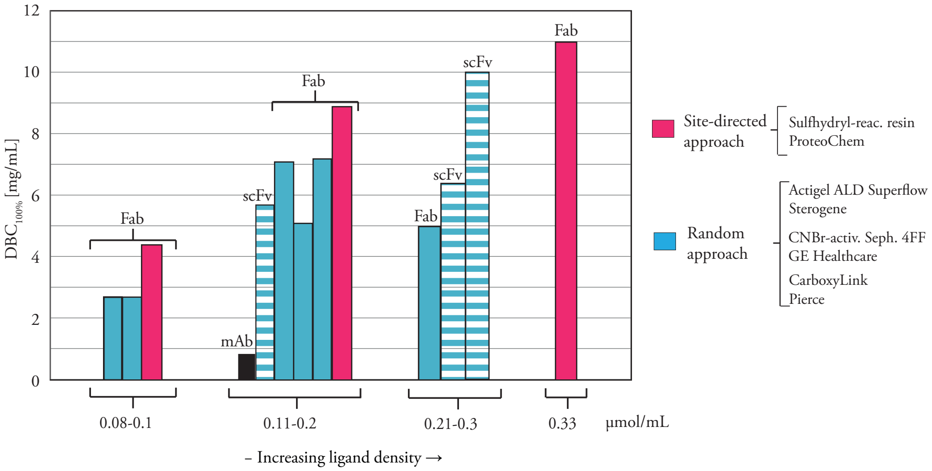


Figure 13. A bar chart displaying $DBC_{100\%}$ of a selection of immunoaffinity adsorbents from paper III. The adsorbents are groups according to ligand density.

In paper III, a systematic study investigating the effect of ligand size, accessibility of binding sites, coupling chemistry, and resin properties on the binding capacity of immunoaffinity resins was performed, as it had not been reported previously. A selection of the developed immunoaffinity adsorbents can be seen in Figure 13. A 3-fold increase in $DBC_{100\%}$ was obtained for Fab immobilized via a random-oriented approach on CNBr-activated Sepharose 4FF as compared to mAb on the same resin and ligand density. However, no difference in capacity was obtained for the Fab and the scFv immobilised via a random-oriented approach on the same resin.

Site-direction of Fab via a C-terminal thiol-group resulted in a further increase in $DBC_{100\%}$ by 1.6-fold relative to immobilisation via amino-groups at the same ligand density. Altogether, highest $DBC_{100\%}$ was obtained for the affinity resins with site-directed Fabs and random-oriented scFvs; 11 mg/mL and 10 mg/mL, respectively. This corresponds to an improvement in $DBC_{100\%}$ by up to 14-fold relative to the mAb-based affinity adsorbent. Altogether, these results suggest that the ligand format, ligand density and the immobilisation approach are three important factors affecting the development of high-capacity affinity adsorbents (paper III).

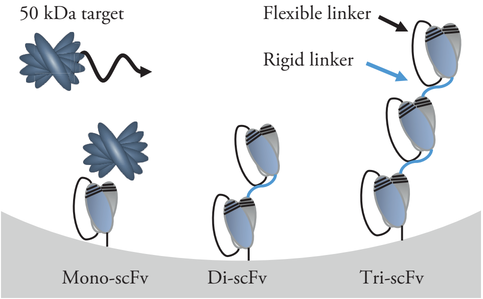


Figure 14. Schematic representation of the multimeric fusion scFv ligands from paper IV.

In paper IV, a proof-of-concept was established for multimeric fusion scFvs as ligands in immunoaffinity adsorbents. The idea was inspired by the hexameric Protein A-based ligands in the MabSelect adsorbent [194]. Three different scFv formats were constructed; mono-, di- and tri-scFvs with N-terminal His_6 tag and C-terminal Cys residue (see Figure 14). The scFvs in the di/tri-scFv constructs were separated by a rigid peptide linker of Pro/Glu repeats with length of 16-18 residues. The ligands were expressed in HEK293 mammalian cells, purified by immobilized-nickel ion affinity chromatography and immobilised to 6% cross-linked agarose resin via their C-terminal thiol-group (site-directed approach). The mono- and multimeric scFv-based immunoaffinity adsorbents resulted in up to 20 mg/mL in $DBC_{100\%}$ and 82% in utilisation yield. This demonstrate that the multimeric scFv-based ligands were just as functional as the mono-scFv ligands.

4.5 Limitation in Regeneration Procedure

Many chromatographic adsorbents, such as MabSelect SuRe [195], can be regenerated with 0.1-0.5 M sodium hydroxide for removal of contaminating proteins and nucleic acids, as well as inactivation of viruses, endotoxins, and microorganisms such as bacteria, yeasts and fungi. For inactivation of endotoxins, the concentration of sodium hydroxide has to be at least 0.5 M, however 0.1 M can work in case the incubation time is prolonged to more than 50 h [196]. Nanobody-based affinity adsorbents are capable of withstanding up to 50 mM sodium hydroxide for 72 h, however at a loss of 10 % in binding capacity [193]. However, immunoaffinity resins cannot withstand these concentrations of sodium hydroxide without losing the binding capacity. This is a major limitation for CIP, long-term stability and re-usability.

5. Concluding Remarks and Future Directions

The work of this thesis covers three main contributions. First, the development of aggregation resistant antibodies with decreased level of non-specific interaction. This is essential for biopharmaceuticals. In this work, negatively charged Asp-substitutions at highly specific positions in the CDRs and FRs, as reported for human V_H domains by Dudgeon and co-workers, were investigated for a model Fab. By using *in silico* screening based on computational models for limitation of number of variants for *in vitro* screening, single mutations and double/triple combinations could be selected and assessed in terms of functionality and developability potential. A majority of the variants were retained or improved in terms of thermodynamic stability compared to wt. In addition, they were improved with regards to non-specificity and had fully retained the affinity to the target protein. Furthermore, the variants containing [28D]-FR1-H, [31D]-CDR1-H and [53D]-CDR2-L were improved in regard to aggregation propensity. Future studies could aim to understand the importance of negative charges to the substantially reduced non-specificity. New model proteins could be assessed. In addition, the roles of colloidal and thermodynamic stabilities on the developability potential could be investigated in the scFv format. The same approach, based on *in silico* screening by computational models to limit the number variants for *in vitro* screening, could be applied.

Second, purification of scFvs can be challenging when conventional methodologies are lacking. A non-conventional two-step purification process by MMC was developed for a scFv with kappa V_L II. Capture and polishing were performed by MEP HyperCel and Capto Adhere, respectively. The polish step was developed and optimised in a statistical approach by screening and optimisation DoE. Optimal conditions were bind/elute-mode with linear salt elution gradient at neutral pH. Optimal purity, yield, and HCP reduction factor were estimated to >98 %, >98 %, and 14, respectively. Future studies could aim to investigate the ratio of negative charge and hydrophobicity for adsorption/desorption, identify and map the residues involved in the binding to Capto Adhere, and examine the transferability of this information to similar proteins that are challenging to purify.

Third, several factors important for the development of high-capacity immunoaffinity adsorbents were systematically investigated. MAb, Fab, scFv and di/tri-scFvs were investigated as affinity ligands against a target protein of 50 kDa. The accessibility of the binding sites had substantial effect on the binding capacity. It was largely impacted by the ligand format, ligand density, and orientation of ligand. Out of the developed immunoaffinity adsorbents, the highest binding capacity, up to 20 mg/mL, was obtained for site-directed mono- and tri-scFv ligands. Future studies could aim to investigate (i) the benefit of higher order scFv fusions and higher ligand densities for further maximization of the binding capacity, and (ii) whether these scFv ligands can be stabilized recombinantly to withstand CIP with sodium hydroxide (50 mM to 0.1 M). In a directed evolution approach, a phage display library could be constructed and screened for this type of stability while maintaining binding affinity to the target protein.

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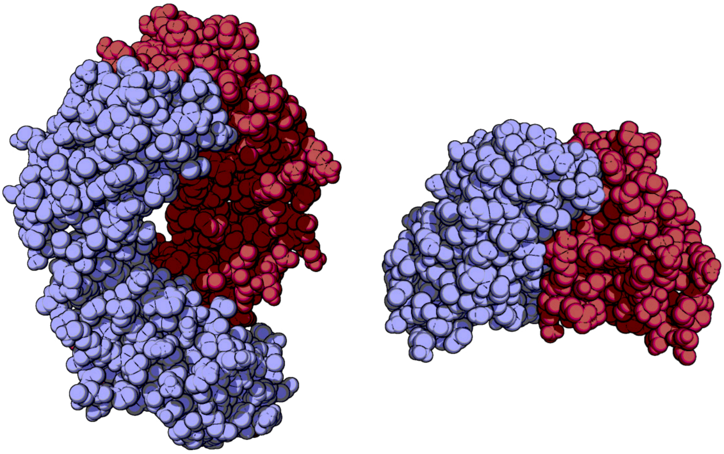

In the biopharmaceutical industry, antibody fragments, such as the antigen binding fragment (Fab, *left*) and the single-chain variable fragment (scFv, *right*), have become the format of choice in various applications, e.g. therapeutic anti-cancer drugs, probes in diagnostic applications and affinity ligands in immunoaffinity adsorbents. Their smaller size enables several advantages over their full-length counterpart, e.g. improved tissue penetration, faster blood clearance and improved binding capacity. However, limitations in their developability and processability can greatly impact manufacturing, stability and safety.

This doctoral thesis aims to review the developability, processability and applicability of various antibody fragments as well as new emerging antibody-based formats. In addition, four different studies are presented; (i) a semi-rational design study for improving the developability potential a Fab, (ii) a downstream processability study of a scFv based on statistical design of experiments and proteomics approach, (iii) an applicability study of Fab and scFv as affinity ligands in immunoaffinity adsorbents, and (iv) development of multimeric fusion scFvs as novel affinity ligands in immunoaffinity adsorbents.

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