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Manufacturing of Fab antibody fragments – refolding and purification from inclusion bodies expressed in *E.coli*

Master thesis

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STATUTORY DECLARATION

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Abstract

In recent years lots of scientific work has been dedicated to the engineering of antibody fragments as a promising alternative to full size antibodies. Due to their lower molecular weight, the expression in different host systems is facilitated. In the biopharmaceutical industry *Escherichia coli (E. coli)* still represents one of the most attractive systems for cost-effective large scale manufacturing. However overexpression in *E. coli* often leads to the formation of inclusion bodies. Therefore a sophisticated refolding process is necessary to achieve considerable amounts of correctly refolded protein. The objective of the present study was the establishment of a refolding and purification process of three different Fab fragments (A4, 3H6, B1). The heavy and light chains of the Fab fragments were expressed separately in *E. coli* cells, their inclusion bodies were resolubilised and finally tried to be refolded to their active form. Refolding by dilution, which is the most widely used method in industrial scale, was utilized to perform these experiments. Different affinity media were investigated for the subsequent capturing step and purification of the correctly and partly folded Fab fragments. As an alternative to manual preparation, refolding experiments, using a Freedom Evo® 150 robot and a Design of Experiments approach, were conducted and evaluated. The refolding experiments with 3H6 and B1 showed only successful refolding on the single light chain. No correctly folded Fab fragment could be generated. With the Fab A4 molecule good refolding yields could be achieved from 1-250 mL scale. Furthermore an automatic refolding screening could be successfully established for Fab fragments. It was tested with the Fab A4 molecule and the yield was comparable to the standard screening procedure.

Kurzfassung

Innerhalb der letzten Jahre wurde die Entwicklung von Antikörperfragmenten, als Alternative zu den herkömmlichen Antikörpern, unter großem wissenschaftlichen Einsatz vorangetrieben. Antikörperfragmente lassen sich aufgrund ihres niedrigeren Molekulargewichtes leichter in verschiedenen Wirtszellen produzieren. In der biopharmazeutischen Industrie wird jedoch noch immer die Herstellung unter Verwendung von *E. coli*, aufgrund der geringen Kosten und stabiler Skalierbarkeit, bevorzugt. Die Überexpression von Proteinen führt in *E.coli* jedoch leicht zur Bildung von Einschlusskörperchen. Ein gut entwickelter Aufreinigungsprozess ist unumgänglich um eine gute Ausbeute an korrekt gefaltetem Protein zu erzielen. Das Ziel dieser Studie war es einen Faltungs- und Reinigungsprozess von drei verschiedenen Fab Fragmenten (A4, 3H6 und B1) zu etablieren. Die schweren und leichten Ketten der Fab-Fragmente wurden in *E.coli* exprimiert, resolubilisiert und in ihre native Form zurückgefaltet. Im industriellen Maßstab ist die Proteinrückfaltung durch Verdünnen die am weitesten verbreitete Technik und wurde auch für diese Studie verwendet. Um die korrekt gefalteten Fragmente von Intermediaten abzutrennen, wurden verschiedene Affinitätsmedien untersucht. In weiterer Folge wurden Rückfaltungsmethoden mit dem Freedom Evo® 150 Roboter unter der Verwendung eines Design of Experiments mit herkömmlichen Rückfaltungsstrategien verglichen. In den Experimenten mit den Proteinen 3H6 und B1 konnten nur die einzelnen leichten Ketten erfolgreich gefaltet werden. Mit A4 sind gute Rückfaltungsausbeuten bei einem Maßstab bis zu 250 mL erzielt worden. Des Weiteren konnte ein automatisches Screeningverfahren für Fab Fragmente etabliert werden. Die Experimente mit dem untersuchten A4 Molekül lieferten Rückfaltungsausbeuten, die mit Ergebnissen aus Standardscreeningverfahren vergleichbar waren.

Contents

1 Introduction

1.1 Biopharmaceuticals in recent years

At the turn of the millennium eighty-four biopharmaceuticals were in general medical use accounting for a global market value of $12 billion. These numbers have constantly risen to over 200 products by the end of 2010 and an estimated global market value of $99 billion in 2009. Their therapeutic potential lies in treatment of cardiovascular diseases, cancer, different types of diabetes as well as other infectious diseases [1]. Recent studies state that between 2006 and 2010 only 40 % of all approved pharmaceuticals were genuinely new, showing the increase in approved biosimilars. Among five of the ten top-selling products, four are based on monoclonal Antibodies (mAb). In 2009 seven new mAb products entered the market in the United States and/or Europe, among them the first bispecific antibody. For the upcoming seven years the worldwide market for protein-based therapies is estimated to be annually growing at between 7 % and 15 % [2].

Only a very small percentage of new biopharmaceuticals will pass clinical trials and enter the market. As a result, large amounts of money are pumped into biomedical research (over 100 billion US $ in 2008 the U.S.A alone) [3] [4].

Biopharmaceuticals are well suited to treat enzyme deficiencies and metabolic conditions, which presumably occur in orphan diseases like cystic fibrosis or Crohn´s disease, whereas cancers and inherited genetic disorders are still target of intensive scientific research [5].

1.2 Expression of recombinant proteins

Nowadays the production of recombinant proteins can be carried out either in bacteria, yeasts, fungi, cell cultures and even in genetically modified plants or animals. Each system provides certain advantages, but there are some limitations to these platforms one has to account for. Recombinant proteins vary heavily in their properties like size, origin and protein characteristics, so their production favors specific organisms.

The prokaryotic bacterium *E. coli* has been one of the most widely used expression systems in biotechnological history. In 2009 seventeen out of fifty-eight approved products were produced in *E.coli*, thirty-two in mammalian cell lines, four in *Saccharomyces cerevisiae*, one in *Pichia pastoris*, two in plant cells, one in an insect cell-based system and one

biopharmaceutical was produced in transgenic animals [1] [6]. In 2006 about 40 % of all biopharmaceuticals were produced in *E. col*i [7]. It needs simple growth media, but therefore it is able to achieve high growth- and expression rates. *E. coli* lacks the ability to perform extensive post translational modifications on expressed proteins and the production of soluble and correctly folded proteins proves difficult. Yeasts like *Saccharomyces cerevisiae* tend to hyperglycosylate expressed proteins, which can possibly trigger an autoimmune response, once the drug is administered to patients. If complex post translational modifications are required for the functionality of the expressed protein, yeasts or mammalian cell cultures (e.g. CHO cells) still remain the systems of choice. Immunoglobulins of the G family require mammalian cell expression due to their large size and generic glycosylation site in the C_H2 domain [8]. However the increased cell culture productivity has shifted the attention of process development to operations downstream of the production reactor. Advances in mammalian cell culture have taken titers beyond the 5 g/L mark and new platform technologies for efficient protein recovery are urgently sought for.

1.2.1 Recombinant protein production in *E.coli*

In general *E. coli* has the ability to produce large quantities of recombinant proteins with titers over 10 g/L. Laboratory procedures can be kept simple and the transformation of *E. coli* is very efficient. One may choose different features of the bacterial strain, such as the antibiotic resistance type, which facilitates the biochemical screening and selection procedures [9]. If possible, the secretion of protein to the periplasmic space, via the usage of signal peptides, for instance pelB or ompA, is the method of choice, if acceptable yields can be achieved. The periplasmic space provides a more oxidizing milieu than the cytoplasm, and chaperons may facilitate the correct refolding of polypeptides and hence prevent their aggregation or degradation by proteases. Nowadays even extracellular protein secretion has been realized with certain *E.coli* strains. It takes less effort in the downstream processing of soluble proteins although the final yield will most likely be lower than with insoluble proteins. High density *E.coli* cultures grown in laboratory fermenters or on industrial scales can yield periplasmic Fab and Fab´ fragments in the 1 g/L range [8]. Therefore this way of recombinant protein production is still the most efficient. However, there have been studies that investigated the expression of antibody molecules in the oxidizing cytoplasmic *milieu* of specially engineered strains [10]. More commonly proteins are accumulated in the cytoplasm as

IBs. IBs are very dense structures of incorrectly folded protein. The formation of IBs occurs during the intracellular refolding process of proteins and therefore they consist of partly folded and aggregated protein [11]. Experimental data suggest, that protein expression rates are too high and deny sufficient time for the nascent polypeptide to fold into its native conformation. Purified inclusion bodies show a typically cylindrical shape and are about 1.5 µm long [12]. IBs make up to 40% of the total cellular protein and can be observed under a light microscope due to their light scattering properties [13]. Optical microscopes provide vision of larger inclusions as particles reflecting light, which are called "refractile bodies". The formation of inclusion bodies is not host-specific, because other host cells like *Saccharomyces sp.* or insect lines produce them as well [14] [15]. If the recombinant polypeptide contains cysteine residues, the formation of intra- and intermolecular disulphide bridges is widely impeded in the cytoplasm, due to its reducing environment and the obtained proteins are biologically inactive. Aggregated IBs can be separated from cell debris by centrifugation and they need to undergo certain washing steps to be pure enough for subsequent processing. Another positive aspect of IB formation is shown in the recombinant production of immunotoxins, which normally may intoxicate *E. coli* cells, but when expressed as IBs they cannot function properly and larger yields are obtained. In order to obtain high yields of a recombinant protein it is necessary to find the most promising conditions for the resolubilisation and refolding of proteins from IBs.

1.3 Downstream processing of IBs

If the targeted protein cannot be efficiently produced in a soluble form, the intracellular formation of IBs will most likely be ineluctable [16]. This will lead to a more complicate downstream process containing an additional IB preparation, IB resolubilization and refolding step. The potentially increased product loss and higher costs in material and longer production times are usually compensated by the high IB expression levels in *E. coli* cells. Figure 1 compares downstream processes with soluble and insoluble proteins.

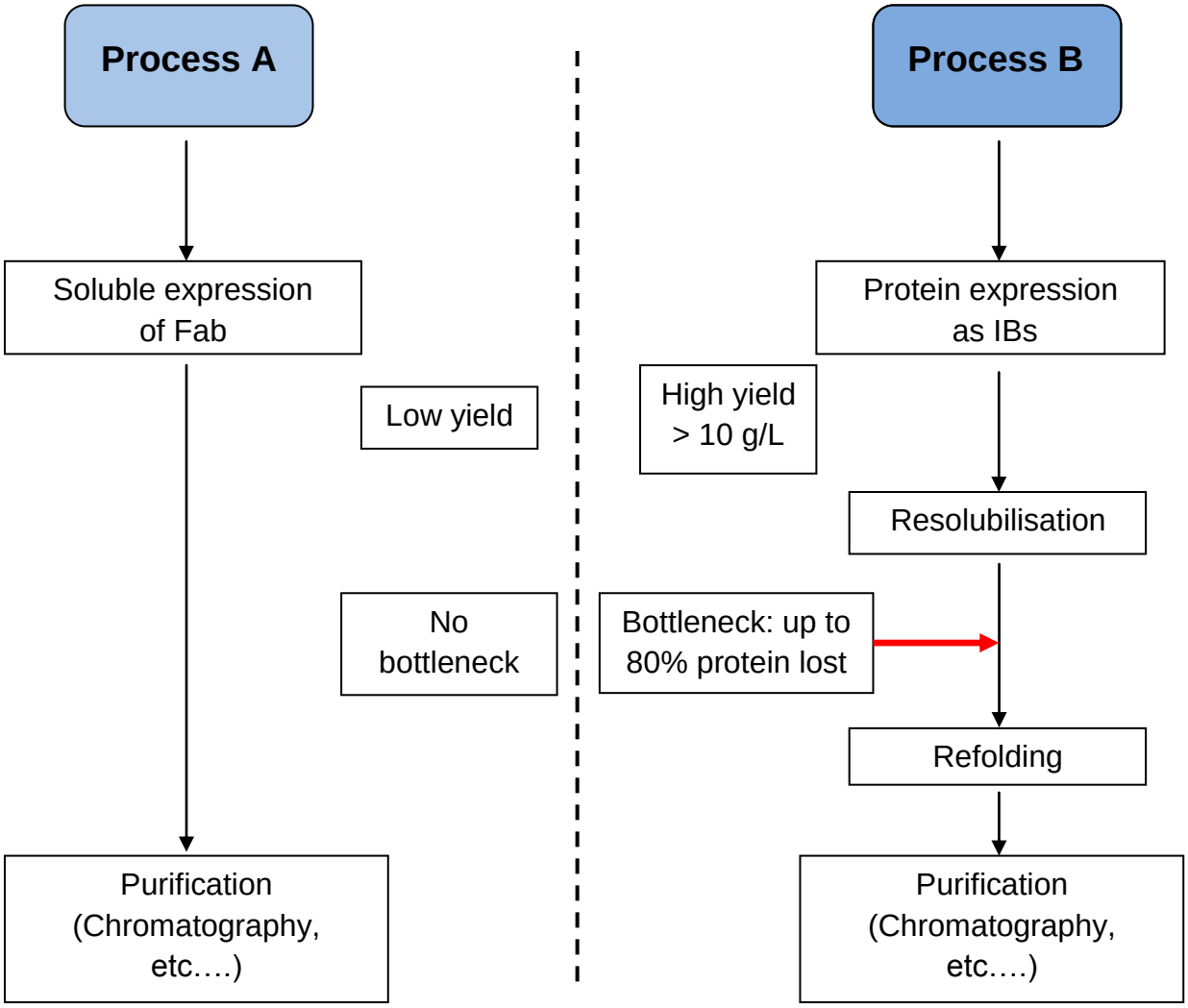


Figure 1: Comparison of downstream processes with solubly and insolubly expressed proteins

Process B involves at least two additional purification steps, which are crucial concerning product yield. Due to their specific density of about 1.3 g/mL IBs can be separated from other cell components by centrifugation after cell disruption. Extensive washing steps including the utilization of detergents like Triton-X 100 and denaturants (e.g. urea) are often necessary to purify the crude inclusion bodies. This partly explains the high ratio of downstream costs, which can make up to 80 % of overall production cost, besides high equipment expanses and long process times. When IBs are sufficiently pure they may contain between 50 to 90 % of recombinant protein, the rest is composed of cell debris and host proteins [17].

1.4 Antibodies

1.4.1 General information about immunoglobulins

In humans there are five different antibody isotypes or classes of antibodies, IgA, IgD, IgE, IgG and IgM respectively. These immunoglobulins differ in their biological properties and act at different sites in a human body. Human B cells possess the ability to present antibodies with unique paratopes. After their maturation in the bone marrow they circulate in the blood and lymph stream and bind to specific antigens. This procedure causes them to differentiate into either antigen producing plasma cells or into memory cells, which play an important role during the secondary immune response. Antibodies are glycoproteins and are counted among the heavy plasma proteins. The basic functional subunit is an immunoglobulin monomer like in IgD, IgE and IgG, contrariwise IgA forms dimers with two Ig subunits and IgM even forms pentamers. An Ig monomer has a "Y"-shaped form and consists of four polypeptide chains: Two identical heavy chains as well as two identical light chains. These chains are connected via disulfide bridges and further stabilized by noncovalent bonds like van der Waals forces and hydrogen bonds. Five types of mammalian heavy chains are known, which are denoted by the Greek letters α, δ, ε, γ and μ. These heavy chains define the isotypes of the antibody, whether it is IgA, IgD, IgE, IgG or IgM. Heavy chains as well as light chains have a constant and a variable region. The constant region of heavy chains is made up of either three or four constant domains (C_H), the variable region by one variable domain (V_H), while the light chain constant region has one constant domain (C_L) and the variable region possesses one variable domain (V_L). The individual domains are made of about 100 amino acids, which form β-sheet barrels. The number of constant domains is different between Ig classes and they determine

the flexibility of the antibody at its hinge regions, which is needed for proper antigen binding capabilities. The light chains in mammals are divided into either kappa (κ) or lamda (λ) type. The binding sites for the complement system and various cell surface receptors are located at the bottom of the "Y" shaped antibody stem, the so called Fc region (Fragment crystallizable region). The antigen binding sites are located at the N-terminal region of the Fab fragment (Fragment antigen binding). The domains and disulfide bridges of an antibody are shown in figure 2.

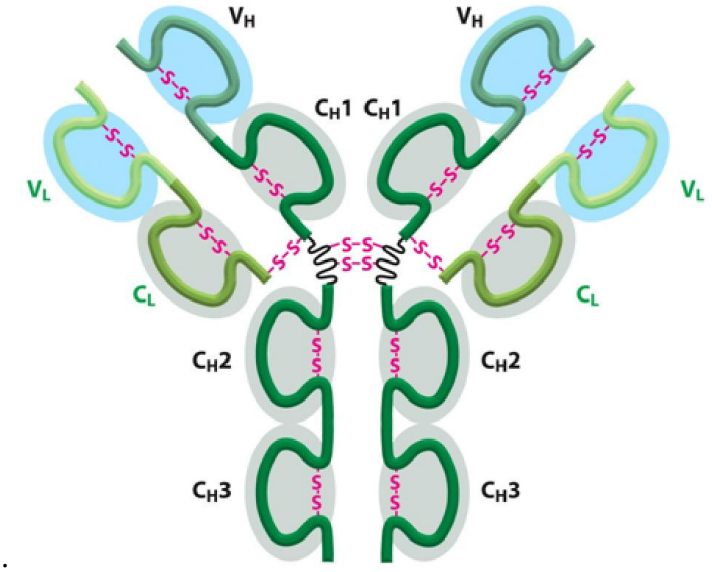


Figure 2: Domains and disulfide bridges of an antibody [18]

The N-terminal domains of both chains contain hypervariable regions with a length of about ten amino acids, which provide the large number of possible paratopes via V(D)J recombination also known as somatic recombination. In developing B cells segments of variable (V), diverse (D), and joining (J) genes are nearly-randomly combined during transcription. Light chains possess only variable and joining genes, whereas heavy chains have additional diverse genes [19]. The hypervariable regions within a Fab fragment are outlined in figure 3.

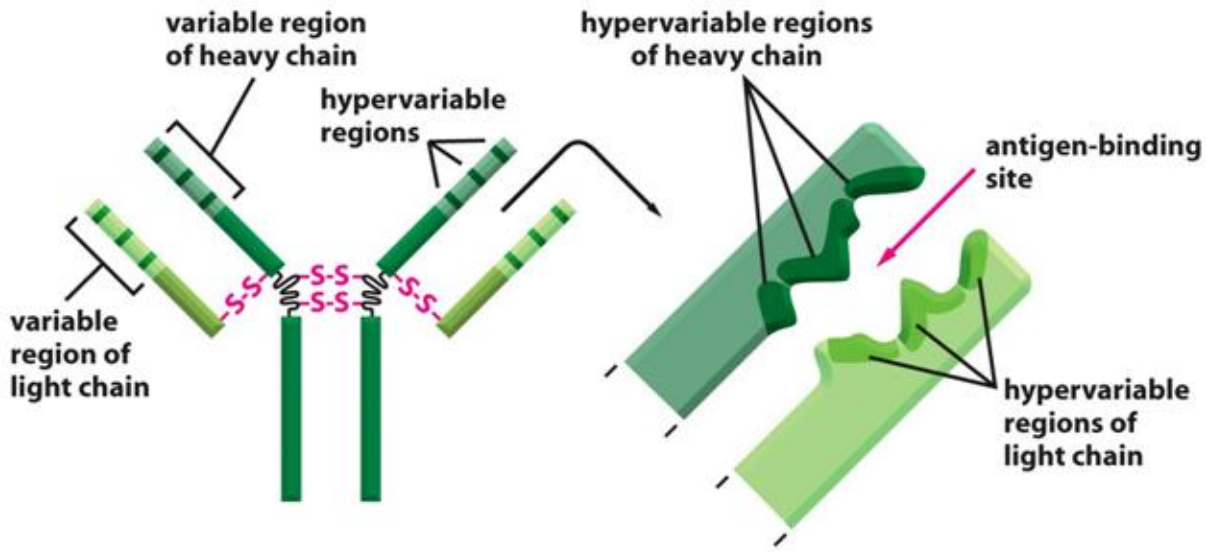


Figure 3: Hypervariable regions within a Fab fragment [18]

The binding between an antibody and an antigen follows the same principle as the binding of an enzyme to a substrate. Hydrogen bonds, ionic bonds, van der Waals forces and hydrophobic interactions make sure that the equilibrium between bound and free antibodies and antigens is established.
The reaction can be expressed as

Ab+Ag ↔AbAg

[Ab] represents the concentration of free antibody and [Ag] the concentration of free antigen, further the reaction is specified by the affinity constant K_a:

$$K_a = \frac{[AbAg]}{[Ab] * [Ag]}$$

1.4.2 Fab fragments and their applications

Fab fragments own some genuinely positive characteristics, which render them valuable for certain clinical applications. They provide a highly selective antigen binding capability leading to their utilisation as clinical therapeutics, medical diagnostics and generally in scientific research. Due to their smaller size compared do full size antibodies they can be produced more easily and more cost-effectively in bacteria than in mammalian cells and

therefore it is easier to supply a large market [20]. The Fab molecules are missing the C_H1 and C_H2 Ig domains and can be combined to a F(ab´)$_2$ with an identical Fab fragment via a stabilising disulfide bridge between cysteines near the C-terminal region. Attachment of therapeutic effector molecules such as radionuclides, enzymes or toxins is also possible via cysteine-linkage [21]. Originally Fab fragments were produced by enzymatic digestion of full size antibodies with papain or pepsin, resulting in the cleavage of the Fc fragment, and subsequent purification of the digestion mix [14] [15]. This leads to F(ab´)$_2$ molecules and mild reduction yields two identical Fab fragments, each about 50 kDa in size. If dimeric antigen binding species are required, the simplest way is direct disulphide or chemical cross-linking of Fab´ in vitro after purification [22] [23] [24]. It was also reported, that cleavage of F(ab´)$_2$ in circulating blood or elsewhere in vivo generates Fab´ molecules, which suffer from loss of avidity and rapid clearance from the circulation. Increasing the number of hinge disulfides to two as found in the hinge regions of F(ab´)$_2$ generated by proteolytic cleavage of IgG1 increases the stability of F(ab´)$_2$ molecules, which effects greater serum survival [25] [26]. Fab fragments are of lower molecular weight and therefore pharmacokinetics for these molecules are different to single-chain variable fragments (scFv) and full size antibodies [22] [27]. ScFv contain only the variable domains of heavy and light chain (V_H and V_L) and are linked together by a short polypeptide chain. The smaller the antibody fragment is, the better its tissue penetration is. In this aspect scFv penetrate faster than Fab fragments, which themselves are faster than full size antibodies. Concerning the half life time of antibody fragments the order is vice versa. Fab fragments as clinical therapeutics cover a wide range of possible applications. Polyclonal horse and sheep Fab and F(ab´)$_2$ generated from IgG with papain or pepsin act as potent antidotes. Crotalidae Polyvalent Immune Fab (Ovine;CroFab) is a polyspecific antivenom, which has become the mainstay of treating rattlesnake bites since its approval by the FDA in 2000 [8]. Other Fabs and F(ab´)$_2$ counter for example the cardiac glycoside digoxin or the famous and ambiguous botulinum toxin. Abciximab (ReoPro) is a monoclonal Fab, that consists of mouse variable and human constant domains. It inhibits platelet aggregation and is indicated for the prevention of restenosis in patients undergoing coronary angioplasty [4] [28] [57]. Ranibizumab (Lucentis) was approved by the FDA in 2006 for the treatment of macular degeneration by binding to the vescular endothelial growth factor A (VEGF-A). In this case the higher mobility and tissue penetration capability allows the drug to be administered locally through inocular injection and therefore circumventing intravenous injection. Ranibizumab is expressed in *E. coli* as periplasmic Fab [28] [58]. Besides the classical monovalent antibodies like scFv or Fab, other engineered variants as diabodies, triabodies,

tetrabodies, single-domain antibodies and minibodies deliver a wider range of therapeutic and analytical applications. They still possess unique antibody specificities; nevertheless they are easier to produce, due to their smaller size compared to full size antibodies. Figure 4 shows the diversity of antibody molecules.

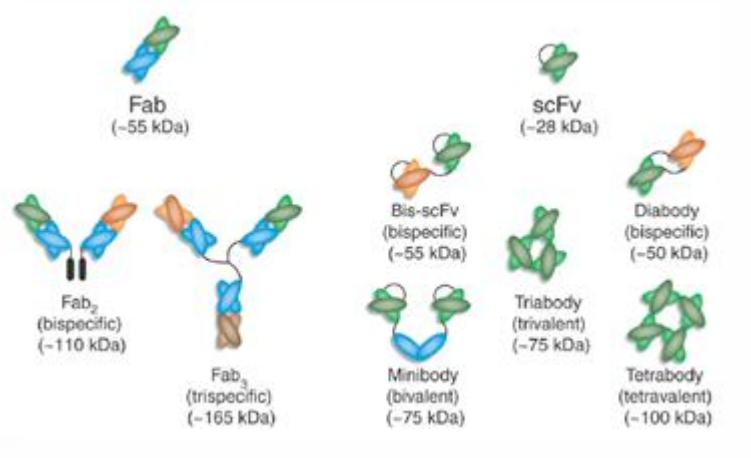


Figure 4: Diversity of antibody molecules [18]

Fab fragments can be engineered to be bispecific so they can bind to different epitopes e.g. a tumor cell and a T-helper cell at the same time. In 2009 the first bispecific antibody was approved in the European Union, than can bind to a Tumor cell and a T cell with its hypervariable region at the Fab fragment and to accessory cells (Macrophages, dendritic cells etc) with its Fc receptor [1]. Since the mid 1990s, several mouse monoclonal antibodies labeled with γ-emitting radionuclides were approved by the FDA for diagnostic imaging of various cancers [8]. If necessary, the plasma half life of Fab fragments can be extended by PEGylation although shorter circulatory half-life may be desired for highly toxic Fab-appendices. PEGylation increases the circulatory half-life from a few hours to two weeks allowing subcutaneous administration once a month [29]. Fabs can facilitate crystallization of hydrophobic proteins by "locking in" or "freezing" certain protein conformations and therefore allowing determination of three-dimensional protein structures e.g. transmembrane proteins. Co-crystallization has supported focused mutagenesis strategies in the directed evolution of humanized and human Fab to human VEGF by phage display [30]. It has to be pointed out, that their ability to bind antigens monovalently without mediating antibody effector functions as well as their lower immunogenicity are important features of Fab fragments [8] [31].

1.4.3 Purification of Fab fragments - Affinity Chromatography

Protein purification via chromatography has been established as a very potent strategy to achieve high yields combined with high purities. Together with protein crystallization, chromatography is sufficiently researched and in the majority of cases usable for industrial scale production. The great variety within chromatography has made it extensively used in analytical as well as preparative processes. For the specific purification of proteins in biotechnology, the so called Affinity chromatography was established. Several affinity media were developed for purification of antibodies and antibody fragments, utilizing selective non-covalent interaction between the analyte and specific molecules. Proteins, antibody fragments, and polypeptides function as ligands. Antibodies and antibody fragments bind to these ligands specifically and reversibly and can be separated from other biomolecules under the right elution conditions [32]. The most commonly used affinity ligands are Protein A, G and L, but nanobodies or specifically designed polypeptides can also be used as ligands. Protein G as well as protein A, are bacterial proteins from *Staphylococcus aureus*, protein L originates from *Peptostreptococcus magnus* and V_HH domains are extracted from camelids [33-37].

Table 1 shows a list of commonly used affinity chromatography media for purfication of antibodies and antibody derivatives [59-61].

Table 1: List of commonly used media in affinity chromatography

Media	Vendor	Ligand	Binding site	Matrix
MabSelect, Mab Select Xtra, Mab Select Sure	GE Healthcare	Protein A	Fc region and human V_H3	Sepharose
Protein G	Sigma-Aldrich	Protein G	Fc region	Sepharose
Protein L	Sigma-Aldrich	Protein L	Kappa LC	Sepharose
CaptureSelect IgG-C_H1	BAC	Camelid VHH	C_H1	Agarose
CaptureSelect Fab kappa	BAC	Camelid VHH	Kappa LC	Sepharose
CaptureSelect Fab lambda	BAC	Camelid VHH	Lambda LC	Sepharose

Figure 5 shows the different binding sites of the used resins:

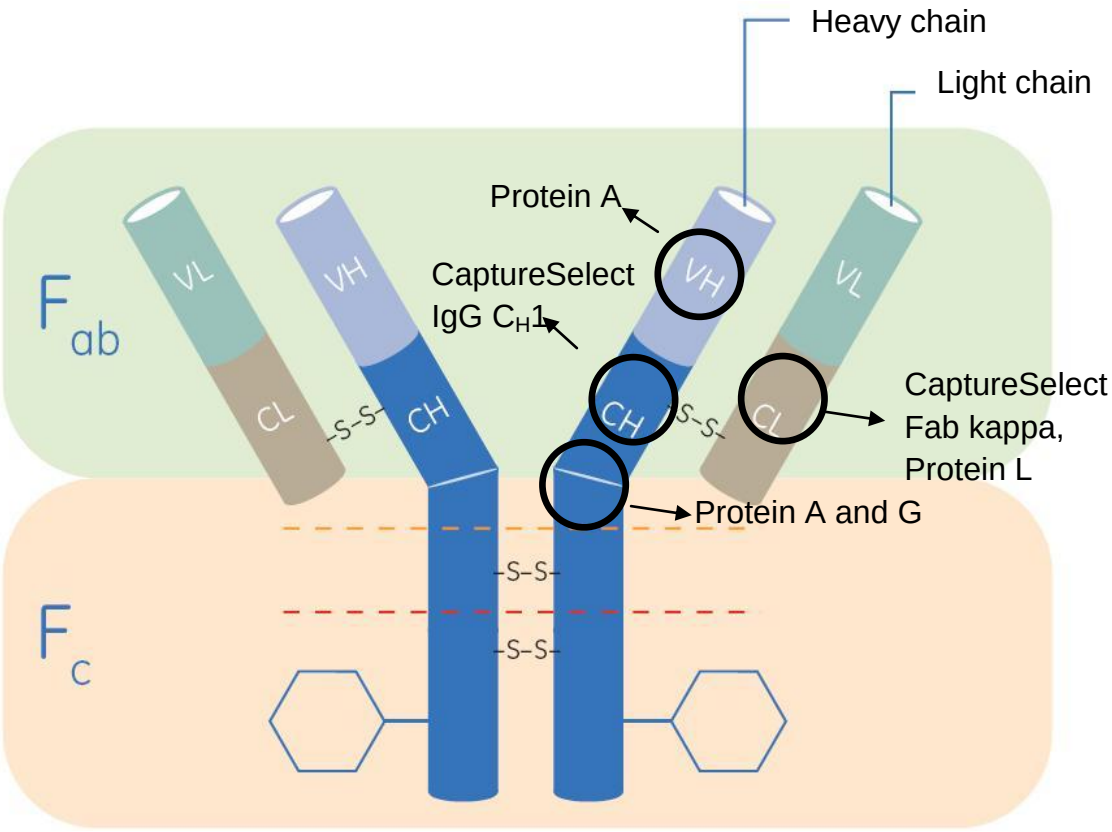
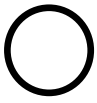


Figure 5: Binding sites of affinity resins

 marks binding sites for resins

- - - - - - - = Papain digestion site

- - - - - - - = Pepsin digestion site

2 Material and methods

2.1 Equipment

For solid media different balances were used:
A Mettler Toledo PB3002-S/FACT Classic Plus or a Mettler Toledo Excellence Plus XP analytical balance were used, as well as a Sartorius LP34000P or a Sartorius Mc1 Laboratory LC 12000 S scale.
Media were sterilised using a Systec D65, 2005, S/N D0559, 3x400 V, 68 L autoclave. In cases when autoclaving was impracticable, solutions were sterile filtrated with Nalgene® filter units (MF75™ Series).

Sterile operations were performed in a HeraSafe/Kendro Type KS15 laminar flow workbench+ (order No. 51022731. S/N 40373470. 2004.04 EN 12469: 2000 class II, working volume1500x780x627 mm).

Biomass production was carried out in Biostat® C-plus fermenters type CT5-2 from Sartorius stedim biotech with a working volume of 5 liters.
Protein purification was performed with the liquid chromatography systems ÄKTApurifier™, ÄKTAexplorer™ or ÄKTA™ avant, GE Lifescience.

A Freedom Evo® 150 robot from Tecan Group Ltd. (Seestrasse 103, 8708 Männedorf Switzerland) was used for automated refolding experiments.

Protein analysis was performed using a LabChip® GX II system with corresponding HT Protein Express chips, both purchased from Caliper LifeSciences with (Hopkinton, MA 01748 U.S.A.)

HQ-water was provided by a Millipore Elix Advantage 10 water purification system.

Table 2 summarizes the equipment used during the practical work.

Table 2: Equipment list

Equipment	Manufacturer
Homogenizer APV 1000	APV
Centrifuge 5415R	Eppendorf
Centrifuge 5810R	Eppendorf
Centrifuge 5430	Eppendorf
Centrifuge Avanti J-26 XP	Beckman Coulter
Fermenter	Biostat® C-plus 5L
ULTRA TURRAX®	IKA®
pH-meter SevenMulti	Mettler Toledo
Incubator	Biocenter 2001 Renggli
Shaker Multitron II with humidification system	Infors
Thermomixer compact	Eppendorf
Systec D65, 2005, S/N D0559, 3x400 V, 68 L autoclav	Systec
Xcell SureLockTM protein electrophoresis cell	Invitrogen
ÄKTA Purifier	GE Healthcare
ÄKTA Explorer	GE Healthcare
ÄKTA Avant	GE Healthcare
Robot	Freedom Evo® 150
Lab chip GX II	Caliper Life science

2.2 Materials

2.2.1 Chemicals

All chemicals were purchased from Merck (Germany) or Sigma-Aldrich (USA), unless stated otherwise. Bacto yeast extract and bacto agar were purchased from BD (USA).

2.2.2 Bacterial strains and plasmids

Escherichia coli BL 21 (DE3) (F– *ompT hsdS*B(r_B– m_B–) *gal dcm)* bacterial strain, purchased from Novagen, was used for fermentations in flask and in the 5 L fermenters.

Figure 6 depicts the plasmid with the 3H6 HC coding gene

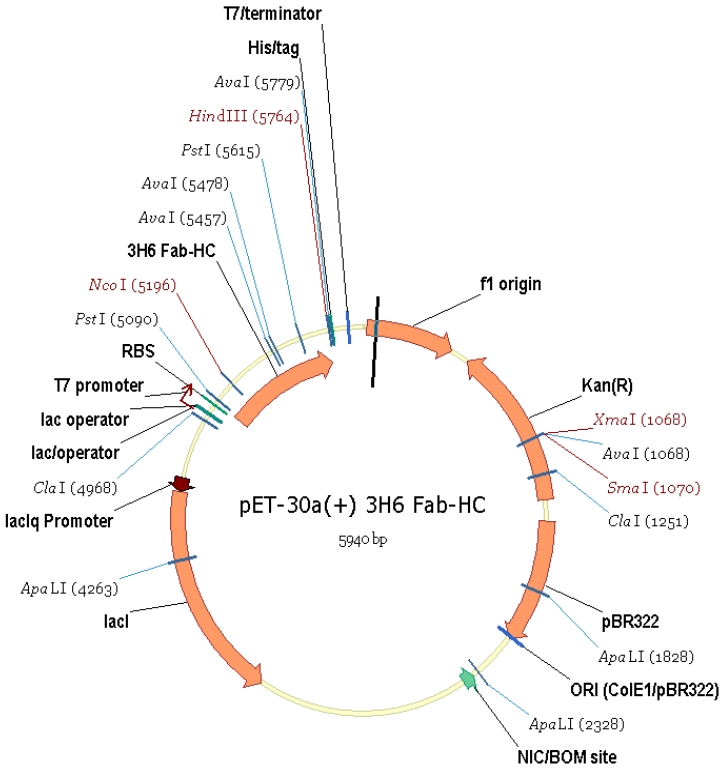


Figure 6: pET -30a plasmid containing the 3H6 Fab HC coding gene

2.2.3 Buffers and Media

The tables 3 -15 show the media and composition of buffers and solutions.

Table 3: Soy LB medium

Soy LB Medium (SLB)	Concentration [g L^{-1}]
Bacto yeast extract	5
Sodium chloride	10
Soy peptone	10

Table 4: Antibiotic stock solution

Antibiotic stock solutions 1000x	Concentration [g L^{-1}]
Kanamycin (sulfate)	50

Table 5: OD buffer

OD-buffer	Concentration [g L^{-1}]
Na_2HPO_4*$12H_2O$ Disodium hydrogen phosphate dodecahydrate	20.73
Potassium dihydrogen phosphate	5.7
Sodium chloride	11.6

Table 6: TB-Medium

TB-Medium	Concentration [g L^{-1}]
Yeast Extract	10
Soy Peptone	20
Potassium dihydrogen phosphate	9.4
Dipotassium hydrogen phosphate	2.2
Glycerol	4

Table 7: IPTG- solution

Isopropyl β-D-1-thiogalactopyranoside-solution	100 mM	Concentration [g L^{-1}]
IPTG		23,83

Table 8: Homogensation buffer

Homogenisation buffer	Concentration [mM]
Tris-HCl	100
NaCl	150
EDTA	5

All components were dissolved in dH_2O and the pH was adjusted to 8.0.

Table 9: IB Wash buffer 1

IB Wash buffer 1	Concentration [mM]
Tris-HCl	20
NaCl	150
EDTA	5

All components were dissolved in dH_2O and the pH was adjusted to 8.0.

Table 10: IB Wash buffer 2

IB Wash buffer 2	Concentration [mM]
Tris-HCl	20
NaCl	150
EDTA	5
Urea	2000

All components were dissolved in dH_2O, 0.5 % (v/v) Triton® X-100 (Serva Electrophoresis, Germany) was added and the pH was adjusted to 8.0.

Table 11: Electrophoresis buffer

Electrophoresis buffers	Composition
Running buffer 1xMES	100 mL from 20 x MES and 1900 mL dH_2O
Buffer for fixation	1 L Ethanol, 860 mL dH_2O and 140 mL glacial acetic acid
Washing buffer	dH_2O
Staining solution	Simply Blue™ Safe Stain
Destaining solution	dH_2O

Table 12: Main culture medium

Main culture medium	Concentration [g L^{-1}] or volume mL L^{-1}
Glucose	600
Potassium dihydrogen phosphate	3
Dipotassium hydrogen phosphate	4.58
Sodium citrate dihydrate	66
$MgSO_4*7H_2O$ Magnesium sulfate	26.4

heptahydrate	
$CaCl_2$*2H_2O Calcium dichloride dihydrate	5.28
Trace element solution	13.2 mL
10 M sodium hydroxide	4 mL
L-Methionine	1
dH_2O	Ad 1000 mL

Table 13: Trace element solution

Trace element solution	Concentration [g L^{-1}]
85 % phosphoric acid	173
Iron(II) sulfate heptahydrate	40
Manganese sulfate dihydrate	10
Aluminium chloride hexahydrate	10
Cobalt chloride hexahydrate	4
Zinc sulfate heptahydrate	2
Sodium molybdate dihydrate	2
Copper(II) chloride dihydrate	1
Boric acid	0.5
dH_2O	Ad 1000 mL

Table 14: Blotting buffer

Blotting buffers	Composition
3 % milk powder solution	3 g non-fat dry milk powder in 100 mL 1x PBS and 0.1 % Tween 20
Washing buffer	1x PBS and 0.1 % Tween 20
Antibody solution	1x PBS and 0.1 % Tween 20, 1% Blocker™ BSA, Anti-Human IgG (Fab specific)-Alkaline Phosphatase 1:5000
Staining solution	Western Blue® Stabilized Substrate for Alkaline Phosphatase

Blocker™ BSA (10%) in PBS solution was purchased from Thermo Scientific, 3747 N. Meridian Rd., Rockford, IL61101, U.S.A.

Protein standards and markers for SDS Page and Western Blot:

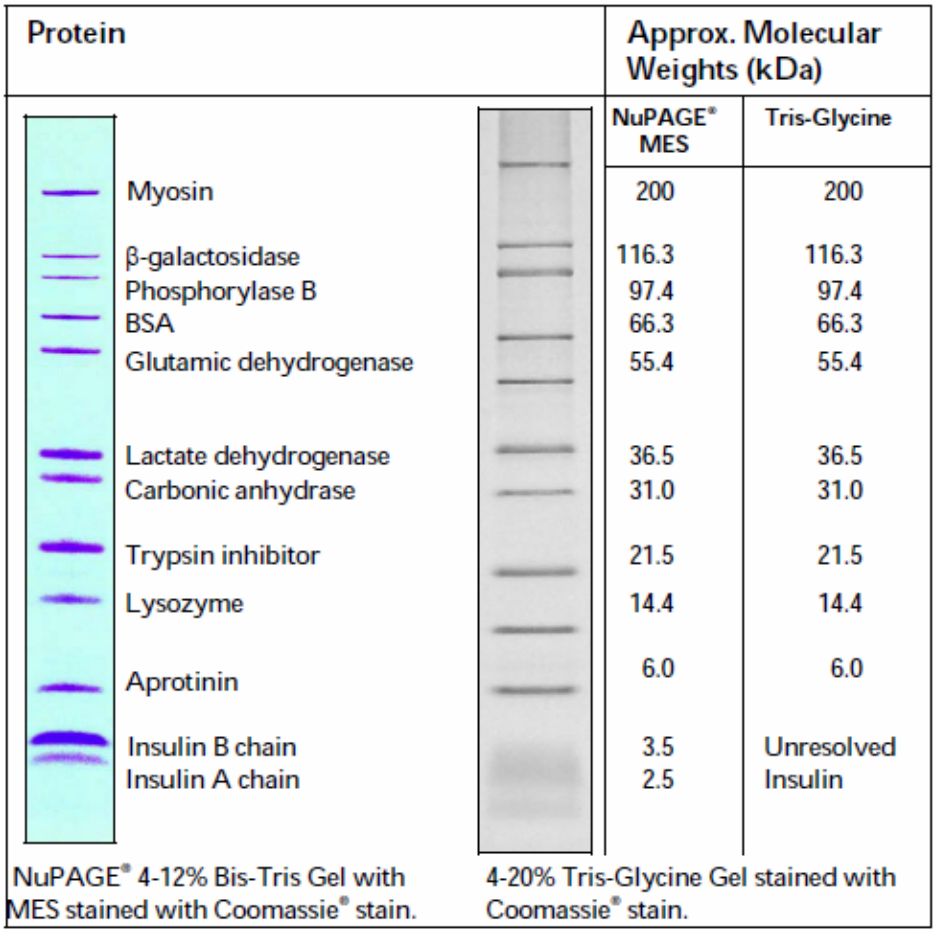


Figure 7: Protein size standards

Table 15: List of used standards and stock solutions

Name	Manufacturer
Mark 12™	Invitrogen
MagicMark™ XP Western Protein Standard	Invitrogen
NuPAGE® LDS Sample Buffer (4X)	Invitrogen
Albumin Standard 2 mg/mL	Thermo Scientific

2.2.5 Fab model-proteins

Three different Fab model-proteins were provided by Boehringer Ingelheim RCV GmbH & Co KG for the experiments. They are called 3H6, B1 and A4. From 3H6 and B1 parent cultures were provided and fermentations still had to be conducted to receive IBs, whereas with A4 IBs were provided.

Table 16 shows some of the proteins´ characteristics.

Table 16: Characteristics of the single chains

Protein	Size (kD)	pI	Number of amino acids
3H6 HC	23.9	9.04	225
3H6 LC	23.7	4.72	215
B1 HC	24.8	9.24	230
B1 LC	24.5	6.91	221
A4 HC	23.2	9.04	219
A4 LC	23.1	5.73	215

All polypeptide chains are fairly evenly sized and that the heavy chains have higher pI values than the light chains, due to their different amino acid composition. The data was generated by entering the exact sequences into Vector NTI (Invitrogen, U.S.A) and calculation of the desired values.

In comparison to the individual protein chains Table 17 gives an overview over the characteristics of the complete Fab fragments. Size and pI were again calculated with Vector NTI.

Table 17: Characteristics of Fab fragments

Fab fragment	Size (kD)	pI	Number of amino acids
3H6	47.6	6.35	440
B1	49.3	8.8	441
A4	46.3	8.4	434

2.3 Methods

2.3.1. Clone screening

Four different parent cultures for each light and heavy chain of each Fab fragment were used for the clone screening. The necessary preparative microbiological work had been performed at the BI Process Science–Microbial Fermentation unit. The pre cultures were grown in 50 mL Greiner tubes containing 10 mL soy lysogeny broth (SLB) medium and 10 µL of Kanamycin stock (see table 6) as antibiotic. 50 µL of the parent cultures were added under sterile conditions and the bacteria grew at 37°C and 300 rpm over night in a Multitron Incubator Shaker II (ATR, U.S.A.). In the morning the optical density (OD) was measured at 550 nm using a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, U.S.A) If necessary the samples were diluted using OD-buffer (see table 5) to reach an absorbance in the linear range from 0.2-0.6. Pure OD-buffer was used as blank. The necessary amount of pre culture to inoculate the main cultures with an OD of 0.2 was calculated. For the main cultures 250 mL flasks were filled with 50 mL SLB medium and 50 µL of Kanamycin were added under sterile conditions using a laminar flow box. Once the OD reached 1-1.5, the cultures were induced using 1 mL of a 50 mM IPTG stock, which led to a concentration of 1 mM. 4 hours after induction 2 mL of fermention broths were taken, centrifuged at 13000 rpm at 4°C using a type 5415R centrifuge (Eppendorf, Germany). The supernatants were discarded and the pellet was stored at -80°C. For protein analysis with gel electrophoresis the pellets were mixed with 0.5 mL of BugBuster® Mix and 1µL lysonase, vortexed and shaken at 550 rpm for 15 minutes at room temperature using the Thermomixer compact (Eppendorf, Germany). The samples were vortexed again and centrifuged at 13000 rpm for 15 minutes. 20 µL of the supernatant were diluted with 40µL of 1xLDS buffer to give a 1:3 dilution ratio, denatured at 80°C at 450 rpm for 5 minutes and cooled to room temperature again. The Eppendorf tubes were spinned down and 10 µL were loaded onto the gel to analyse the soluble fraction of the proteins.

In order to analyse the insoluble fraction, the centrifuged pellet after the BugBuster® Mix digestion was dissolved in 0.5 mL of 1x LDS buffer and ultrasonicated using a Misonix sonicator XL (Qsonica, U.S.A). For sonication the samples were cooled with ice and the sonication 5 cycles were performed with 30 % of the sonicator´s maximum power and each cycle lasted 1 minute. Afterwards the samples were diluted in a ratio of 1:3 with 1x reducing LDS buffer, containing 2.5 % (v/v) β-mercaptoethanol, and heated to 80°C for 5 minutes and

cooled to room temperature. The Eppendorf tubes were spinned down and 10 µL were loaded onto the gel.

2.3.2 Fermentation in 1L flasks

Pre cultures were grown in 50 mL SLB medium with 50 µL Kanamycin stock as antibiotic and 50 µL of the best parent cultures. The pre cultures were kept at 37°C and 300 rpm over night. Three 1 L flasks per clone, containing 300 mL terrific broth medium and 300 µL Kanaymcin stock each, were provided for the main cultures. The OD of the pre cultures was measured and the necessary amout of pre culture to inoculate the main cultures with an OD of 0.2 was calculated. Inoculation was performed using a laminar flow box to provide sterile conditions. Once the OD reached 2-3 each culture was induced with 3 mL of a 100 mM IPTG stock to reach a concentration of 1 mM. Due to poor growth performance during the next 4 hours at 37°C and 300 rpm, the cultures were grown for 12 more hours. Cell harvesting was performed at 4°C by centrifuging the 900 mL of each construct at 10000 rpm using an Evolution RC centrifuge (Sorvall, U.S.A) with a FiberLite® F8-6x1000y rotor (Thermo Scientific, U.S.A).

2.3.3 Fermentation in Biostat® C-plus fermenters

To provide enough biomass for further experiments larger fermentations in Biostat® C-plus (Sartorius, Germany) fermenters had to be conducted. One 1 L Erlenmeyer flasks was filled with 300 mL main culture medium for each pre culture containing 1 mM Kanamycin. 300 µL of the parent cultures were added and the flasks were kept at 37°C and 300 rpm until an OD of about 2 was reached.

The 5 L fermenter was filled with 2250 mL main culture medium and inoculated with the calculated amount of pre culture to reach an OD of 0.2 at the start. After 8 hours an exponentional glucose feed was started and after 20 hours a constant feedrate of 154 g/h was used for 10 hours. 30 hours after the fermentation start protein production was induced by adding 10 mL of a 0,5 M IPTG solution resulting in an IPTG concentration of 1 mM. The fermentation was ended after 36 hours and the final broth volumen was 5 L. Cell harvesting

was performed at 4°C at 10000 rpm using an Evolution RC centrifuge (Sorvall, U.S.A) with a FiberLite® F8-6x1000y rotor (Thermo Scientific, U.S.A). The cells were stored at -20°C.

2.3.4 Polyacryl gel electrophoresis

For protein analysis polyacryl gel electrophoresis was performed using Xcell SureLock™ protein electrophoresis cells (Invitrogen, U.S.A.). The cells were assembled according to the supplier´s manual. NuPAGE® 12% Bis-Tris gels (Invitrogen, U.S.A.) were washed with 1x MES buffer and placed into the chambers. 1x MES buffer was filled into the chambers according to the manual. Protein samples were diluted to final concentrations between 0.05 and 0.15 µg/µL. NuPAGE® LDS sample buffer (4x) was used to dilute the samples. Lithiumdodecylsulfate binds to the proteins and provides a constant negative charge allowing the proteins to migrate in the electrical field. The samples were heated to 80°C for 5 minutes and 450 rpm with a Thermomixer compact (Eppendorf, Germany) to denature the protein. Afterwards the samples were centrifuged for 5 minutes at 13000 rpm using a miniSpin PLUS centrifuge (Eppendorf, Germany) and 10-20 µL were loaded onto the gel. The proteins are separated by their charge to mass ratio. If reduction of disulfide bonds was desired β-mercaptoethanol was added to the LDS buffer before dilution. The voltage for electrophoresis was set to 200 V and the running time was between 45 and 60 minutes depending on the desired resolution. Afterwards the gels were treated with fixation buffer for 15 minutes before dyeing with Simply Blue™ Safe Stain (Invitrogen, U.S.A) was performed according to the manufacturer´s manual. Subsequently the optical density of the stained gels was measured by laser densitometry using a Personal Laser Densitometer SI (Amersham Bioscience, UK) with the software PDSI Scanner Control v5.03 (Amersham Bioscience, UK) and protein quantification was performed using the software TotalLab TL 120 (Nonlinear Dynamics®, UK).

2.3.5 IB Preparation

The frozen biomass was suspended in homogenization buffer in a ratio of 1:8 using an ULTRA TURRAX® with adequate power. Three homogenization passages were applied at a pressure of 650 bar using an APV 1000 homogenizer (APV, Germany). During the process the biomass was permanently cooled to 8-12°C. After homogenization the biomass was

centrifuged for 30 minutes at 5000 rpm using the Avanti J-26 XP centrifuge (Beckman COULTER® U.S.A.) with a JLA 8.100 Rotor (J-Lite® series, Beckman, U.S.A.). The supernatant was discarded and the crude inclusion bodies were further purified.

The pellet was suspended in wash buffer 2 (see table 10) in a ratio of 1:8 and washed by using an ULTRA TURRAX® for 2 minutes. Hereupon the IBs were centrifuged at 6000 rpm for 30 minutes using the Avanti J-26 XP centrifuge (Beckman COULTER® U.S.A.) with a JLA 8.100 Rotor (J-Lite® series, Beckman, U.S.A.) The supernatant was discarded and the step was repeated two times. Then the pellet was resuspended in wash buffer 1 (see table 9) in a ratio of 1:8 and washed by using an ULTRA TURRAX® for 2 minutes. Hereupon the IBs were centrifuged at 7000 rpm for 40 minutes using the Avanti J-26 XP centrifuge (Beckman COULTER® U.S.A.) with a JLA 8.100 Rotor (J-Lite® series, Beckman, U.S.A.) The supernatant was discarded and the step was repeated once. The IBs were weighed and stored at -20°C. Protein solubilisation in the washing steps should be kept at a minimum and product loss in the supernatant was monitored by SDS-PAGE analysis.
To analyse the protein content of the inclusion bodies 100 mg were weighed in a 1.5 mL Eppendorf tube. 880 µL of 6 M Guanidine-HCl were added and the pellet was resuspended using an ULTRA TURRAX® T-50 basic at 12000 rpm for 1 minute. Afterwards 20 µL of a 1 M DTT solution were pipetted into the tube to give a 20 mM DTT concentration. The tubes were placed on a roller for 45 minutes and subsequently spinned down for 5 minutes. The supernatant was diluted with 8 M Urea in ratios from 1:30 to 1:50. The diluted samples were mixed with reducing 2x LDS buffer, heated for 5 minutes at 80°C at 450 rpm and cooled to room temperature. The samples were spinned down and 10 µL were loaded onto the gel.

2.3.6 Resolubilisation

The best resolubilisation conditions were separately developed for heavy and light chains of 3H6 and B1. Different Guanidine-HCl and Urea concentrations were tested and the buffer pH varied between 7.5 and 10.5 all containing 5 mM EDTA. High Guanidine-HCl concentrations are to be avoided, because they cause problems with SDS-PAGE analysis and increase conductivity so subsequent IEC cannot be performed without lowering the conductivity. For the resolubilisation of the heavy chains different surfactants like N-lauroylsarcosine or SDS were tested. In the end one buffer for both heavy and light chain was used. The amount of resolubilised IBs should provide a protein concentration of about 4.5 mg/mL and was

suspended in a ratio of 1:5-1:10 in resolubilisation buffer. The inclusion bodies were stirred with an ULTRATURRAX® for about 60 seconds. Then 1 M DTT stock solution was added to provide a final DTT concentration of 20 mM in the tube. The tubes were placed on a roller for 45 minutes to assure denaturation and were afterwards centrifuged at 12000 rpm for 15 minutes using a 5810 R centrifuge (Eppendorf, Germany). The supernatant was used for further experiments.

2.3.7 Refolding in 1.5 mL Eppendorf tubes

Standard refolding experiments were conducted in 1.5 mL Eppendorf tubes with a total working volume of 1 mL. The corresponding amounts of refolding components were pipetted into the tubes and mixed. The resolubilised protein was pipetted onto the tube´s lid. Immediately after closing the lids the tubes were vortexed for a few seconds to assure rapid dispersion of the protein. The incubation time varied between 24 and 72 hours and different temperatures and agitation speeds were tested. Varying buffer components, additives, pH values and Redox-systems were tested. Standard SDS-PAGE gel analysis or analysis with the HT Protein Express LabChip was used for protein determination.

2.3.8 Refolding in 100 mL scale

In order to investigate refolding behaviour in larger scale defined experiments were upscaled to a volume of 100 mL. All buffer components were pipetted into a beaker (listed separately for each experiment below) and the protein was added via pumping it into the refolding buffer under constant stirring. The feed rate was about 2 mL/hour.
Refolding times lasted between 48 and 72 hours and analysis was performed via SDS-PAGE or HT Protein Express LabChip.

2.3.9 Refolding experiments using the Tecan Freedom EVO® 150 robot

The aim of these experiments was to establish a robot based refolding screening system. Automatic refolding experiments were conducted using the freedom EVO 150 system

equipped with the Freedom Evoware 2 standard software. Automatic pipetting was performed comparable to the manual procedure. Manually and automatically pipetted refoldings should be compared regarding time, sample number throughput and accuracy etc. 2-fold concentrated stock solutions, dH2O, stocks containing the Redox system and the solubilised heavy and light chains of the Fab fragments were prepared. The robot was programmed to add all components under constant shaking into a 24-deep well plate with a maximum volume of 2 mL. The protein solution was pipetted last and the refolding time was 48 hours. The refolding experiments were analysed with SDS-PAGE or capillary electrophoresis using the HT Protein Express LabChip.

2.3.10 Protein analysis with Caliper system

Protein analysis by using an HT Protein Express LabChip in a Caliper Lifescience system was an alternative method to analyse and quantify the refolding experiments besides SDS-PAGE. The samples were treated accordingly to the supplier´s manual and the software LabChip GX II was used to quantify the detected bands. The Caliper system was also used to compare manual pipetting accuracy and the pipetting accuracy of the Tecan Freedom EVO® robot and whether automatic sample preparation provides reproducible results.

2.3.11 Western blot

Western blotting was performed by using the iBlot™ Gel Transfer Device (Invitrogen, U.S.A.) to prove the correct folding of the Fab fragments of A4 and 3H6. SDS-PAGE gels were loaded with samples of refolded Fab fragments and they were run at 200 V for 45 minutes. Afterwards the gels were placed in the iBlot™ Gel Transfer Device, and blotting was performed following the supplier´s manual. The steps after blotting onto the nitrocellulose membrane were done as described in Table 18.

Table 18: Western blot procedure

Step	Solution	Volume [mL]	Duration [minutes]
1	3 % milk powder solution	40	60
2	1x PBS and 0.1 % Tween 20	40	10
3	1x PBS and 0.1 % Tween 20	40	10
4	1x PBS and 0.1 % Tween 20	40	10
5	1x PBS and 0.1 % Tween 20, 1 % Blocker™ BSA, Anti-Human IgG (Fab specific)-Alkaline Phosphatase 1:5000	10	60
6	1x PBS and 0.1 % Tween 20	40	10
7	1x PBS and 0.1 % Tween 20	40	10
8	1x PBS and 0.1 % Tween 20	40	10
9	Western Blue® Stabilized Substrate for Alkaline Phosphatase	10	5

For each step the membrane was placed in a small plastic box and put on a shaker at 100 Mot/min to assure that the membrane was permanently submerged in the respective solutions. After dyeing the membrane was dried and scanned by laser densitometry using a Personal Laser Densitometer SI (Amersham Bioscience, UK) with the software PDSI Scanner Control v5.03 (Amersham Bioscience, UK).

3 Results and discussion

3.1 Experimental outline

The aim of this study was to establish refolding processes in order to obtain correctly folded Fab fragments of three different proteins: 3H6, B1 and A4. The heavy and light chains of the Fab fragments were separately expressed as inclusion bodies in *Escherichia coli* The effective solubilisation of the proteins was the first important step to generate functional Fab fragments by optimising subsequent refolding conditions. Various conditions were tested by altering refolding parameters like buffer composition, temperature, pH, redox conditions, metal ion catalysis, refolding volume or refolding time length.

Furthermore an automated, robot based refolding procedure was to be evaluated during this thesis concerning time-saving, sample number throughput and robustness compared to manually performed experiments. The pipetting steps were carried out in 24-well plates with a Tecan Freedom EVO® robot using a total volume of up to1.5 mL and a protein concentration of 0.1 to 0.2 mg/mL. As an alternative quantification method to SDS-PAGE, capillary gel electrophoresis in a Caliper detection system was used.

3.2 Results of the upstream processes

3.2.1 Results of the clone screening

For each light and heavy chain of the proteins 3H6 and B1 four *E.coli* strains were provided, which were tested for their expression capabilities. The cell cultures were grown in 250 mL flasks, with a total fermentation volume of 50 mL. Four hours after induction with IPTG 2 mL samples were drawn and centrifuged to pellet. The cells were visually checked for IB production using a microscope and the protein content was determined. Figure 8 shows IBs in the *E.coli* cells of 3H6 expressing the light chain in blue color.

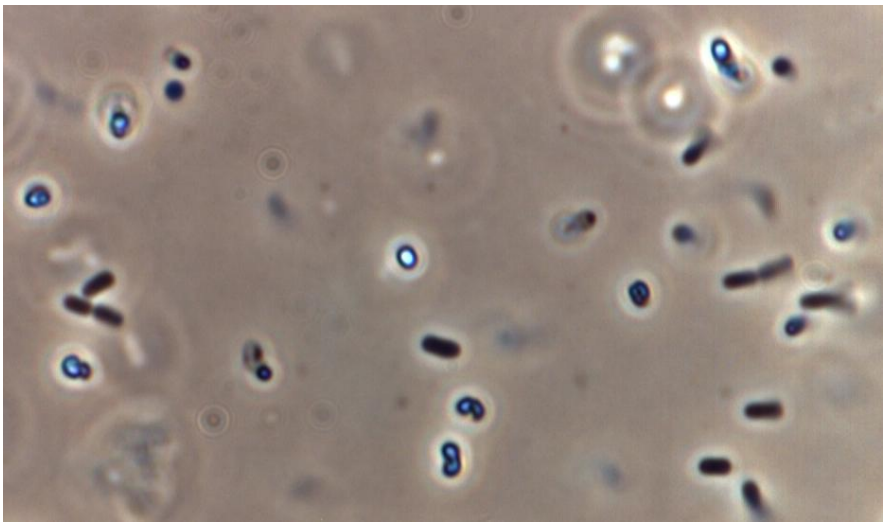
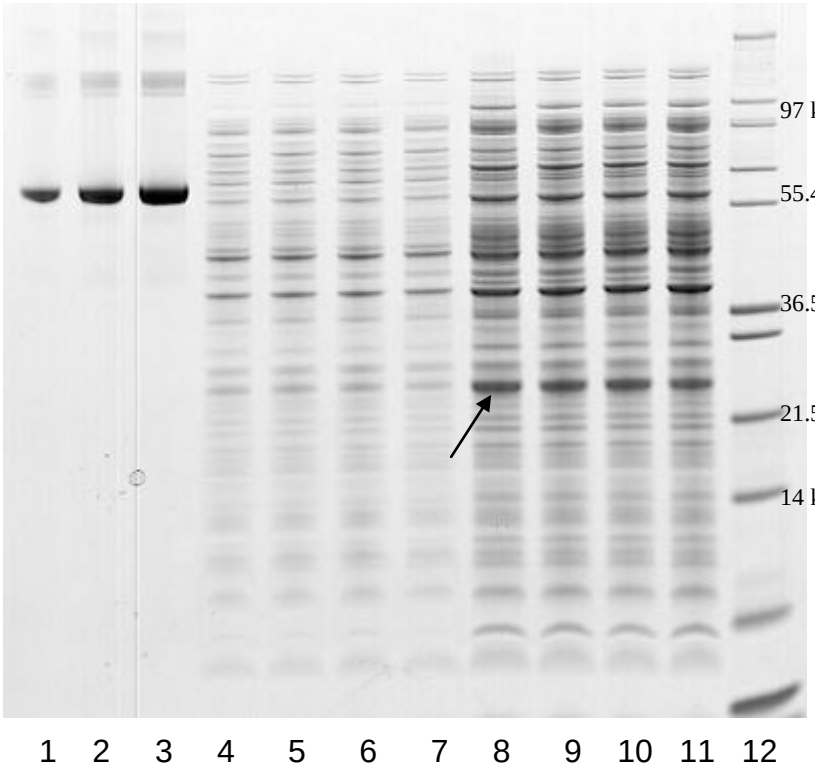


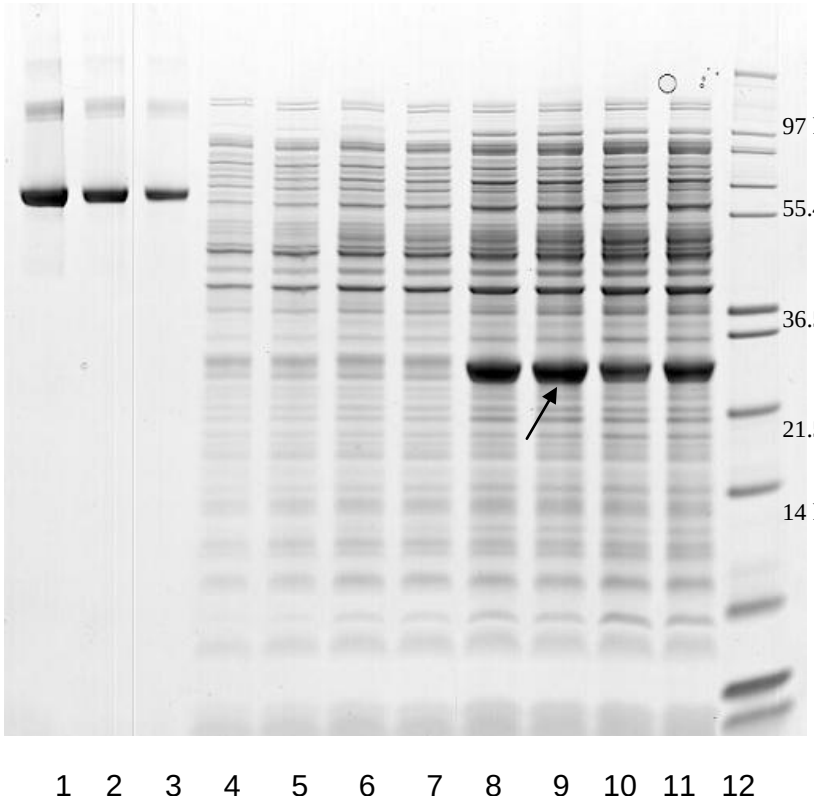
Figure 8: Microscopy: *E. coli* cells containing IBs

Figures 9-13 show the results of protein determination with SDS-PAGE highlighting the best clones of each construct and marking the samples at end of fermentation in grey.



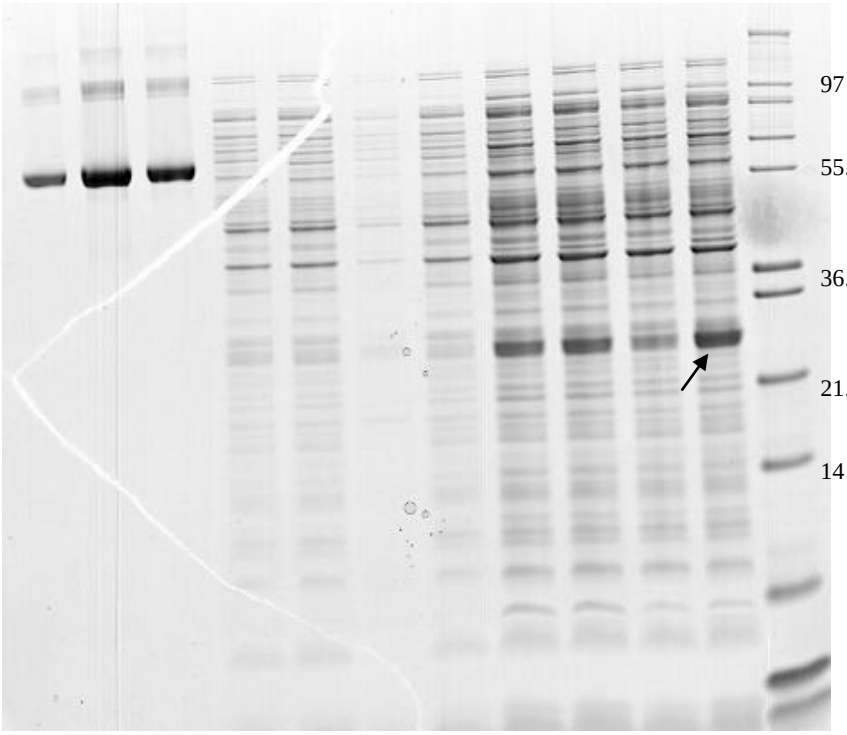
Lane	Description
1-3	BSA
4	HC clone 1
5	HC clone 2
6	HC clone 3
7	HC clone 4
8	HC clone 1
9	HC clone 2
10	HC clone 3
11	HC clone 4
12	Mark 12

Figure 9: Clone screening for 3H6 HC: samples at end of fermentation are highlighted in grey



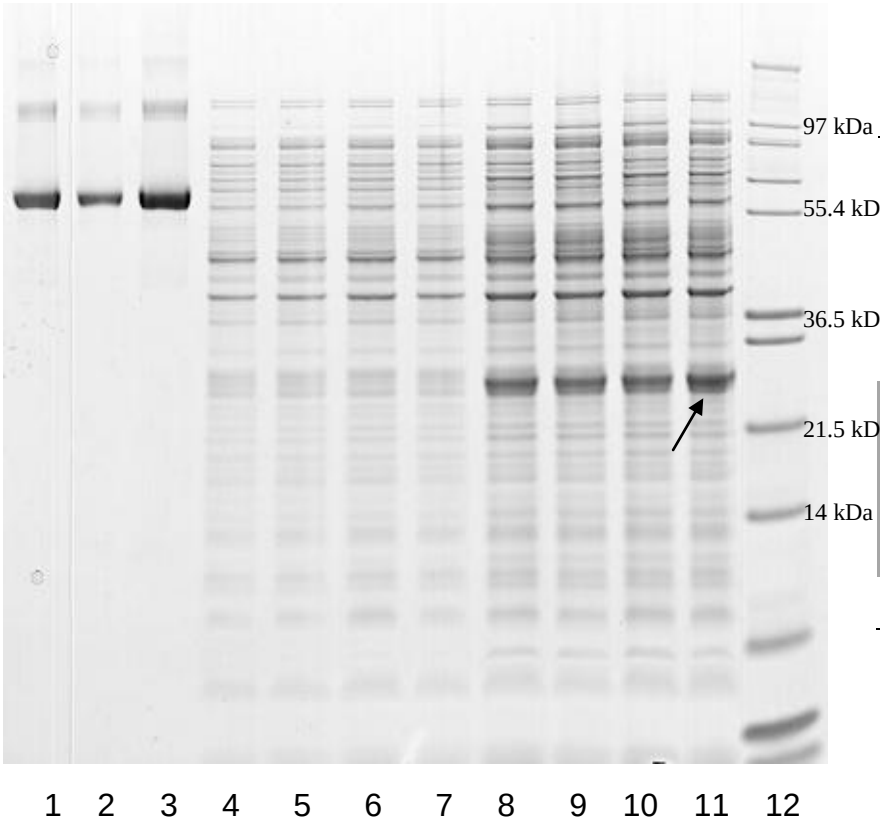
Lane	Description
1-3	BSA
4	LC clone 1
5	LC clone 2
6	LC clone 3
7	LC clone 4
8	LC clone 1
9	LC clone 2
10	LC clone 3
11	LC clone 4
12	Mark 12

Figure 10: Clone screening for 3H6 LC: samples at end of fermentation are highlighted in grey



Lane	Description
1-3	BSA
4	HC clone 1
5	HC clone 2
6	HC clone 3
7	HC clone 4
8	HC clone 1
9	HC clone 2
10	HC clone 3
11	HC clone 4
12	Mark 12

Figure 11: Clone screening for B1 HC: samples at end of fermentation are highlighted in grey



Lane	Description
1-3	BSA
4	LC clone 1
5	LC clone 2
6	LC clone 3
7	LC clone 4
8	LC clone 1
9	LC clone 2
10	LC clone 3
11	LC clone 4
12	Mark 12

Figure 12: Clone screening for B1 LC: samples at end of fermentation are highlighted in grey

Table 19 shows the titers of the clone screenings:

Table 19: Titers of the clonescreenings

Clone	Protein content [mg/mL]
3H6 HC	0.05
3H6 LC	0.16
B1 HC	0.08
B1 LC	0.09

Further analysis showed that all protein was produced as inclusion bodies. No soluble target protein after expression could be detected (data not shown).

3.2.2 Results of fermentation in 1L flasks and IB preparation

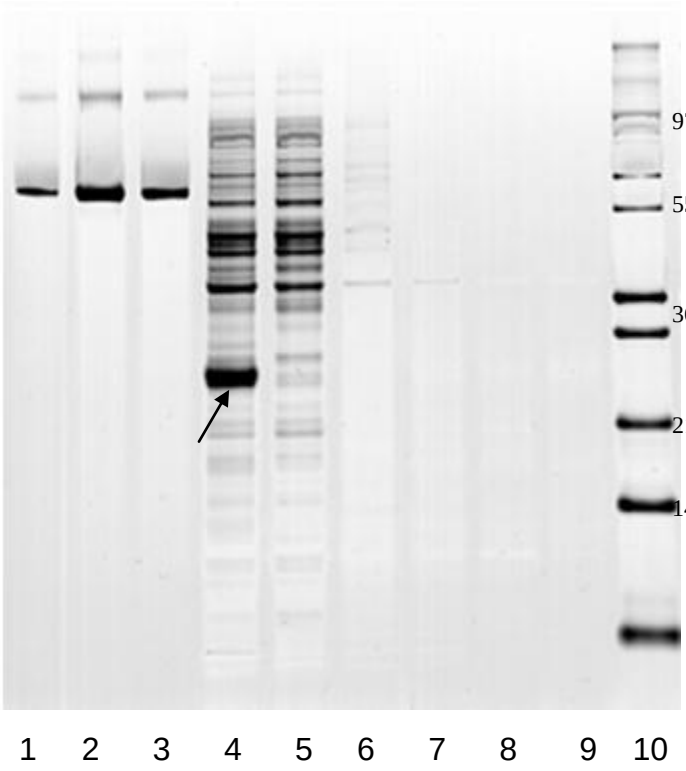
Table 20 shows the yield of biomasses for each fermentation as well as the percentage of inclusion bodies compared to the total biomasses.

Table 20: Biomass yields of fermentations in 1L flasks

Fermentation 1L flask	yield [g/L]	% inclusion bodies (w/w)
3H6 HC	27,3	53
3H6 LC	30,9	51
B1 HC	27,2	48
B1 LC	22,0	44

After dilution with homogenisation buffer, the biomasses from these 1 L fermentations were homogenized using a Constant cell disruption systems homogenizer. (Constant systems, UK). The pressure was set to 650 bar and three passages were applied on every passage. Inclusion bodies can make up to 40 % of total cellular protein [13] which is in the range of our experiments. Regarding the yield and the purity though, it can be assumed that the homogenisation has not worked highly efficiently. More passages could have been applied to disrupt a higher percentage of cells and more washing steps could have been used to gain purer IBs. It has to be considered that with more passages through the homogenizer and intense washing, product loss may also be significantly higher.

Figure 13 shows SDS-PAGE samples from homogenization and following washing steps of B1 HC.



Lane	Sample
1-3	BSA
4	Homogenate
5	Supernatant of Homogenate
6	Supernatant washing step 1
7	Supernatant washing step 2
8	Supernatant washing step 3
9	Supernatant washing step 4
10	Mark 12

Figure 13: SDS-PAGE of the purification of B1 HC IBs

It was observed that the supernatant of the homogenate from B1´s heavy chain included only small amounts of target protein. During the washing steps no target protein was lost. The SDS-PAGE gels of the other homogenizations and washing steps looked very similar so the data is not shown.

Table 21 shows the protein content of the inclusions bodies from the fermentations in 1 L flasks.

Table 21: Protein content of IBs

Protein	Protein content [mg/g IBs]
3H6 HC	30.9
3H6 LC	36.9
B1 HC	33.0
B1 LC	39.1

The protein content was not very high, which was probably due to poor homogenization efficiency and long centrifugation times. Cell debris and host cell proteins were still mixed with inclusion bodies.

3.2.3 Results of fermentations in 5 L scale and IB preparation

In order to obtain enough material for subsequent experiments fermentations in 5 L scale were conducted for each heavy and light chain.

Table 22 shows the wet biomass weight of every 5 L fermentation after cell harvest and the CDW of each fermentation.

Table 22: Biomass and CDW of the 5 L fermentations

	3H6 HC	3H6 LC	B1 HC	B1 LC
Biomass [g]	1444	1516	1614	1487
DCW [g/L]	280	296	314	284

SDS-PAGE was used to determine the amount of expressed protein.
Table 23 provides an overview of protein expression before induction and at the end of each fermentation.

Table 23: Titers of 5L fermentations

	3H6 HC	3H6 LC	B1 HC	B1 LC
Titers before induction [g/L]	0	0	0	0
Titers at end of fermentation [g/L]	2.7	10.4	2.6	8.0

Before induction no target protein was detectable, showing good protein expression control with the used promoter.

Table 24 shows the yield of inclusion bodies compared to wet cell paste for each protein.

Table 24: Inclusion body yields

	3H6 HC	3H6 LC	B1 HC	B1 LC
Yield IBs %	8.9-14.7	5.3-17.8	7.4	8.8

The results of protein preparation with 3H6 varied, because more inclusion body preparations were performed to generate material for experiments. B1 biomass was only prepared once and therefore there is only one set of data available.

Table 25 lists the protein contents per gram purified inclusion bodies.

Table 25: Protein content in IBs

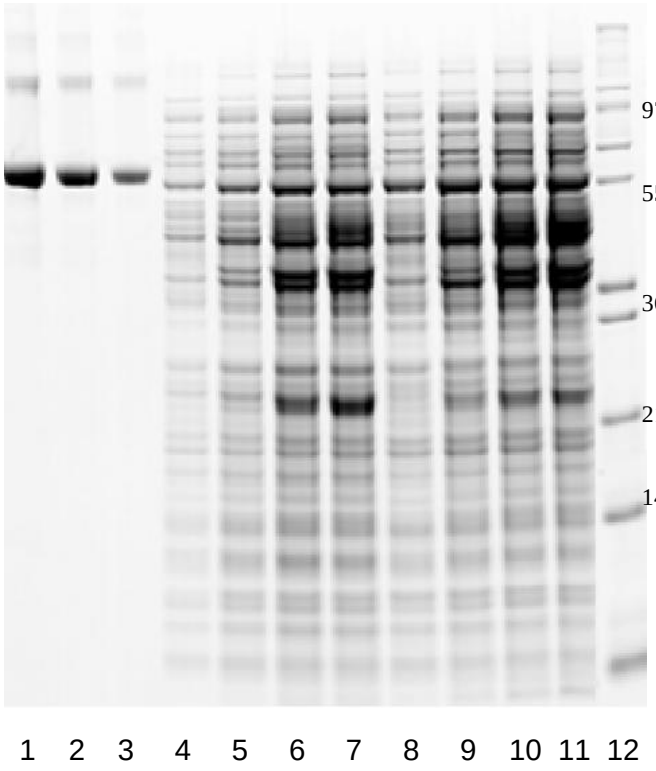
	3H6 HC	3H6 LC	B1 HC	B1 LC	A4 HC	A4 LC
Protein content [mg/g IBs]	42	105	22	95	110	140

It was observed that IBs from heavy chains were more difficult to prepare. The protein yield was lower by a factor of about 2.3 for 3H6 and about 4.3 for B1 compared to the light chains´ content. As a consequence a significantly higher amount of inclusion bodies of the heavy chains was necessary for solubilisation to yield a defined protein concentration compared to light chain IBs.

3.3 Results of the downstream processes

3.3.1 Resolubilisation experiments

The goal of the resolubilisation experiments was to determine the best solubilisation conditions for HC and LC protein from inclusion bodies. Chaotropic agents like Urea and Guanidine-Hydrochloride (GdnHCl), assure the dissolution of the three dimensional structure in proteins and are used to keep the protein soluble. Different concentrations of Urea (4, 6, 8 and 10 M) and 6 M GdnHCl were tested at different pH values. The experiments were performed separately with the heavy and light chains to gain information about the individual solubilisation properties. At pH 7.5 and 8.5 Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol) was used as buffering component and glycine was used at pH 9.5 and 10.5. Each buffer contained 5 mM EDTA, which denied protease activities catalyzed by metal ions.
Figure 14 shows the resolubilisation results with different molar concentrations of urea at pH 7.5 and 9.5.



Lane	Sample	Urea [M]	pH
1-3	BSA	-	-
4	3H6 HC	4	7.5
5	3H6 HC	6	7.5
6	3H6 HC	8	7.5
7	3H6 HC	10	7.5
8	3H6 HC	4	9.5
9	3H6 HC	6	9.5
10	3H6 HC	8	9.5
11	3H6 HC	10	9.5
12	Mark 12	-	-

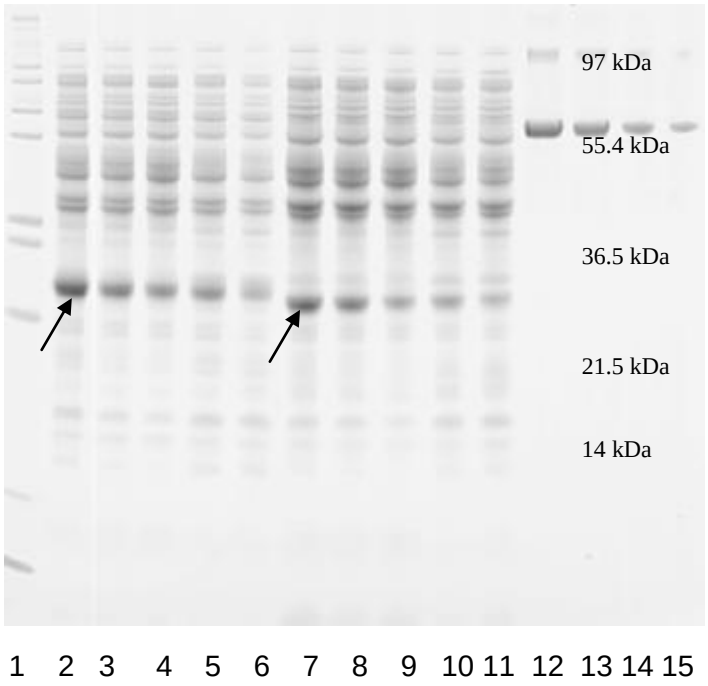
Figure 14: Resolubilisation results of 3H6 HC

The best solubilisation conditions were observed at high concentrations of urea (lanes 7 and 11) for the heavy chains of 3H6 and B1. The best pH value was screened in detail in further experiments.

This bias held true also for the light chains, yielding better solubilisations at higher urea conditions, although the light chains of all Fab fragments were already solubilised to a satisfying amout at 8 M urea (data not shown).

Different additives were added to improve the solubilisation of the heavy chains with urea as chaotropic agent. SDS (sodium dodecyl sulfate) and SLS (sodium lauroyl sarcosinate) were tested as ionic detergents at concentrations of 0.5 and 1% (v/v) with the resolubilisation buffers, resulting in no further increase of solubilised protein. A NaCl concentration of 0.3 M, a DTT (Dithiothreitol) concentration of 0.1 M and an EDTA concentration of 20 mM were also tested separately. None of these effected a significant improvement on the solubilisation (data not shown).

Figure 15 displays the different resolubilisation results for the heavy chains at different pH values with an urea concentration of 10 M.



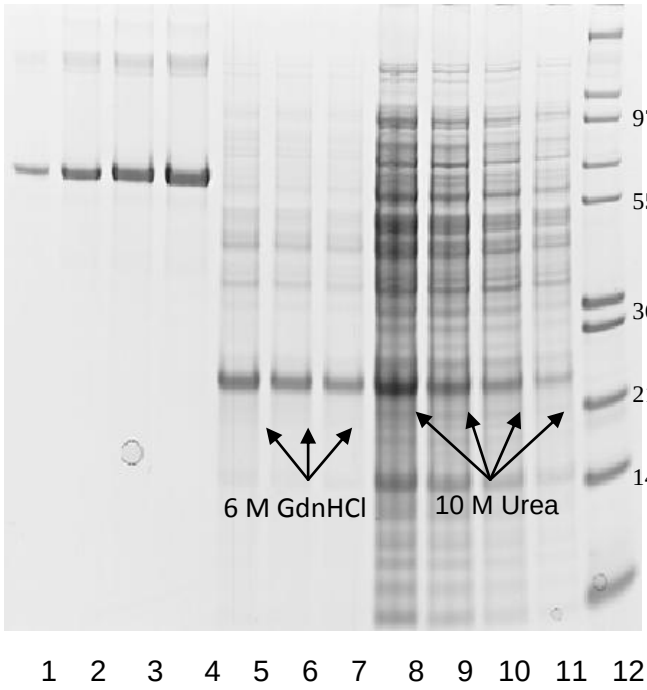
Lane	Sample	pH
1	Mark 12	-
2	B1 HC	10.5
3	B1 HC	10
4	B1 HC	9.5
5	B1 HC	8.5
6	B1 HC	7.5
7	3H6 HC	10.5
8	3H6 HC	10
9	3H6 HC	9.5
10	3H6 HC	8.5
11	3H6 HC	7.5
12-15	BSA	

Figure 15: pH screening of HC solubilisations

Resolubilisation yields increased with rising pH values so pH 10.5 turned out to be the best condition.

In comparison to urea Guanidine hydrochloride was tested at a concentration of 6M.

Figure 16 shows a comparison of the two chaotropic reagents for the solubilisation of the heavy chain of 3H6.



Lane	Sample
1-4	BSA
5	3H6 HC 1:60
6	3H6 HC 1:80
7	3H6 HC 1:100
8	3H6 HC 1:5
9	3H6 HC 1:10
10	3H6 HC 1:20
11	3H6 HC 1:50
12	Mark 12

Figure 16: Comparison of urea and GdnHCl with 3H6 HC

Resolubilisation yields using GdnHCl were much higher than when using urea, because GdnHCl dissolved much more of the heavy chain than urea. Urea facilitates as well the solubilisation of other proteins to a higher degree compared to GdnHCl. These additionally solubilised proteins may interfere with the subsequent refolding process and therefore 6 M GdnHCl was chosen for the solubilisation buffer at the beginning of the present study.

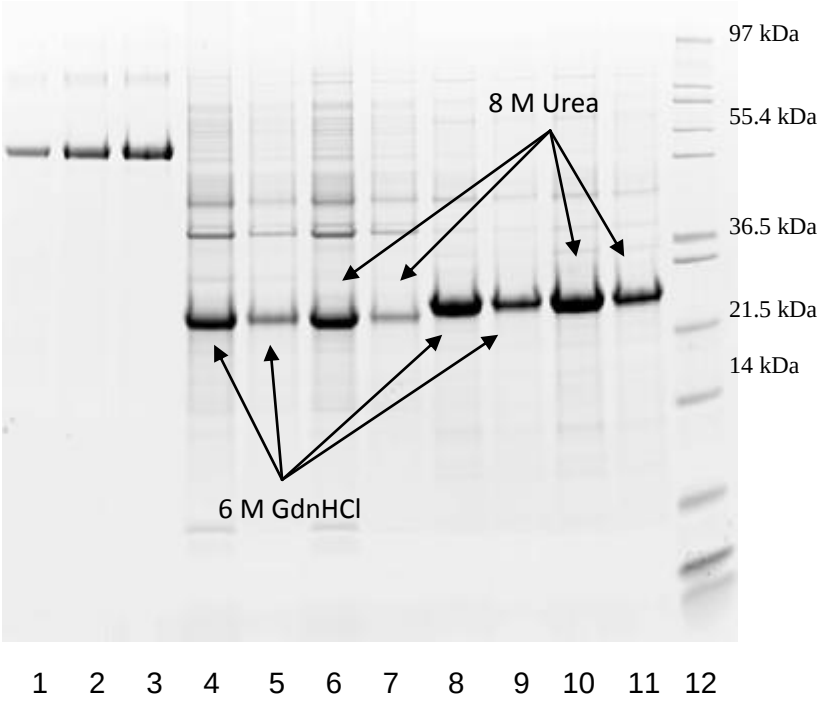
These initial resolubilisation experiments were carried out with inclusion bodies from the 1L flask fermentations. These may not have been as pure as the inclusion bodies, which were prepared from the 5L fermentation scale, so GdnHCl seemed to be the better choice for effective protein solubilisation.

Finally a better inclusion body purification procedure for 3H6 was introduced to improve the purity of the IBs. In this case a standard resolubilisation buffer containing urea instead of GdnHCl was tested and the results of urea where comparable with the buffer containing GdnHCl. The improvements concerned reduced centrifugal forces (by 1000 rpm) and centrifugal times (by 5-10 minutes). This resulted in higher purities with the drawback of

lower yields.

The buffer composition was 8 M urea, 200 mM glycine, 5 mM EDTA and had a pH value of 10.5.

Figure 17 displays a comparison of this buffer to one containing 6 M GdnHCl.



Lane	Sample
1-3	BSA
4	3H6 HC 1:20
5	3H6 HC 1:50
6	3H6 HC 1:20
7	3H6 HC 1:50
8	3H6 LC 1:30
9	3H6 LC 1:60
10	3H6 LC 1:30
11	3H6 LC 1:60
12	Mark 12

Figure 17: Urea and GdnHCl solubilisation with purer IBs

The samples in lanes 4, 5, 8, 9, contained GdnHCl and the lanes 6, 7, 10, and 11 contained urea and are highlighted in grey.

The SDS PAGE analysis results of the tested buffers were comparable and host protein solubilisation seemed to be only slightly enhanced by GdnHCl compared to urea. Hence urea was able to provide the same concentrations of solubilised protein as GdnHCl. This would provide some serious advantages, because urea is prefered over GdnHCl in large scale production as it is not corroding steel components and it does not interfere with SDS PAGE analysis. Guanidine precipitates with LDS sample buffer for electrophoresis at concentrations higher than 0.2 M. GdnHCl also enhances the conductivity in a significant way and therefore interferes heavily with any sort of ion-chromatography. This limits the possibilities for chromatographic purification and the GdnHCl would have to be removed via an diafiltration step. This would enhance costs in a considerable amount in production scale.

All resolubilisation buffers contained 20 mM DTT, which acted as a reducing agent and reduced the disulfide bridges within the inclusion bodies. The corresponding amounts of DTT were pipetted from a freshly thawed DTT stock solution (1 M DTT).
The light chains were solubilised in the same buffer as the heavy chains although they could have been solubilised with urea. However, the requirement was to find a resolubilisation buffer in which heavy and light chain could be solubilised together. So GdnHCl was the reagent of choice for the 3H6 and B1 experiments. The scope of the study was to find and optimise refolding strategies and not primarily to improve resolubilisation conditions. Therefore GdnHCl provided reliable solubilisation properties even with different qualities in HC IB preparations.

3.3.2 Refolding experiments

The aim of the refolding experiments was to find a buffer composition, which was on one hand able to keep the protein soluble and on the other hand providing the necessary circumstances to allow correct protein refolding.
Figure 18 provides an overview of a possible optimization procedure for a refolding buffer, which was also applied on my experiments with 3H6 and B1.

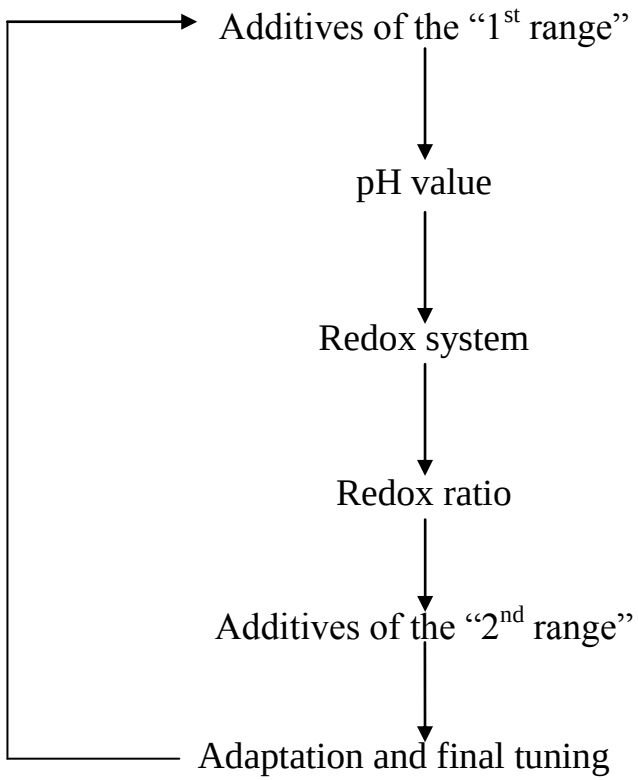


Figure 18: Optimising procedure for refolding buffers

Additives of the "1st range" are used to keep the protein soluble and to support refolding after transferring the protein from the resolubilisation buffer, where the protein is highly concentrated, into the refolding buffer.
Urea for example was also used in the refolding buffers, but at lower concentrations than in the resolubilisation buffers. Here again, its chaotropic and protein stabilizing properties were exploited.
Another important aspect was to lower the DTT concentration from the resolubilising puffer (20 mM) to a concentration below 2 mM to allow formation of disulfide bridges. L-arginine is an additional refolding agent known to facilitate protein refolding and recovery [38]. It impedes protein aggregation at low concentrations and may increase the protein solubility in combination with urea, which does not necessarily entail increased Fab formation [39] [40]. L-arginine however has the not negligible drawback to be expensive and therefore not well suited for large scale production. Other simple chemicals like sucrose or sodium chloride were also tested during the "1st range", but none of these led to a verified Fab formation.

As additives of the "2nd range" various detergents and additives were counted, which might have a positive influence on the refolding behavior of certain proteins. They can significantly reduce protein aggregation during refolding *in vitro* and stabilize proteins during the refolding process. Drawbacks are the high costs of these additives as well as potentially difficult separation from the protein in the downstream process. The concentrations for these components in the refolding buffer were set above the critical micelle concentration (CMC). At the CMC, the detergents will form micelles, creating a more hydrophobic environment, thus preventing protein aggregation and even allowing unfolding. The formation of micelles is depending on temperature and pressure. The lower the CMC is the more stable they are, which reveals the hydrophobic binding strengths of the detergent. Detergents with a higher CMC can be separated more easily via dialysis than detergents with a low CMC. This is of importance, when it comes to the removal of the detergent, which is often necessary for further downstream processing.

Table 26 shows the additives, which were categorized as additives of the "2nd range".

Table 26: Additives of the "2nd range"

Detergent	Category	Concentration [mM]
Tween 20	nonionic	0.27
Tween 40	nonionic	0.069
Tween 80	nonionic	0.03
Brij 35	nonionic	0.18
Triton X-100	nonionic	0.9
Brij 58	nonionic	0.021
SDS	anionic	24
Sodium-1-Heptanesulfonate	anionic	0.9
Zwittergent 3-14	zwitterionic	0.015
CAPS	zwitterionic	300
Sodiumdeoxychelate	anionic	15
NSDB-195	nondetergent	0.3
3-1(1-Pyridinio)-1-Propanesulfonate	nondetergent	0.3
N-Laurylsarcosine	anionic	43.8
Sodiumcaprylate	anionic	1000
N-Dodecylpyridiniumchloride	zwitterionic	45.6
CHAPS	zwitterionic	18
Tetradecytrimethylammoniumbromide	cationic	12
CTAB (Hexadecyltrimethylammoniabromide)	cationic	3
N-Dodecyl-N,N-dimethylammonio-1-propanesulfonate (SB12)	zwitterionic	9
α-Cyclodextrin	nonionic	0-100
β-Cyclodextrin	nonionic	0-100
γ-Cyclodextrin	nonionic	0-100

As redox systems the components cysteine/cystine, cysteamine/cystamine as well as reduced glutathione/oxidized glutathione were tested with 3H6 and B1. The redox system promotes

the formation of disulfide bridges, which is initiated by the oxidized form and is also called thiol-disulfide exchange [41].

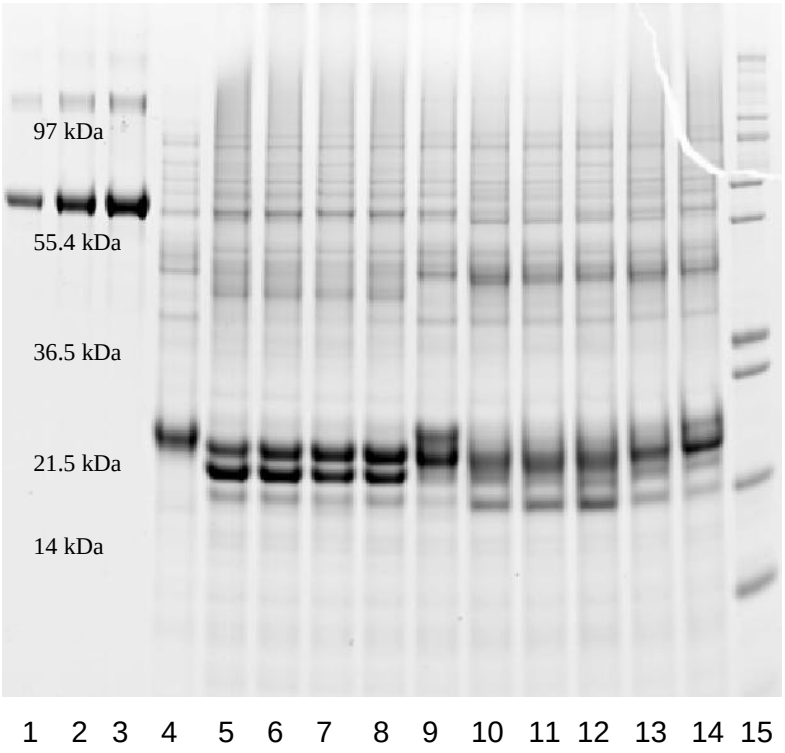
3.3.3 3H6 refolding experiments with separate light and heavy chain

Urea was tested in a range between 1 and 2 M for individual refolding for heavy and light chain.

L-arginine was the second buffer component, which was tested for refolding. The final concentration ranged from 0 to 0.7 M for both chains.

At the beginning the pH was set to 9.5 for these experiments and should be tested in detail at a later stage. Cysteine and cystine were used as redox system at a concentration of 2 mM for the heavy chain. The light chain was tested with redox ratios of 4:1-1:4 resulting in cysteine and cystine concentrations of 2 – 8 mM.

Figure 19 displays 3H6 light chain refolding experiments with varying L-arginine concentrations and varying redox ratios.



Lane	Sample	L-arginine [M]	Redox ratio
1-3	BSA	-	-
4	3H6 LC red.	-	-
5	3H6 LC	0	1:1
6	3H6 LC	0	1:2
7	3H6 LC	0	1:4
8	3H6 LC	0	2:1
9	3H6 LC	0	4:1
10	3H6 LC	0.7	1:1
11	3H6 LC	0.7	1:2
12	3H6 LC	0.7	1:4
13	3H6 LC	0.7	2:1
14	3H6 LC	0.7	4:1
15	Mark 12	-	-

Figure 19: 3H6 LC refolding with different L-arginine concentrations

The light chain could be kept soluble at low urea concentrations and the redox system did not play an important role. Figure 19 shows also, that 3 differently folded conformations of 3H6 light chains could be found via SDS PAGE. L-arginine showed a significant effect on refolding behavior of the light chain. Bands on lanes 5-9 have a higher intensity compared to lanes 10-14.

The heavy chain could not be kept sufficiently soluble by using only urea and L-arginine at pH 9.5 so different pH values (pH 8 and 10) were tested as well as different additives, which should increase solubility.

Table 27 shows the buffer composition, the additives as well as the concentrations used for 3H6 heavy chain early refolding experiments.

Table 27: Starting composition of 3H6 HC refolding buffer

Additive	Concentration [mM]
Glycine/Tris	50
Urea	2000
L-arginine	0-400
Cysteine	2
Cystine	2
α-cyclodextrin	0-100
β-cyclodextrin	0-100
γ-cyclodextrin	0-100
Zwittergent 3-14	0-5
N-lauroylsarcosine	15
Brij 35	100
CHAPS	5

Zwittergent 3-14, N-lauroylsarcosine and β-cyclodextrin led to an improved solubility of 3H6 heavy chain (data not shown).

3.3.4 3H6 refolding experiments in 1mL scale

For all experiments in 1 mL scale the buffer components were pipetted from stock solutions into a 1.5 ml Eppendorf tube and water was added until the required volume was reached. All components were vortexed and afterwards the protein resolubilisate was pipetted onto the lid. After closing the lid the tubes were vortexed immediately. The final refolding volume was set to 1 mL.

During the first refolding experiments with 3H6 heavy and light chain the pH value and the urea concentration of the refolding buffer were tested. At the pH values 7.5 and 8.5 50 mM Tris was used as buffering substance and 50 mM glycine was used for pH 9.5 and 10.5. The refolding buffers contained a concentration of 1, 2, 3, or 4 M urea and 2 mM Cysteine and 2 mM Cystine were used as redox system. Under these circumstances no Fab fragment could be found (data not shown).

At least 2 M urea was necessary to prevent substantial protein aggregation during refolding. pH 9.5 was sufficient, pH 10.5 brought no further improvement.

In the subsequent experiment different L-arginine concentrations were tested (00.5 M) and the redox ratios of cysteine and cystine was varied between 1:4 and 4:1 (data not shown)

There was still protein aggregation visible, caused by the low solubility of the heavy chain, so β-cyclodextrin (0-100 mM) or Zwittergent 3-14 (0-5 mM) were tested in the refolding buffer.

Zwittergent 3-14 showed slightly positive effects on solubility but no Fab fragment could be detected. β-cyclodextrin did not improve the results, when heavy and light chain were refolded simultaneously.

At this point other redox systems besides cysteine/cystine were tested. The redox systems cysteamine/cystamine and reduced glutathione (GSH) and oxidized glutathione (GSSG) were tested in ratios between 1:4 and 4:1. One part corresponded to a concentration of 2 mM of redox reagent.

The redox system GSH/GSSG showed a new band on the SDS PAGE gel. The band was strongest at a concentration of 2 mM for GSH and GSSG (data not shown). However, it could not be proven, that it was a correctly folded Fab fragment and therefore more experiments were planned and performed.

An effective way to accelerate protein refolding is the usage of positive metal ions. Copper can facilitate the oxidation of thiolgroups to disulfide bonds [42] and also prevents protein aggregation during the refold process [43].

Copper was tested as an additive in the range of 0.1-0.5 µM. The basis refolding buffer contained 50 mM glycine at pH 9.5, 3 M urea, 0.2 M L-arginine and 5 mM Zwittergent 3-14. After a refolding time of 48 hours no Fab fragment could be detected on SDS PAGE gel.

4°C and room temperature (22°C) were tested for 3H6 refolding in order to figure out if temperature had a significant influence on the refolding behavior of the Fab fragment. It turned out that no improvement could be detected after refolding at 4°C for 48 h compared to 22° C. Therefore room temperature remained the general refolding temperature.

3.3.5 3H6 refolding in 10-250 mL scale

Larger refolding experiments were carried out to investigate the refolding behavior beside 1 mL Eppendorf tubes experiments and to generate correctly folded Fab fragments. It was intended to purify Fab fragments using different affinity chromatography steps afterwards.The first refolding experiment in 10 mL scale was carried out with 3H6 LC. 1 mL scale experiments showed different intermediates of the LC. For this reason the first 10 mL refoldings with the LC were used to confirm this. Refolding conditions were 50 mM glycine pH 9.5, 1 M Urea, 0.7 M Arginin and a redox ratio of cysteine/cystine of 1:4. The refolding time was 48 h and refolding temperature was 22°C. The refolding volume was diluted 1:2 with equilibration buffer to lower the pH and to facilitate binding to the Kappa select column. Before loading onto the column, the refolding volume was filtered with a 0.22 µm filter. Elution was performed by applying a pH shift from pH 7.4 to pH 2.8. In order to increase the pH value immediately after elution 2 M Tris buffer with a pH of 7.5 was provided in each fractionation tube. The volume was 5 % of the total elution volume and this should prevent protein denaturation by acidic conditions. Analysis was done with SDS PAGE. Process parameters and results are displayed in Figures 20-22 and Table 28.

Table 28: 3H6 chromatography with Kappa select

Resin	Kappa select	
Column volume [mL]	1.12	
Step	**Buffer**	**Volume [CV]**
Equilibration	50 mM NaH_2PO_4, 150 mM NaCl pH 7.4	5
Load	Filtrated refolding volume	14.3
Wash	50 mM NaH_2PO_4, 150 mM NaCl pH 7.4	5
Elution	50 mM Glycine pH 2.5	3
Regeneration	100 mM GdnHCl pH 2.5	3
Neutralisation	dH_2O	2
Storage	20% Ethanol	3

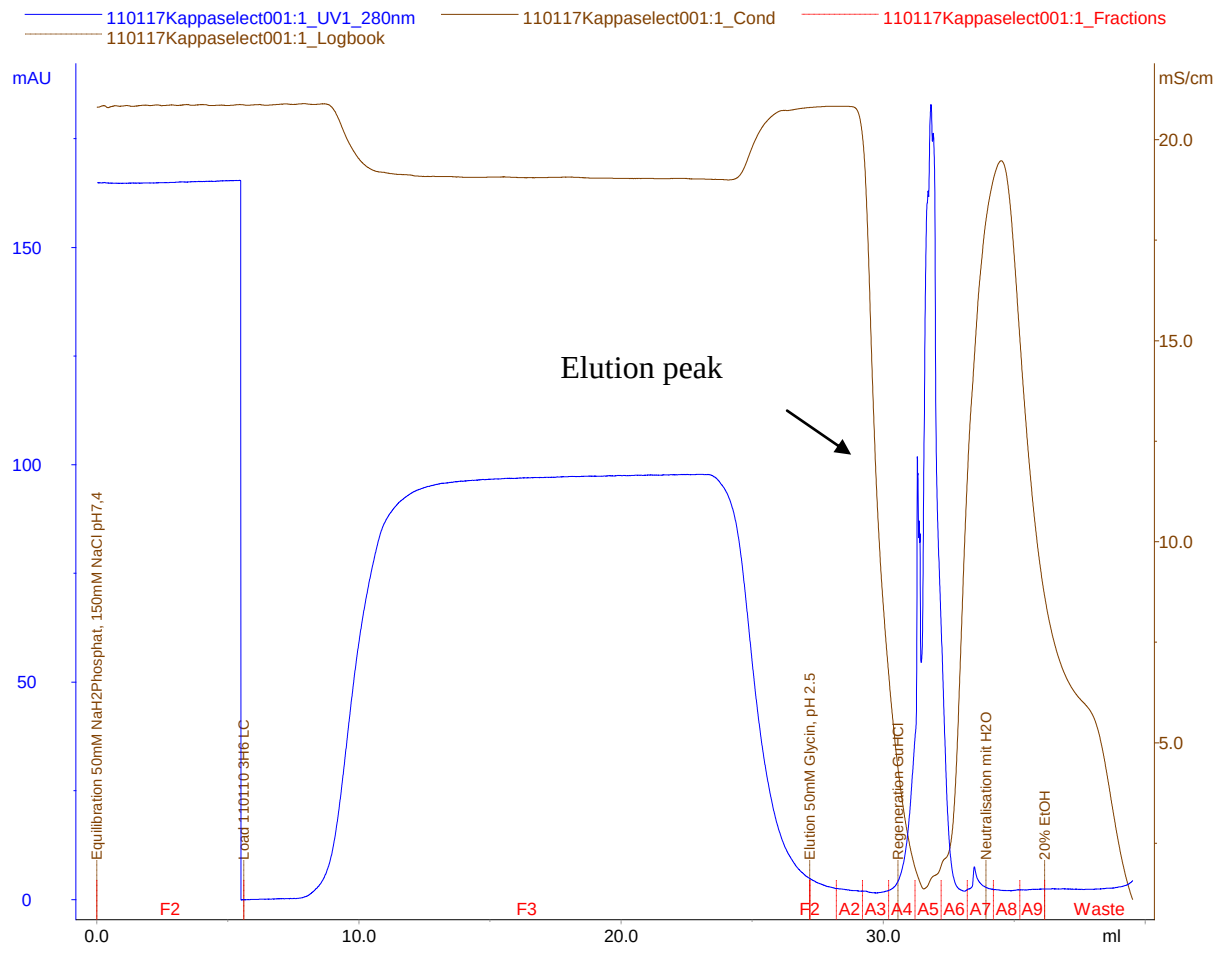


Figure 20: Chromatogram of 3H6 LC refolding with Kappa select

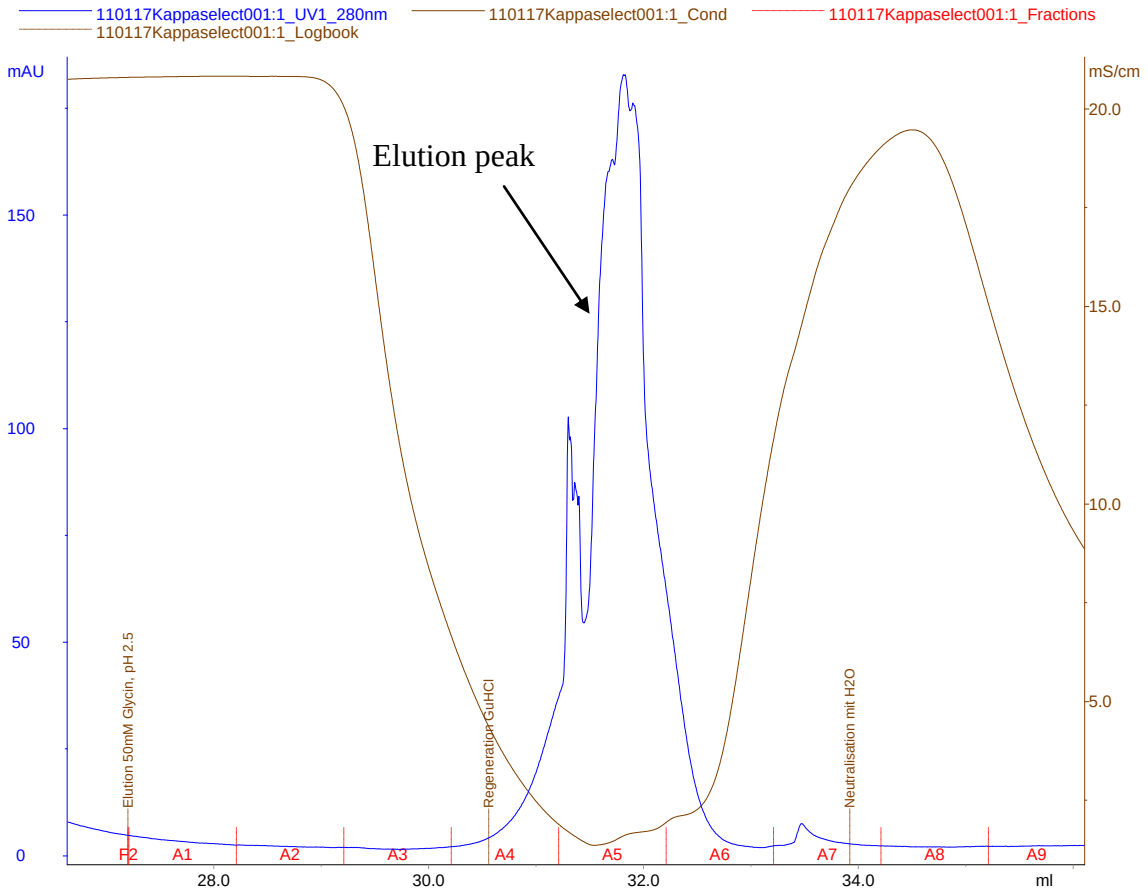
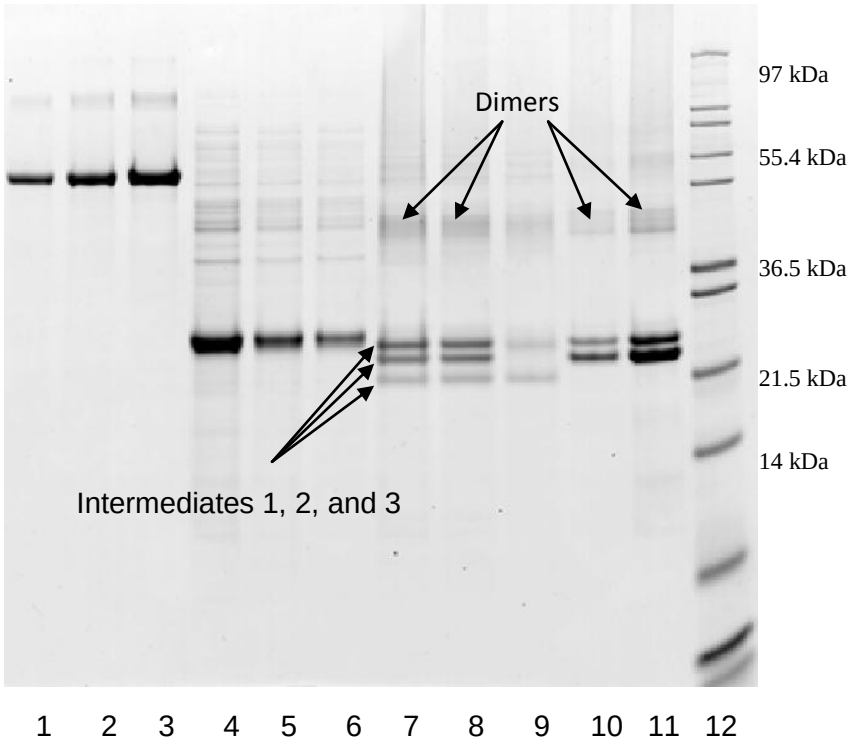


Figure 21: Elution peak of 3H6 LC refolding with Kappa select



Lane	Sample
1-3	BSA
4 -6	3H6 LC red.
7	Refolding volume
8	Load
9	FT
10	A5
11	A6
12	Mark 12

Figure 22: SDS-PAGE gel of 3H6 after Kappa select

On the SDS PAGE gel three differently folded intermediates (I1, I2 and I3 respectively) of 3H6 light chain were observed. Intermediate 2 had bound completely to the resin, together with parts of intermediate 1. Intermediate 3 did not bind to the column, which could only be found in the flowthrough fraction together with the rest of unbound intermediate 1. Gel bands 10 and 11 show successful elution of the light chain intermediates 1 and 2 by the pH shift. It can also be observed, that possible LC dimers are formed during the refolding. The size of the band on the SDS gel correlates with the size of LC dimers with about 47 kDa and they also bind to the affinity resin and can be eluted. After all it was proven, that it was possible to refold 3H6 light chain and to assure binding to a Kappa select resin as well as controlled elution.

In order to clarify, whether the problem lies at the refolding step or the purification step of the HC, Protein G was used as an additional affinity resin. If the HC was correctly folded it should bind to the Protein G resin.In this experiment the 3H6 Fab fragment should be refolded under the following conditions in a 10 mL scale: 3 M Urea, 50 mM glycine, 0.2 M L-arginine, 5 mM Zwittergent, 2 mM reduced glutathione and 2 mM oxidised glutathione, pH 9.5, 0.22 mg/mL HC/LC protein concentration, 22°C, 24 hours. Hereafter the refolding volume was diluted with equilibration buffer at a ratio of 1:2 and filtrated with a 0.22 µm filter. Elution was performed by applying a pH shift from pH 7.4 to pH 2.8. In order to increase the pH value immediately after elution 2 M Tris buffer with a pH of 7.5 was provided in each fractionation tube. The volume was 5% of the total elution volume and SDS-PAGE was used for analysis. The process parameter and results are displayed in figures 23-24 and table 29.

Table 29: Protein G chromatography with 3H6

Resin	Protein G	
Column volume [mL]	1.57	
Step	**Buffer**	**Volume [CV]**
Equilibration	50mM NaH_2PO_4, 50 mM NaCl pH 7.4	1
Load	Filtrated refolding volume	10.8
Wash	50mM NaH_2PO_4, 150 mM NaCl pH 7.4	5
Elution	50 mM Glycine pH 2.5	8
Regeneration	100 mM GdnHCl pH 2.5	3
Neutralisation	dH_2O	2
Storage	20% Ethanol	3

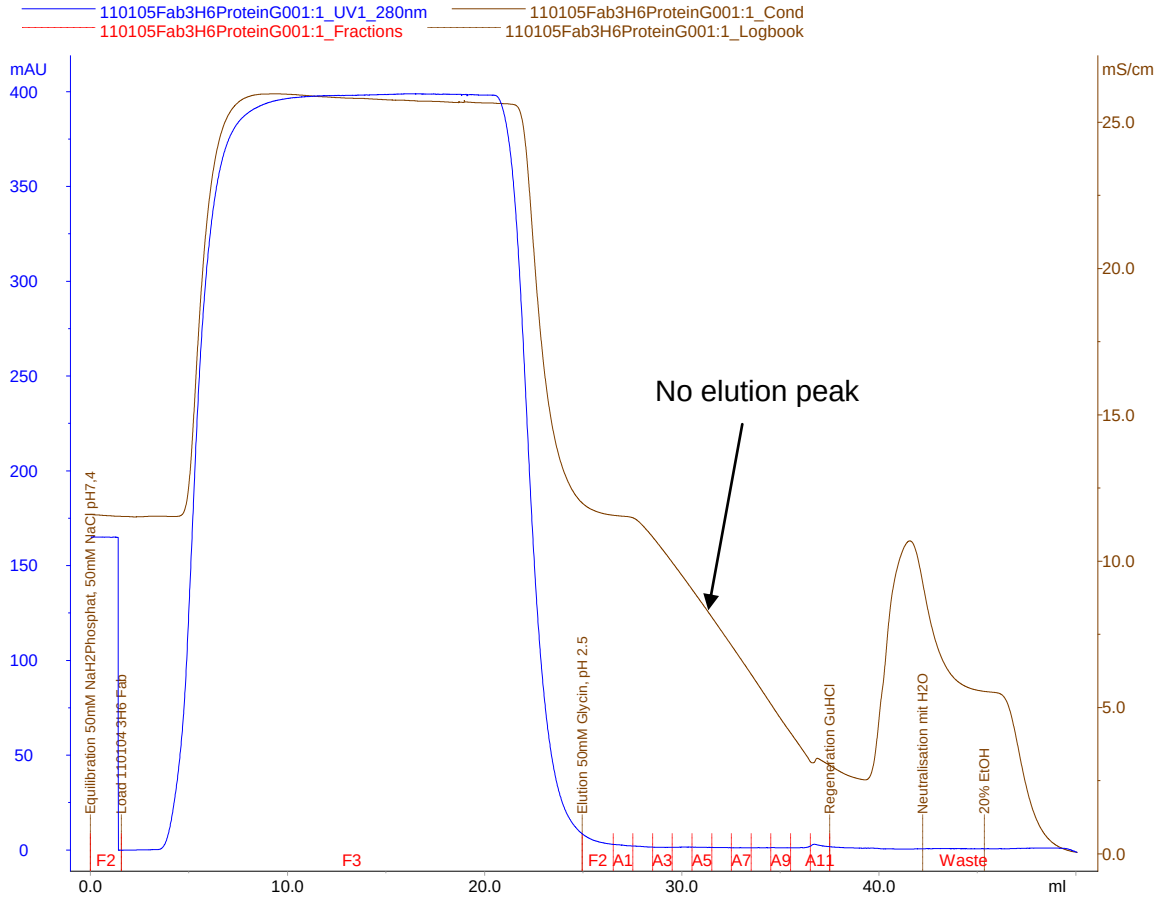
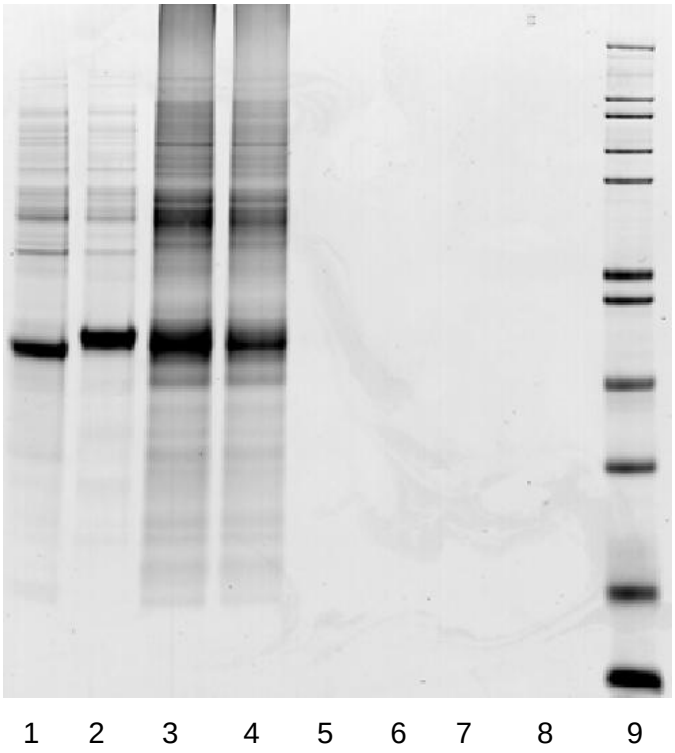


Figure 23: Chromatogram of 3H6 with Protein G



Lane	Sample
1	3H6 HC red.
2	3H6 LC red.
3	Load
4	FT
5	A2
6	A4
7	A6
8	A8
9	Mark 12

Figure 24: SDS-PAGE results of 3H6 after Protein G

The results show that no Fab fragment has bound to the Protein G column. As the heavy chain is supposed to bind to a Protein G resin it can be deduced, that the heavy chain was not folded correctly. Therefore the heavy chain could not form disulphide bridges with the light chain and the formation of the Fab fragment was impeded. Lane 1 and lane 2 in the SDS-PAGE analysis show, that heavy and light chain were weighed correctly and no mistake was made by pipetting them together. Lane 3 shows the column load sample, already diluted with elution buffer and lane 4 displays the FT. Four fractions from the elution step were singled out and analysed with SDS-PAGE. No protein could be detected, which can also be deduced by the not increasing UV line during the elution step on the chromatogram.

It was known from previous experiments, that the Kappa select resin has the capability to bind the light chain of the 3H6 Fab fragment. In theory a correctly refolded Fab fragment should also be purified via its light chain. A refolding volume of 100 ml was set up in a beaker on the following terms: 3 M urea, 50 mM glycine, 0.2 M L-arginine, 5 mM Zwittergent, 2 mM reduced glutathione and 2 mM oxidised glutathione and the pH value was set to 9.5. The protein concentration of the heavy chain was 0.34 mg/mL and of the light chain it was 0.43 mg/mL. So the limiting factor for the protein yield was the concentration of the heavy chain, allowing a maximum Fab fragment concentration of 0.34 mg/mL, if the yield would have been 100 %. The aim of this experiment was to determine, whether long refolding time had a significant influence on the refolding behaviour of the 3H6 Fab fragment. After 40 hours, 50 mL of the total refolding volume of 100 mL were loaded onto a Kappa select resin, eluted and analysed via SDS-PAGE. The other 50 mL were let to refold for another 25 hours, resulting in a total refolding time of 65 hours, purified with the Kappa select column and also analysed via SDS-PAGE. Before loading onto the column the refolding samples were diluted with equilibration buffer in a volume of 1:2 and filtrated with a 0.22 µm filter.

Table 30 shows the chromatography results for the 40 and 65 hours refolding experiments with 3H6.

Table 30: Chromatography of 3H6 with Kappa select after 40 and 65 hours

Resin	Kappa select	
Column volume [mL]	4.71	
Step	**Buffer**	**Volume [CV]**
Equilibration	50mM NaH_2PO_4, 150 mM NaCl pH 7.4	2
Load	Filtrated refolding volume	18
Wash	50mM NaH_2PO_4, 150 mM NaCl pH 7.4	5
Elution	50 mM Glycine pH 2.5	3
Regeneration	100 mM GdnHCl pH 2.5	3
Neutralisation	dH_2O	2
Storage	20% Ethanol	3

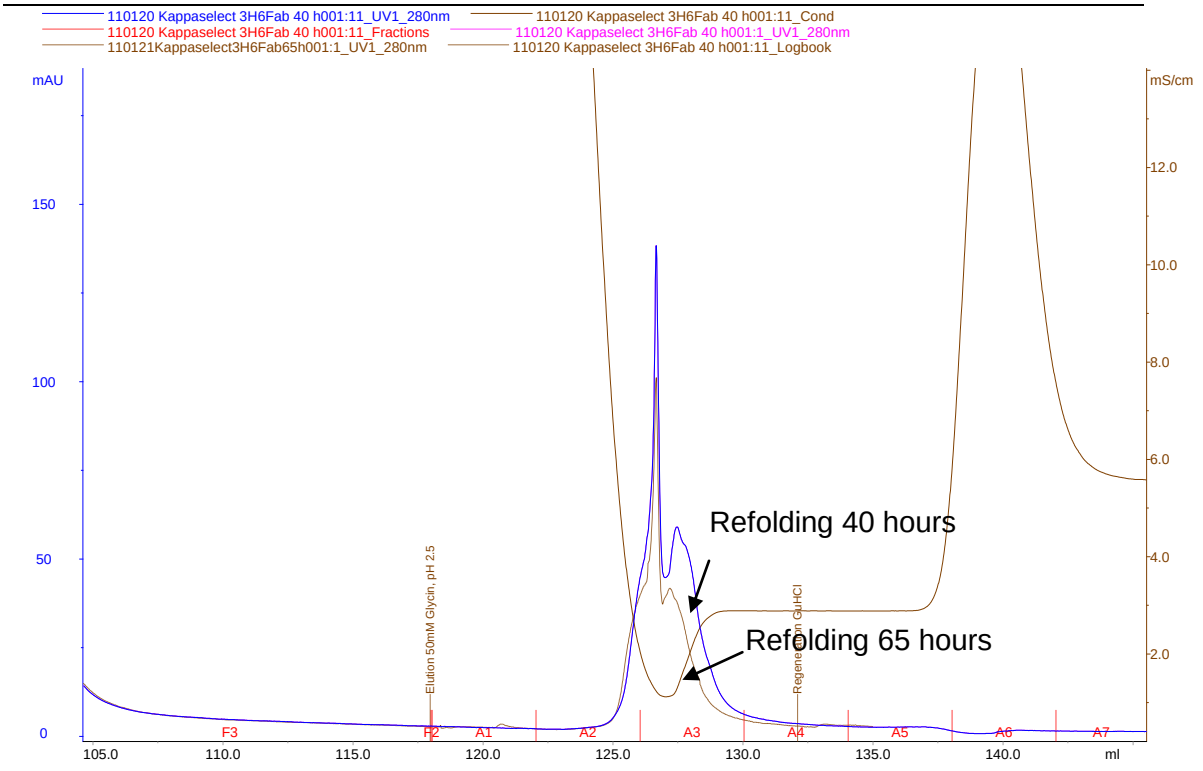


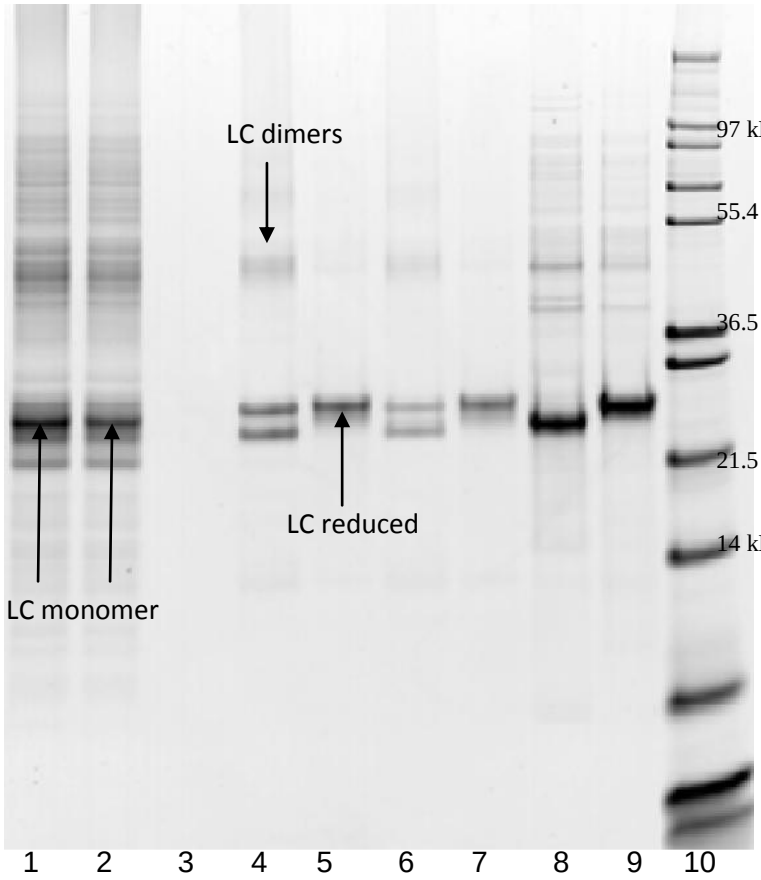
Figure 25: Chromatogram of 3H6 with Kappa select after 40 and 65 hours

Figure 25 shows the UV absorption graphs of the chromatography runs with 40 hours and 65 hours refolding time. The load volume was identical for both runs with 84 mL. Elution was performed by applying a pH shift from pH 7.4 to pH 2.8. In order to increase the pH value immediately after elution Tris buffer with a pH of 7.5 was provided in each fractionation tube. The volume was 5% of the total elution volume.

The UV profiles are nearly identical although the flowthrough UV-signal of the 65 hours refolding was slightly higher compared to the 40 hours refolding (data not shown). The

elution peaks have almost identical shape and the retention times on the column are also equal.

The results of the SDS-PAGE analysis with the 65 hours samples are provided in figure 26.



Lane	Sample
1	Load
2	FT
3	A4
4	A5
5	A5 red.
6	A6
7	A6 red.
8	3H6 HC red.
9	3H6 LC red
10	Mark 12

Figure 26: SDS-PAGE results of 3H6 after 40 and 65 hours

The most distinctive band in lane 1 is the LC monomer, which diminishes in the flowthrough, because it binds to the Kappa select resin. Lane 4 shows the first elution fraction containing protein. At the size of about 23 kDa two differently folded LC monomer configurations were detected. At about 46 kDa the LC dimers was observed. The proteins of each elution fraction were reduced and also analysed on the SDS gel. After reduction only LC monomer band was observed (lane 4), which leads to the assumption that only LC dimers are formed during the refolding process and no Fab fragment. The same can be said for lanes 5 and 6. The differently folded bands in lane 5 generate one single LC monomer band after treatment with β-mercaptoethanol, which can be seen in lane 6. As the heavy chain alone does not bind to the Kappa select column during chromatography, the band cannot be seen on the SDS gel. As reference the HC and LC solubilisate was applied on the gel (lane 7 and 8). The SDS-PAGE

data for the 40 hours refolding nearly equals the one of the 65 hours refolding process and is not shown here.
Summing up it can be said, that elongation of the refolding time from 40 hours to 65 hours brought no detectable formation of the 3H6 Fab fragment. The formation of LC dimers and multimers seems to be preferred to the formation of the Fab fragment, but they can be reduced to monomers by a simple reduction step. The chromatogram of both experiments are nearly identical, as displayed in figure 25, showing that long refolding times will not trigger Fab fragment formation with 3H6.

In order to be able to detect even small amounts of correctly refolded 3H6 Fab fragment, an ultrafiltration step was tested to increase the protein concentration of the elution fractions before the analysis with SDS-PAGE was performed. The refolding volume was 50 mL with the following conditions: 3 M urea, 50 mM glycine, 0.2 M L-arginine, 5 mM Zwittergent, 2 mM reduced glutathione and 2 mM oxidised glutathione. The pH value was set to 9.5 and the refolding time was 68 hours. The protein concentration was 0.23 mg/mL for heavy and light chain of 3H6. Before loading 85 mL onto the column the refolding volumes were diluted with equilibration buffer in a ratio of 1:2 and filtrated with a 0.22 µm filter. The chromatography conditions were the same as used for earlier Kappa select chromatography runs. In order to increase the pH value immediately after elution 2 M Tris buffer with a pH of 7.5 was provided in each fractionation tube. The volume was 5 % of the total elution volume.

The results can be seen in figure 27.

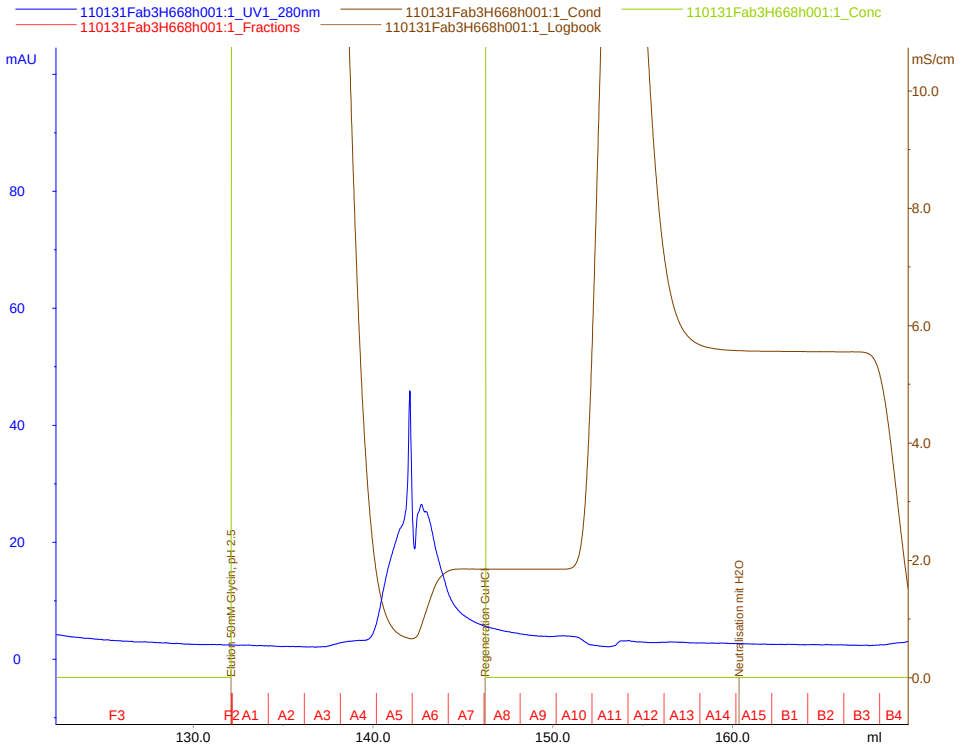
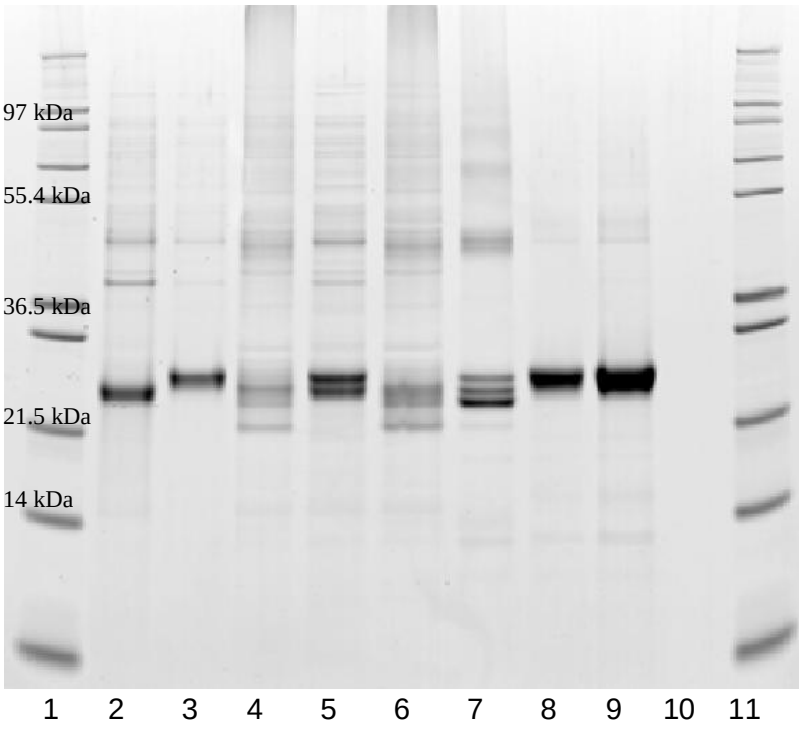


Figure 27: Elution peak of 3H6 with Kappa select after UF step

The elution fractions A5-A7 with a total volume of 2 mL were concentrated by using a spin column with a 5 kDa cut off membrane to a factor of 10.

The results of the SDS-PAGE analysis are shown in figure 29.



Lane	Sample
1	Mark 12
2	3H6 HC red.
3	3H6 LC red.
4	Load
5	Load red.
6	FT
7	Retentate
8	Retentate red. 1:4
9	Retentate red. 1:2
10	Permeat
11	Mark 12

Figure 28: SDS-PAGE results after the UF step

The ultrafiltration step brought no significant improvement for the SDS-PAGE analysis. Lane 5 shows that under reduced conditions both heavy and light chain are found in the same concentrations in the refolding volume as it was intended for the experiments. The difference between lane 4 (Load) and lane 6 (flowthrough) is not significant on the SDS gel because only parts of the light chain bind to the Kappa select resin and not correctly folded protein usually not binds. Lane 7 shows the three differently folded light chain conformation at about the size of 23 kDa as well as LC dimers and LC multimers. Reduction and application on the gel leads again to a single LC monomer band at the size of 23 kDa without a band from the HC. This confirms, that only the LC binds to the column and that the band at the size where the Fab fragment should be is made up of LC dimers and not the Fab fragment itself. The cut off size of the membrane for the ultrafiltration was small enough and able to hold back all the protein. This is proven by the fact that in lane 10 no bands can be seen, because here the permeate of the ultrafiltration step was applied on the gel.

As an additional affinity resin Mabselect Xtra was tested to capture 3H6 Fab fragments. For this experiment a refolding experiment with a volume of 250 mL was set up with the following conditions: 3 M urea, 50 mM glycine, 0.2 M L-arginine, 5 mM Zwittergent, 2 mM reduced glutathione and 2 mM oxidised glutathione. The pH value was set to 9.5 and the refolding time was 96 hours at a refolding temperature of 4°C. Before loading onto the Mabselect Xtra column the refolding volume was diluted in a ratio of 1:2 with equilibration buffer and filtrated using a filter with a pore size of 0.22 µm. In order to increase the pH value immediately after elution 2 M Tris buffer with a pH of 7.5 was provided in each fractionation tube. The volume was 5% of the total elution volume.

Table 31 and figure 29 depict the chromatography data of 3H6 with Mabselect Xtra.

Table 31: Chromatography data of 3H6 with Mabselect Xtra

Resin	Mabselect Xtra	
Column volume	4.08 mL	
Step	**Buffer**	**Volume (CV)**
Equilibration	50mM NaH_2PO_4, 150 mM NaCl pH 7.4	5
Load	Filtrated refolding volume	110
Wash	50mM NaH_2PO_4, 150 mM NaCl pH 7.4	5
Elution	50 mM Glycine pH 2.5	4
Regeneration	100 mM GdnHCl pH 2.5	3
Neutralisation	dH_2O	4
Storage	20% Ethanol	3

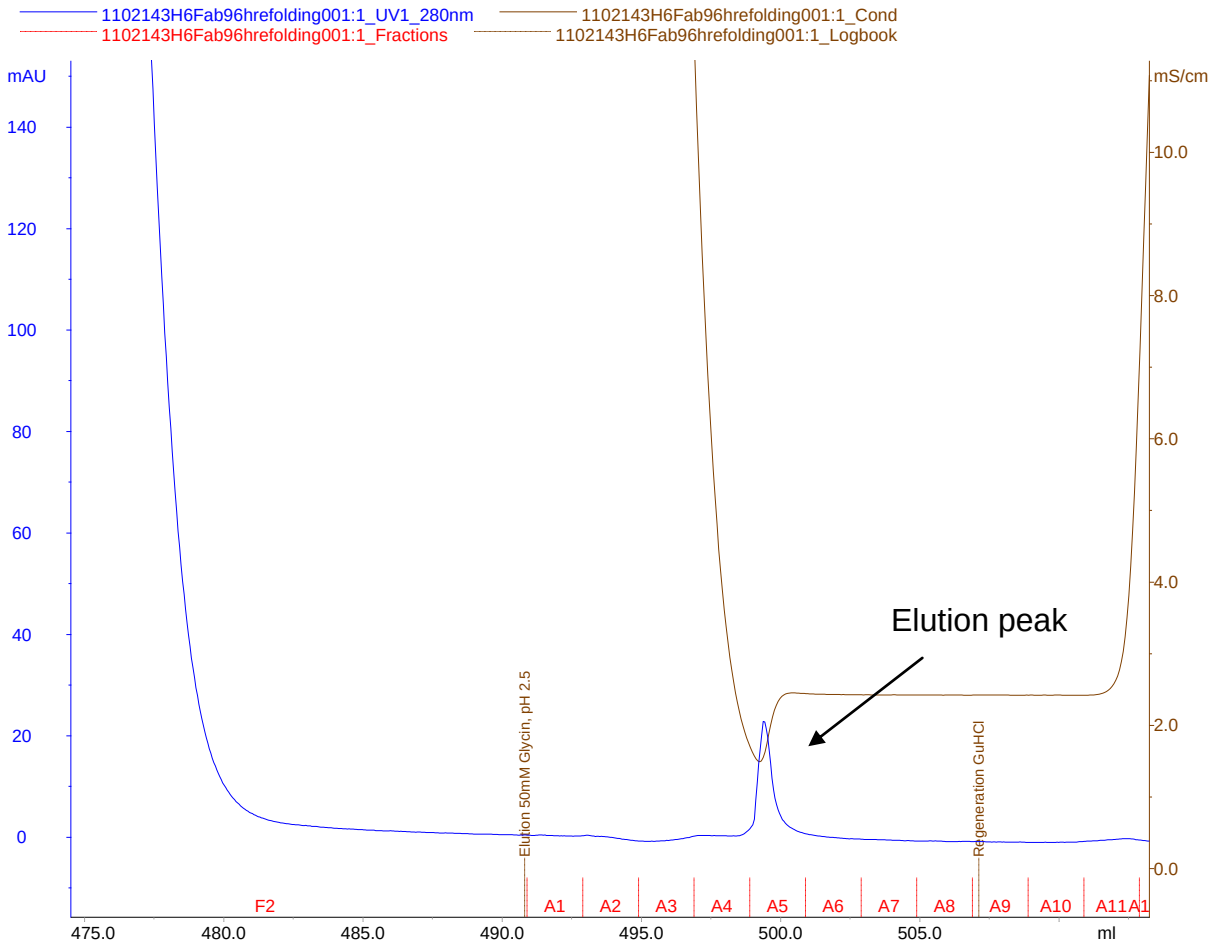
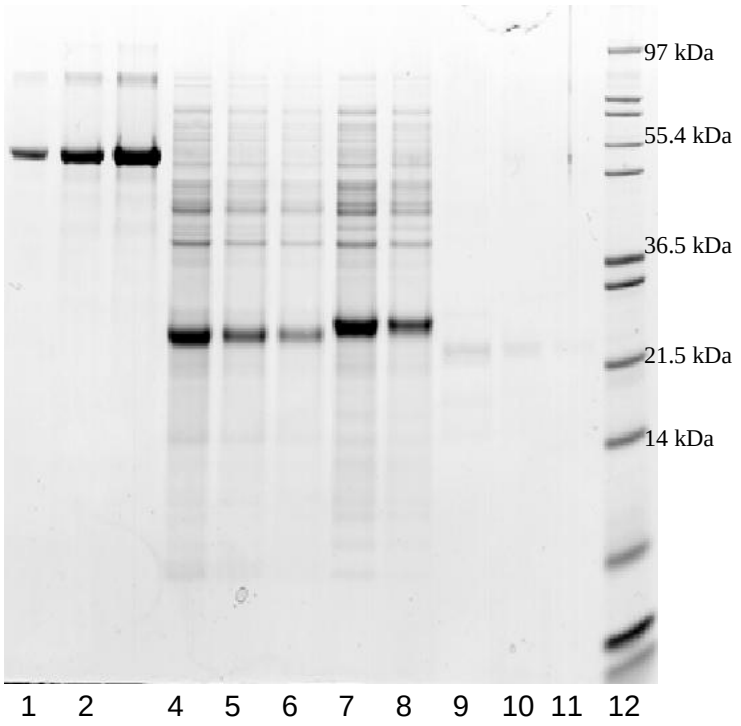
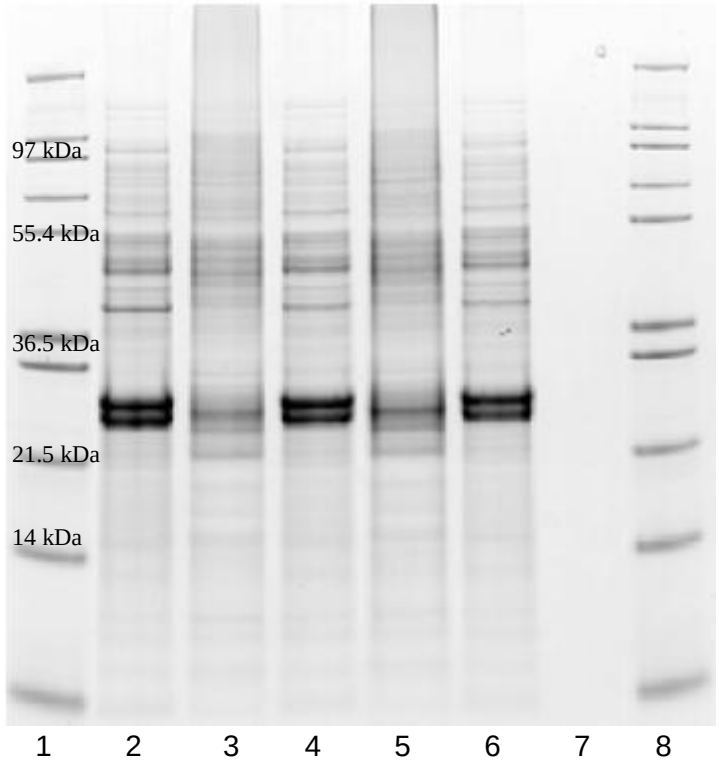


Figure 29: Chromatogram of 3H6 with Mabselect Xtra



Lane	Sample
1-3	BSA
4-6	3H6 HC red.
7-8	3H6 LC red.
9	A5
10	A5 red. 1:2
11	A5 red. 1:4
12	Mark 12

Figure 30: SDS-PAGE results of 3H6 after MabselectXtra 1/2



Lane	Sample
1	Mark 12
2	Refolding volume red.
3	Load
4	Load red.
5	FT
6	FT red.
7	Permeate
8	Mark 12

Figure 31: SDS-PAGE results of 3H6 after MabselectXtra 2/2

Figures 30 and 31 show different concentrations of the HC and LC solubilisates as well as the elution peak fraction A5. The elution fraction A5 was concentrated by a factor of four in a spin tube with a membrane containing a cut off pore size of 5kDa before analysis with SDS-PAGE. No correctly folded Fab fragment can be found in this fraction. It shows only the

faintest bands, which are possible HC monomers, but they are not strong enough to postulate a positive binding ability of 3H6 HC to the Mabselect Xtra resin. Reduction of load and flowthrough results in the expected monomers of HC and LC. The higher molecular bands are probably host cell proteins and are an indication, that the used inclusion bodies should undergo more extensive washing procedures to receive purer protein for further refolding experiments. On Lane 7 the permeate of the ultrafiltration step was applied and shows that the cut off of 5kDa was enough to retain what little protein was in the elution fraction A5. It was not possible to purify the 3H6 Fab fragment with the Mabselect Xtra resin, due to little or no correctly refolded heavy chain, which cannot bind to the resin under these circumstances.

As fourth affinity resin the Capture select resin from BAC was tested for the purification of the 3H6 Fab fragment. As mentioned before in the results of the small scale experiments, N-laurylsarcosine showed some positive effects on increasing the solubility of the 3H6 heavy chain. Due to this fact it was also used in larger scale experiments.

The refolding conditions for this experiment in a 50 mL scale were 3 M urea, 50 mM glycine, 0.2 M L-arginine, 14.6 mM N-laurylsarcosine, 2 mM reduced glutathione and 2 mM oxidised glutathione. The pH value was set to 9.5 and the refolding time was 77 hours at a refolding temperature of 22°C. Before loading onto the Capture select column the refolding volume was diluted in a ratio of 1:2 with equilibration buffer and filtrated with a filter with a pore size of 0.22 µm. In order to increase the pH value immediately after elution Tris buffer with a pH of 7.5 was provided in each fractionation tube. The volume was 5 % of the total elution volume.

Table 32 shows the chromatography date for 3H6 with Capture select.

Table 32: Chromatography data for 3H6 with Capture select

Resin	Capture select	
Column volume	1.5 mL	
Step	**Buffer**	**Volume (CV)**
Equilibration	50mM NaH_2PO_4, 150 mM NaCl pH 7.4	5
Load	Filtrated refolding Ansatz	53
Wash	50mM NaH_2PO_4, 150 mM NaCl pH 7.4	5
Elution	50 mM Glycine pH 2.5	3
Regeneration	100 mM GdnHCl pH 2.5	4
Neutralisation	dH_2O	4
Storage	20% Ethanol	5

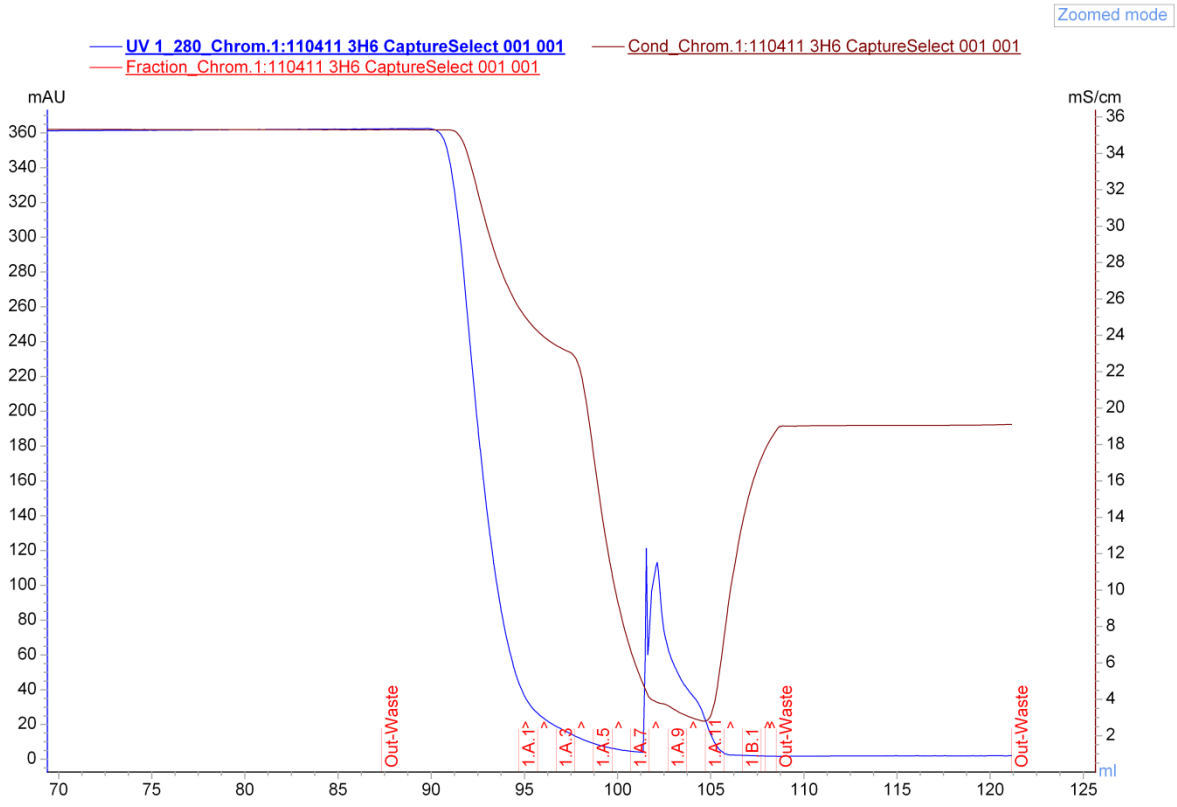
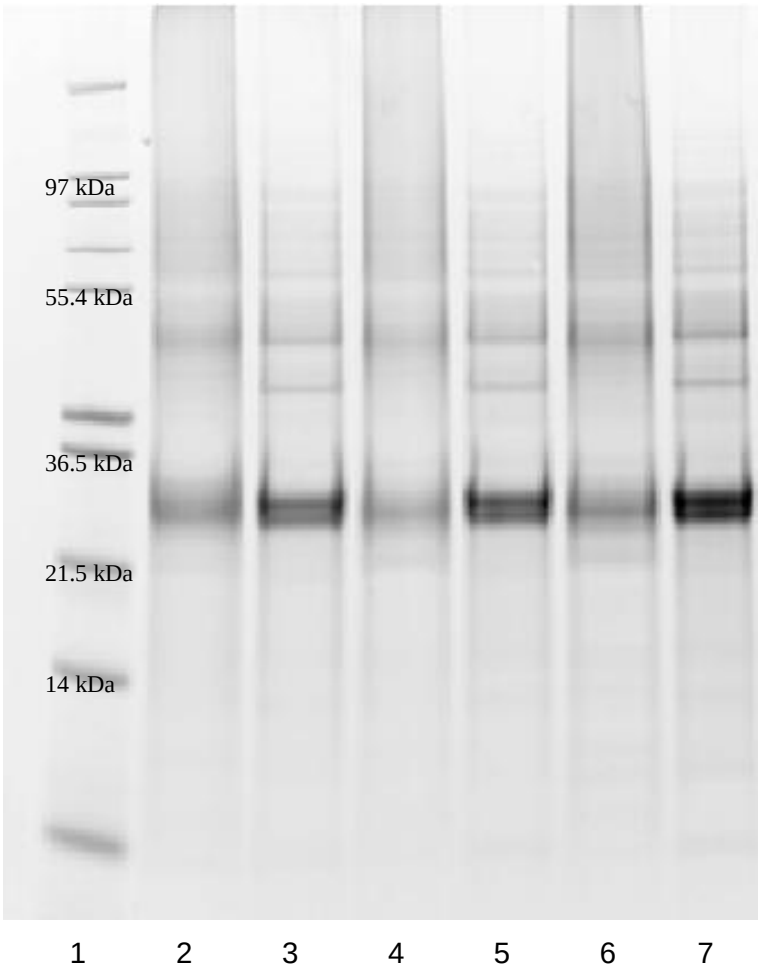
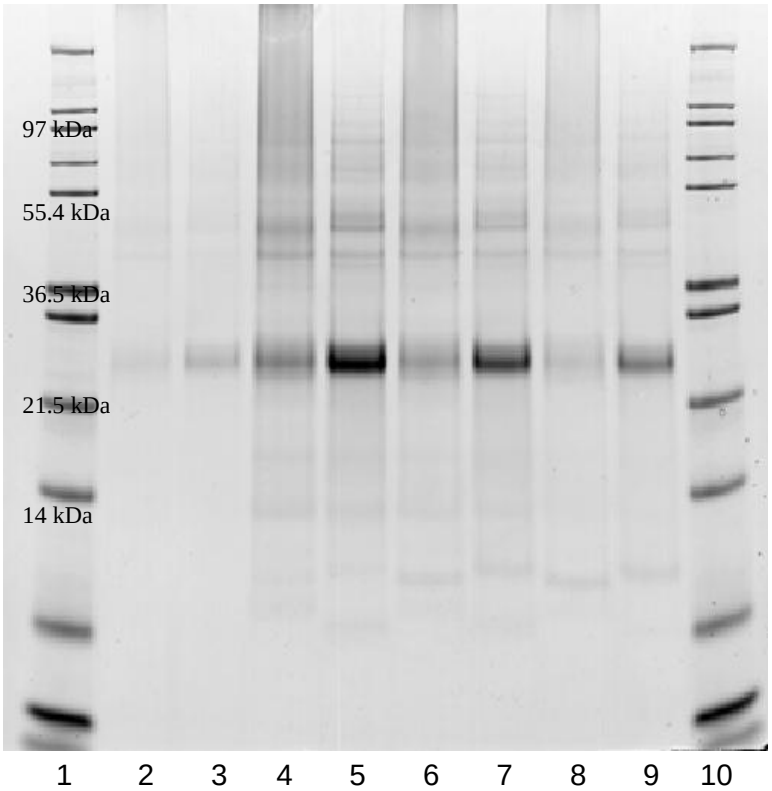


Figure 32: Chromatogram of 3H6 with Capture select



Lane	Sample
1	Mark 12
2	Refolding volume
3	Refolding volume red.
4	Load
5	Load red.
6	FT
7	FT red.

Figure 33: SDS-PAGE result of 3H6 after Capture select 1/2



Lane	Sample
1	Mark 12
2	A7
3	A7 red.
4	A8
5	A8 red.
6	A9
7	A9 red.
8	A10
9	A 10 red.
10	Mark 12

Figure 34: SDS-PAGE result of 3H6 after Capture select 2/2

Figures 32-34 show the results of 3H6 after the Capture select chromatography. Under reduced conditions the monomers of 3H6 and LC can be detected easily (lanes 3, 5 and 7 figure 33). In lanes 2, 4 and 6 at the molecular size of the Fab fragment host cell bands can be seen but no Fab fragment in particular. These host cell bands are still at the same position under reduced SDS-PAGE conditions, which would be different if the bands would have been the Fab fragment.

The analysis of the elution fractions shows, that the heavy chain of 3H6 is found in every fraction as the Capture select resins binds to the C_H1 domain. Reduction (lanes 3, 5, 7 and 9) show the reduced HC monomer with the expected size. Lanes 2, 4, 6 and 8 show protein aggregation, causing the smear at the higher molecular weight regions and partly refolded 3H6 HC at the size of about 24 kDa. The Fab fragment is not found in clearly detectable amounts so the bands at the size of the Fab fragment are either host cell proteins or agglomerates of the HC.
It could be shown, that the Capture select resin binds to the HC of 3H6 and therefore is theoretically suited to purify 3H6 Fab fragments. The bigger issue seems to be protein aggregation during the refolding process, because no Fab fragment formation can be detected and the single monomers cannot be seen until reduction after elution.

Table 33 provides an overview over the tested affinity resins for the purification of the 3H6 Fab fragment and the binding capabilities for heavy of light chain.

Table 33: Binding capabilities of affinity resins tested with 3H6

	Protein G	Mabselect Xtra	Captureselect	Kappa select
Fab fragment	-	-	-	-
HC	-	+	+	-
LC	-	-	-	+

3.3.6 B1 refolding experiments in 1mL scale

Due to time restrains the experiments with B1 were limited to 1 mL batch experiments allowing only basic screening conditions to be tested.

During the first screening urea concentrations between 1 and 4 M as well as pH values of 7.5, 8.5, 9.5 and 10 were tested. Tris buffer was used for pH 7.5 and 8.5 and glycine was used as buffer for the pH values 9.5 and 10 respectively. 2 mM cysteine and 2 mM cystine were used as standard redox system for all refolding experiments within this first basic screening. Refolding time was 48 hours at room temperature.
It turned out, that a high pH value of 10 and at least 2 M urea were needed to keep the heavy chain partly soluble, but no Fab fragment could be detected. (data not shown)

During the second screening basic refolding additives were tested which are listed in table 34 together with the tested concentrations.

Table 34: Concentrations of the second refolding screening with B1

Additive	Concentration [mM]
L-arginine	100-500
Zwittergent	2-10
α-cyclodextrin	20-100
β-cyclodextrin	20-100
γ-cyclodextrin	20-100

The urea concentration was set to 2 M and the pH value was 10. 2 mM cysteine and 2 mM cystine served again as redox system and the refolding time was 48 hours at room temperature.
L-arginine showed improvements concerning the protein solubility, whereas Zwittergent did not influence the solubility in a positive way. Protein aggregation was still predominant and therefore the Fab fragment formation was not possible. α-cyclodextrin and β-cyclodextrin affected the protein solubility in a positive way, more than γ-cyclodextrin although not as good as L-arginine. (Data not shown)
A wider screening for common detergents was conducted like it had been done with 3H6.

Table 35 shows the used detergents for B1 refolding experiments.

Table 35: Detergents for B1 refolding experiments

Detergent	Category	Concentration [mM]
Tween 20	nonionic	0.27
Tween 40	nonionic	0.069
Tween 80	nonionic	0.03
Brij 35	nonionic	0.18
Triton X-100	nonionic	0.9
Brij 58	nonionic	0.021
SDS	anionic	24
Sodium-1-Heptanesulfonate	anionic	0.9
Sucrose	nonionic	300
NSDB-195	nondetergent	0.3
3-1(1-Pyridinio)-1-Propanesulfonate	nondetergent	0.3
N-Lauroylsarcosine	anionic	43.8
Sodiumcaprylate	anionic	1000
N-Dodecylpyridiniumchloride	zwitterionic	45.6
CHAPS	zwitterionic	18
Tetradecytrimethylammoniumbromide	cationic	12
CTAB (Hexadecyltrimethylammoniabromide)	cationic	3
N-Dodecyl-N,N-dimethylammonio-1-propanesulfonate (SB12)	zwitterionic	9

In general it can be said, that nonionic detergents like Brij or CHAPS proved to be enhancing the solubility of the proteins. The other additives had no positive influence on the refolding behaviour or the solubility of the single chains. The tendency to form aggregated protein intermediates of the heavy chain seems to be more likely with the HC of B1 than with the HC of 3H6.

3.4 Results of A4 1mL refolding experiments with the Tecan Freedom EVO® 150 robot

A further aim of this study was to test and evaluate a robot based screening system for Fab refolding. In addition results from manually pipetted refoldings should be compared with the automatically prepared refoldings. Fab A4 was used for these experiments, due to the positive refolding results in the work of Gerald Bieder, with the following buffer: 1 M urea, 0.1 M L-arginine, 40 mM α-cyclodextrin, 1 mM cysteine, 3mM cystine and 50 mM glycine at pH 9.5 [50].

A previously established screening model (shown in figure 36), which can be used for up to 24 different refolding experiments on a single 24 well plate, served as a basis. Using this model one can test up to six different buffers, four different redox systems, two different protein concentrations and/or two optional refolding agents.

The programme for the robot divides the 24 well plate into 4 rows (A-D) and 6 columns (1-6). Figure 35 shows the refolding agent distribution possibilities on a 24 well plate.

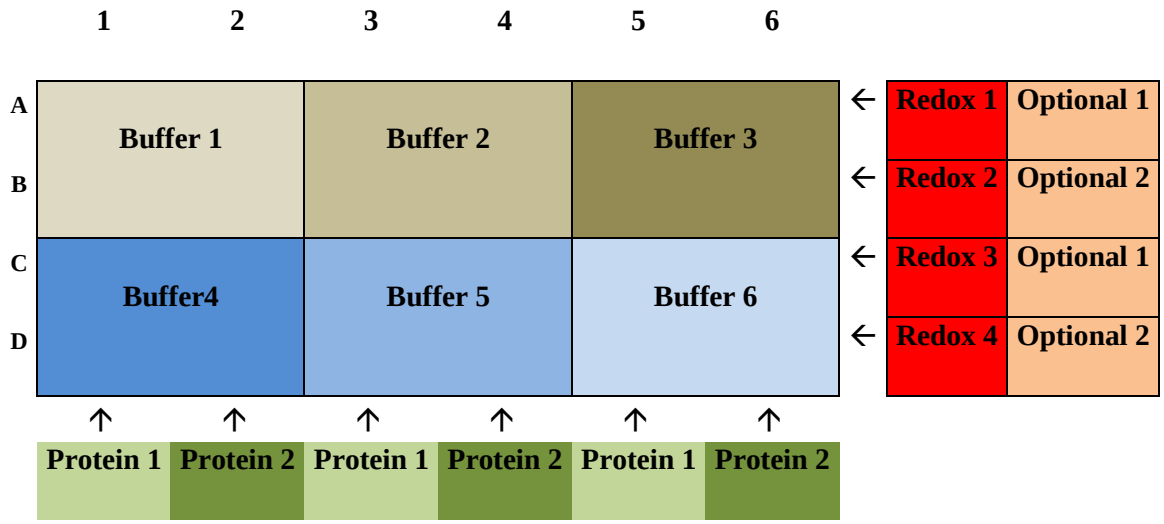


Figure 35: Agent distribution on a 24 well plate

The used buffers still have to be prepared manually like the redox system solutions and the protein stock solutions but the pipetting is done automatically by the robot. The buffers were 2-fold concentrated stocks, the redox solutions were 20-fold concentrated and the protein solutions were about 22-fold concentrated. In principal the concentrations one can use are only limited by the maximum solubility of the agent and the total volume in the well of the plate. It should be avoided to prepare stock solutions with a high viscosity, which may cause problems with the pipetting accuracy of the robot and finally the concentration in the

refolding volume. One could choose a total refolding volume (limited by the well volume) and the final concentration of each refolding agent. The programme automatically calculated the amount of water to be added to reach the desired concentrations. The pipetting sequence with the robot was buffer first, then redox system, optional agent, and water before finally the protein solution was added. All pipetting steps were conducted under shaking on the plate shaker to assure immediate dilution of any newly added agent. This guarantees also immediate distribution as soon as the protein solution is added to the refolding volume, which minimizes the protein aggregation during this dilution step.

For these experiments a rack with 16 positions was filled with the desired agents in 20 mL tubes, from which the robot conducted the pipetting. Figure 37 depicts the robot work station for refolding experiments with the buffer rack, handling arms, manipulation plate and shaker.

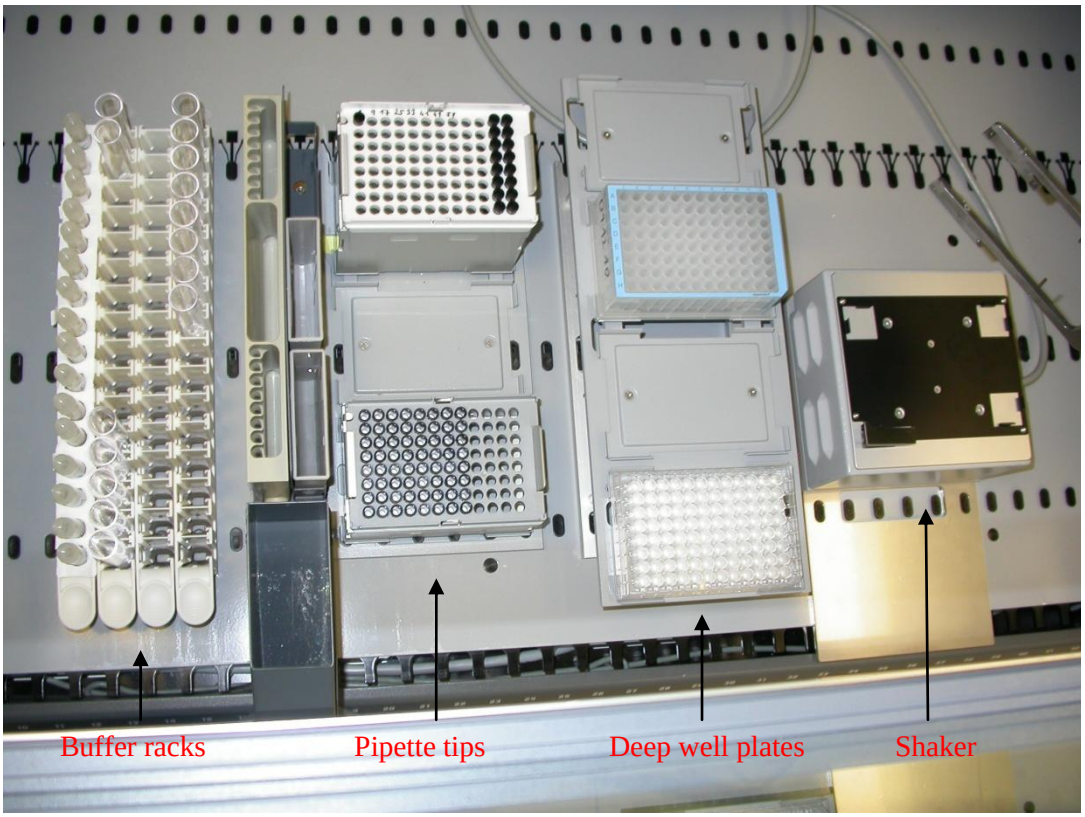


Figure 36: Robot setup for refolding experiments

Table 36 shows the sequence of the agents in the robot rack.

Table 36: Agent distribution in the robot buffer rack

Rack	Agents (10 mL)
1	Buffer 1
2	Buffer 2
3	Buffer 3
4	Buffer4
5	Buffer 5
6	Buffer 6
7	H2O
8	H2O
9	Redox 1
10	Redox 2
11	Redox 3
12	Redox 4
13	Optional 1
14	Optional 2
15	Protein 1
16	Protein 2

The advantage of this setup was, that the programme could be used for any number of refolding experiments without the drawback of writing a new pipetting programme for every experiment, which is very time consuming and error-prone. One only had to put the pre-made buffers and agents into the rack and the robot automatically performed the pipetting. With the Tecan Freedom EVO® 150 robot the following buffer components listed in table 37 have been tested for the refolding with A4.

Table 37: Buffer components tested with A4 on the robot

Additive	Concentration [mM]
Urea	1000-3000
L-arginine	50-200
Tris	0-500
α-cyclodextrin	0-40
Cysteine	0-3
Cystine	0-3

In this particular experiment different pH values (7.5, 8.5 and 9.5), different L-ariginine concentrations (0.05 M and 0.1 M) and different redox systems (1mM cysteine/3mM cystine

and 2 mM cysteine/2mM cystine) were tested for the refolding behaviour of A4. The analysis was performed by using the Caliper system. Furthermore it should be tested, if robot pipetting is as accurate as manual pipetting when preparing samples for Caliper analysis.

Figure 37 shows the experimental setup on the 24 well plate and in table 38 the buffer compositions are displayed.

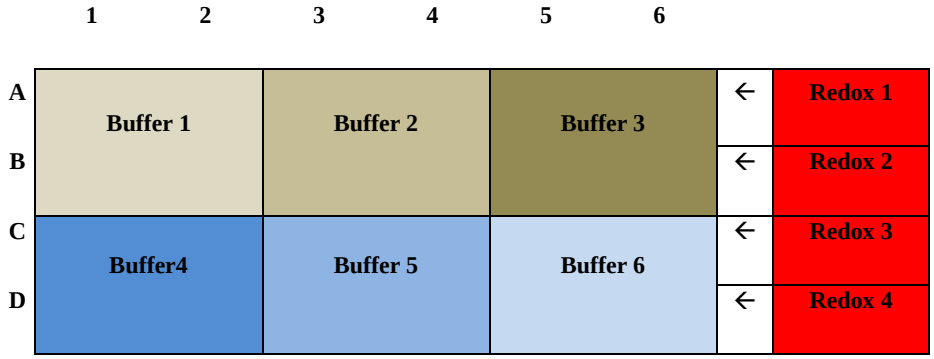


Figure 37: The experimental setup on the 24 well plate

Table 38: Experimental setup of a 24 well plate

Concentrations	
2 M urea, 0.05 M L-arginine, 50 mM Tris, pH 7.5	Buffer 1
2 M urea, 0.1 M L-arginine, 50 mM Tris, pH 7.5	Buffer 2
2 M urea, 0.05 M L-arginine, 50 mM Tris, pH 8.5	Buffer 3
2 M urea, 0.1 M L-arginine, 50 mM Tris, pH 8.5	Buffer4
2 M urea, 0.05 M L-arginine, 50 mM glycine, pH 9.5	Buffer 5
2 M urea, 0.1 M L-arginine, 50 mM glycine, pH 9.5	Buffer 6
1 mM cysteine / 3 mM cystine	Redox 1
2 mM cysteine / 2 mM cystine	Redox 2
1 mM cysteine / 3 mM cystine	Redox 3
2 mM cysteine / 2 mM cystine	Redox 4
0.2 mg / mL	Protein

The protein concentration was 0.2 mg/mL in every well, the final refolding volume was set to 1.5 mL and the refolding time was 48 hours.

Figure 38 shows the results of the Caliper analysis.

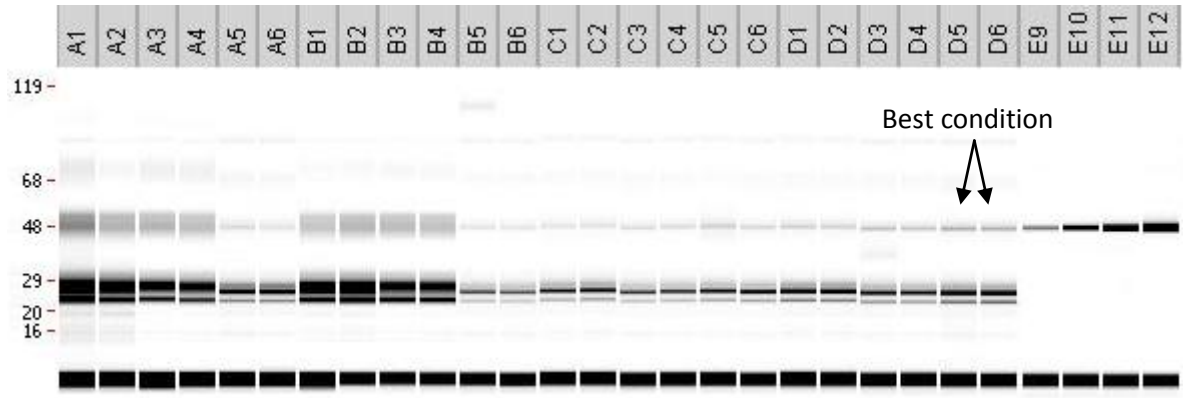


Figure 38: Caliper results for A4 refolding on the robot

The experiments in lanes A1-A4 and B1-B4 are at pH 7.5 and show diffuse bands at the expected size of about 46 kDa as the protein starts do precipitate due to the proximity to the isoelectric point.

The lanes A5-A6, B5-6, C1-C2 and D1-D2 show the bands at pH 8.5 and lanes C3-C6 and D3-D6 show the results at pH 9.5. The A4 standard is displayed on lanes E9-E10 with concentrations between 0.025 mg/mL and 0.43 mg/mL.

The best yield was reached in lanes D5 and D6 at pH 9.5, 2 M urea, 0.1 M L-arginine and 2 mM cysteine / 2 mM cystine as redox system with 7.4 %.

Figure 39 shows the comparison between robot pipetting and manual pipetting for Caliper analysis.

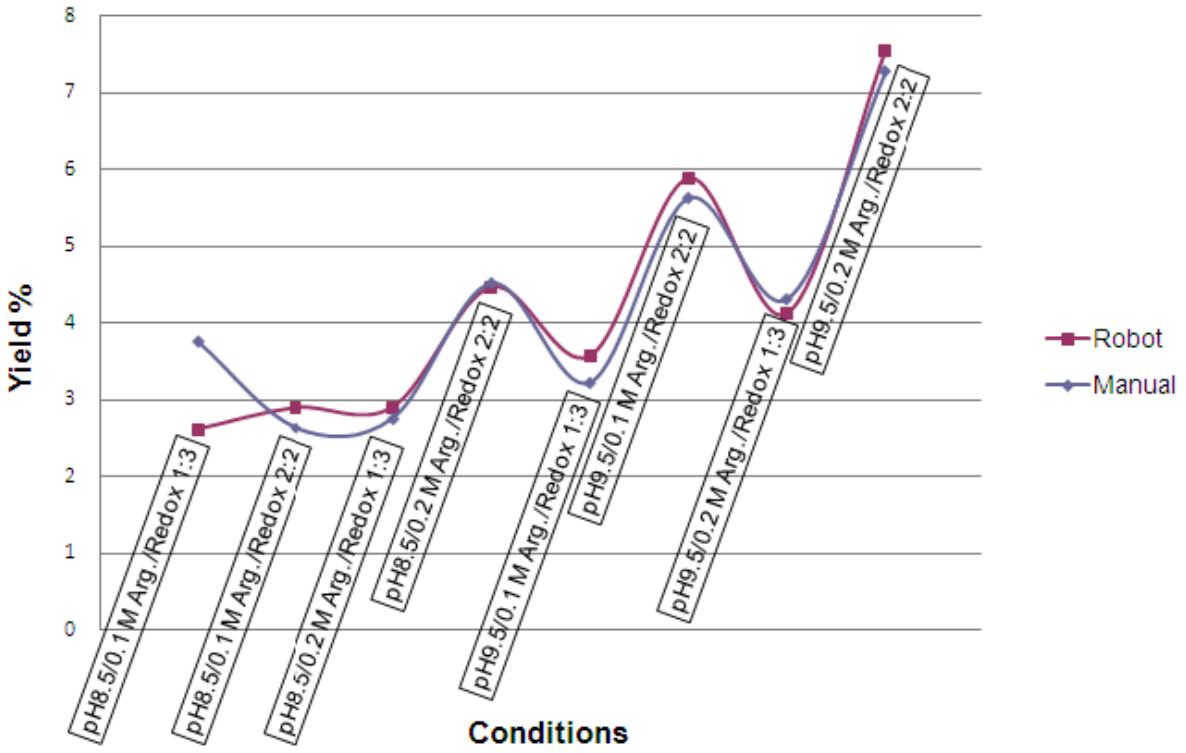


Figure 39: Comparison of manual and robot pipetting

The data of pH 7.5 is not shown here as the protein starts to precipitate at this pH. One can see that a redox ratio of 2mM cysteine/2mM cystine produces higher yields than 1 mM cysteine/3 mM cystine and that the yield increases with the pH value as well as with the L-arginine concentration. The accuracy of the robot is comparable with manual pipetting, but the sample throughput is much higher than with manual refolding. Time saving would even increase, if the buffers could be made directly out of stock solutions, as this is the most time consuming factor. It is still necessary to set the pH manually, if buffer ingredients affect the pH, which is often the case in screening experiments.

3.5 A4 refoldings in 100 mL scale

After testing the refolding conditions for A4 in small scale experiments on the robot, the next aim was to conduct the experiments in a 100 mL scale and to purify the Fab fragment via Protein A chromatography.

The basic buffer for these refolding experiments contained 50 mM glycine, 1 M urea, 0.1 M L-arginine, 2 mM cysteine and 2 mM cystine. The pH was set to 9.5 and the solubilised protein was pumped into the refolding volume at a rate of 2 mL per hour under constant stirring at 22 °C. The refolding time was 90 hours. The load was filtrated with a 0.22 µm filter and loaded directly onto the MabSelect^{TM} column. 150 µL 2 M Tris base buffer at pH 11 were pipetted into every elution fraction to increase the pH after the acidic elution step. The size of total elution fraction was therefore 1.15 mL.

The second buffer contained 200 mM Tris base besides the basic buffer and the buffer for the third experiment contained 40 mM α-cyclodextrin instead of Tris base. Just by comparison of the elution peak, one can say that the refolding buffer with 40 mM α-cyclodextrin leads to the best refolding condition.

The chromatogram of the protein A affinity step using the refolding solution containing 40 mM α-cyclodextrin is displayed in figure 40.

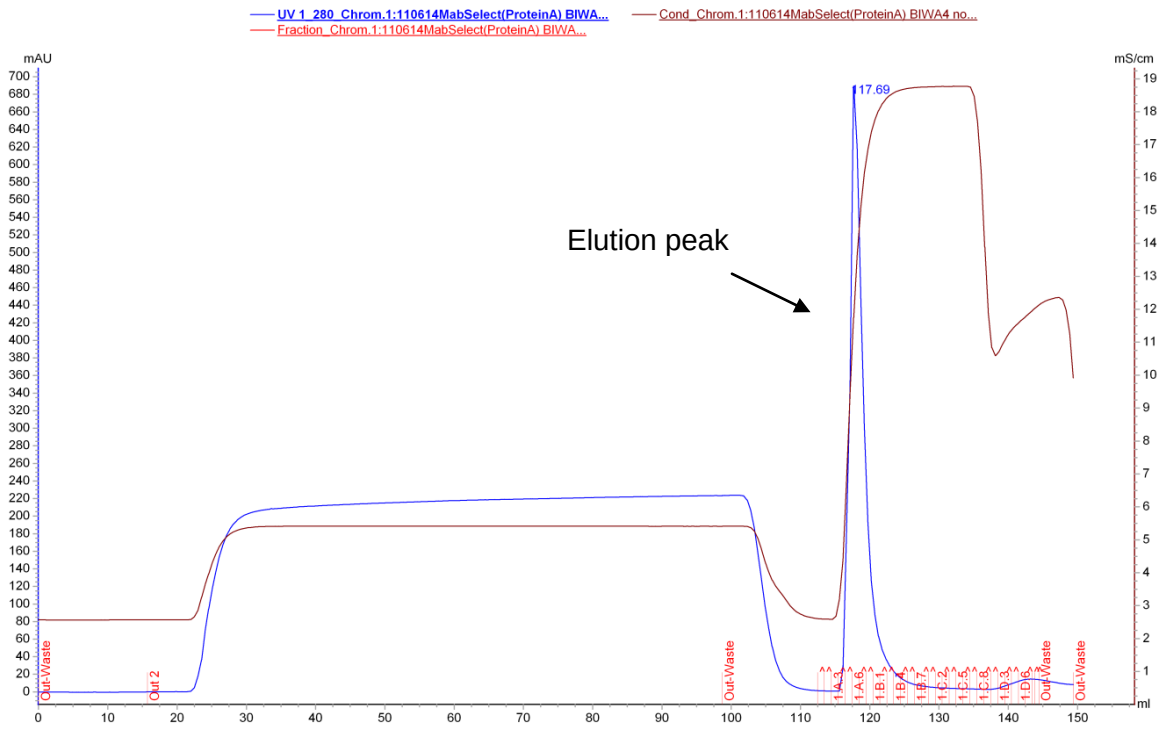
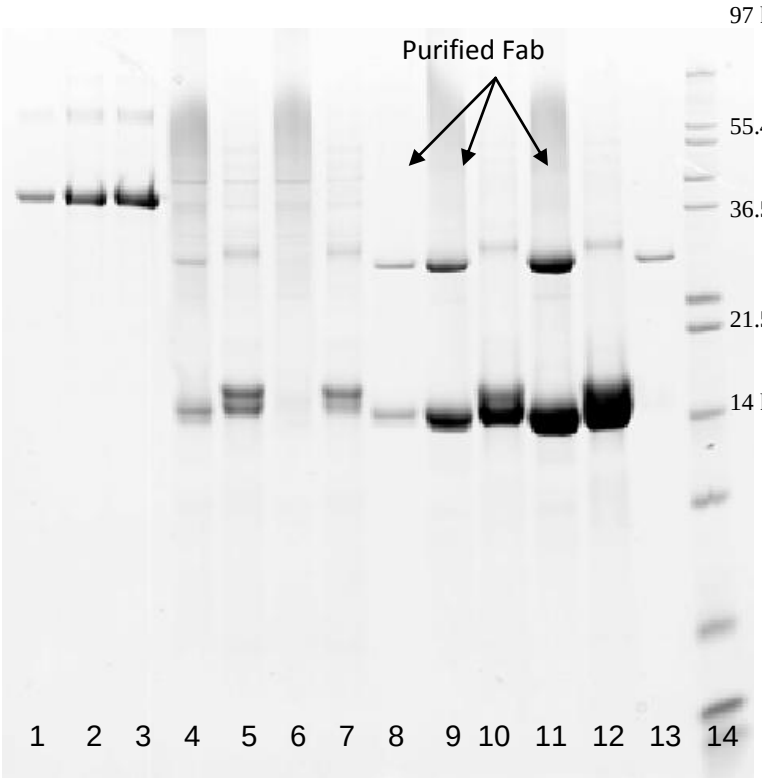


Figure 40: Chromatogram of A4 after Mabselect

The analysis for each experiment was done with SDS-PAGE and figure 41 shows the results of the best refolding experiment with 40 mM α-cyclodextrin.



Lane	Sample
1-3	BSA
4	Refolding volume
5	Refolding volume red.
6	FT
7	FT red
8	A4
9	A5
10	A5 red.
11	A6
12	A6 red
13	A4 Std.
14	Mark 12

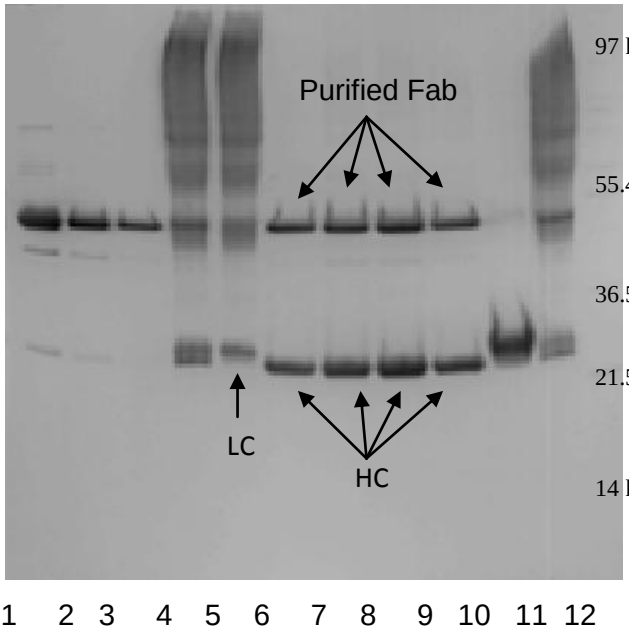
Figure 41: SDS-PAGE results of A4 after Mabselect

In these experiments the Fab molecule was already visible before purification by chromatography (lane 4). Reduction led again to the separate heavy and light chains in the same molar ratio (lane 5). Lane 7 shows, that after reduction heavy and light chain could be found in the flowthrough. This may be due to incorrectly folded Fab fragments or possible dimers, which did bind to the MabSelect^{TM} resin. Not all elution fractions are displayed here but the correctly folded Fab fragments are clearly visible.
MabSelect^{TM} binds to the Fc region and the heavy chain and therefore the single heavy chains are found in the elution fractions in a higher ratio than light chains, which derived from the bound Fab fragment.

With these experiments it was shown that the A4 Fab fragment can be correctly refolded and purified by affinity chromatography.

3.5.1 Western blot A4

As an additional verification of the correct folding of A4 a Western blot with a Fab specific antibody was carried out. The samples were taken from a Protein A chromatography experiment. The results are outlined in figure 42.



Lane	Sample
1-3	A4 Standard
4	Refolding sample
5	FT
6	A4
7	A5
8	B1
9	B2
10	Solubilisation 1:40
11	Refolding sample
12	Magic Mark XP

Figure 42: Western Blot of A4

The Western blot shows the correctly folded and purified Fab fragment in lanes 6, 7, 8, and 9. It can also be observed that only the heavy chain and the Fab fragment are binding to the Protein A resin, which was expected to be like that. Therefore the FT in lane 5 is showing no HC in lane 5. In lanes 4, 10, and 11 the bands of heavy and light chain at about 23 kDa start merging together due to their nearly equal size and the resolution decreases in these lanes.

We also see that the specificity of the Fab specific antibody is not the best, as it binds also to the heavy chain and other intermediates. This could be overcome by using an antibody which specifically targets the heavy chain so that only the correctly folded Fab fragment and the purified heavy chain fragments would be stained. Other staining parameters like a higher dilution of the antibody or a shorter staining time could also be targets for further Western blot optimisation.

3.6 Design of Experiments with A4

After completion of the experiments with A4 from 1 mL scale to 250 mL scale, another approach to determine the best refolding conditions was considered. Previous screening experiments were conducted using a step by step approach. The best concentrations or conditions were determined one after the other, for example after finding the optimum L-arginine concentration in one screening series, the optimum urea concentration was screened in the next series. The new aim now was to test a Design of Experiments (DoE) setup using a central composite design to find the best refolding concentration for the three factors urea, L-arginine and a-cyclodextrin. DoE provides strategies and methods of experimental design for performing and analyzing test series in a systematic and efficient way. All experiment parameters are varied to maximize the information gained from the experimental results. DoE is able to determine the dependency of the yield on the tested components and of the tested components on each other [75]. Figure 43 shows a central composite design, which was chosen for the experiments with A4.

In this 3D model the red center point represents the starting buffer composition chosen for these experiments and every axis represents the concentration of one of the tested components. The other fourteen points are generated automatically by the DoE program and cover the predetermined range of concentrations for each buffer component (see table 39). The composition of the center point will be tested several times to recognize any deviation by

the experimental setup through statistical analysis. The central composite design was chosen, because the concentrations of the various buffer components were known before and should be confirmed by this experiment. However for most experiments the final buffer composition will not be known and the DoE approach should also be able to find it faster than a step by step screening system.

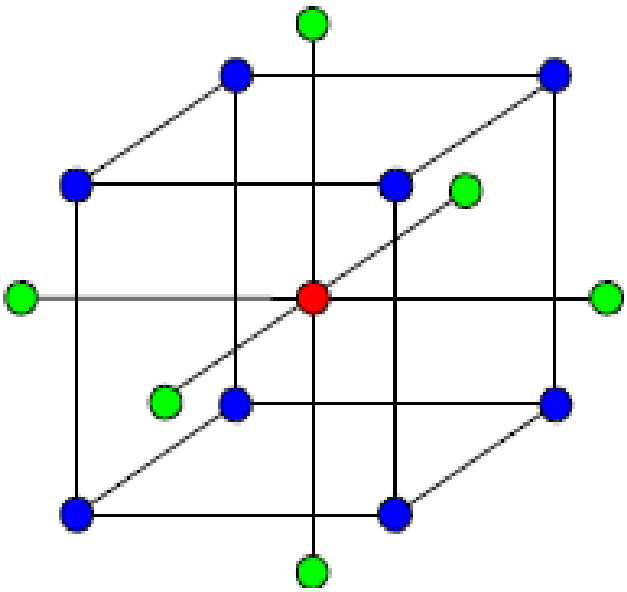


Figure 43: Central composite design with center point in red

Table 39 shows the range of the tested buffer components.

Table 39: Buffer concentration tested with Design of Experiments

Factor	Concentration [mM]
Urea	500-4000
L-arginine	100-600
α-cyclodextrin	10-80

The pH for all experiments was set to 9.5 and the glycine concentration was 50 mM. The final refolding volume was 1 mL and the protein concentration was 0.2 mg/mL for HC and LC so the highest possible yield of Fab fragment would also be 0.2 mg/mL. Twenty different refolding buffers were calculated by the programme and each composition is displayed in table 40. The refolding time was 48 hours and the refolding temperature was set to 22°C.

Table 40: Refolding conditions calculated by the DoE program

Std	Run	Type	Factor 1 A:Urea M	Factor 2 B:Arginine M	Factor 3 C:Cyclodextr... M
1	1	Factorial	0.50	0.10	0.10
16	2	Center	2.25	0.35	0.45
10	3	Axial	4.18	0.35	0.45
6	4	Factorial	4.00	0.10	0.80
3	5	Factorial	0.50	0.60	0.10
2	6	Factorial	4.00	0.10	0.10
14	7	Axial	2.25	0.35	0.84
7	8	Factorial	0.50	0.60	0.80
8	9	Factorial	4.00	0.60	0.80
5	10	Factorial	0.50	0.10	0.80
18	11	Center	2.25	0.35	0.45
17	12	Center	2.25	0.35	0.45
19	13	Center	2.25	0.35	0.45
4	14	Factorial	4.00	0.60	0.10
11	15	Axial	2.25	0.07	0.45
15	16	Center	2.25	0.35	0.45
9	17	Axial	0.32	0.35	0.45
12	18	Axial	2.25	0.63	0.45
13	19	Axial	2.25	0.35	0.06
20	20	Center	2.25	0.35	0.45

Figures 44 and 45 show parts of the Caliper analysis file.

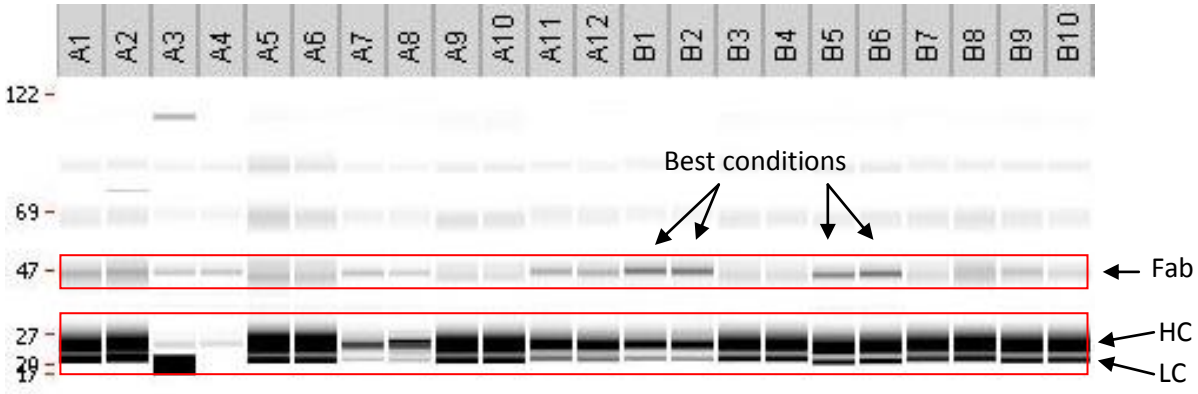


Figure 44: Caliper results of DoE with A4 1/2

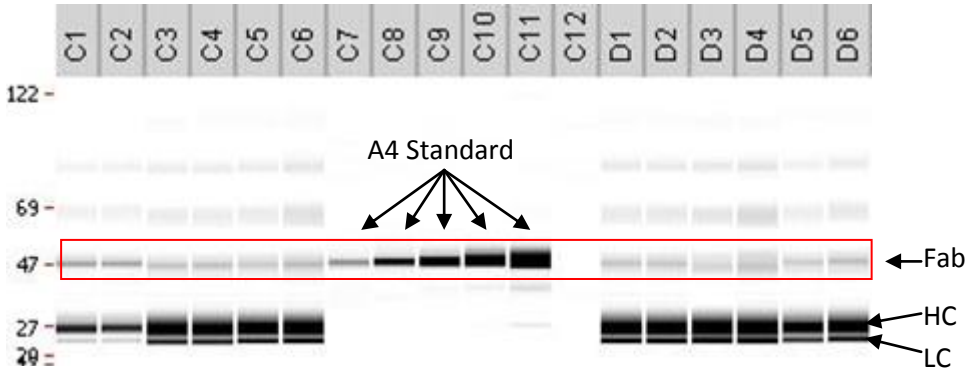


Figure 45: Caliper results of DoE with A4 2/2

The marked lanes B1, B2 and B5, B6 (both are repeated determinations) show the two conditions with significantly higher yields.
In B1 and B2 the buffer additives were 0.5 M urea, 0.6 M L-arginine and 0.01 M α-cyclodextrin and the final yield was 10.1 % in this case.

In B5 and B6 the additives concentrations were 2.25 M urea, 0.07 M L-arginine and 0.045 M α-cyclodextrin. This resulted in a yield of 9.5 %.

Although it was possible to find two different conditions which showed superior refolding yields compared to the other tested conditions, there was still no single conditions that leads to outstanding results. Furthermore the found conditions did not match the condition, which was found in previous step by step screenings (50 mM glycine, 1 M urea, 0.1 M L-arginine, 40 mM α-cyclodextrin, 2 mM cysteine and 2 mM cystine).
Afterwards this can be ascribed to problems with the Caliper analysis system. The analytical method led to incoherent results when the buffers contained α-cyclodextrin and resulted in defective data. With this data DoE was not able to find an optimum for each of the screened components. In the previous step by step screening experiments SDS-PAGE was used to quantify the yield and led to an optimized buffer.

Due to time restrains it was not possible to repeat the DoE experiments again in combination with SDS-PAGE analysis to verify the previously found results but the DoE approach shows a sophisticated way to design screening experiments from scratch.

4 Conclusion and Outlook

The upstream processes of the proteins 3H6 and B1 were successful in small scale experiments as well as with 5L fermentations. It could be observed that the titers of the fermentations containing the light chains of the Fab fragments were about 3.9 times higher with 3H6 and 3 times higher in the case of B1, than with their heavy chains respectively. The expression yield is influenced by numerous factors like plasmid copy number, mRNA stability, the leader sequence or tightly repressible promoters [44] [45]. Concerning the lower titers of the fermentations containing the plasmids with the heavy chains, the genome sequences of the heavy chains are possible targets for optimisation. The engineering of heterologous protein expression should take the codon usage bias of the host cell organism, in this case *E.coli*, into account [62]. Changing the protein coding sequence of a gene can dramatically affect its expression [63]. Nowadays, synthetic biology provides the necessary means to generate artificial DNA sequences, which can enhance gene expression rates significantly [64]. The pET system, which was used in these experiments, is a very powerful system for cloning and expression of recombinant proteins in *E.coli.* Its features are a very strong promoter (T7), a Kanamycin resistance and the possibility of adding a His tag to the expressed protein. The T7 RNA polymerase is so selective and active, that under full induction up to 50 % of the total cellular protein can be made of the desired product. The Kanamycin resistance offers a practical tool for clone screening and the expression level can be attenuate simply by adjusting the inducer concentration. A second screening system is provided by the ability to screen for blue/white screening through interruption of the alkaline phosphatase gene [46-48].

There was no soluble protein expression detectable and therefore the formation of inclusion bodies was pushed. The process of inclusion body purification after homogenisation should be investigated more intensively as the standard washing procedures do not always yield the required purity for further refolding experiments. An alternative strategy to the performed washing and centrifugation steps would be the utilisation of Trimethylammoniopropyl-methanethiosulfonate (TAPS-sulfonate). The reversible derivatization of cysteine residues leaves the solubilised protein with a positive net charge strong enough to enable purification by cation exchange using e.g. an S-Sepharose based resin [49] [50].

The protein content of the light chains in mg/g IBs was also higher than with the heavy chains by a factor of about 4. Host cell proteins still present in inclusion bodies can interfere with the

refolding process and cause aggregation or misfolding of the expected protein. Due to the higher hydrophobicity of the HC, its´ solubility is also hindered in aqueous environments [51].

This may also influence the composition of the resolubilisation buffer. If less host cell proteins are present, urea can be used more easily as chaotropic agent instead of Guanidine-hydrochloride, because less impurities are co-solubilised. Urea is also preferred for large scale applications, because Gdn-HCl is more corrosive and due to its high conductivity any form of ion-chromatography for further protein purification is widely inhibited.

The refolding experiments with B1 did not lead to any correctly refolded Fab fragment. B1 was not studied as intensively as 3H6 or A4 and therefore leaves plenty of room for future investigations. The preparation of the inclusion bodies is regarded as one of the crucial steps to receive a good starting position for refolding experiments. It became clear that this Fab fragment could not be refolded by applying "standard experiments" and will need more scientific input. The solubilisation issues concerning the heavy chain have to be overcome first to obtain two soluble chains which could finally be refolded. One has to determine the minimal concentration of chaotropic reagents and additives to keep the chains soluble but still providing the possibility to refold and undergo the protein´s tendency to denature under too high concentrations.

Facing problems to find a functioning refolding buffer for the HC of two model proteins with the widely used chaotropic components urea and Gdn-HCl, alternative solubilisation conditions should be taken into account. Extreme pH values have been successfully used for the solubilisation and subsequent refolding of inclusion bodies from *E.coli* [65] [66]. Organic solvents like alcohols could also help in the solubilisation of HC of B1 and 3H6 [67]. Another technique to solubilise IB from *E. coli* is to apply high hydrostatic pressure (up to 300 MPa) in combination with low concentrations of denaturants [68]. The following refolding process is accomplished by lowering the pressure stepwise to atmospheric pressure with low concentrations of redox shuffling agents [69]. Despite the costs for such an experimental setup, it could bring advantages over the conventional refolding procedures used for B1 and 3H6.

The Fab fragment 3H6 showed positive effects concerning the refolding behavior of the light chain. As with B1 the inclusion body preparation process and the composition of the solubilisation buffer should be investigated in more detail. However, the formation of LC-LC dimers seems to be preferred over the formation of the Fab fragment, which is confirmed by the fact that the elution fractions break down into LC monomers after reduction with β-

mercaptoethanol. Excess of any chain will bias the refolding process: excess LC will facilitate dimerisation and too much of HC will catalyze the aggregation of the HC, which is still a challenging task to overcome. The assumption, that only the LC folded correctly limited the number of affinity media and excludes e.g. Protein A from the pool of possible purification resins at the moment. The final refolding buffer contained 3 M urea, 0.2 M L-arginine, 15 mM N-laurylsarcosine, 50 mM glycine, 2 mM GSH and 2 mM GSSG at pH 9.5. Zwittergent 3-14 also showed positive aspects on the HC solubility and it should be tested more intensively. Positive metal ions like copper should also be tested more thoroughly together with various redox systems and ratios to receive a controllable and complete oxidation, which enables the formation of disulphide bridges.

The main focus of attention for the refolding experiments with A4 was on the establishment of a robot based screening system. An optimised refolding buffer had been elaborated by Gerald Bieder [52] and led to a buffer composition of 1 M urea, 0.1 M L-arginine, 40 mM α-cyclodextrin, 1 mM cysteine, 3mM cystine and 50 mM glycine at pH 9.5 by a conventional step by step screening system in 1 mL Eppendorf tubes. The refolding experiments using the Tecan® Freedom 150 robot led to a slightly different redox ratio of 2 mM cysteine and 2 mM cystine.

The robot experiments should also evaluate the possibilities to use the robot for automated refolding experiments on one hand and automated preparation of 96 well plates for Caliper analysis. The experiments showed that the robot is able to perform the pipetting steps as accurately as a person, but the saving in time is still to be increased. Although the robot can perform all pipetting steps autonomously, each buffer has to be individually prepared and cannot be made out of stock solutions by the robot itself yet. This is due to the need to set the pH right for many experiments and poses a time consuming step. The error rate is also lower when using the robot and if the limitations like e. g. too high viscosity of solutions are considered, robot based experiments can allow a higher sample throughput than conventional screening systems.

The potential to perform experiments following the Design of Experiments approach on a refolding robot leads to fast data acquisition and purposive experiments [70]. In this study the concentrations of urea, L-arginine and α-cyclodextrin were investigated concerning their influence on the refolding behavior of A4. The highest yields reached about 10%, but the results highly differed from the results, which were found during the step-by-step screening on the robot without DoE guidance. This could be traced back to analytical problems with the

Caliper analysis system, when using α-cyclodextrin in the refolding buffers. Earlier experiments had been analysed with SDS-PAGE. Due to time restrains it was not possible to repeat the DoE experiments with another analytical system but the experimental design is ready-to-use.

Alternative refolding methods like reverse dilution can be used if the protein intermediates are soluble. This would lead to a prolonged interaction of unfolded protein with constantly diminishing concentrations of the denaturant and finally result in a higher yield of correctly folded protein.

Various on-column refolding techniques are described in literature, which could help with refolding experiments. A possible method could be the removal of the denaturant via buffer exchange on a size exclusion chromatography (SEC) resin. A feed pulse of denatured protein, reducing agent and denaturant is injected to the SEC column. Through the differences in the distribution coefficient, these components separate during migration in the column. This leads to a decrease of denaturant and reducing agent around the protein, which induces protein refolding. Although this setup is not used for high scale applications in general it could help in lab scale refolding experiments. The resin could also be used to immediately separate correctly folded protein from intermediates and aggregated proteins, by its different diffusion properties [53] [71] [72].

Another way would be to bind the protein onto an ion-exchange chromatography resin and to perform the refolding by controlling the oxidative refolding environment and adjacent elution [54] [73].

Chaperone mimics like the GroEL/GroELs system are also used to facilitate on-column refolding of proteins. GroEL/GroES binds to unfolded polypeptides or the folding intermediates, preventing improper interactions of polypeptide chains that lead to aggregation [55] [56] [74].

Parallel to further refolding experiments the establishment of another robust analytical system besides SDS-PAGE and Caliper analysis would make sense. Reversed phase HPLC could assure the accuracy of the analytical data and one could detect even small improvements during the still challenging task of Fab fragment refolding.

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5 Appendix

5.1 Tables

5.2 Figures

5.3 References

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